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UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

October 20, 2022

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APPLICATION NUMBER: *16/055,847*
FILING DATE: *August 6, 2018*
PATENT NUMBER: *10,857,205*
ISSUE DATE: *December 8, 2020*

By Authority of the
Under Secretary of Commerce for Intellectual Property
and Director of the United States Patent and Trademark Office

Curtis Goffe
Certifying Officer

DocCode – SEQ.TXT

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Application Number: 16055847 Document Date: 08/06/2018

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Form Revision Date: August 26, 2013

Electronically Filed

PRELIMINARY AMENDMENT Under CFR 1.115 Address to: Mail Stop Patent Application Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Attorney Docket No.	REGN-008CIPCON3
	Confirmation No.	To Be Assigned
	First Named Inventor	YANCOPOULOS, GEORGE D.
	Application Number	To Be Assigned
	Filing Date	August 6, 2018
	Group Art Unit	To Be Assigned
	Examiner Name	To Be Assigned
	Title: *"Use of a VEGF Antagonist to Treat Angiogenic Eye Disorders"*	

Sir:

Prior to the examination of the above-referenced application on the merits, please enter the amendments below.

AMENDMENTS TO THE SPECIFICATION

Please amend paragraph [0001] on page1 of the specification to read as follows:

[0001] This application **is a continuation of U.S. Patent Application Serial No. 15/471,506, filed March 28, 2017 (now allowed) which is a continuation of U.S. Patent Application Serial No. 14/972,560, filed December 17, 2015, now U.S. Patent No. 9,669,069 issued June 6, 2017 which is a continuation of U.S. Patent Application Serial No. 13/940,370 filed July 12, 2013, now U.S. Patent No. 9,254,338 issued February 9, 2016 which** is a continuation-in-part of International Patent Application No. PCT/US2012/020855, filed on January 11, 2012, which claims the benefit of US Provisional Application Nos. 61/432,245, filed on January 13, 2011, 61/434,836, filed on January 21, 2011, and 61/561,957, filed on November 21, 2011, the contents of which are hereby incorporated by reference in their entireties.

AMENDMENTS TO THE CLAIMS

1. - 20. (**Canceled**)

21. (**New**) A method for treating macular edema following retinal vein occlusion in a human subject comprising administering 2 mg aflibercept to the subject by intravitreal injection once every 4 weeks.

22. (**New**) The method of claim 21 wherein the aflibercept is administered in a volume of 0.05 ml.

23. (**New**) The method of claim 22 wherein the aflibercept is in a pharmaceutical formulation comprising a pharmaceutically acceptable carrier.

REMARKS UNDER 37 CFR § 1.115

Formal Matters

Claims 21-23 are pending after entry of the amendments set forth herein.

Claims 1-20 are canceled without prejudice.

Claims 21-23 are added.

Support for new claim 21 can be found at paragraph [0010] and throughout the specification.

Support for new claim 22 can be found at paragraph [0070] and throughout the specification.

Support for new claim 23 can be found at paragraph [0027] and throughout the specification.

The specification has been amended to update the cross-reference to related application section.

No new matter has been added.

PARENT APPLICATION

The parent application has been allowed. Further, as indicated above, correspondence and support for the current claims relative to those of the parent application can be reviewed and confirmed. In the event the Examiner has any questions with respect to claim support or other issues in connection with the application, the Examiner is respectfully requested to contact the undersigned attorney at the indicated telephone number to arrange for an interview to expedite this position of this application.

STATEMENT UNDER 37 C.F.R. §§1.56 AND 1.2

Applicants hereby advise the Examiner of the status of a co-pending application in compliance with the Applicant's duty to disclose under 37 C.F.R. §§1.56 and 1.2 (see also MPEP §2001.06(b)) as discussed in *McKesson Info. Soln. Inc., v. Bridge Medical Inc.*, 487 F.3d 897; 82 USPQ2d 1865 (Fed. Cir. 2007).

The Applicants wish to bring to the Examiner's attention U.S. Patent Application No. 13/940,370, filed July 12, 2013 which issued on February 9, 2016 as U.S. Patent 9,254,338.

The Applicants wish to bring to the Examiner's attention U.S. Patent Application No. 14/972,560, filed December 17, 2015 which issued on June 6, 2018 as U.S. Patent No. 9,669,069.

The Applicants wish to bring to the Examiner's attention that a Notice of Allowance was mailed on July 26, 2018 in co-pending U.S. Patent Application No. 15/471,506, filed March 28, 2018.

These documents are available on PAIR, and thus are not provided with this communication. Please inform the undersigned if there is any difficulty in obtaining the documents from PAIR.

CONCLUSION

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number REGN-008CIPCON3.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: 6 August 2018

By: /Karl Bozicevic, Reg. No. 28,807/
Karl Bozicevic
Registration No. 28,807

Bozicevic, Field & Francis LLP
201 Redwood Shores Parkway, Suite 200
Redwood City, California 94065
Telephone: (650) 327-3400
Direct: (650) 833-7735
Facsimile: (650) 327-3231

Electronically Filed 8/6/2018

INFORMATION DISCLOSURE STATEMENT	Attorney Docket No.	REGN-008CIPCON3
	Confirmation No.	To Be Assigned
	First Named Inventor	GEORGE D. YANCOPOULOS
	Application Number	To Be Assigned
	Filing Date	August 6, 2018
	Group Art Unit	
	Examiner Name	
Address to: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Title: "*Use of a VEGF Antagonist to Treat Angiogenic Eye Disorders*"	

Sir:

Applicants submit herewith documents which may be material to the examination of this application and in respect of which there may be a duty to disclose in accordance with 37 C.F.R. § 1.56. This submission is not intended to constitute an admission that any document referred to therein is "prior art" for this invention unless specifically designated as such. A listing of the documents is shown on enclosed Form PTO/SB/08A.

The Examiner is requested to make the documents listed on the enclosed PTO/SB/08A of record in this application. Applicants would appreciate the Examiner initialing and returning the initialed copy of form PTO/SB/08A, indicating the documents cited therein have been considered and made of record herein.

All of the references identified herein were disclosed in parent application serial number 15/471,506, and as such, copies thereof are not included pursuant to the provisions of 37 CFR § 1.98(d).

Statements

☒ **No statement**

☐ **PTA Statement under 37 CFR § 1.704(d)(1):** Each item of information contained in the information disclosure statement filed herewith:

(i) Was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the information disclosure statement; or

(ii) Is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the information disclosure statement.

☐ **IDS Statement under 37 CFR § 1.97(e)(1):** Each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement; or

☐ **IDS Statement under 37 CFR § 1.97(e)(2):** No item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in § 1.56(c) more than three months prior to the filing of the information disclosure statement.

Fees

☒ No fee is believed to be due.

☐ The appropriate fee set forth in 37 C.F.R. §1.17(p) accompanies this information disclosure statement.

The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number REGN-008CIPCON3.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: August 6, 2018

By: /Karl Bozicevic, Reg. No. 28,807/
Karl Bozicevic
Reg. No. 28,807

BOZICEVIC, FIELD & FRANCIS LLP
201 Redwood Shores Parkway, Suite 200
Redwood City, CA 94065
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	To Be Assigned
				Filing Date	August 6, 2018
				First Named Inventor	YANCOPOULOS, GEORGE D.
				Art Unit	N/A
				Examiner Name	N/A
Sheet	1	of	3	Attorney Docket Number	REGN-008CIPCON3

U.S. PATENT DOCUMENTS

Examiner Initial*	Cite No.	Patent Number Number-Kind Code (*if known*)	Issue Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1	7396664	2008-07-08	Daly et al.	

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No.	Publication Number **Number-Kind Code** (*if known*)	Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1	20050163798	2005-07-28	Papadopoulos et al.	
	2	20050260203	2005-11-24	Wiegand et al.	
	3	20060058234	2006-03-16	Daly et al.	
	4	20060172944	2006-08-03	Wiegand et al.	
	5	20070190058	2007-08-16	Shams	
	6	20030171320	2003-09-11	Guyer	

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No.	Foreign Document Number Country Code-Number-Kind Code (*if known*)	Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T
	1	WO 2000/75319	2000-12-14	Regeneron Pharmaceuitcals, Inc.		
	2	WO 2007/022101 A2	2007-02-22	Regeneron Pharmaceuticals, Inc.		
	3	WO 2008/063932	2008-05-29	Genentech, Inc.		
	4	JP 2010-509369	2010-03-25	Genentech, Inc.	See WO 2008/063932 for English Equivalent	

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
	1	ANONYMOUS "Lucentis (rangibizymab injection) Intravitreal Injection" pp. 103 (June 2006)	
	2	Information from ClinicalTrials.gov archive View of NCT00637377 "Vascular Endothelial Growth Factor (VEGF) Trap-Eye: Investigation of Efficacy and Safety in Wet Age-Related Macular Degeneration (AMD) (VIEW 2)" *ClinicalTrials.gov*. Web. 2010-11-30.	
	3	CENTER FOR DRUG EVALUATION AND RESEARCH APPLICATION NUMBER: 21-756 MEDICAL REVIEW(S) (December 17, 2004) <URL:https://www.accessdata.fda.gov/drugsatfda_docs/nda/2004/21-756_Macugen_medr.pdf>	
	4	CENTER FOR DRUG EVALUATION AND RESEARCH BLA APPLICATION NUMBER: 125156 MEDICAL REVIEW, (June 2006) <URL:https://www.accessdata.fda.gov/drugsatfda_docs/nda/2006/125156s000_Lucentis_MedR.pdf>	
	5	CHARLES, Steve (Guest Lecturer) "VEGF Trap Has Positive DME Data" Tenth Annual Retina Fellows Forum Jan 29 and 30, Chicago, Article Date 03/01/2010	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	To Be Assigned
				Filing Date	August 6, 2018
				First Named Inventor	YANCOPOULOS, GEORGE D.
				Art Unit	N/A
				Examiner Name	N/A
Sheet	2	of	3	Attorney Docket Number	REGN-008CIPCON3

NON PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
	6	DIXON et al., "VEGF Trap-Eye for the treatment of neobascular age-related macular degeneration" Expert Opin. Investig. Drugs (2009) 18 (10): 1-8.	
	7	DO et al., "An exploratory study of the safety, tolerability and bioactivity of a single intravitreal injection of vascular endothelial growth factor Trap-Eye in patients with diabetic macular oedema" Br J Opthamol. 93(2):144-1449 (February 2009)	
	8	DO et al., "The DA VINCI Study: phase 2 primary results of VEGF Trap-Eye in patients with diabetic macular edema" Opthamology 118(9):1819-1826 (September 2011)	
	9	THE EYETECH STUDY GROUP, "Anti-Vascular Endothelial Growth Factor Therapy for Subfoveal Choroidal Neovascularization Secondary to Age-related Macular Degeneration" American Academy of Ophthamology, 110(5):979-986 (May 2003)	
	10	HEIERet al., " rhuFab V2 (anti-VEGF Antibody) for Treatment of Exudative AMD" Symposium 8:Experimental and Emerging Treatments for Choroidal Neovascularization, 10 pp (2002)	
	11	HEIER et al., "RhuFab V2 in Wet AMD - 6 Month Continued Improvement Following Multiple Intravitreal Injections" Invest Ophthalmol Vis Sci, 44:E-Abstract 972 (2003)	
	12	HEIER et al., "Intravitreal Aflibercept (VEGF Trap-Eye) in Wet Age-related macular Degeneration," Ophthalmology, 119:2537-2548 (2012)	
	13	Information from ClinicalTrials.gov archive on the VIEW 2 study (NCT00637377) "VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet AMD (VIEW 2)" version available and updated on 17 March 2008.	
	14	Information from ClinicalTrials.gov archive on the view of NCT00509795 "Vascular Endothelial Growth Factor (VEGF) Trap-Eye: Investigation of Efficacy and Safety in Wet Age-Related Macular Degeneration (AMD)" (12-01-2009)	
	15	Information from ClinicalTrials.gov archive on the view of NCT00789477 "DME and VEGF Trap-Eye: Investigation of Clinical Impact" (11-18-2010)	
	16	Information from ClinicalTrials.gov archive on the view of NCT00509795 "Vascular Endothelial Growth Factor (VEGF) Trap-Eye: Investigation of Efficacy and Safety in Wet Age-Related Macular Degeneration (AMD)" (01-07-2011)	
	17	KRZYSTOLIK et al., "Prevention of Experimental Choroidal NEovascularization With Intravitreal Anti-Vascular Endothelial Growth Factor Antibody Fragment" Arch Ophthamol., 120:338-346 (Mar. 2002)	
	18	MITRA et al., "Review of anti-vascular endothelial growth factor therapy in macular edema secondary to central retinal vein occlusions" Expert Review in Ophthalmo, Taylor & Francis, GB (January 1, 2011) 6(6):623-629	
	19	MOUSA AND MOUSA, "Current Status of Vascular Endothelial Growth Factor Inhibition in Age-Related Macular Degeneration" Biodrugs 2010; 24(3); 183-194.	
	20	NGUYEN et al., "A Phase I Study of Intravitreal Vascular Endothelial Growth Factor Trap-Eye in Patients with Neovascular Age-Related Macular Degeneration" Opthamology, J.B. Lippincott Co., Philadelphia, PA, US, 116(11):2141-2148 (November 1, 2009) 116(11):2141-2148 (November 1, 2009)	
	21	NGUYEN et al., "A phase I trial of an IV-administered vascular endothelial growth factor trap for treatment in patients with choroidal neovascularization due to age-related macular degeneration" Ophthalmology (Sept 2006) 113(9):1522e1-1522e14 (epub July 28,2006)	

Examiner Signature		Date Considered	

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				First Named Inventor	YANCOPOULOS, GEORGE D.
				Art Unit	N/A
				Examiner Name	N/A
Sheet	3	of	3	Attorney Docket Number	REGN-008CIPCON3

NON PATENT LITERATURE DOCUMENTS			
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	22	NICHOLS, EARL R., "AAO: Ranibizumab (rhuRab) May Improve Vision in Age-Related Macular Degeneration" Doctor's Guide Global Edition, www.pslgroup.com/dg/23f2aa.htm, pp. 1-2 (November 24, 20013)	
	23	OLIVERA et al., "VEGF Trap R1R2 suppresses experimental corneal angiogenesis" European Journal of Ophthalmology (January 1, 2010) 20(1):48-54	
	24	PAI et al., "Current concepts in intravitreal drug therapy for diabetic retinopathy" Saudi Journal of Opthamology 24(4):143-149 (June 30, 2010)	
	25	Regeneron Pharmaceuticals, Inc. FORM 10-Q, published on 7 November 2007 for the period ending 30 September 2007	
	26	Regeneron, Press release "Regeneron Reports First Quarter 2008 Financial and Operating Results", May 1, 2008.	
	27	Regeneron Press Release "Bayer and Regeneron Report Positive Top-Line Results of Two Phase 3 Studies with VEGF Trap-Eye in Wet Age-related Macular Degeneration" November 22, 2010	
	28	Regeneron Press Release "Regeneron and Bayer Report Positive Results for VEGF Trap-Eye in Phase 3 Study in Central Retinal Vein Occlusion (CRVO) and in Phase 2 Study in Diabetic Macular Edema (DME)" December 20, 2010	
	29	Regeneron Pharmaceuticals Inc., "VEGF Trap-Eye Final Phase 2 Results in Age-related Macular Degeneration Presented at 2008 Retina Society Meeting" (September 28, 2008) (XP-002770952)	
	30	SIMO AND HERNANDEZ, "Advances in Medical Treatment of Diabetic Retinopathy" Diabetes Care, Volume 32, Number 8, August 2009	
	31	Slides for the 2008 Retina Society Meeting "VEGF Trap-Eye in Wet AMD CLEAR-IT 2: Summary of One-Year Key Results", September 28, 2008.	
	32	STEWART, "THe expanding role of vascular endothelial growth factor inhibitors in opthamology" Mayo Clin Proc. 87(1):77-88 (January 2012)	
	33	THOMAS REUTERS INTEGRITY "VEGF Trap-Eye final phase II results in age-related macular degeneration presented at 2008 Retina Society Meeting" (September 28, 2008)	
	34	WHO Drug Information, "International Nonproprietary Names for Pharmaceutical Substances (INN)" Vol. 20, No. 2, 2006, pages 115-119.	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Electronically Filed

NOTIFICATION OF PRIOR SEQUENCE LISTING		
	Attorney Docket	REGN-008CIPCON3
	First Named Inventor	YANCOPOULOS, GEORGE D.
	Application Number	To Be Assigned
	Filing Date	6 August 2018
Address to: Mail Stop Patent Application Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Confirmation Number	To Be Assigned
	Group Art Unit	To Be Assigned
	Examiner Name	To Be Assigned
	Title: "USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS"	

Sir:

The above-identified patent application contains sequences of nucleic acid and polypeptides. A sequence listing was prepared for parent application, **15/471,506,** filed **March 28, 2018**, in paper and computer-readable format. The sequence information in the paper or compact disk copy of the sequence listing (required by 1.821(c)) of this application is identical to the sequence information in the computer-readable format (CRF) of the above-identified other application. No new matter has been added. Therefore, please transfer to this application, in accordance with 37 CFR § 1.821(e), the fully compliant computer readable copy from applicants' other application. A paper (.txt) copy of this sequence listing is enclosed.

Applicants respectfully submit that the present patent application is now in compliance with 37 CFR §§ 1.821 - 1.825. The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number REGN-008CIPCON3.

Respectfully submitted,

BOZICEVIC, FIELD & FRANCIS LLP

Dated: 6 August 2018

By: /Karl Bozicevic, Reg. No. 28,807/
Karl Bozicevic
Registration No. 28,807

BOZICEVIC, FIELD & FRANCIS LLP
201 Redwood Shores Parkway, Suite 201
Redwood City, California 94065
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

SEQUENCE LISTING

<110> George D. Yancopoulos

<120> Use of a VEGF Antagonist to Treat Angiogenic Eye Disorders

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Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
            340                 345                 350
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
        355                 360                 365
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
    370                 375                 380
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
385                 390                 395                 400
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
                405                 410                 415
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
            420                 425                 430
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
        435                 440                 445
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Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    450                 455
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Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS
First Named Inventor/Applicant Name:	George D. YANCOPOULOS
Filer:	Karl Bozicevic
Attorney Docket Number:	REGN-008CIPCON3

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
UTILITY APPLICATION FILING	1011	1	300	300
UTILITY SEARCH FEE	1111	1	660	660
UTILITY EXAMINATION FEE	1311	1	760	760
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1720**

Electronic Acknowledgement Receipt

EFS ID:	33381499
Application Number:	16055847
International Application Number:	
Confirmation Number:	3451
Title of Invention:	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS
First Named Inventor/Applicant Name:	George D. YANCOPOULOS
Customer Number:	96387
Filer:	Karl Bozicevic
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Attorney Docket Number:	REGN-008CIPCON3
Receipt Date:	06-AUG-2018
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RAM confirmation Number	080718INTEFSW16264200
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		REGN-008CIPCON3_2018-08-06_Appln_as_fld.pdf	153933 6121ec056faa53a9a0bc99105653645a20021d96	yes	24
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Specification		1	21	
	Claims		22	23	
	Abstract		24	24	
Warnings:					
Information:					
2	Drawings-only black and white line drawings	REGN-008CIPCON3_Figure.pdf	105393 2d582f645d0c5d17d717e589b029a39331991bdb	no	1
Warnings:					
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3	Oath or Declaration filed	REGN-008CIPCON3_declaration.pdf	173097 6bda7272374e6af80c8c3d8cf30d012e4657b588	no	2
Warnings:					
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4	Application Data Sheet	WebADS.pdf	134486 9e50bc4532e6ef832deb156f7f785c1f632bfb97	no	8
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Information:					

5		REGN-008CIPCON3_2018-08-06_pre_amend_asfld.pdf	49231 607e9ab4330cfdd274b05f3c2d1cec6eb54503f4	yes	5

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Preliminary Amendment	1	1
	Specification	2	2
	Claims	3	3
	Applicant Arguments/Remarks Made in an Amendment	4	5

Warnings:

Information:

6	Transmittal Letter	REGN-008CIPCON3_2018-08-06_IDS_trans.pdf	49233 fedcc17b94c47a3263f07960b33082af55e068af	no	2

Warnings:

Information:

7	Information Disclosure Statement (IDS) Form (SB08)	REGN-008CIPCON3_2018-08-06_IDS_SB08A.pdf	48234 e3d76034ef5c2ff318267b6338671cd3818062ab	no	3

Warnings:

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8	CRF Statement Paper and CRF are the same	REGN-008CIPCON3_2018-08-06_seq_list_trans.pdf	27044 e860080ee4b9f783b69836fa5b45657b2ed0fd8b	no	1

Warnings:

Information:

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Warnings:

Information:

10	Sequence Listing (Text File)	REGN-008CIPCON3_SeqList.txt	6076 	no	-
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Warnings:					
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			Total Files Size (in bytes):	798154	

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National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of International Patent Application No. PCT/US2012/020855, filed on January 11, 2012, which claims the benefit of US Provisional Application Nos. 61/432,245, filed on January 13, 2011, 61/434,836, filed on January 21, 2011, and 61/561,957, filed on November 21, 2011, the contents of which are hereby incorporated by reference in their entireties.

FIELD OF THE INVENTION

[0002] The present invention relates to the field of therapeutic treatments of eye disorders. More specifically, the invention relates to the administration of VEGF antagonists to treat eye disorders caused by or associated with angiogenesis.

BACKGROUND

[0003] Several eye disorders are associated with pathological angiogenesis. For example, the development of age-related macular degeneration (AMD) is associated with a process called choroidal neovascularization (CNV). Leakage from the CNV causes macular edema and collection of fluid beneath the macula resulting in vision loss. Diabetic macular edema (DME) is another eye disorder with an angiogenic component. DME is the most prevalent cause of moderate vision loss in patients with diabetes and is a common complication of diabetic retinopathy, a disease affecting the blood vessels of the retina. Clinically significant DME occurs when fluid leaks into the center of the macula, the light-sensitive part of the retina responsible for sharp, direct vision. Fluid in the macula can cause severe vision loss or blindness. Yet another eye disorder associated with abnormal angiogenesis is central retinal vein occlusion (CRVO). CRVO is caused by obstruction of the central retinal vein that leads to a back-up of blood and fluid in the retina. The retina can also become ischemic, resulting in the growth of new, inappropriate blood vessels that can cause further vision loss and more serious complications. Release of vascular endothelial growth factor (VEGF) contributes to increased vascular permeability in the eye and inappropriate new vessel growth. Thus, inhibiting the angiogenic-promoting properties of VEGF appears to be an effective strategy for treating angiogenic eye disorders.

[0004] FDA-approved treatments of angiogenic eye disorders such as AMD and CRVO include the administration of an anti-VEGF antibody called ranibizumab (Lucentis®, Genentech, Inc.) on a monthly basis by intravitreal injection.

[0005] Methods for treating eye disorders using VEGF antagonists are mentioned in, *e.g.*, US 7,303,746; US 7,306,799; US 7,300,563; US 7,303,748; and US 2007/0190058. Nonetheless,

there remains a need in the art for new administration regimens for angiogenic eye disorders, especially those which allow for less frequent dosing while maintaining a high level of efficacy.

BRIEF SUMMARY OF THE INVENTION

[0006] The present invention provides methods for treating angiogenic eye disorders. The methods of the invention comprise sequentially administering multiple doses of a VEGF antagonist to a patient over time. In particular, the methods of the invention comprise sequentially administering to the patient a single initial dose of a VEGF antagonist, followed by one or more secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonists. The present inventors have surprisingly discovered that beneficial therapeutic effects can be achieved in patients suffering from angiogenic eye disorders by administering a VEGF antagonist to a patient at a frequency of once every 8 or more weeks, especially when such doses are preceded by about three doses administered to the patient at a frequency of about 2 to 4 weeks. Thus, according to the methods of the present invention, each secondary dose of VEGF antagonist is administered 2 to 4 weeks after the immediately preceding dose, and each tertiary dose is administered at least 8 weeks after the immediately preceding dose. An example of a dosing regimen of the present invention is shown in Figure 1. One advantage of such a dosing regimen is that, for most of the course of treatment (*i.e.*, the tertiary doses), it allows for less frequent dosing (*e.g.*, once every 8 weeks) compared to prior administration regimens for angiogenic eye disorders which require monthly administrations throughout the entire course of treatment. (*See*, *e.g.*, prescribing information for Lucentis® [ranibizumab], Genentech, Inc.).

[0007] The methods of the present invention can be used to treat any angiogenic eye disorder, including, *e.g.*, age related macular degeneration, diabetic retinopathy, diabetic macular edema, central retinal vein occlusion, corneal neovascularization, etc.

[0008] The methods of the present invention comprise administering any VEGF antagonist to the patient. In one embodiment, the VEGF antagonist comprises one or more VEGF receptor-based chimeric molecule(s), (also referred to herein as a "VEGF-Trap" or "VEGFT"). An exemplary VEGF antagonist that can be used in the context of the present invention is a multimeric VEGF-binding protein comprising two or more VEGF receptor-based chimeric molecules referred to herein as "VEGFR1R2-FcΔC1(a)" or "aflibercept."

[0009] Various administration routes are contemplated for use in the methods of the present invention, including, *e.g.*, topical administration or intraocular administration (*e.g.*, intravitreal administration).

[0010] Aflibercept (EYLEA™, Regeneron Pharmaceuticals, Inc) was approved by the FDA in November 2011, for the treatment of patients with neovascular (wet) age-related macular degeneration, with a recommended dose of 2 mg administered by intravitreal injection every 4

weeks for the first three months, followed by 2 mg administered by intravitreal injection once every 8 weeks.

[0011] Other embodiments of the present invention will become apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE FIGURE

[0012] Figure 1 shows an exemplary dosing regimen of the present invention. In this regimen, a single "initial dose" of VEGF antagonist ("VEGFT") is administered at the beginning of the treatment regimen (*i.e.* at "week 0"), two "secondary doses" are administered at weeks 4 and 8, respectively, and at least six "tertiary doses" are administered once every 8 weeks thereafter, *i.e.*, at weeks 16, 24, 32, 40, 48, 56, etc.).

DETAILED DESCRIPTION

[0013] Before the present invention is described, it is to be understood that this invention is not limited to particular methods and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0014] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. As used herein, the term "about," when used in reference to a particular recited numerical value, means that the value may vary from the recited value by no more than 1%. For example, as used herein, the expression "about 100" includes 99 and 101 and all values in between (*e.g.*, 99.1, 99.2, 99.3, 99.4, etc.).

[0015] Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

DOSING REGIMENS

[0016] The present invention provides methods for treating angiogenic eye disorders. The methods of the invention comprise sequentially administering to a patient multiple doses of a VEGF antagonist. As used herein, "sequentially administering" means that each dose of VEGF antagonist is administered to the patient at a different point in time, *e.g.*, on different days separated by a predetermined interval (*e.g.*, hours, days, weeks or months). The present invention includes methods which comprise sequentially administering to the patient a single initial dose of a VEGF

antagonist, followed by one or more secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonist.

[0017] The terms "initial dose," "secondary doses," and "tertiary doses," refer to the temporal sequence of administration of the VEGF antagonist. Thus, the "initial dose" is the dose which is administered at the beginning of the treatment regimen (also referred to as the "baseline dose"); the "secondary doses" are the doses which are administered after the initial dose; and the "tertiary doses" are the doses which are administered after the secondary doses. The initial, secondary, and tertiary doses may all contain the same amount of VEGF antagonist, but will generally differ from one another in terms of frequency of administration. In certain embodiments, however, the amount of VEGF antagonist contained in the initial, secondary and/or tertiary doses will vary from one another (*e.g.*, adjusted up or down as appropriate) during the course of treatment.

[0018] In one exemplary embodiment of the present invention, each secondary dose is administered 2 to 4 (*e.g.*, 2, 2½, 3, 3½, or 4) weeks after the immediately preceding dose, and each tertiary dose is administered at least 8 (*e.g.*, 8, 8½, 9, 9½, 10, 10½, 11, 11½, 12, 12½, 13, 13½, 14, 14½, or more) weeks after the immediately preceding dose. The phrase "the immediately preceding dose," as used herein, means, in a sequence of multiple administrations, the dose of VEGF antagonist which is administered to a patient prior to the administration of the very next dose in the sequence with no intervening doses.

[0019] In one exemplary embodiment of the present invention, a single initial dose of a VEGF antagonist is administered to a patient on the first day of the treatment regimen (*i.e.*, at week 0), followed by two secondary doses, each administered four weeks after the immediately preceding dose (*i.e.*, at week 4 and at week 8), followed by at least 5 tertiary doses, each administered eight weeks after the immediately preceding dose (*i.e.*, at weeks 16, 24, 32, 40 and 48). The tertiary doses may continue (at intervals of 8 or more weeks) indefinitely during the course of the treatment regimen. This exemplary administration regimen is depicted graphically in Figure 1.

[0020] The methods of the invention may comprise administering to a patient any number of secondary and/or tertiary doses of a VEGF antagonist. For example, in certain embodiments, only a single secondary dose is administered to the patient. In other embodiments, two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, or more) secondary doses are administered to the patient. Likewise, in certain embodiments, only a single tertiary dose is administered to the patient. In other embodiments, two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, or more) tertiary doses are administered to the patient.

[0021] In embodiments involving multiple secondary doses, each secondary dose may be administered at the same frequency as the other secondary doses. For example, each secondary dose may be administered to the patient 4 weeks after the immediately preceding dose. Similarly, in embodiments involving multiple tertiary doses, each tertiary dose may be administered at the same frequency as the other tertiary doses. For example, each tertiary dose may be administered

to the patient 8 weeks after the immediately preceding dose. Alternatively, the frequency at which the secondary and/or tertiary doses are administered to a patient can vary over the course of the treatment regimen. For example, the present invention includes methods which comprise administering to the patient a single initial dose of a VEGF antagonist, followed by one or more secondary doses of the VEGF antagonist, followed by at least 5 tertiary doses of the VEGF antagonist, wherein the first four tertiary doses are administered 8 weeks after the immediately preceding dose, and wherein each subsequent tertiary dose is administered from 8 to 12 (*e.g.*, 8, 8½, 9, 9½, 10, 10½, 11, 11½, 12) weeks after the immediately preceding dose. The frequency of administration may also be adjusted during the course of treatment by a physician depending on the needs of the individual patient following clinical examination.

VEGF ANTAGONISTS

[0022] The methods of the present invention comprise administering to a patient a VEGF antagonist according to specified dosing regimens. As used herein, the expression "VEGF antagonist" means any molecule that blocks, reduces or interferes with the normal biological activity of VEGF.

[0023] VEGF antagonists include molecules which interfere with the interaction between VEGF and a natural VEGF receptor, *e.g.*, molecules which bind to VEGF or a VEGF receptor and prevent or otherwise hinder the interaction between VEGF and a VEGF receptor. Specific exemplary VEGF antagonists include anti-VEGF antibodies, anti-VEGF receptor antibodies, and VEGF receptor-based chimeric molecules (also referred to herein as "VEGF-Traps").

[0024] VEGF receptor-based chimeric molecules include chimeric polypeptides which comprise two or more immunoglobulin (Ig)-like domains of a VEGF receptor such as VEGFR1 (also referred to as Flt1) and/or VEGFR2 (also referred to as Flk1 or KDR), and may also contain a multimerizing domain (*e.g.*, an Fc domain which facilitates the multimerization [*e.g.*, dimerization] of two or more chimeric polypeptides). An exemplary VEGF receptor-based chimeric molecule is a molecule referred to as VEGFR1R2-FcΔC1(a) which is encoded by the nucleic acid sequence of SEQ ID NO:1. VEGFR1R2-FcΔC1(a) comprises three components: (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130 to 231 of SEQ ID NO:2; and (3) a multimerization component ("FcΔC1(a)") comprising amino acids 232 to 457 of SEQ ID NO:2 (the C-terminal amino acid of SEQ ID NO:2 [*i.e.*, K458] may or may not be included in the VEGF antagonist used in the methods of the invention; *see e.g.*, US Patent 7,396,664). Amino acids 1-26 of SEQ ID NO:2 are the signal sequence.

[0025] The VEGF antagonist used in the Examples set forth herein below is a dimeric molecule comprising two VEGFR1R2-FcΔC1(a) molecules and is referred to herein as "VEGFT." Additional

VEGF receptor-based chimeric molecules which can be used in the context of the present invention are disclosed in US 7,396,664, 7,303,746 and WO 00/75319.

ANGIOGENIC EYE DISORDERS

[0026] The methods of the present invention can be used to treat any angiogenic eye disorder. The expression "angiogenic eye disorder," as used herein, means any disease of the eye which is caused by or associated with the growth or proliferation of blood vessels or by blood vessel leakage. Non-limiting examples of angiogenic eye disorders that are treatable using the methods of the present invention include age-related macular degeneration (*e.g.*, wet AMD, exudative AMD, etc.), retinal vein occlusion (RVO), central retinal vein occlusion (CRVO; *e.g.*, macular edema following CRVO), branch retinal vein occlusion (BRVO), diabetic macular edema (DME), choroidal neovascularization (CNV; *e.g.*, myopic CNV), iris neovascularization, neovascular glaucoma, post-surgical fibrosis in glaucoma, proliferative vitreoretinopathy (PVR), optic disc neovascularization, corneal neovascularization, retinal neovascularization, vitreal neovascularization, pannus, pterygium, vascular retinopathy, and diabetic retinopathies.

PHARMACEUTICAL FORMULATIONS

[0027] The present invention includes methods in which the VEGF antagonist that is administered to the patient is contained within a pharmaceutical formulation. The pharmaceutical formulation may comprise the VEGF antagonist along with at least one inactive ingredient such as, *e.g.*, a pharmaceutically acceptable carrier. Other agents may be incorporated into the pharmaceutical composition to provide improved transfer, delivery, tolerance, and the like. The term “pharmaceutically acceptable” means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly, in humans. The term “carrier” refers to a diluent, adjuvant, excipient, or vehicle with which the antibody is administered. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences (15th ed, Mack Publishing Company, Easton, Pa., 1975), particularly Chapter 87 by Blaug, Seymour, therein. These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as LIPOFECTIN™), DNA conjugates, anhydrous absorption pastes, oil-in-water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. Any of the foregoing mixtures may be appropriate in the context of the methods of the present invention, provided that the VEGF antagonist is not inactivated by the formulation and the formulation is physiologically compatible and tolerable with the route of administration. See also Powell et al. PDA (1998) J Pharm Sci

Technol. 52:238-311 and the citations therein for additional information related to excipients and carriers well known to pharmaceutical chemists.

[0028] Pharmaceutical formulations useful for administration by injection in the context of the present invention may be prepared by dissolving, suspending or emulsifying a VEGF antagonist in a sterile aqueous medium or an oily medium conventionally used for injections. As the aqueous medium for injections, there are, for example, physiological saline, an isotonic solution containing glucose and other auxiliary agents, etc., which may be used in combination with an appropriate solubilizing agent such as an alcohol (e.g., ethanol), a polyalcohol (e.g., propylene glycol, polyethylene glycol), a nonionic surfactant [e.g., polysorbate 80, HCO-50 (polyoxyethylene (50 mol) adduct of hydrogenated castor oil)], etc. As the oily medium, there may be employed, e.g., sesame oil, soybean oil, etc., which may be used in combination with a solubilizing agent such as benzyl benzoate, benzyl alcohol, etc. The injection thus prepared can be filled in an appropriate ampoule if desired.

MODES OF ADMINISTRATION

[0029] The VEGF antagonist (or pharmaceutical formulation comprising the VEGF antagonist) may be administered to the patient by any known delivery system and/or administration method. In certain embodiments, the VEGF antagonist is administered to the patient by ocular, intraocular, intravitreal or subconjunctival injection. In other embodiments, the VEGF antagonist can be administered to the patient by topical administration, *e.g.*, via eye drops or other liquid, gel, ointment or fluid which contains the VEGF antagonist and can be applied directly to the eye. Other possible routes of administration include, *e.g.*, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral.

AMOUNT OF VEGF ANTAGONIST ADMINISTERED

[0030] Each dose of VEGF antagonist administered to the patient over the course of the treatment regimen may contain the same, or substantially the same, amount of VEGF antagonist. Alternatively, the quantity of VEGF antagonist contained within the individual doses may vary over the course of the treatment regimen. For example, in certain embodiments, a first quantity of VEGF antagonist is administered in the initial dose, a second quantity of VEGF antagonist is administered in the secondary doses, and a third quantity of VEGF antagonist is administered in the tertiary doses. The present invention contemplates dosing schemes in which the quantity of VEGF antagonist contained within the individual doses increases over time (*e.g.*, each subsequent dose contains more VEGF antagonist than the last), decreases over time (*e.g.*, each subsequent dose contains less VEGF antagonist than the last), initially increases then decreases, initially decreases then increases, or remains the same throughout the course of the administration regimen.

[0031] The amount of VEGF antagonist administered to the patient in each dose is, in most cases, a therapeutically effective amount. As used herein, the phrase "therapeutically effective amount" means a dose of VEGF antagonist that results in a detectable improvement in one or more symptoms or indicia of an angiogenic eye disorder, or a dose of VEGF antagonist that inhibits, prevents, lessens, or delays the progression of an angiogenic eye disorder. In the case of an anti-VEGF antibody or a VEGF receptor-based chimeric molecule such as VEGFR1R2-FcΔC1(a), a therapeutically effective amount can be from about 0.05 mg to about 5 mg, *e.g.*, about 0.05 mg, about 0.1 mg, about 0.15 mg, about 0.2 mg, about 0.25 mg, about 0.3 mg, about 0.35 mg, about 0.4 mg, about 0.45 mg, about 0.5 mg, about 0.55 mg, about 0.6 mg, about 0.65 mg, about 0.7 mg, about 0.75 mg, about 0.8 mg, about 0.85 mg, about 0.9 mg, about 1.0 mg, about 1.05 mg, about 1.1 mg, about 1.15 mg, about 1.2 mg, about 1.25 mg, about 1.3 mg, about 1.35 mg, about 1.4 mg, about 1.45 mg, about 1.5 mg, about 1.55 mg, about 1.6 mg, about 1.65 mg, about 1.7 mg, about 1.75 mg, about 1.8 mg, about 1.85 mg, about 1.9 mg, about 2.0 mg, about 2.05 mg, about 2.1 mg, about 2.15 mg, about 2.2 mg, about 2.25 mg, about 2.3 mg, about 2.35 mg, about 2.4 mg, about 2.45 mg, about 2.5 mg, about 2.55 mg, about 2.6 mg, about 2.65 mg, about 2.7 mg, about 2.75 mg, about 2.8 mg, about 2.85 mg, about 2.9 mg, about 3.0 mg, about 3.5 mg, about 4.0 mg, about 4.5 mg, or about 5.0 mg of the antibody or receptor-based chimeric molecule.

[0032] The amount of VEGF antagonist contained within the individual doses may be expressed in terms of milligrams of antibody per kilogram of patient body weight (*i.e.*, mg/kg). For example, the VEGF antagonist may be administered to a patient at a dose of about 0.0001 to about 10 mg/kg of patient body weight.

TREATMENT POPULATION AND EFFICACY

[0033] The methods of the present invention are useful for treating angiogenic eye disorders in patients that have been diagnosed with or are at risk of being afflicted with an angiogenic eye disorder. Generally, the methods of the present invention demonstrate efficacy within 104 weeks of the initiation of the treatment regimen (with the initial dose administered at "week 0"), *e.g.*, by the end of week 16, by the end of week 24, by the end of week 32, by the end of week 40, by the end of week 48, by the end of week 56, etc. In the context of methods for treating angiogenic eye disorders such as AMD, CRVO, and DME, "efficacy" means that, from the initiation of treatment, the patient exhibits a loss of 15 or fewer letters on the Early Treatment Diabetic Retinopathy Study (ETDRS) visual acuity chart. In certain embodiments, "efficacy" means a gain of one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or more) letters on the ETDRS chart from the time of initiation of treatment.

EXAMPLES

[0034] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[0035] The exemplary VEGF antagonist used in all Examples set forth below is a dimeric molecule having two functional VEGF binding units. Each functional binding unit is comprised of Ig domain 2 from VEGFR1 fused to Ig domain 3 from VEGFR2, which in turn is fused to the hinge region of a human IgG1 Fc domain (VEGFR1R2-FcΔC1(a); encoded by SEQ ID NO:1). This VEGF antagonist is referred to in the examples below as "VEGFT". For purposes of the following Examples, "monthly" dosing is equivalent to dosing once every four weeks.

Example 1: Phase I Clinical Trial of Intravitreally Administered VEGF Receptor-Based Chimeric Molecule (VEGFT) in Subjects with Neovascular AMD

[0036] In this Phase I study, 21 subjects with neovascular AMD received a single intravitreal (IVT) dose of VEGFT. Five groups of three subjects each received either 0.05, 0.15, 0.5, 2 or 4 mg of VEGFT, and a sixth group of six subjects received 1 mg. No serious adverse events related to the study drug, and no identifiable intraocular inflammation was reported. Preliminary results showed that, following injection of VEGFT, a rapid decrease in foveal thickness and macular volume was observed that was maintained through 6 weeks. At Day 43 across all dose groups, mean excess retinal thickness [excess retinal thickness = (retinal thickness – 179μ)] on optical coherence tomography (OCT) was reduced from 119μ to 27μ as assessed by Fast Macular Scan and from 194μ to 60μ as assessed using a single Posterior Pole scan. The mean increase in best corrected visual acuity (BCVA) was 4.75 letters, and BCVA was stable or improved in 95% of subjects. In the 2 highest dose groups (2 and 4 mg), the mean increase in BCVA was 13.5 letters, with 3 of 6 subjects demonstrating improvement of ≥ 3 lines.

Example 2: Phase II Clinical Trial of Repeated Doses of Intravitreally Administered VEGF Receptor-Based Chimeric Molecule (VEGFT) in Subjects with Neovascular AMD

[0037] This study was a double-masked, randomized study of 3 doses (0.5, 2, and 4 mg) of VEGFT tested at 4-week and/or 12-week dosing intervals. There were 5 treatment arms in this study, as follows: 1) 0.5 mg every 4 weeks, 2) 0.5 mg every 12 weeks, 3) 2 mg every 4 weeks, 4) 2 mg every 12 weeks and 5) 4 mg every 12 weeks. Subjects were dosed at a fixed interval for the

first 12 weeks, after which they were evaluated every 4 weeks for 9 months, during which additional doses were administered based on pre-specified criteria. All subjects were then followed for one year after their last dose of VEGFT. Preliminary data from a pre-planned interim analysis indicated that VEGFT met its primary endpoint of a statistically significant reduction in retinal thickness after 12 weeks compared with baseline (all groups combined, decrease of 135μ, $p < 0.0001$). Mean change from baseline in visual acuity, a key secondary endpoint of the study, also demonstrated statistically significant improvement (all groups combined, increase of 5.9 letters, $p < 0.0001$). Moreover, patients in the dose groups that received only a single dose, on average, demonstrated a decrease in excess retinal thickness ($p < 0.0001$) and an increase in visual acuity ($p = 0.012$) at 12 weeks. There were no drug-related serious adverse events, and treatment with the VEGF antagonists was generally well-tolerated. The most common adverse events were those typically associated with intravitreal injections.

Example 3: Phase I Clinical Trial of Systemically Administered VEGF Receptor-Based Chimeric Molecule (VEGFT) in Subjects with Neovascular AMD

[0038] This study was a placebo-controlled, sequential-group, dose-escalating safety, tolerability and bioeffect study of VEGFT by IV infusion in subjects with neovascular AMD. Groups of 8 subjects meeting eligibility criteria for subfoveal choroidal neovascularization (CNV) related to AMD were assigned to receive 4 IV injections of VEGFT or placebo at dose levels of 0.3, 1, or 3 mg/kg over an 8-week period.

[0039] Most adverse events that were attributed to VEGFT were mild to moderate in severity, but 2 of 5 subjects treated with 3 mg/kg experienced dose-limiting toxicity (DLT) (one with Grade 4 hypertension and one with Grade 2 proteinuria); therefore, all subjects in the 3 mg/kg dose group did not enter the study. The mean percent changes in excess retinal thickness were: -12%, -10%, -66%, and -60% for the placebo, 0.3, 1, and 3 mg/kg dose groups at day 15 (ANOVA $p< 0.02$), and -5.6%, +47.1%, and -63.3% for the placebo, 0.3, and 1 mg/kg dose groups at day 71 (ANOVA $p< 0.02$). There was a numerical improvement in BCVA in the subjects treated with VEGFT. As would be expected in such a small study, the results were not statistically significant.

Example 4: Phase III Clinical Trials of the Efficacy, Safety, and Tolerability of Repeated Doses of Intravitreal VEGFT in Subjects with Neovascular Age-Related Macular Degeneration

A. Objectives, Hypotheses and Endpoints

[0040] Two parallel Phase III clinical trials were carried out to investigate the use of VEGFT to treat patients with the neovascular form of age-related macular degeneration (Study 1 and Study 2). The primary objective of these studies was to assess the efficacy of IVT administered VEGFT

compared to ranibizumab (Lucentis®, Genentech, Inc.), in a non-inferiority paradigm, in preventing moderate vision loss in subjects with all subtypes of neovascular AMD.

[0041] The secondary objectives were (a) to assess the safety and tolerability of repeated IVT administration of VEGFT in subjects with all sub-types of neovascular AMD for periods up to 2 years; and (b) to assess the effect of repeated IVT administration of VEGFT on Vision-Related Quality of Life (QOL) in subjects with all sub-types of neovascular AMD.

[0042] The primary hypothesis of these studies was that the proportion of subjects treated with VEGFT with stable or improved BCVA (<15 letters lost) is similar to the proportion treated with ranibizumab who have stable or improved BCVA, thereby demonstrating non-inferiority.

[0043] The primary endpoint for these studies was the prevention of vision loss of greater than or equal to 15 letters on the ETDRS chart, compared to baseline, at 52 weeks. Secondary endpoints were as follows: (a) change from baseline to Week 52 in letter score on the ETDRS chart; (b) gain from baseline to Week 52 of 15 letters or more on the ETDRS chart; (c) change from baseline to Week 52 in total NEI VFQ-25 score; and (d) change from baseline to Week 52 in CNV area.

B. Study Design

[0044] For each study, subjects were randomly assigned in a 1:1:1:1 ratio to 1 of 4 dosing regimens: (1) 2 mg VEGFT administered every 4 weeks (2Q4); (2) 0.5 mg VEGFT administered every 4 weeks (0.5Q4); (3) 2 mg VEGFT administered every 4 weeks to week 8 and then every 8 weeks (with sham injection at the interim 4-week visits when study drug was not administered (2Q8); and (4) 0.5 mg ranibizumab administered every 4 weeks (RQ4). Subjects assigned to (2Q8) received the 2 mg injection every 4 weeks to week 8 and then a sham injection at interim 4-week visits (when study drug is not to be administered) during the first 52 weeks of the studies. (No sham injection were given at Week 52).

[0045] The study duration for each subject was scheduled to be 96 weeks plus the recruitment period. For the first 52 weeks (Year 1), subjects received an IVT or sham injection in the study eye every 4 weeks. (No sham injections were given at Week 52). During the second year of the study, subjects will be evaluated every 4 weeks and will receive IVT injection of study drug at intervals determined by specific dosing criteria, but at least every 12 weeks. (During the second year of the study, sham injections will not be given.) During this period, injections may be given as frequently as every 4 weeks, but no less frequently than every 12 weeks, according to the following criteria: (i) increase in central retinal thickness of ≥100 μm compared to the lowest previous value as measured by optical coherence tomography (OCT); or (ii) a loss from the best previous letter score of at least 5 ETDRS letters in conjunction with recurrent fluid as indicated by OCT; or (iii) new or persistent fluid as indicated by OCT; or (iv) new onset classic neovascularization, or new or persistent leak on fluorescein angiography (FA); or (v) new macular hemorrhage; or (vi) 12 weeks

have elapsed since the previous injection. According to the present protocol, subjects must receive an injection at least every 12 weeks.

[0046] Subjects were evaluated at 4 weeks intervals for safety and best corrected visual acuity (BCVA) using the 4 meter ETDRS protocol. Quality of Life (QOL) was evaluated using the NEI VFQ-25 questionnaire. OCT and FA examinations were conducted periodically.

[0047] Approximately 1200 subjects were enrolled, with a target enrollment of 300 subjects per treatment arm.

[0048] To be eligible for this study, subjects were required to have subfoveal choroidal neovascularization (CNV) secondary to AMD. "Subfoveal" CNV was defined as the presence of subfoveal neovascularization, documented by FA, or presence of a lesion that is juxtafoveal in location angiographically but affects the fovea. Subject eligibility was confirmed based on angiographic criteria prior to randomization.

[0049] Only one eye was designated as the study eye. For subjects who met eligibility criteria in both eyes, the eye with the worse VA was selected as the study eye. If both eyes had equal VA, the eye with the clearest lens and ocular media and least amount of subfoveal scar or geographic atrophy was selected. If there was no objective basis for selecting the study eye, factors such as ocular dominance, other ocular pathology and subject preference were considered in making the selection.

[0050] Inclusion criteria for both studies were as follows: (i) signed Informed consent; (ii) at least 50 years of age; (iii) active primary subfoveal CNV lesions secondary to AMD, including juxtafoveal lesions that affect the fovea as evidenced by FA in the study eye; (iv) CNV at least 50% of total lesion size; (v) early treatment diabetic retinopathy study (ETDRS) best-corrected visual acuity of: 20/40 to 20/320 (letter score of 73 to 25) in the study eye; (vi) willing, committed, and able to return for all clinic visits and complete all study-related procedures; and (vii) able to read, understand and willing to sign the informed consent form (or, if unable to read due to visual impairment, be read to verbatim by the person administering the informed consent or a family member).

[0051] Exclusion criteria for both studies were as follows: 1. Any prior ocular (in the study eye) or systemic treatment or surgery for neovascular AMD except dietary supplements or vitamins. 2. Any prior or concomitant therapy with another investigational agent to treat neovascular AMD in the study eye, except dietary supplements or vitamins. 3. Prior treatment with anti-VEGF agents as follows: (a) Prior treatment with anti-VEGF therapy in the study eye was not allowed; (b) Prior treatment with anti-VEGF therapy in the fellow eye with an investigational agent (not FDA approved, e.g. bevacizumab) was allowed up to 3 months prior to first dose in the study, and such treatments were not allowed during the study. Prior treatment with an approved anti-VEGF therapy in the fellow eye was allowed; (c) Prior systemic anti-VEGF therapy, investigational or FDA/Health Canada approved, was only allowed up to 3 months prior to first dose, and was not allowed during

the study. 4. Total lesion size > 12 disc areas (30.5 mm2, including blood, scars and neovascularization) as assessed by FA in the study eye. 5. Subretinal hemorrhage that is either 50% or more of the total lesion area, or if the blood is under the fovea and is 1 or more disc areas in size in the study eye. (If the blood is under the fovea, then the fovea must be surrounded 270 degrees by visible CNV.) 6. Scar or fibrosis, making up > 50% of total lesion in the study eye. 7. Scar, fibrosis, or atrophy involving the center of the fovea. 8. Presence of retinal pigment epithelial tears or rips involving the macula in the study eye. 9. History of any vitreous hemorrhage within 4 weeks prior to Visit 1 in the study eye. 10. Presence of other causes of CNV, including pathologic myopia (spherical equivalent of –8 diopters or more negative, or axial length of 25 mm or more), ocular histoplasmosis syndrome, angioid streaks, choroidal rupture, or multifocal choroiditis in the study eye. 11. History or clinical evidence of diabetic retinopathy, diabetic macular edema or any other vascular disease affecting the retina, other than AMD, in either eye. 12. Prior vitrectomy in the study eye. 13. History of retinal detachment or treatment or surgery for retinal detachment in the study eye. 14. Any history of macular hole of stage 2 and above in the study eye. 15. Any intraocular or periocular surgery within 3 months of Day 1 on the study eye, except lid surgery, which may not have taken place within 1 month of day 1, as long as it was unlikely to interfere with the injection. 16. Prior trabeculectomy or other filtration surgery in the study eye. 17. Uncontrolled glaucoma (defined as intraocular pressure greater than or equal to 25 mm Hg despite treatment with anti-glaucoma medication) in the study eye. 18. Active intraocular inflammation in either eye. 19. Active ocular or periocular infection in either eye. 20. Any ocular or periocular infection within the last 2 weeks prior to Screening in either eye. 21. Any history of uveitis in either eye. 22. Active scleritis or episcleritis in either eye. 23. Presence or history of scleromalacia in either eye. 24. Aphakia or pseudophakia with absence of posterior capsule (unless it occurred as a result of a yttrium aluminum garnet [YAG] posterior capsulotomy) in the study eye. 25. Previous therapeutic radiation in the region of the study eye. 26. History of corneal transplant or corneal dystrophy in the study eye. 27. Significant media opacities, including cataract, in the study eye which might interfere with visual acuity, assessment of safety, or fundus photography. 28. Any concurrent intraocular condition in the study eye (e.g. cataract) that, in the opinion of the investigator, could require either medical or surgical intervention during the 96 week study period. 29. Any concurrent ocular condition in the study eye which, in the opinion of the investigator, could either increase the risk to the subject beyond what is to be expected from standard procedures of intraocular injection, or which otherwise may interfere with the injection procedure or with evaluation of efficacy or safety. 30. History of other disease, metabolic dysfunction, physical examination finding, or clinical laboratory finding giving reasonable suspicion of a disease or condition that contraindicates the use of an investigational drug or that might affect interpretation of the results of the study or render the subject at high risk for treatment complications. 31. Participation as a subject in any clinical study

within the 12 weeks prior to Day 1. 32. Any systemic or ocular treatment with an investigational agent in the past 3 months prior to Day 1. 33. The use of long acting steroids, either systemically or intraocularly, in the 6 months prior to day 1. 34. Any history of allergy to povidone iodine. 35. Known serious allergy to the fluorescein sodium for injection in angiography. 36. Presence of any contraindications indicated in the FDA Approved label for ranibizumab (Lucentis®). 37. Females who were pregnant, breastfeeding, or of childbearing potential, unwilling to practice adequate contraception throughout the study. Adequate contraceptive measures include oral contraceptives (stable use for 2 or more cycles prior to screening); IUD; Depo-Provera®; Norplant® System implants; bilateral tubal ligation; vasectomy; condom or diaphragm plus either contraceptive sponge, foam or jelly.

[0052] Subjects were not allowed to receive any standard or investigational agents for treatment of their AMD in the study eye other than their assigned study treatment with VEGFT or ranibizumab as specified in the protocol until they completed the Completion/Early Termination visit assessments. This includes medications administered locally (e.g., IVT, topical, juxtascleral or periorbital routes), as well as those administered systemically with the intent of treating the study and/or fellow eye.

[0053] The study procedures are summarized as follows:

[0054] Best Corrected Visual Acuity: Visual function of the study eye and the fellow eye were assessed using the ETDRS protocol (The Early Treatment Diabetic Retinopathy Study Group) at 4 meters. Visual Acuity examiners were certified to ensure consistent measurement of BCVA. The VA examiners were required to remain masked to treatment assignment.

[0055] Optical Coherence Tomography: Retinal and lesion characteristics were evaluated using OCT on the study eye. At the Screen Visit (Visit 1) images were captured and transmitted for both eyes. All OCT images were captured using the Zeiss Stratus OCT™ with software Version 3 or greater. OCT images were sent to an independent reading center where images were read by masked readers at visits where OCTs were required. All OCTs were electronically archived at the site as part of the source documentation. A subset of OCT images were read. OCT technicians were required to be certified by the reading center to ensure consistency and quality in image acquisition. Adequate efforts were made to ensure that OCT technicians at the site remained masked to treatment assignment.

[0056] Fundus Photography and Fluorescein Angiography (FA): The anatomical state of the retinal vasculature of the study eye was evaluated by funduscopic examination, fundus photography and FA. At the Screen Visit (Visit 1) funduscopic examination, fundus photography and FA were captured and transmitted for both eyes. Fundus and angiographic images were sent to an independent reading center where images were read by masked readers. The reading center confirmed subject eligibility based on angiographic criteria prior to randomization. All FAs and

fundus photographs were archived at the site as part of the source documentation. Photographers were required to be certified by the reading center to ensure consistency and quality in image acquisition. Adequate efforts were made to ensure that all photographers at the site remain masked to treatment assignment.

[0057] Vision-Related Quality of Life: Vision-related QOL was assessed using the National Eye Institute 25-Item Visual Function Questionnaire (NEI VFQ-25) in the interviewer-administered format. NEI VFQ-25 was administered by certified personnel at a contracted call center. At the screening visit, the sites assisted the subject and initiated the first call to the call center to collect all of the subject's contact information and to complete the first NEI VFQ-25 on the phone prior to randomization and IVT injection. For all subsequent visits, the call center called the subject on the phone, prior to IVT injection, to complete the questionnaire.

[0058] Intraocular Pressure: Intraocular pressure (IOP) of the study eye was measured using applanation tonometry or Tonopen. The same method of IOP measurement was used in each subject throughout the study.

[0059]

C. Results Summary (52 Week Data)

[0060] The primary endpoint (prevention of moderate or severe vision loss as defined above) was met for all three VEGFT groups (2Q4, 0.5Q4 and 2Q8) in this study. The results from both studies are summarized in Table 1.

Table 1

	Ranibizumab 0.5 mg monthly (RQ4)	VEGFT 0.5 mg monthly (0.5Q4)	VEGFT 2 mg monthly (2Q4)	VEGFT 2 mg every 8 weeks[a] (2Q8)
Maintenance of vision* (% patients losing <15 letters) at week 52 versus baseline				
Study 1	94.4%	95.9%**	95.1%**	95.1%**
Study 2	94.4%	96.3%**	95.6%**	95.6%**
Mean improvement in vision* (letters) at 52 weeks versus baseline (p-value vs RQ4)***				
Study 1	8.1	6.9 (NS)	10.9 (p<0.01)	7.9 (NS)
Study 2	9.4	9.7 (NS)	7.6 (NS)	8.9 (NS

[a] Following three initial monthly doses
* Visual acuity was measured as the total number of letters read correctly on the Early Treatment Diabetic Retinopathy Study (ETDRS) eye chart.
** Statistically non-inferior based on a non-inferiority margin of 10%, using confidence interval approach (95.1% and 95% for Study 1 and Study 2, respectively)
*** Test for superiority
NS = non-significant

[0061] In Study 1, patients receiving VEGFT 2mg monthly (2Q4) achieved a statistically significant greater mean improvement in visual acuity at week 52 versus baseline (secondary endpoint), compared to ranibizumab 0.5mg monthly (RQ4); patients receiving VEGFT 2mg monthly on average gained 10.9 letters, compared to a mean 8.1 letter gain with ranibizumab 0.5mg dosed every month ($p<0.01$). All other dose groups of VEGFT in Study 1 and all dose groups in Study 2 were not statistically different from ranibizumab in this secondary endpoint.

[0062] A generally favorable safety profile was observed for both VEGFT and ranibizumab. The incidence of ocular treatment emergent adverse events was balanced across all four treatment groups in both studies, with the most frequent events associated with the injection procedure, the underlying disease, and/or the aging process. The most frequent ocular adverse events were conjunctival hemorrhage, macular degeneration, eye pain, retinal hemorrhage, and vitreous floaters. The most frequent serious non-ocular adverse events were typical of those reported in this elderly population who receive intravitreal treatment for wet AMD; the most frequently reported events were falls, pneumonia, myocardial infarction, atrial fibrillation, breast cancer, and acute coronary syndrome. There were no notable differences among the study arms.

Example 5: Phase II Clinical Trial of VEGFT in Subjects with Diabetic Macular Edema (DME)

[0063] In this study, 221 patients with clinically significant DME with central macular involvement were randomized, and 219 patients were treated with balanced distribution over five groups. The control group received macular laser therapy at baseline, and patients were eligible for repeat laser treatments, but no more frequently than at 16 week intervals. The remaining four groups received VEGFT by intravitreal injection as follows: Two groups received 0.5 or 2 mg of VEGFT once every four weeks throughout the 12-month dosing period (0.5Q4 and 2Q4, respectively). Two groups received three initial doses of 2 mg VEGFT once every four weeks (*i.e.*, at baseline, and weeks 4 and 8), followed through week 52 by either once every 8 weeks dosing (2Q8) or as needed dosing with very strict repeat dosing criteria (PRN). Mean gains in visual acuity versus baseline were as shown in Table 2:

Table 2

	n	Mean change in visual acuity at week 24 versus baseline (letters)	Mean change in visual acuity at week 52 versus baseline (letters)
Laser	44	2.5	-1.3
VEGFT 0.5 mg monthly (0.5Q4)	44	8.6**	11.0**
VEGFT 2 mg monthly (2Q4)	44	11.4**	13.1**
VEGFT 2 mg every 8	42	8.5**	9.7**

weeks[a] (2Q8)			
VEGFT 2 mg as needed[a] (PRN)	45	10.3**	12.0**

[a] Following three initial monthly doses
** $p < 0.01$ versus laser

[0064] In this study, the visual acuity gains achieved with VEGFT administration at week 24 were maintained or numerically improved up to completion of the study at week 52 in all VEGFT study groups, including 2 mg dosed every other month

[0065] As demonstrated in the foregoing Examples, the administration of VEGFT to patients suffering from angiogenic eye disorders (*e.g.*, AMD and DME) at a frequency of once every 8 weeks, following a single initial dose and two secondary doses administered four weeks apart, resulted in significant prevention of moderate or severe vision loss or improvements in visual acuity.

Example 6: A Randomized, Multicenter, Double-Masked Trial in Treatment Naïve Patients with Macular Edema Secondary to CRVO

[0066] In this randomized, double-masked, Phase 3 study, patients received 6 monthly injections of either 2 mg intravitreal VEGFT (114 patients) or sham injections (73 patients). From Week 24 to Week 52, all patients received 2 mg VEGFT as-needed (PRN) according to retreatment criteria. Thus, "sham-treated patients" means patients who received sham injections once every four weeks from Week 0 through Week 20, followed by intravitreal VEGFT as needed from Week 24 through Week 52. "VEGFT-treated patients" means patients who received VEGFT intravitreal injections once every four weeks from Week 0 through Week 20, followed by intravitreal VEGFT as needed from Week 24 through Week 52. The primary endpoint was the proportion of patients who gained ≥15 ETDRS letters from baseline at Week 24. Secondary visual, anatomic, and Quality of Life NEI VFQ-25 outcomes at Weeks 24 and 52 were also evaluated.

[0067] At Week 24, 56.1% of VEGFT-treated patients gained ≥15 ETDRS letters from baseline vs 12.3% of sham-treated patients ($P<0.0001$). Similarly, at Week 52, 55.3% of VEGFT-treated patients gained ≥15 letters vs 30.1% of sham-treated patients ($P<0.01$). At Week 52, VEGFT-treated patients gained a mean of 16.2 letters vs 3.8 letters for sham-treated patients ($P<0.001$). Mean number of injections was 2.7 for VEGFT-treated patients vs 3.9 for sham-treated patients. Mean change in central retinal thickness was -413.0 µm for VEGFT-treated patients vs -381.8 µm for sham-treated patients. The proportion of patients with ocular neovascularization at Week 24 were 0% for VEGFT-treated patients and 6.8% for sham-treated patients, respectively; at Week 52 after receiving VEGFT PRN, proportions were 0% and 6.8% for VEGFT-treated and sham-treated. At Week 24, the mean change from baseline in the VFQ-25 total score was 7.2 vs 0.7 for the

VEGFT-treated and sham-treated groups; at Week 52, the scores were 7.5 vs 5.1 for the VEGFT-treated and sham-treated groups.

[0068] This Example confirms that dosing monthly with 2 mg intravitreal VEGFT injection resulted in a statistically significant improvement in visual acuity at Week 24 that was maintained through Week 52 with PRN dosing compared with sham PRN treatment. VEGFT was generally well tolerated and had a generally favorable safety profile.

Example 7: Dosing Regimens

[0069] Specific, non-limiting examples of dosing regimens within the scope of the present invention are as follows:

[0070] VEGFT 2 mg (0.05 mL) administered by intravitreal injection once every 4 weeks (monthly).

[0071] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 8 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0072] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 8 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0073] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 8 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0074] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 12 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0075] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 12 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0076] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 12 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0077] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 16 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0078] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 16 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on

visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0079] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 16 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0080] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 20 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0081] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 20 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0082] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 20 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0083] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 24 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0084] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 24 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0085] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 24 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0086] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 28 weeks, followed by 2 mg (0.05 mL) via intravitreal injection once every 8 weeks.

[0087] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 28 weeks, followed by 2 mg (0.05 mL) via intravitreal injection on a less frequent basis based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0088] VEGFT 2 mg (0.5 mL) administered by intravitreal injection once every 4 weeks for the first 28 weeks, followed by 2 mg (0.05 mL) via intravitreal injection administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0089] VEGFT 2 mg (0.05 mL) administered by intravitreal injection as a single initial dose, followed by additional doses administered *pro re nata* (PRN) based on visual and/or anatomical outcomes (as assessed by a physician or other qualified medical professional).

[0090] Variations on the above-described dosing regimens would be appreciated by persons of ordinary skill in the art and are also within the scope of the present invention. For example, the amount of VEGFT and/or volume of formulation administered to a patient may be varied based on patient characteristics, severity of disease, and other diagnostic assessments by a physician or other qualified medical professional.

[0091] Any of the foregoing administration regimens may be used for the treatment of, *e.g.*, age-related macular degeneration (*e.g.*, wet AMD, exudative AMD, etc.), retinal vein occlusion (RVO), central retinal vein occlusion (CRVO; *e.g.*, macular edema following CRVO), branch retinal vein occlusion (BRVO), diabetic macular edema (DME), choroidal neovascularization (CNV; *e.g.*, myopic CNV), iris neovascularization, neovascular glaucoma, post-surgical fibrosis in glaucoma, proliferative vitreoretinopathy (PVR), optic disc neovascularization, corneal neovascularization, retinal neovascularization, vitreal neovascularization, pannus, pterygium, vascular retinopathy, etc.

SEQUENCES

[0092] SEQ ID NO:1 (DNA sequence having 1377 nucleotides):

ATGGTCAGCTACTGGGACACCGGGGTCCTGCTGTGCGCGCTGCTCAGCTGTCTGCTTCTCAC
AGGATCTAGTTCCGGAAGTGATACCGGTAGACCTTTCGTAGAGATGTACAGTGAAATCCCCGA
AATTATACACATGACTGAAGGAAGGGAGCTCGTCATTCCCTGCCGGGTTACGTCACCTAACAT
CACTGTTACTTTAAAAAAGTTTCCACTTGACACTTTGATCCCTGATGGAAAACGCATAATCTGG
GACAGTAGAAAGGGCTTCATCATATCAAATGCAACGTACAAAGAAATAGGGCTTCTGACCTGT
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TCATAGATGTGGTTCTGAGTCCGTCTCATGGAATTGAACTATCTGTTGGAGAAAAGCTTGTCTT
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ACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCT

GCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCG
GAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGC
AAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCA
TGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGA

[0093] SEQ ID NO:2 (polypeptide sequence having 458 amino acids):

MVSYWDTGVLLCALLSCLLLTGSSSGSDTGRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVTLK
KFPLDTLIPDGKRIIWDSRKGFIISNATYKEIGLLTCEATVNGHLYKTNYLTHRQTNTIIDVVLSPSHGI
ELSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTLTIDGVTRS
DQGLYTCAASSGLMTKKNSTFVRVHEKDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEV
TCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN
NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

[0094] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. A method for treating an angiogenic eye disorder in a patient, said method comprising sequentially administering to the patient a single initial dose of a VEGF antagonist, followed by one or more secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonist;

wherein each secondary dose is administered 2 to 4 weeks after the immediately preceding dose; and

wherein each tertiary dose is administered at least 8 weeks after the immediately preceding dose.

2. The method of claim 1, wherein only a single secondary dose is administered to the patient, and wherein the single secondary dose is administered 4 weeks after the initial dose of the VEGF antagonist.

3. The method of claim 1, wherein only two secondary doses are administered to the patient, and wherein each secondary dose is administered 4 weeks after the immediately preceding dose.

4. The method of claim 3, wherein each tertiary dose is administered 8 weeks after the immediately preceding dose.

5. The method of claim 1, wherein at least 5 tertiary doses of the VEGF antagonist are administered to the patient, and wherein the first four tertiary doses are administered 8 weeks after the immediately preceding dose, and wherein each subsequent tertiary dose is administered 8 or 12 weeks after the immediately preceding dose.

6. The method of claim 1, wherein the angiogenic eye disorder is selected from the group consisting of: age related macular degeneration, diabetic retinopathy, diabetic macular edema, central retinal vein occlusion, branch retinal vein occlusion, and corneal neovascularization.

7. The method of claim 6, wherein the angiogenic eye disorder is age related macular degeneration.

8. The method of claim 1, wherein the VEGF antagonist is an anti-VEGF antibody or fragment thereof, an anti-VEGF receptor antibody or fragment thereof, or a VEGF receptor-based chimeric molecule.

9. The method of claim 8, wherein the VEGF antagonist is a VEGF receptor-based chimeric molecule.

10. The method of claim 9, wherein the VEGF receptor-based chimeric molecule comprises VEGFR1R2-FcΔC1(a) encoded by the nucleic acid sequence of SEQ ID NO:1.

11. The method of claim 9, wherein the VEGF receptor-based chimeric molecule comprises (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130-231 of SEQ ID NO:2; and (3) a multimerization component comprising amino acids 232-457 of SEQ ID NO:2.

12. The method of claim 1, wherein all doses of the VEGF antagonist are administered to the patient by topical administration or by intraocular administration.

13. The method of claim 12, wherein all doses of the VEGF antagonist are administered to the patient by intraocular administration.

14. The method of claim 13, wherein the intraocular administration is intravitreal administration.

15. The method of claim 11, wherein all doses of the VEGF antagonist are administered to the patient by topical administration or by intraocular administration.

16. The method of claim 15, wherein all doses of the VEGF antagonist are administered to the patient by intraocular administration.

17. The method of claim 16, wherein the intraocular administration is intravitreal administration.

18. The method of claim 17, wherein all doses of the VEGF antagonist comprise from about 0.5 mg to about 2 mg of the VEGF antagonist.

19. The method of claim 18, wherein all doses of the VEGF antagonist comprise 0.5 mg of the VEGF antagonist.

20. The method of claim 18, wherein all doses of the VEGF antagonist comprise 2 mg of the VEGF antagonist.

ABSTRACT

The present invention provides methods for treating angiogenic eye disorders by sequentially administering multiple doses of a VEGF antagonist to a patient. The methods of the present invention include the administration of multiple doses of a VEGF antagonist to a patient at a frequency of once every 8 or more weeks. The methods of the present invention are useful for the treatment of angiogenic eye disorders such as age related macular degeneration, diabetic retinopathy, diabetic macular edema, central retinal vein occlusion, branch retinal vein occlusion, and corneal neovascularization.

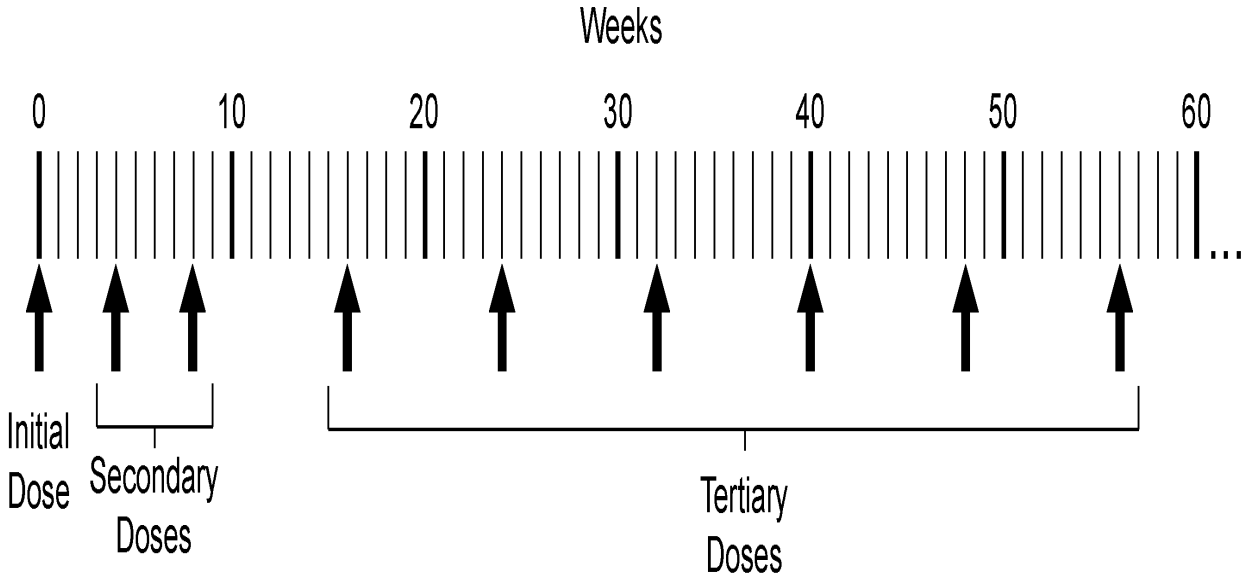
Weeks
0
10
20
30
40
50
60
...
Initial Dose
Secondary Doses
Tertiary Doses

Figure 1

PTO/AIA/01 (06-12)
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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT International application number 13/940,370

filed on July 12, 2013.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: YANCOPOULOS, GEORGE D. Date (Optional): 10/20/13

Signature: X

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form. Use an additional PTO/AIA/01 form for each additional inventor.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.
10.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	George	D.	YANCOPOULOS	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Yorktown Heights	State/Province	NY	Country of Residence i	US

Mailing Address of Inventor:

Address 1	c/o Regeneron Pharmaceuticals, Inc.
Address 2	777 Old Saw Mill River Road

City	Tarrytown	State/Province	CA
Postal Code	10591	Country i	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	96387		
Email Address	docket@bozpat.com	Add Email	Remove Email

Application Information:

Title of the Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS		
Attorney Docket Number	REGN-008CIPCON3	Small Entity Status Claimed	☐
Application Type			
Subject Matter			
Total Number of Drawing Sheets (if any)	1	Suggested Figure for Publication (if any)	1

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	96387		

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix	Remove
Registration Number					
Prefix	**Given Name**	Middle Name	**Family Name**	Suffix	Remove
Registration Number					

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	15471506	2017-03-28

Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
15471506	Continuation of	14972560	2015-12-17	9669069	2017-06-06

Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14972560	Continuation of	13940370	2013-07-12	9254338	2016-02-09

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13940370	Continuation in part of	PCT/US2012/020855	2012-01-11

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2012/020855	Claims benefit of provisional	61432245	2011-01-13

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2012/020855	Claims benefit of provisional	61434836	2011-01-21

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
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Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2012/020855	Claims benefit of provisional	61561957	2011-11-21

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Application Number	Country[i]	Filing Date (YYYY-MM-DD)	Access Code[i] (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

◯ Assignee	◯ Legal Representative under 35 U.S.C. 117	◯ Joint Inventor
◉ Person to whom the inventor is obligated to assign.	◯ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:	

If the Applicant is an Organization check here. ☒

Organization Name	Regeneron Pharmaceuticals, Inc.

Mailing Address Information For Applicant:

Address 1	777 Old Saw Mill River Road		
Address 2			
City	Tarrytown	**State/Province**	NY
Country[i]	US	Postal Code	10591
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☒

Organization Name	Regeneron Pharmaceuticals, Inc.

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1	777 Old Saw Mill River Road		
Address 2			
City	Tarrytown	**State/Province**	NY
Country i	US	Postal Code	10591
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e. g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Karl Boziceivc/			Date (YYYY-MM-DD)	
First Name	Karl	Last Name	Bozicevic	Registration Number	28807

Additional Signature may be generated within this form by selecting the Add button.

```
============================================================
Sequence Listing was accepted.
See attached Validation Report.
If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
Reviewer: Wheat Jr, Scott (ASRC)
Timestamp: [year=2018; month=8; day=8; hr=11; min=49; sec=24; ms=155; ]
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Application No: 16055847 Version No: 1.0

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Finished: 2018-08-06 16:40:31.511
Elapsed: 0 hr(s) 0 min(s) 0 sec(s) 174 ms
Total Warnings: 2
Total Errors: 1
No. of SeqIDs Defined: 2
Actual SeqID Count: 2

Error code		Error Description
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<120> Use of a VEGF Antagonist to Treat Angiogenic Eye Disorders

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United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/055,847	08/06/2018		1720	REGN-008CIPCON3	3	1

CONFIRMATION NO. 3451

96387
Regeneron - Bozicevic, Field & Francis
201 REDWOOD SHORES PARKWAY
SUITE 200
REDWOOD CITY, CA 94065

FILING RECEIPT

OC000000101727627

Date Mailed: 08/22/2018

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

George D. Yancopoulos, Yorktown Heights, NY;

Applicant(s)

Regeneron Pharmaceuticals, Inc., Tarrytown, NY

Assignment For Published Patent Application

Regeneron Pharmaceuticals, Inc., Tarrytown, NY

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 15/471,506 03/28/2017
which is a CON of 14/972,560 12/17/2015 PAT 9669069
which is a CON of 13/940,370 07/12/2013 PAT 9254338
which is a CIP of PCT/US2012/020855 01/11/2012
which claims benefit of 61/432,245 01/13/2011
and claims benefit of 61/434,836 01/21/2011

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 08/21/2018
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/055,847**
Projected Publication Date: 11/29/2018
Non-Publication Request: No
Early Publication Request: No
Title

USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/055,847

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	660
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	3 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 460 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1720

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
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	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

To: docket@bozpat.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 96387

Aug 24, 2018 04:30:32 AM

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Application	Document	Mailroom Date	Attorney Docket No.
16055847	APP.FILE.REC	08/22/2018	REGN-008CIPCON3

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Electronically filed 10/2/2018		
REQUEST FOR CORRECTED FILING RECEIPT	Attorney Docket No.	REGN-008CIPCON3
	Confirmation No.	3451
	First Named Inventor	GEORGE D. YANCOPOULOS
	Application Number	16/055,847
	Filing Date	August 6, 2018
Address to:	Group Art Unit	
Commissioner for Patents	Examiner Name	Jon McClelland Lockard
P.O. Box 1450 Alexandria, VA 22313-1450	Title: "*Use of a VEGF Antagonist to Treat Angiogenic Eye Disorders*"	

Sir:

A filing receipt for the above-identified patent application has been issued by the U.S. Patent and Trademark Office (copy attached) and has been found to contain the following error(s):

(1) Please correct the "Domestic Priority data as claimed by application" to include U.S. Provisional Patent Application No. 61/561,957 as indicated on the originally filed Application Data Sheet.

If for any reason a fee is found to be necessary, the Commissioner is authorized to charge such fee to Deposit Account No. 50-0815.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: October 2, 2018

By: /Karl Bozicevic, Reg. No. 28,807/
Karl Bozicevic
Reg. No. 28,807

BOZICEVIC, FIELD & FRANCIS LLP
201 Redwood Shores Parkway, Suite 200
Redwood City, CA 94065
Telephone: (650) 327-3400
Direct: (650) 833-7735
Facsimile: (650) 327-3231

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/055,847	08/06/2018		1720	REGN-008CIPCON3	3	1

CONFIRMATION NO. 3451

96387
Regeneron - Bozicevic, Field & Francis
201 REDWOOD SHORES PARKWAY
SUITE 200
REDWOOD CITY, CA 94065

FILING RECEIPT

Date Mailed: 08/22/2018

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

George D. Yancopoulos, Yorktown Heights, NY;

Applicant(s)

Regeneron Pharmaceuticals, Inc., Tarrytown, NY

Assignment For Published Patent Application

Regeneron Pharmaceuticals, Inc., Tarrytown, NY

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 15/471,506 03/28/2017
which is a CON of 14/972,560 12/17/2015 PAT 9669069
which is a CON of 13/940,370 07/12/2013 PAT 9254338
which is a CIP of PCT/US2012/020855 01/11/2012
which claims benefit of 61/432,245 01/13/2011
and claims benefit of 61/434,836 01/21/2011
and claims the benefit of 61/561,957 11/21/2011

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 08/21/2018
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/055,847**
Projected Publication Date: 11/29/2018
Non-Publication Request: No
Early Publication Request: No
Title

USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

Inventor Information:

Inventor 1

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	George	D.	YANCOPOULOS	

Residence Information (Select One) ◉ US Residency ○ Non US Residency ○ Active US Military Service

City	Yorktown Heights	State/Province	NY	Country of Residence	US

Mailing Address of Inventor:

Address 1	c/o Regeneron Pharmaceuticals, Inc.
Address 2	777 Old Saw Mill River Road

City	Tarrytown	State/Province	CA
Postal Code	10591	**Country**	US

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below.
For further information see 37 CFR 1.33(a).

☐ **An Address is being provided for the correspondence Information of this application.**

Customer Number	96387		
Email Address	docket@bozpat.com	Add Email	Remove Email

Application Information:

Title of the Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS		
Attorney Docket Number	REGN-008CIPCON3	**Small Entity Status Claimed**	☐
Application Type			
Subject Matter			
Total Number of Drawing Sheets (if any)	1	**Suggested Figure for Publication (if any)**	1

PTO/AIA/14 (08-15)
Approved for use through 04/30/2017. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	◉ Customer Number	○ US Patent Practitioner	○ Limited Recognition (37 CFR 11.9)
Customer Number	96387		

Prefix	**Given Name**	Middle Name	**Family Name**	Suffix	
					Remove
Registration Number					
Prefix	**Given Name**	Middle Name	**Family Name**	Suffix	
					Remove
Registration Number					

Additional Representative Information blocks may be generated within this form by selecting the **Add** button.

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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status	Pending		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
	Continuation of	15471506	2017-03-28

Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
15471506	Continuation of	14972560	2015-12-17	9669069	2017-06-06

Prior Application Status	Patented				Remove
Application Number	Continuity Type	Prior Application Number	Filing Date (YYYY-MM-DD)	Patent Number	Issue Date (YYYY-MM-DD)
14972560	Continuation of	13940370	2013-07-12	9254338	2016-02-09

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
13940370	Continuation in part of	PCT/US2012/020855	2012-01-11

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2012/020855	Claims benefit of provisional	61432245	2011-01-13

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2012/020855	Claims benefit of provisional	61434836	2011-01-21

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCT/US2012/020855	Claims benefit of provisional	61561957	2011-11-21

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX) the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Application Number	Country	Filing Date (YYYY-MM-DD)	Access Code (if applicable)

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

PTO/AIA/14 (08-15)
Approved for use through 04/30/2017. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

PTO/AIA/14 (08-15)
Approved for use through 04/30/2017. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant 1

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

○ Assignee	○ Legal Representative under 35 U.S.C. 117	○ Joint Inventor
◉ Person to whom the inventor is obligated to assign.	○ Person who shows sufficient proprietary interest	

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:	

If the Applicant is an Organization check here. ☒

Organization Name	Regeneron Pharmaceuticals, Inc.

Mailing Address Information For Applicant:

Address 1	777 Old Saw Mill River Road		
Address 2			
City	Tarrytown	**State/Province**	NY
Country [i]	US	Postal Code	10591
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button.

PTO/AIA/14 (08-15)
Approved for use through 04/30/2017. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Assignee 1

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☒

Organization Name	Regeneron Pharmaceuticals, Inc.

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1	777 Old Saw Mill River Road		
Address 2			
City	Tarrytown	**State/Province**	NY
Country i	US	Postal Code	10591
Phone Number		Fax Number	
Email Address			

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

PTO/AIA/14 (08-15)
Approved for use through 04/30/2017. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Application Data Sheet 37 CFR 1.76	Attorney Docket Number	REGN-008CIPCON3
	Application Number	
Title of Invention	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS	

Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e. g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Karl Boziceivc/			Date (YYYY-MM-DD)	
First Name	Karl	Last Name	Bozicevic	Registration Number	28807

Additional Signature may be generated within this form by selecting the Add button.

Electronic Acknowledgement Receipt

EFS ID:	33891453
Application Number:	16055847
International Application Number:	
Confirmation Number:	3451
Title of Invention:	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS
First Named Inventor/Applicant Name:	George D. Yancopoulos
Customer Number:	96387
Filer:	Karl Bozicevic
Filer Authorized By:	
Attorney Docket Number:	REGN-008CIPCON3
Receipt Date:	02-OCT-2018
Filing Date:	06-AUG-2018
Time Stamp:	18:04:14
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Corrected Filing Receipt	REGN-008CIPCON3_2018-10-02_Request_OFR_completesubmission.pdf	762318 ef2db058337a314821f5284f8483be1237fb0e83	no	12

Warnings:

Information:	
Total Files Size (in bytes):	762318

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/055,847	08/06/2018	1647	1720	REGN-008CIPCON3	3	1

CONFIRMATION NO. 3451

CORRECTED FILING RECEIPT

96387
Regeneron - Bozicevic, Field & Francis
201 REDWOOD SHORES PARKWAY
SUITE 200
REDWOOD CITY, CA 94065

OC000000102823734

Date Mailed: 10/05/2018

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

George D. Yancopoulos, Yorktown Heights, NY;

Applicant(s)

Regeneron Pharmaceuticals, Inc., Tarrytown, NY

Assignment For Published Patent Application

Regeneron Pharmaceuticals, Inc., Tarrytown, NY

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 15/471,506 03/28/2017
which is a CON of 14/972,560 12/17/2015 PAT 9669069
which is a CON of 13/940,370 07/12/2013 PAT 9254338
which is a CIP of PCT/US2012/020855 01/11/2012
which claims benefit of 61/432,245 01/13/2011
and claims benefit of 61/434,836 01/21/2011
and claims benefit of 61/561,957 11/21/2011

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 08/21/2018

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/055,847**

Projected Publication Date: 11/29/2018

Non-Publication Request: No

Early Publication Request: No

Title

USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS

Preliminary Class

424

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific

countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

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The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

To: docket@bozpat.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 96387

Oct 05, 2018 04:09:21 AM

Dear PAIR Customer:

Regeneron - Bozicevic, Field & Francis
201 REDWOOD SHORES PARKWAY
SUITE 200
REDWOOD CITY, CA 94065
UNITED STATES

The following USPTO patent application(s) associated with your Customer Number, 96387 , have new outgoing correspondence. This correspondence is now available for viewing in Private PAIR.

The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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Application	Document	Mailroom Date	Attorney Docket No.
16055847	APP.FILE.REC	10/05/2018	REGN-008CIPCON3

To view your correspondence online or update your email addresses, please visit us anytime at https://sportal.uspto.gov/secure/myportal/privatepair.

If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

Monday - Friday 6:00 a.m. to 12:00 a.m.

Thank you for prompt attention to this notice,

UNITED STATES PATENT AND TRADEMARK OFFICE

PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

Doc Code: PA..
Document Description: Power of Attorney

PTO/AIA/82B (07-13)
Approved for use through 01/31/2018. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number

POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date
16/055,847	August 06, 2018

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☑ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above: 96387

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☑ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name					
Address					
City		State		Zip	
Country					
Telephone		Email			

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Regeneron Pharmaceuticals, Inc.

☐ Inventor or Joint Inventor (title not required below)
☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)
☑ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)
☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature	/Frank R. Cottingham/	Date (Optional)	October 4, 2018
Name	Frank R. Cottingham		
Title	Executive Director, Assistant General Counsel, Patents, Regeneron Pharmaceuticals, Inc.		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☑ Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: **Regeneron Pharmaceuticals, Inc.**

Application No./Patent No.: **16/055,847** Filed/Issue Date: **August 6, 2018**

Titled: **Use of a VEGF Antagonist to Treat Angiogenic Eye Disorders**

Regeneron Pharmaceuticals, Inc., a **corporation**
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. ☒ The assignee of the entire right, title, and interest.

2. ☐ An assignee of less than the entire right, title, and interest (check applicable box):

   ☐ The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   ☐ There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. ☐ The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. ☐ The recipient, via a court proceeding or the like (*e.g.,* bankruptcy, probate), or an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel **047080**, Frame **0383**, or for which a copy thereof is attached.

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____

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STATEMENT UNDER 37 CFR 3.73(c)

3. From: _____ To: _____

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☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☒ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

/Karl Bozicevic, Reg. No. 28,807/	**October 10, 2018**
Signature	Date
Karl Bozicevic	**28,807**
Printed or Typed Name	Title or Registration Number

Electronic Acknowledgement Receipt

EFS ID:	33960203
Application Number:	16055847
International Application Number:	
Confirmation Number:	3451
Title of Invention:	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS
First Named Inventor/Applicant Name:	George D. Yancopoulos
Customer Number:	96387
Filer:	Karl Bozicevic
Filer Authorized By:	
Attorney Docket Number:	REGN-008CIPCON3
Receipt Date:	10-OCT-2018
Filing Date:	06-AUG-2018
Time Stamp:	12:20:48
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	0725US04_POA.pdf	169058 354f234da9a8c8f7fb7b5ba9fb5e574d3572b58c	no	1

Warnings:

Information:					
2	Assignee showing of ownership per 37 CFR 3.73	REGN-008CIPCON3_2018-10-10_373_C_stmt.pdf	32228 d7c8c52ab1287dbd49dd00e1d9c948c1c3b6615a	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	201286	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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United States Patent and Trademark Office
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www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/055,847	08/06/2018	George D. Yancopoulos	REGN-008CIPCON3

CONFIRMATION NO. 3451

96387
Regeneron - Bozicevic, Field & Francis
201 REDWOOD SHORES PARKWAY
SUITE 200
REDWOOD CITY, CA 94065

POA ACCEPTANCE LETTER

OC000000103120052

Date Mailed: 10/18/2018

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 10/10/2018.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/sleutchit/

To: docket@bozpat.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 96387

Oct 18, 2018 10:16:14 AM

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Regeneron - Bozicevic, Field & Francis
201 REDWOOD SHORES PARKWAY
SUITE 200
REDWOOD CITY, CA 94065
UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
16055847	N570	10/18/2018	REGN-008CIPCON3

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/055,847	08/06/2018	George D. Yancopoulos	REGN-008CIPCON3

CONFIRMATION NO. 3451

96387
Regeneron - Bozicevic, Field & Francis
201 REDWOOD SHORES PARKWAY
SUITE 200
REDWOOD CITY, CA 94065

PUBLICATION NOTICE

OC00000104130787

Title:USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS

Publication No.US-2018-0339018-A1
Publication Date:11/29/2018

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

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Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

To: docket@bozpat.com,,
From: PAIR_eOfficeAction@uspto.gov
Cc: PAIR_eOfficeAction@uspto.gov
Subject: Private PAIR Correspondence Notification for Customer Number 96387

Nov 30, 2018 04:38:44 AM

Dear PAIR Customer:

Regeneron - Bozicevic, Field & Francis
201 REDWOOD SHORES PARKWAY
SUITE 200
REDWOOD CITY, CA 94065
UNITED STATES

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Application	Document	Mailroom Date	Attorney Docket No.
16055847	NTC.PUB	11/29/2018	REGN-008CIPCON3

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If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov with 'e-Office Action' on the subject line or call 1-866-217-9197 during the following hours:

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PATENT APPLICATION INFORMATION RETRIEVAL SYSTEM

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Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/055,847	08/06/2018	George D. Yancopoulos	REGN-008CIPCON3	3451

96387 7590 05/01/2019

Regeneron - Bozicevic, Field & Francis
201 REDWOOD SHORES PARKWAY
SUITE 200
REDWOOD CITY, CA 94065

EXAMINER	
LOCKARD, JON MCCLELLAND	

ART UNIT	PAPER NUMBER
1647	

NOTIFICATION DATE	DELIVERY MODE
05/01/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

docket@bozpat.com

UNITED STATES DEPARTMENT OF COMMERCE
U.S. Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450

APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR/ PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
16/055,847	08/06/2018	Yancopoulos, George D.	REGN-008CIPCON3

		EXAMINER	
Regeneron - Bozicevic, Field & Francis 201 REDWOOD SHORES PARKWAY SUITE 200 REDWOOD CITY, CA 94065		MARIANNE C SEIDEL	
		ART UNIT	PAPER
		1600	20190429

DATE MAILED: _____

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner for Patents

The third-party submission under 37 CFR 1.290 filed on 4/21/19 and 4/27/19 for the instant application has been determined to be compliant with 35 U.S.C. 122(e) and 37 CFR 1.290 and is being entered in the application. Please allow a few days for the submission to be visible in the Patent Application Information Retrieval (PAIR) system.

/MARIANNE C SEIDEL/
Quality Assurance Specialist, Art Unit 1600

PTO-90C (Rev.04-03)

THIRD-PARTY SUBMISSION UNDER 37 CFR 1.290
CONCISE DESCRIPTION OF RELEVANCE

Application Number	16055847

U.S. PATENTS

Cite No	Patent Number	Concise Description of Relevance

U.S. PATENT APPLICATION PUBLICATION

Cite No	Publication Number	Concise Description of Relevance

FOREIGN PATENT DOCUMENTS		
CiteNo	**Foreign Document Number**	**Concise Description of Relevance**

NON-PATENT PUBLICATIONS		
Cite No	**Reference**	**Concise Description of Relevance**
1	Peter AUTHER, Ranibizumab for Macular Edema Due to Retinal Vein Occlusions Implication of VEGF as a Critical Stimulator title, 9 pages , 30/08/2008	See Attached

2	Lucentis Label title ,7 pages, 30/06/2010	See Attached

PTO/SB/429(08-12)
Approved for use through 07/31/2015. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

THIRD-PARTY SUBMISSION UNDER 37 CFR 1.290	Application Number	16055847

U.S. PATENTS				
Cite No	Patent Number	Kind Code[1]	Issue Date (YYYY-MM-DD)	First Named Inventor

U.S. PATENT APPLICATION PUBLICATIONS				
Cite No	Publication Number	Kind Code[1]	Publication Date (YYYY-MM-DD)	First Named Inventor

FOREIGN PATENTS AND PUBLISHED FOREIGN PATENT APPLICATIONS						
Cite No	Foreign Document Number[3]	Country Code[2]	Kind Code[1]	Publication Date (YYYY-MM-DD)	Applicant, Patentee or First Named Inventor	T[5]
						☐

NON-PATENT PUBLICATIONS (e.g., journal article, Office action)			
Cite No	Author (if any), title of the publication, page(s) being submitted, publication date, publisher (where available), place of publication (where available).	T[5]	E[6]

THIRD-PARTY SUBMISSION UNDER 37 CFR 1.290	Application Number	16055847

1	Lucentis Label title ,7 pages, 30/06/2010	☐	☒
2	Peter AUTHER, Ranibizumab for Macular Edema Due to Retinal Vein Occlusions Implication of VEGF as a Critical Stimulator title, 9 pages , 30/08/2008	☐	☒

STATEMENTS

The party making the submission is not an individual who has a duty to disclose information with respect to the above-identified application under 37 CFR 1.56.

This submission complies with the requirements of 35 U.S.C. 122(e) and 37 CFR 1.290.

☐ The fee set forth in 37 CFR 1.290(f) has been submitted herewith.

☒ The fee set forth in 37 CFR 1.290(f) is not required because this submission lists three or fewer total items and, to the knowledge of the person signing the statement after making reasonable inquiry, this submission is the first and the only submission under 35 U.S.C 122(e) filed in the above-identified application by the party making the submission or by a party in privity with the party.

☐ This resubmission is being made responsive to a notification of non-compliance issued for an earlier filed third-party submission. The corrections in this resubmission are limited to addressing the non-compliance. As such, the party making this resubmission: (1) requests that the Office apply the previously-paid fee set forth in 37 CFR 1.290(f), or (2) states that no fee is required to accompany this resubmission as the undersigned is again making the fee exemption statement set forth in 37 CFR 1.290(g).

THIRD-PARTY SUBMISSION UNDER 37 CFR 1.290	Application Number	16055847

Signature	/Elizabeth Thompson/		
Name/Print	Elizabeth	Registration Number (if applicable)	

Examiner Signature		Date Considered	

*EXAMINER: Signature indicates all documents listed above have been considered,except for citations through which a line is drawn. Draw line through citation if not considered. Include a copy of this form with next communication to applicant. 1. If known, enter kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16. See MPEP 901.04(a). 2. Enter the country or patent office that issued the document, by two-letter code under WIPO standard ST.3. See MPEP 1851. 3. For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. 4. If known, enter the kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 . See MPEP 901.04(a). 5. Check mark indicates translation attached. 6. Check mark indicates evidence of publication attached.

Electronic Acknowledgement Receipt

EFS ID:	35783629
Application Number:	16055847
International Application Number:	
Confirmation Number:	3451
Title of Invention:	
First Named Inventor/Applicant Name:	
Correspondence Address:	- - - - - - - - - -
Filer:	Elizabeth Thompson
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Concise Description of Relevance	Concise-description-generated.pdf	30185 1fd17ba5e73b9f30386d2803b71e9bc73fd08cb4	no	3
Warnings:					
Information:					
2	Third-Party Submission Under 37 CFR 1.290	Third-party-preissuance-submission.pdf	43151 79d1d8f0f8ebf66c58ca2108d13dcc72cab805d0	no	3
Warnings:					
Information:					
3	Request for Notification of Non-compliant Third-Party Submission	Third-party-notification-request.pdf	20074 40d5d061fee7b35a11a9fd0caf93f69559afcb7d	no	1
Warnings:					
Information:					
		Total Files Size (in bytes):	93410		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

THIRD-PARTY SUBMISSION UNDER 37 CFR 1.290
CONCISE DESCRIPTION OF RELEVANCE

Application Number	16055847

U.S. PATENTS

Cite No	Patent Number	Concise Description of Relevance

U.S. PATENT APPLICATION PUBLICATION

Cite No	Publication Number	Concise Description of Relevance

FOREIGN PATENT DOCUMENTS		
CiteNo	**Foreign Document Number**	**Concise Description of Relevance**

NON-PATENT PUBLICATIONS		
Cite No	**Reference**	**Concise Description of Relevance**
1	Vascular Endothelial Growth Factor Trap‐Eye Investigation of Efficacy and Safety in Central Retinal Vein Occlusion title, 8 pages, 11/12/2009, US	See Attached

2	Niral Karia Author, Retinal vein occlusion: pathophysiology and treatment options title, 8 pages, 07/31/2010, Clinical Ophthalmology publisher	See Attached

3	RAFAEL Author, Advances in the Medical Treatment of Diabetic Retinopathy, 7 pages, 08/31/2009, DIABETES CARE publisher	See Attached

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Approved for use through 07/31/2015. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

THIRD-PARTY SUBMISSION UNDER 37 CFR 1.290	Application Number	16055847

U.S. PATENTS				
Cite No	Patent Number	Kind Code[1]	Issue Date (YYYY-MM-DD)	First Named Inventor

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THIRD-PARTY SUBMISSION UNDER 37 CFR 1.290	Application Number	16055847

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THIRD-PARTY SUBMISSION UNDER 37 CFR 1.290	Application Number	16055847

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☐	This resubmission is being made responsive to a notification of non-compliance issued for an earlier filed third-party submission. The corrections in this resubmission are limited to addressing the non-compliance. As such, the party making this resubmission: (1) requests that the Office apply the previously-paid fee set forth in 37 CFR 1.290(f), or (2) states that no fee is required to accompany this resubmission as the undersigned is again making the fee exemption statement set forth in 37 CFR 1.290(g).

Signature	/Joe Reynolds/		
Name/Print	Joe	Registration Number (if applicable)	

Examiner Signature		Date Considered	

*EXAMINER: Signature indicates all documents listed above have been considered,except for citations through which a line is drawn. Draw line through citation if not considered. Include a copy of this form with next communication to applicant. 1. If known, enter kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16. See MPEP 901.04(a). 2. Enter the country or patent office that issued the document, by two-letter code under WIPO standard ST.3. See MPEP 1851. 3. For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. 4. If known, enter the kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 . See MPEP 901.04(a). 5. Check mark indicates translation attached. 6. Check mark indicates evidence of publication attached.

Concise Description of Relevance

The following references are being submitted:

P1: Vascular Endothelial Growth Factor (VEGF) Trap-Eye: Investigation of Efficacy and Safety in Central Retinal Vein Occlusion (CRVO); web site: https://clinicaltrials.gov/ct2/history/NCT01012973?V_1=View#StudyPageTop; Published on Nov 12, 2009;

P2: Niral Karia, Retinal vein occlusion: pathophysiology and treatment options. Clinical Ophthalmology 2010:4 809–816, Published on Jul, 2010;

P3: RAFAEL SIM ´O and CRISTINA HERN´ANDEZ, Advances in the Medical Treatment of Diabetic Retinopathy. DIABETES CARE, VOLUME 32, NUMBER 8, AUGUST 2009, page 1559, Published on Aug, 2009.

As the claim chart shows below, **P1** discloses study description of clinical trial NCT01012973, which aims to determine the efficacy of vascular endothelial growth factor (VEGF) Trap-Eye injected into the eye on vision function in subjects with macular edema as a consequence of central retinal vein occlusion. The only difference is that Claim 21 uses "2 mg aflibercept" instead of "VEGF Trap-Eye" used in P1;

P2 discloses that Retinal vein occlusion (RVO) is the most common retinal vascular disease after diabetic retinopathy. Depending on the area of retinal venous drainage effectively occluded it is broadly classified as either central retinal vein occlusion (CRVO), hemispheric retinal vein occlusion (HRVO), or branch retinal vein occlusion (BRVO). (*see* page 809 introduction section first two sentences);

P3 discloses that aflibercept also known as a VEGF Trap-Eye because its ability to block VEGF proteins, is a fusion protein comprised of segments of the extracellular domains of human VEGF receptor 1 and 2 fused to the constant region of human IgG. (s*ee* page 1559 right column third paragraph);

Besides, as it is mentioned in the present invention, the VEGF antagonist comprises one or more VEGF receptor-based chimeric molecule(s), (also referred to herein as a "VEGF-Trap" or "VEGFT"). An exemplary VEGF antagonist that can be used in the context of the present invention is a multimeric VEGF-binding protein comprising two or more VEGF receptor-based chimeric molecules referred to herein as "VEGFR1R2-FcΔC1(a)" or "aflibercept." (*see* paragraph [0008]);

	U.S. Application No. 16/055,847	Concise Description of Relevance
Claim #	Claim Elements	Vascular Endothelial Growth Factor (VEGF) Trap-Eye: Investigation of Efficacy and Safety in Central Retinal Vein Occlusion (CRVO)
21	A method for treating macular edema following retinal vein occlusion in a human subject comprising administering 2 mg aflibercept to the subject	To determine the efficacy of vascular endothelial growth factor (VEGF) Trap-Eye injected into the eye on vision function in subjects with macular edema as a consequence of central retinal vein occlusion. [Study Description]
	by intravitreal injection	Intravitreal injection. [Assigned Interventions]
	once every 4 weeks.	Weeks 0 to 20 injection of VEGF Trap-Eye every 4 weeks [Assigned Interventions]

Advances in the Medical Treatment of Diabetic Retinopathy

RAFAEL SIMÓ, MD, PHD
CRISTINA HERNÁNDEZ, MD, PHD

Proliferative diabetic retinopathy (PDR) remains the leading cause of blindness among working-age individuals in developed countries (1). Diabetic macular edema (DME), another important event that occurs in diabetic retinopathy, is more frequent in type 2 than type 1 diabetes (2). Whereas PDR is the most common sight-threatening lesion in type 1 diabetes, DME is the primary cause of poor visual acuity in type 2 diabetes. Because of the high prevalence of type 2 diabetes, DME is the main cause of visual impairment for diabetic patients (2). In addition, DME is almost invariably present when PDR is detected in type 2 diabetes (3). Neovascularization caused by severe hypoxia is the hallmark of PDR, whereas vascular leakage caused by the breakdown of the blood retinal barrier (BRB) is the main event involved in the pathogenesis of DME (4,5).

STANDARD TREATMENT — Although tight control of both blood glucose levels and hypertension is essential to prevent or arrest progression of the disease, the recommended goals are difficult to achieve in many patients and, consequently, diabetic retinopathy develops during the evolution of the disease. When PDR or clinically significant DME do appear, argon-laser photocoagulation is currently indicated, which the efficacy of has been widely demonstrated (6). However, the optimal period for laser treatment has frequently passed; moreover, it is not uniformly successful in halting visual decline. In addition, argon-laser photocoagulation is associated with moderate visual loss, some diminished visual field, reduced color vision, and reduced contrast sensitivity. The presence of these symptoms led to the prevailing thinking that laser treatment prevents vision loss but rarely results in visual improvement.

Intravitreal corticosteroids have been successfully used in the eyes of patients with persistent DME and loss of vision following the failure of conventional treatment (i.e., focal laser treatment and attention to systemic risk factors). However, reinjections are commonly needed, and there are substantial adverse effects such as infection, glaucoma, and cataract formation (6). In addition, recent reports have shown that focal/grid photocoagulation is more effective and has fewer side effects than intravitreal triamcinolone for DME (7,8).

Vitreoretinal surgery is an expensive and complicated treatment that should be carried out only by vitreoretinal specialists experienced in this procedure, and it is normally reserved for the ultimately blinding complications of PDR, such as severe vitreous hemorrhage and secondary retinal detachment. For these reasons, new pharmacological treatments based on the understanding of the pathophysiological mechanisms of diabetic retinopathy are needed.

The paucity of relevant clinical studies addressed to testing new drugs in diabetic retinopathy is due, in part, to the necessity of long-term studies performed in large cohorts of diabetic patients by means of standardized masked grading of retinal photographs. Although there is no fixed rule, the duration of the trial must be consistent with the natural history of diabetic retinopathy and, consequently, at least 5 years seems to be necessary for separating the behavior of retinopathy in the intervention and control groups. In addition, most clinical trials have been aimed at evaluating the progression of diabetic retinopathy, whereas there have been few studies targeting prevention. All these caveats should be kept in mind when analyzing clinical trials on diabetic retinopathy because they can significantly contribute to false-negative results. The presence of diabetic retinopathy in nondiabetic subjects is another challenge. Wong et al. (9), in a study that included more than 11,000 participants from three population cohorts, provide evidence that with the current fasting plasma glucose cutoff of 7.0 mmol/l used to diagnose diabetes, 7.4–13.4% of nondiabetic patients had diabetic retinopathy. This finding, apart from questioning the current diagnostic criteria of diabetes, suggests a potential limit to the risk reduction for diabetic retinopathy that should be taken into consideration when interpreting the results of clinical trials.

Recently, two pivotal studies have been published regarding the beneficial effects of two types of drugs (fenofibrate and candesartan) on diabetic retinopathy (10–12). These studies fulfill all the main requirements for obtaining a valid result: long-term follow-up (~5 years), a large cohort of diabetic patients, retinopathy assessed by standardized methods, and a significant number of patients without diabetic retinopathy at study entry, thus allowing evaluation of the effectiveness of prevention. In advanced stages of diabetic retinopathy, intravitreous anti–vascular endothelial growth factor (VEGF) agents have emerged as new treatments. These drugs are yet to be approved for diabetic retinopathy treatment, but they are currently used by ophthalmologists in selected cases of PDR and DME (13,14). This article discusses the current state of knowledge concerning these novelties in the medical treatment of diabetic retinopathy and highlight areas where further studies and evidence are required.

FENOFIBRATE — Fenofibrate is a peroxisome proliferactor–activated receptor (PPAR)-α agonist indicated for the treatment of hypertriglyceridemia and mixed dislipidemia. Its main action is to lower plasma triglyceride levels, but it also reduces total and LDL cholesterol, raises HDL cholesterol, and decreases

From the CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Madrid, Spain, and the Diabetes and Metabolism Research Unit, Institut de Recerca Hospital Universitari Vall d'Hebron, Barcelona, Spain.
Corresponding author: Rafael Simó, rsimo@ir.vhebron.net.
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concentration of small LDL cholesterol particles and apolipoprotein B (15). Recently, Keech et al. (10) have reported results concerning laser treatment for diabetic retinopathy from the FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study. The main aim of this randomized controlled trial was to assess whether long-term lipid-lowering therapy using fenofibrate (a PPAR-α agonist) could reduce the need for laser treatment in a large cohort (n = 9,795) of type 2 diabetic patients. The average follow-up was 5 years, and the end point was the need for laser treatment (a tertiary end point of the main study). In an intention-to-treat analysis, fenofibrate (200 mg once daily) reduced the frequency of laser treatment for macular edema by 31% and for proliferative retinopathy by 30%. In addition, in a substudy performed on patients in whom retinal status was graded by fundus photography, fenofibrate was able to reduce the progression of existing retinopathy. Although this study has some limiting factors (16,17), the substantial benefits obtained from reducing the need for laser treatment argue for consideration of using fenofibrate in the management of diabetic retinopathy. However, our poor knowledge of the mechanisms involved in its beneficial effects in diabetic retinopathy might limit its potential impact on clinical practice. Theoretically, another PPAR-α apart from fenofibrate can also be beneficial for diabetic retinopathy; however, at present this has been only demonstrated with fenofibrate.

The rationale for FIELD was that elevated lipid levels in systemic circulation constitute a risk factor for diabetic retinopathy; therefore, long-term lipid-lowering therapy with fenofibrate could reduce the progression of diabetic retinopathy and the need for laser treatment in patients with type 2 diabetes. However, no relationship between serum lipids and the appearance or progression of diabetic retinopathy was detected. This is in agreement with other prospective studies showing that serum lipids are unrelated to the progression of diabetic retinopathy or the development of PDR (18,19). In addition, the Collaborative Atorvastatin Diabetes Study (CARDS), a randomized controlled trial of 2,830 patients with type 2 diabetes, did not find atorvastatin to be effective in reducing diabetic retinopathy progression (20). However, this study was limited by substantial missing data (only 65% of patients had retinopathy status recorded at baseline) and lack of photographic grading for diabetic retinopathy. Another randomized trial, the ACCORD-EYE study that is now in progress, could shed light on this issue (21). In this study, the effects of lipid control (statin vs. fenofibrate added to a statin) on the progression of diabetic retinopathy will be evaluated. There will be 4,065 participants recruited to the study at baseline for whom fundus photographs will be taken within 4 months of randomization and again 4 years later. Although in the FIELD study there was no relationship between the quantitative levels of serum lipids and diabetic retinopathy, it is unknown whether the effectiveness of fenofibrate in modulating the qualitative properties of lipoproteins (i.e., reducing remnants and small dense LDL particles) can contribute to its beneficial effects. In addition, it should be noted that the mechanisms regulating intraretinal lipid transport rather than serum levels might be more important in the pathogenesis of diabetic retinopathy. In this regard, we have recently shown that apolipoprotein A1 (apo-A1) is overexpressed in the retina of diabetic patients (22). Apo-A1 is a key factor for the intraretinal transport of lipids, thus preventing lipid deposition and lipotoxicity, and it is also a potent scavenger of reactive oxygen species. Therefore, apo-A1 could play an important role in protecting the retina from oxidative stress. These findings have led us to hypothesize that the retinas from diabetic patients have a higher content of apo-A1 as a protective mechanism; consequently, patients with less capacity for apo-A1 production by the retina will be more prone to develop lipid deposition (hard exudates) and retinal damage induced by oxidative stress. Fenofibric acid was shown to enhance transcription of the gene of apo-A1 in the liver (23), macrophages, and fibroblasts (24), but whether this is also true at the retinal level remains to be elucidated.

Other nonlipidic mechanisms by which fenofibrate could be effective in preventing or arresting diabetic retinopathy might be the following:

1) PPAR-α is present in endothelial cells (25), and its activation by means of PPAR-α agonists has recently been shown to inhibit expression of VEGF receptor 2 (VEGFR2) and neovascularization in human umbilical endothelial cells (26). Varet et al. (27) have demonstrated that fenofibrate inhibits angiogenesis in vitro and in vivo as well as basic fibroblast growth factor–induced angiogenesis in vivo. In addition, in cells derived from human ovarian cancer, clofibric acid (a PPAR-α agonist) downregulates VEGF expression (28). Apart from its antiproliferative effects, fenofibrate inhibits the apoptosis induced by high glucose concentrations in human umbilical endothelial cells (29). Moreover, it has been demonstrated that fenofibrate prevents the apoptosis of human retinal endothelial cells induced by serum deprivation through a PPAR-α–independent but AMP-activated protein kinase–dependent pathway (30). This activation of the AMP-activated protein kinase pathway in endothelial cells could lead to an increase in endothelial nitric oxide synthase phosphorylation and nitric oxide production, thus resulting in beneficial effects on endothelial function (31).
2) PPAR-α is associated with anti-inflammatory and antioxidant activity (32). It has been reported that PPAR-α activation induces the expression and activation of antioxidant enzymes, such as superoxide dismutase and glutation peroxidase (33), and that activation of PPAR-α induces apoptosis of human monocyte–derived macrophages (34). In addition, PPAR-α activators inhibit the expression of vascular cell adhesion molecules on the endothelium (35). This effect might be useful in preventing leukostasis (the inappropriate adherence of leukocytes to the endothelium), which is essential in the pathogenesis of PDR.
3) PPAR-α activation also has a neuroprotective effect (33,36). This could be important in preventing neuroretinal degeneration, an early and crucial event that occurs in diabetic retinopathy even before vascular abnormalities can be detected (37).
4) The breakdown of the BRB, caused by the disruption of tight junctions and subsequent leakage, is the main factor accounting for DME (6). Because of the notable effect of fenofibrate in preventing DME progression, it would be worthwhile to explore whether fenofibrate is able to reduce the increased permeability that exists in diabetic retinopathy.

Future research on the potential effects of fenofibrate in all these areas will be essential for understanding its beneficial effects in diabetic retinopathy, and it will also be critical for using this drug as an adjunct in the management of diabetic retinopathy.

BLOCKING THE RENIN-ANGIOTENSIN SYSTEM — Observational and clinical trials have shown that blood pressure is an important modifiable risk factor for diabetic retinopathy and that lowering high blood pressure significantly reduces the development and progression of retinopathy in both type 1 and type 2 diabetic patients (38,39). The blockade of the renin-angiotensin system (RAS) with an ACE inhibitor or by using angiotensin II type 1–receptor (AT1-R) blockers is one of the most used strategies for hypertension treatment in diabetic patients. Apart from the kidney, the RAS system is expressed in the eye (40). In addition, there is growing evidence that RAS activation in the eye plays an important role in the pathogenesis of diabetic retinopathy (40). Therefore, apart from lowering blood pressure, the blockade of the RAS could also be beneficial per se in reducing the development and progression of diabetic retinopathy.

The major components of RAS have been identified in ocular tissues and are overexpressed in the diabetic retina. Angiotensin II (AT) binds and activates two primary receptors, AT1-R and AT2-R. In adult humans, activation of the AT1-R expressed in endothelial cells and pericytes dominates the pathological states (40). AT1-R activation by AT produced by the retina stimulates several pathways involved in the pathogenesis of diabetic retinopathy such as inflammation, oxidative stress, cell proliferation, pericyte migration, remodelling of extracellular matrix by increasing matrix metalloproteinases, angiogenesis, and fibrosis (40). The RAS is upregulated concomitant with hypoxia-induced retinal angiogenesis and is linked to AT-mediated induction of inflammatory mediators and growth factors, including VEGF and platelet-derived growth factor (40,41). In addition, AT1-R activation by AT promotes leukostasis and neurodegeneration (40), two key elements in the pathogenesis of diabetic retinopathy. Most of these pathogenic actions are inhibited or attenuated by pharmacological blockade of the RAS either at levels of ACE or the AT receptors and are accompanied by downregulation of VEGF and VEGFR-2 (40). Recently, Kim et al. (42) have shown that perindopril (an ACE inhibitor) attenuates VEGF-mediated BRB breakdown in rats with streptozotocin-induced diabetes. In addition, it is also worthy of mention that candesartan inhibited retinal accumulation of the advanced glycation end product pentosidine in spontaneously diabetic Torii rats (43). Apart from reducing microvascular disease, there is growing evidence pointing to neuroprotection as a relevant mechanism involved in the beneficial effects of angiotensin receptor blockers in diabetic retinopathy (44–46).

On these experimental bases, it would be reasonable to postulate that RAS blockade can promote higher beneficial effects in diabetic retinopathy than other antihypertensive agents. However, studies in type 2 diabetic patients with hypertension suggest that ACE inhibitors and angiotensin receptor blockers are not superior in preventing or arresting diabetic retinopathy to other drugs equally effective in reducing blood pressure such as the β-blocker atenolol (47) or calcium channel blocker nisoldipine (48). These prospective randomized studies suggest that lowering blood pressure seems to be much more important than the potential effect of RAS blockade in the diabetic eye. However, the question concerning the potential effect of RAS blockers in normotensive diabetic patients remains to be elucidated. In the EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus (EUCLID), it was reported that in normotensive patients (blood pressure ≤140/90 mmHg), either normoalbuminutic (85% of patients) or microalbuminuric, lisinopril (an ACE inhibitor) had no effect in reducing the incidence of diabetic retinopathy but decreased its progression by two or more grades and decreased the progression to PDR (49). However, these results have been criticized because the placebo group had significantly higher levels of mean A1C than the treatment group. In fact, after adjusting for A1C, the observed differences in progression by two levels and progression to PDR disappear and only the progression by one level remained significant. Other limiting factors of this study were the short period of follow-up (2 years) and the fact that diabetic retinopathy was not the primary end point of the study. Therefore, although the EUCLID study supported the idea of an additional benefit of ACE inhibitors on diabetic retinopathy progression, it was underpowered for the eye-related outcome measures used. Furthermore, in the normotensive type 2 diabetic patients of the Appropriate Blood Pressure Control in Diabetes (ABC) study, Schrier et al. (50) showed that intensive blood pressure control decreased the progression of diabetic retinopathy. However, the results were the same whether enalapril or nisoldipine was used as the initial antihypertensive agent. Therefore, the specific antihypertensive agent again appears to be less important than the achievement of the lower blood pressure values.

The Diabetic Retinopathy Candesartan Trials (DIRECT) program was therefore designed to answer the question of whether the blockade of RAS with AT1-R blocker candesartan could prevent the incidence and progression of retinopathy in type 1 and type 2 diabetes independent of lowering blood pressure (11,12). This program consisted of three randomized double-blind placebo-controlled parallel-group studies: *1*) a primary prevention study involving 1,241 type 1 diabetic patients without diabetic retinopathy (DIRECT-Prevent 1), 2) a secondary prevention study involving 1,905 type 1 diabetic patients with diabetic retinopathy (DIRECT-Protect 1), and 3) a secondary prevention study involving 1,905 type 2 diabetic patients with diabetic retinopathy (DIRECT-Protect 2). In each trial, patients were randomized to receive candesartan (16–32 mg/day) or placebo and the median follow-up was 4.7 years. Patients with type 1 diabetes were eligible for inclusion if they were normoalbuminuric and normotensive (blood pressure ≤130/85 mmHg). For patients with type 2 diabetes, the inclusion criteria were normoalbuminuria and either normal blood pressure without antihypertensive therapy or blood pressure ≤160/90 mmHg during treatment. The primary end point was the incidence of diabetic retinopathy in the primary prevention study and progression of diabetic retinopathy in the secondary prevention studies. In the DIRECT-Prevent 1 study, a nonsignificant reduction (18% relative risk reduction; $P = 0.051$) in the risk of incidence of diabetic retinopathy was observed. However, in a post hoc analysis in which the primary end point was changed from a two-step increase to at least a three-step increase in the ETDRS scale, a significant difference was detected (35% relative risk reduction; $P = 0.003$). This beneficial effect was attenuated but still significant af-

ter the data were adjusted for duration of diabetes, A1C, and systolic blood pressure (26% relative risk reduction; $P = 0.046$) (11). In DIRECT-Protect 1, an identical progression of diabetic retinopathy was found in the placebo and in the candesartan groups, thus suggesting that candesartan is not effective in preventing diabetic retinopathy progression (11). DIRECT-Protect 2 showed a nonsignificant reduction in the progression of diabetic retinopathy (13% relative risk; $P = 0.20$). However, a significant increase in diabetic retinopathy regression was observed (34%, $P = 0.009$), this effect being more evident in patients with mild retinopathy (12). Thus, although the prespecified primary end point was not reached in the DIRECT program, data analysis suggests an overall beneficial effect of candesartan in diabetic retinopathy.

The DIRECT results should be compared with the Action in Diabetes and Vascular Disease (ADVANCE) study, which included 11,140 type 2 diabetic patients (51). In this study, patients randomized to intensive glucose control with glicazide (modified release), as well as other drugs required to achieve A1C ≤6.5% and an ACE inhibitor–diuretic combination (perindopril-indapamide), presented the same 4-year incidence or progression of diabetic retinopathy as the placebo group. These results suggest the possibility that candesartan but not ACE inhibitors might have beneficial effects in diabetic retinopathy. However, it should be noted that unlike DIRECT, ADVANCE did not use standardized retinal photography and there was a lower rate of progression of diabetic retinopathy, thus limiting the power of the study to detect any moderate effects of intervention on microvascular eye disease.

INTRAVITREAL ANTI-VEGF AGENTS — VEGF has been identified as having a major role in the genesis of diabetic retinopathy, with increased levels in animals with experimental diabetes and in the vitreous of patients with diabetic retinopathy. Intravitreal VEGF administration in experimental animals duplicates many features of diabetic retinopathy. Thus, agents that attenuate VEGF action are very attractive because they are able to reduce permeability and neovascularization, the hallmarks of DME and PDR, respectively (4,52).

In general, systemically administered drugs reach the retinochoroidal tissue via blood circulation. However, because the BRB limits the influx of drugs into the retina, large amounts of the drug must be administered to maintain therapeutic concentrations. Regarding anti-VEGF agents, this would lead to systemic inhibition of angiogenesis, which could compromise critical vascular response to ischemic events in diabetic patients with cardiovascular, cerebrovascular, or peripheral vascular disease. Moreover, hypertension and proteinuria (two surrogate markers of systemic VEGF inhibition) as well as the impairment of wound healing are other potential consequences of blocking VEGF and would be particularly worrying to the diabetic population (14). By contrast, the local administration of anti-VEGF agents into the eye by means of intravitreal injections would avoid systemic adverse effects. However, this is invasive and a skilled specialist is required. In addition, in order to maintain effective levels, frequently repeated injections would be necessary, thus increasing local complications such as endophthalmitis, vitreous hemorrhage, retinal detachment, and traumatic cataract. Furthermore, although the eye is thought of as a closed and self-contained system, anti-VEGF drugs injected into the vitreous cavity pass into systemic circulation to varying degrees and could potentially cause the systemic adverse effects mentioned previously (14,52). At present four anti-VEGF agents are available: pegaptamib sodium (macugen; Pfizer), ranibizumab (lucentis; Genentech/Novartis), bevacizumab (avastin; Genentech), and aflibercept (Regeneron Pharmaceuticals/sanofi-aventis).

Pegaptanib is a PEGylated (i.e., conjugated to polyethylene glycol) neutralizing RNA aptamer with an extremely high affinity for isoform 165 of VEGF ($VEGF_{165}$), which is the isoform that participates in pathological but not physiological neovascularization (53). Aptamers are modified nucleotides composed of single-stranded nucleic acids that adopt a specific three-dimensional conformation, allowing them to bind with high specificity and affinity to molecular targets in a manner similar to that of monoclonal antibodies. An important feature of aptamers is that they do not exhibit immunogenicity. Pegaptamib was approved by the U.S. Food and Drug Administration (FDA) for treatment of exudative (wet or neovascular) age-related macular disease (AMD) in December 2004.

Ranibimizumab is a full-length monoclonal antibody directed against VEGF. In contrast to pegaptamib, ranimizumab inhibits the biological activity of all isoforms of human VEGF and could be immunogenic. The FDA approved ranibizumab for wet AMD in June 2006.

Bevacizumab is an anti-VEGF agent similar to ranibizumab and was approved by the FDA in February 2004 for the treatment of disseminated colorectal cancer but not licensed for intraocular use. Nevertheless, intravitreal injection of bevacizumab has become a current off-label treatment by ophthalmologists for neovascular AMD because although it seems to be as effective as pegaptamib or ranimizumab, it is much cheaper.

Aflibercept, also known as a VEGF Trap-Eye because of its ability to block all six VEGF proteins (VEGF-A to VEGF-E as well as placental growth factor), is a fusion protein comprised of segments of the extracellular domains of human VEGF receptors 1 (VEGFR1) and 2 (VEGFR2) fused to the constant region (Fc) of human IgG. Afilbercept is currently being used in clinical trials for both exudative AMD and DME. Aflibercept has a higher binding affinity than other anti-VEGF agents. This higher binding affinity translates into greater activity at lower biological levels and, consequently, a longer duration of action.

The results of prospective clinical trials using pegaptanib and ranibizumab in patients with AMD have been very impressive and have led to the design of specific trials for DME and PDR. At present, only a prospective double-blind multicenter dose-ranging controlled trial has been reported in diabetic patients (54). In this study 172 patients with DME were included, and the patients randomized to receive repeated intravitreal pegaptamib showed better visual outcomes ($P = 0.03$), were more likely to show a reduction in retinal thickness ($P = 0.02$), and needed less additional focal laser ($P = 0.04$) at follow-up (36 weeks) than patients who received intravitreal sham injections. Retrospective data analysis of the eyes of 16 patients with PDR also showed regression of neovascularization (55).

Uncontrolled studies using ranibizumab and bevacizumab have also found a rapid regression of retinal neovascularization, improvement of visual acuity, and decrease of retinal thickness in DME, even in nonresponders to conventional treatment (14,56). However, the response to treatment of DME by VEGF blockade is

not prolonged and is subject to significant variability. This is in distinct contrast to the rapid response of those with both iris and retinal neovascularization in PDR and of those with choroidal neovascularization in wet AMD (57). Interestingly, when the outcomes of intravitreal bevacizumab treatment of DME were compared with those of intravitreal cortisone (triamcinolone acetonide), better outcomes in terms of reduction of foveal thickness and visual results were found with triamcinolone (58). The extent to which VEGF blockade is beneficial for DME is currently being investigated in prospective clinical trials. Apart from their potential as isolated treatments for PDR and DME, intravitreal anti-VEGF agents, in particular bevacizumab, have been shown to be useful in increasing the short-term response to panretinal photocoagulation in high-risk PDR and also seem to be efficacious and safe as an adjuvant treatment to vitrectomy in severe PDR or vitreous hemorrhage (56). This is because intravitreal anti-VEGF agents reduce active neovascularization and vitreous hemorrhage, thus allowing a safe and efficient panretinal photocoagulation or pars plana vitrectomy to be performed while minimizing the risk of complications. Aflibercept has been recently tested in an exploratory study performed in five patients with DME (59). In this study, using a single intravitreal injection, Trap-Eye was well tolerated and preliminary evidence of bioactivity was detected. Taken together, these promising results present a new scenario in the management of diabetic retinopathy. Nevertheless, larger studies investigating not only the effectiveness but also the systemic adverse effects of these agents in the diabetic population are still needed.

It is possible that a drug with more extensive and nonspecific anti-VEGF activity, such as pan-VEGF inhibitors (ranibizumab, bevacizumab, and aflibercept), could be more effective than a drug such as pegaptamib that selectively targets $VEGF_{165}$. In this regard, pegaptamib is substantially less effective than ranibizumab in AMD treatment. By contrast, given that $VEGF_{165}$ plays an essential role in pathological but not physiological neovascularization, pegaptanib could be the best option for avoiding systemic adverse effects in diabetic patients. In addition, long-term intravitreous injections of pan-VEGF inhibitors could lead to retinal neurodegeneration and an increased risk of circulation disturbances in the choriocapillaris (60). However, the theoretical advantage of selective blocking of $VEGF_{165}$ by pegaptamib in terms of both systemic and local side effects remains to be demonstrated in head-to-head clinical trials.

CONCLUDING REMARKS AND FUTURE RESEARCH — Tight control of blood glucose levels and hypertension remains the key element for preventing or arresting diabetic retinopathy. However, two drugs (fenofibrate and candesartan), originally not designed for treatment of diabetic retinopathy, have become new adjuncts in its management. The information drawn from clinical trials indicates that in normotensive diabetic patients, candesartan reduces the incidence of diabetic retinopathy in those with type 1 diabetes and favors diabetic retinopathy regression only in type 2 diabetic patients with mild retinopathy. By contrast, fenofibrate, which has only been tested in type 2 diabetes, has no effect on the incidence of diabetic retinopathy. However, it reduces the progression of existing diabetic retinopathy, thus lessening the need for laser treatment in both DME and PDR, and this beneficial effect is unrelated to changes in serum lipids. Therefore, it would be reasonable to recommend candesartan for type 1 diabetic patients (with or without hypertension) at high risk to develop diabetic retinopathy and for type 2 diabetic patients with mild retinopathy, whereas fenofibrate seems to be a good option for type 2 diabetic patients (with or without dyslipemia) with a wide range of diabetic retinopathy stages (from mild to severe nonproliferative diabetic retinopathy). In addition, the benefit on diabetic retinopathy shown by fenofibrate and candesartan should be considered an extra value when treating dyslipemia and hypertension in diabetic patients. Nevertheless, the mechanisms by which candesartan and, in particular, fenofibrate exert their reported benefits need to be elucidated before these drugs can be launched (alone or in combination) as new tools in the management of diabetic retinopathy. Another question needing specific research is whether such treatments could be administered topically and directly into the eye in order to increase the benefits in diabetic retinopathy.

In advanced stages of diabetic retinopathy, intravitreal delivery of anti-VEGF agents are currently used by many ophthalmologists despite the lack of phase 3 studies supporting their effectiveness and safety. This is due to the successful results obtained in wet AMD and the promising preliminary data in diabetic retinopathy. Intravitreal injection permits antiangiogenic drugs to effectively reach the retina and theoretically overcomes the problem of the systemic blockade of angiogenesis. However, this is an invasive procedure that can have complications such as endophthalmitis and retinal detachment and could even have deleterious effects for the remaining healthy retina. This is especially important in diabetic patients for whom long-term administration is expected. Apart from local side effects, anti-VEGF agents could also produce systemic complications because of their capacity to pass into systemic circulation. The effectiveness and safety of intravitreal anti-VEGF agents are being evaluated in several clinical trials. Meanwhile, in order to minimize systemic adverse effects, it seems reasonable to avoid long-term treatment with anti-VEGF agents for patients with hypertension, proteinuria, renal failure, cardiovascular disease, and foot lesions with wound healing impairment.

A future scenario will involve using a combination of anti-VEGF agents and laser photocoagulation or combining antiangiogenic agents aimed at different steps of angiogenic cascade. This would probably be more successful than single-molecule–specific approaches, would permit a decrease in the frequency of dosing, and would reduce adverse effects. Although it is premature at this stage to advocate such maneuvers, these aspects are certainly worth pursuing in future studies because they may suggest attractive new strategies for improving the treatment of diabetic retinopathy. However, it should be emphasized that, at present, the milestones in diabetic retinopathy treatment are the optimization of blood glucose levels, lowering of blood pressure, and regular fundoscopic screening.

In summary fenofibrate, candesartan, and anti-VEGF agents are now in the armamentarium for diabetic retinopathy treatment. Ophthalmologists and physicians treating diabetic patients should be aware of the potential usefulness of these drugs and work together not only in future research but also in establishing clinical guidelines that will include these newer medical treatments for diabetic retinopathy. Only such coordinated action, as well as competent strategies targeting prevention, will be effective in reducing

the burden and improving the clinical outcome of this devastating complication of diabetes.

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References

1. Congdon NG, Friedman DS, Lietman T. Important causes of visual impairment in the world today. JAMA 2003;290:2057–2060
2. Lightman S, Towler HM. Diabetic retinopathy. Clin Cornerstone 2003;5:12–21
3. Tong L, Vernon SA, Kiel W, Sung V, Orr GM. Association of macular involvement with proliferative retinopathy in type 2 diabetes. Diabet Med 2001;18:388–394
4. Simó R, Carrasco E, García-Ramírez M, Hernández C. Angiogenic and antiangiogenic factors in proliferative diabetic retinopathy. Curr Diabet Rev 2006;2:71–98
5. Joussen A, Smyth N, Niessen C. Pathophysiology of diabetic macular edema. Dev Ophthalmol 2007;39:1–12
6. Mohamed Q, Gillies MC, Wong TY. Management of diabetic retinopathy: a systematic review. JAMA 2007;298:902–916
7. Diabetic Retinopathy Clinical Research Network. A randomized trial comparing intravitreal triamcionolone acetonide and focal/grid photocoagulation for diabetic macular edema. Ophthalmology 2008;115:1447–1449
8. Diabetic Retinopathy Clinical Research Network (DRCR.net), Beck RW, Edwards AR, Aiello LP, Bressler NM, Ferris F, Glassman AR, Hartnett E, Ip MS, Kim JE, Kollman C. Three-year follow-up of a randomized trial comparing focal/grid photocoagulation and intravitreal triamcinolone for diabetic macular edema. Arch Ophthalmol 2009;127:245–251
9. Wong TY, Liew G, Tapp RJ, Schmidt MI, Wang JJ, Mitchell P, Klein R, Klein BE, Zimmet P, Shaw J. Relation between fasting glucose and retinopathy for diagnosis of diabetes: three population-based cross-sectional studies. Lancet 2008;371:736–743
10. Keech AC, Mitchell P, Summanen PA, O'Day J, Davis TM, Moffitt MS, Taskinen MR, Simes RJ, Tse D, Williamson E, Merrifield A, Laatikainen LT, d'Emden MC, Crimet DC, O'Connell RL, Colman PG, FIELD Study Investigators. Effect of fenofibrate on the need for laser treatment for diabetic retinopathy (FIELD study): a randomised controlled trial. Lancet 2007;370:1687–1697
11. Chaturvedi N, Porta M, Klein R, Orchard T, Fuller J, Parving HH, Bilous R, Sjølie AK, DIRECT Programme Study Group. Effect of candesartan on prevention (DIRECT-Prevent 1) and progression (DIRECT-Protect 1) of retinopathy in type 1 diabetes: randomised, placebo-controlled trials. Lancet 2008;372:1394–402
12. Sjølie AK, Klein R, Porta M, Orchard T, Fuller J, Parving HH, Bilous R, Chaturvedi N, DIRECT Programme Study Group. Effect of candesartan on progression and regression of retinopathy in type 2 diabetes (DIRECT-Protect 2): a randomised placebo-controlled trial. Lancet 2008;372:1385–1393
13. Kaiser PK. Antivascular endothelial growth factor agents and their development: therapeutic implications in ocular disesases. Am J Ophthalmol 2006;142:660–668
14. Simó R, Hernández C. Intravitreous anti-VEGF for diabetic retinopathy: hopes and fears for a new therapeutic strategy. Diabetologia 2008;51:1574–1580
15. Rosenson RS. Fenofibrate: treatment of hyperlipidemia and beyond. Expert Rev Cardiovasc Ther 2008;6:1319–1330
16. Simó R, Hernández C. Fenofibrate for diabetic retinopathy. Lancet 2007;370:1667–1668
17. Cheung N, Wong TY. Fenofibrate and diabetic retinopathy. Lancet 2008;371:721–722
18. Miljanovic B, Glynn RJ, Nathan DM, Manson JE, Shaumberd DA. A prospective study of serum lipids and risk of diabetic macular edema in type 1 diabetes. Diabetes 2004;5:2883–2892
19. Leung H, Wang JJ, Rochtchina E, Wong TY, Klein R, Mitchell P. Dyslipidaemia and microvascular disease in the retina. Eye 2005;19:861–868
20. Colhoun HM, Betteridge DJ, Durrington PN, Hitman GA, Neil HA, Livingstone SJ, Thomason MJ, Mackness MI, Charlton-Menys V, Fuller JH, CARDS Investigators. Primary prevention of cardiovascular disease with atorvastatin in type 2 diabetes in the Collaborative Atorvastatin Diabetes Study (CARDS): multicentre randomised placebo-controlled trial. Lancet 2004;364:685–696
21. Chew EY, Ambrosius WT, Howard LT, Greven CM, Johnson S, Danis RP, Davis MD, Genuth S, Domanski M, ACCORD Study Group. Rationale, design, and methods of the Action to Control Cardiovascular Risk in Diabetes Eye Study (ACCORD-EYE). Am J Cardiol 2007;99:103i–111i
22. Simó R, García-Ramírez M, Higuera M, Hernández C. Apolipoprotein A1 is overexpressed in the retina of diabetic patients. Am J Ophthalmol 2009;147:319–325
23. Staels B, Dallongeville J, Auwerx J, Schoojans K, Leitersdorf E, Fruchart JC. Mechanisms of action of fibrates on lipid and lipoprotein metabolism. Circulation 1998;98:2088–2093
24. Arakawa R, Tamehiro N, Nishimaki-Mogami T, Ueda K, Yokoyama S. Fenofibric acid, an active form of fenofibrate, increases apolipoprotein A-I–mediated high-density lipoprotein biogenesis by enhancing transcription of ATP-binding cassette transporter A1 gene in a liver X receptor–dependent manner. Arterioscler Thromb Vasc Biol 2005;25:1193–1197
25. Inoue I, Shino K, Noji S, Awata T, Katayama S. Expression of peroxisome proliferator-activated receptor α (PPARα) in primary cultures of human vascular endothelial cells. Biochem Bioph Res Co 1998;246:370–374
26. Meissner M, Stein M, Urbich C, Reisinger K, Suske G, Staels B, Kaufmann R, Gille J. PPARα activators inhibit vascular endothelial growth factor receptor-2 expression by repressing Sp1-dependent DNA binding and transactivation. Cir Res 2004;94:324–332
27. Varet J, Vincent L, Mirshahi P, Pille JV, Legrand E, Opolon P, Mishal Z, Soria J, Li H, Soria C. Fenofibrate inhibits angiogenesis in vitro and in vivo. Cell Mol Life Sci 2003;60:810–819
28. Yokoyama Y, Xin B, Shigeto T, Umemoto M, Kasai-Sakamoto A, Futagami M, Tsuchida S, Al-Mulla F, Mizunuma H. Clofibric acid, a peroxisome proliferator-activated receptor α, inhibits growth of human ovarian cancer. Mol Cancer Ther 2007;6:1379–1386
29. Zanetti M, Stocca A, Dapas B, Farra R, Uxa L, Bosutti A, Barazzoni R, Bossi F, Giansante C, Tedesco F, Cattin L, Guarnieri G, Grassi G. Inhibitory effects of fenofibrate on apoptosis and cell proliferation in human endothelial cells in high glucose. J Mol Med 2008;86:185–195
30. Kim J, Ahn JH, Kim JH, Yu YS, Kim HS, Ha J, Shinn SH, Oh YS. Fenofibrate regulates retinal endothelial cell survival through the AMPK signal transduction pathway. Exp Eye Res 2007;84:886–893
31. Murakami H, Murakami R, Kambe F, Cao X, Takahashi R, Asai T, Hirai T, Numaguchi Y, Okumura K, Seo H, Murohara T. Fenofibrate activates AMPK and increases eNOS phosphorylation in HUVEC. Biochem Biophys Res Commun 2006;34:973–978
32. Chen XR, Besson VC, Palmier B, Garcia Y, Plotkine M, Marchand-Leroux C. Neurological recovery-promoting, anti-inflammatory, and anti-oxidative effects afforded by fenofibrate, a PPAR α agonist, in traumatic brain injury. J Neurotrauma 2007;24:1119–1131

33. Bordet R, Ouk T, Petrault O, Gelé P, Gautier S, Laprais M, Deplanque D, Duriez P, Staels B, Fruchart JC, Bastide M. PPAR: a new pharmacological target for neuroprotection in stroke and neurodegenerative diseases. Biochem Soc Trans 2006;34: 1341–1346
34. Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, Fruchart JC, Chapman J, Najib J, Staels B. Activation of proliferator-activated receptors α and γ induces apoptosis of human monocyte-derived macrophages. J Biol Chem 1998; 273:25573–25580
35. Israelian-Konaraki Z, Reaven PD. Peroxisome proliferator-activated receptor-alpha and atherosclerosis: from basic mechanisms to clinical implications. Cardiol Rev 2005;13:240–246
36. Deplanque D, Gelé P, Pétrault O, Six I, Furman C, Bouly M, Nion S, Dupuis B, Leys D, Fruchart JC, Cecchelli R, Staels B, Duriez P, Bordet R. Peroxisome proliferator-activated receptor-alpha activation as a mechanism of preventive neuroprotection induced by chronic fenofibrate treatment. J Neurosci 2003;23:6264–6271
37. Antonetti DA, Barber AJ, Bronson SK, Freeman WM, Gardner TW, Jeffersson LS, Kester M, Kimball SR, Krady JK, LaNoue KF, Norbury CC, Quinn PG, Sandirasegrane L, Simpson IA, JDRF Diabetic Retinopathy Center Group. Diabetic retinopathy: seeing beyond glucose-induced microvascular disease. Diabetes 2006;55: 2401–2411
38. Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993;329:977–986
39. UK Prospective Diabetes Study Group. Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes: UKDPS 38. BMJ 1998;317:703–713
40. Wilkinson-Berka JL. Angiotensin and diabetic retinopathy. Int J Biochem Cell Biol 2006;38:752–765
41. Amano S, Yamagishi S, Inagaki Y, Okamoto T. Angiotensin II stimulates platelet-derived growth factor-B gene expression in cultured retinal pericytes through intracellular reactive oxygen species generation. Int J Tissue React 2003;25:51–55
42. Kim JH, Kim JH, Yu YS, Cho CS, Kim KW. Blockade of AT attenuates VEGF-mediated blood-retinal barrier breakdown in diabetic retinopathy. J Cereb Blood Flow Metab 2009;29:621–628
43. Sugiyama T, Okuno T, Fukuhara M, Oku H, Ikeda T, Obayashi H, Ohta M, Fukui M, Hasegawa G, Nakamura N. Angiotensin II receptor blocker inhibits abnormal accumulation of advanced glycation end products and retinal damage in a rat model of type 2 diabetes. Exp Eye Res 2007;85:406–412
44. Krikov M, Thone-Reineke C, Müller S, Villringer A, Unger T. Candesartan but not ramipril pretreatment improves outcome after stroke and stimulates neurotrophin BNDF/TrkB system in rats. J Hypertens 2008;26:544–552
45. Kurihara T, Ozawa Y, Nagai N, Shinoda K, Noda K, Imamura Y, Tsubota K, Okano H, Oike Y, Ishida S. Angiotensin II type 1 receptor signaling contributes to synaptophysin degradation and neuronal dysfunction in the diabetic retina. Diabetes 2008;57:2191–2198
46. Silva KC, Rosales MA, Biswas SK, Lopes de Faria JB, Lopes de Faria JM. Diabetic retinal neurodegeneration is associated with mitochondrial oxidative stress and is improved by an angiotensin receptor blocker in a model combining hypertension and diabetes. Diabetes 2009;58: 1382–1390
47. UKDPS Study Group. Efficacy of atenolol and captopril in reducing risk of macrovascular and microvascular complications in type 2 diabetes: UKDPS 39. UK Prospective Diabetes Study Group. BMJ 1998;317:713–720
48. Estacio RO, Jeffers BJ, Guifford N, Schrier RW. Effect of blood pressure control on diabetic microvascular complications in patients with hypertension and type 2 diabetes. Diabetes Care 2000;23(Suppl.2): B54–B64
49. Chaturvedi N, Sjolie AK, Stephenson JM, Abrahamian H, Keipes M, Castellarin A, Rogulja-Pepeonik Z, Fuller JH. Effect of lisinopril on progression of retinopathy in normotensive people with type 1 diabetes: the EUCLID Study Group EURODIAB controlled trial of lisinopril in insulin-dependent diabetes mellitus. Lancet 1998;35:28–31
50. Schrier RW, Estacio RO, Esler A, Mehler P. Effects of aggressive blood pressure control in normotensive type 2 diabetic patients on albuminuria, retinopathy and strokes. Kidney Int 2002;61:1086–1097
51. ADVANCE Collaborative Group, Patel A, MacMahon S, Chalmers J, Neal B, Billot L, Woodward M, Marre M, Cooper M, Glasziou P, Grobbee D, Hamet P, Harrap S, Heller S, Liu L, Mancia G, Mogensen CE, Pan C, Poulter N, Rodgers A, Williams B, Bompoint S, de Galan BE, Joshi R, Travert F. Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. N Engl J Med 2008;358:2560–2572
52. Wirostko B, Wong TY, Simó R. Vascular endothelial growth factor and diabetic complications. Prog Retin Eye Res 2008;27:608–621
53. Ishida S, Usui T, Yamashiro K, Kaji Y, Amano S, Ogura Y, Hida T, Oguchi Y, Ambati J, Miller JW, Gragoudas ES, Ng YS, D'Amore PA, Shima DT, Adamis AP. VEGF164-mediated inflammation is required for pathological, but not physiological, ischemia-induced retinal neovascularization. J Exp Med 2003;198: 483–489
54. Cunningham ET Jr, Adamis AP, Altaweel M, Aiello LP, Bressler NM, D'Amico DJ, Goldbaum M, Guyer DR, Katz B, Patel M, Schwartz SD, Macugen Diabetic Retinopathy Study Group. A phase II randomized double-masked trial of pegaptanib, an anti-vascular endothelial growth factor aptamer, for diabetic macular edema. Ophthalmology 2005;112:1747–1757
55. Adamis AP, Altaweel M, Bressler NM, Cunningham ET Jr, Davis MD, Goldbaum M, Gonzales C, Guyer DR, Barrett K, Patel M, Macugen Diabetic Retinopathy Study Group. Changes in retinal neovascularization after pegaptamib (Macugen) therapy in diabetic individuals. Ophthalmology 2006;113:23–28
56. Arevalo JF, Garcia-Amaris RA. Intravitreal bevacizumab for diabetic retinopathy. Curr Diabetes Rev 2009;5:39–46
57. Sang DN, D'Amore PA. Is blockade of vascular endothelial growth factor beneficial for all types of diabetic retinopathy? Diabetologia 2008;51:1570–1573
58. Shimura M, Nakazawa T, Yasuda K, Shiono T, Iida T, Sakamoto T, Nishida K. Comparative therapy evaluation of intravitreal bevacizumab and triamcinolone acetonide on persistent diffuse diabetic macular edema. Am J Ophthalmol 2008; 145:854–861
59. Do DV, Nguyen QD, Shah SM, Browning DJ, Haller JA, Chu K, Yang K, Cedarbaum JM, Vitti RL, Ingerman A, Campochiaro PA. An exploratory study of the safety, tolerability and bioactivity of a single intravitreal injection of vascular endothelial growth factor Trap-Eye in patients with diabetic macular oedema. Br J Ophthalmol 2009;93:144–149
60. D'Amore PA. Vascular endothelial cell growth factor-a: not just for endothelial cells anymore. Am J Pathol 2007;171: 14–18

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REVIEW

Retinal vein occlusion: pathophysiology and treatment options

Niral Karia

Department of Ophthalmology, Southend Hospital, Prittlewell Chase, Westcliff on Sea, Essex, United Kingdom

Abstract: This paper reviews the current thinking about retinal vein occlusion. It gives an overview of its pathophysiology and discusses the evidence behind the various established and emerging treatment paradigms.

Keywords: central, hemispheric, branch, retinal vein occlusion, visual loss

Introduction

Retinal vein occlusion (RVO) is the most common retinal vascular disease after diabetic retinopathy.[1] Depending on the area of retinal venous drainage effectively occluded it is broadly classified as either central retinal vein occlusion (CRVO), hemispheric retinal vein occlusion (HRVO), or branch retinal vein occlusion (BRVO). Hayreh observed that each of these has two subtypes.[2] The former two can be subdivided into ischemic and nonischemic CRVO or HRVO, with each having distinct clinical features and prognosis. A number of parameters can be used to assess the degree of ischemia such as the degree of visual loss, presence of a relative afferent pupillary defect, extent of retinal capillary nonperfusion on fluorescein angiography, and electrodiagnostics showing reduced b wave amplitude, reduced b:a ratio and prolonged b-wave implicit time.

BRVO can be considered a major BRVO where a quarter or more of the retina is affected or a macular BRVO where only part of the macular is affected.

Presentation of RVO in general is with variable painless visual loss with any combination of fundal findings consisting of retinal vascular tortuosity, retinal hemorrhages (blot and flame shaped), cotton wool spots, optic disc swelling and macular edema. In a CRVO, retinal hemorrhages will be found in all four quadrants of the fundus, whilst these are restricted to either the superior or inferior fundal hemisphere in a HRVO. In a BRVO, hemorrhages are largely localized to the area drained by the occluded branch retinal vein. Vision loss occurs secondary to macular edema or ischemia.

Epidemiology

The true incidence of RVO in a population as a whole is difficult to establish, as many RVOs are silent where the condition is mild, the patient is asymptomatic, and it is only detected incidentally. However, longitudinal population based studies have helped in providing an estimate of this incidence. The Blue Mountains Eye Study[1] found that the 10-year cumulative incidence of RVO was 1.6% and was significantly associated with increasing age, especially over the age of 70 years. However there was

Correspondence: Niral Karia
Department of Ophthalmology, Southend Hospital, Prittlewell Chase, Westcliff on Sea, Essex SS00RY, United Kingdom
Tel +44 1702 435 555
Fax +44 1702 385 877
Email niral.karia@southend.nhs.uk

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no predilection for gender or race.[3] The Beaver Dam Eye Study[4] reported a 15-year cumulative incidence of CRVO of 0.5%. For a BRVO this was approximately three times more at 1.8%. Applying this to United Nations projected UK population figures for 2010 gives approximately 47,000 new cases annually.[5] This figure is greater than 150,000 for the United States.[6] Rogers et al[7] carried out a pooled analysis of population based studies from the United States, Europe, Asia, and Australia and projected that approximately 16 million people worldwide may have RVO in at least one eye worldwide. The pooled data showed a higher prevalence of BRVO in Asians and Hispanics compared to whites, although this was not statistically significant, and there was no gender predilection. Whilst less common, it is now generally accepted that (idiopathic) RVO does also occur in the younger (under 50 years) age group, where CRVO tends to be more of the nonischemic type.[2]

Etiology

Although the exact etiology of RVO remains elusive, it is likely to follow a thrombotic event. In CRVO this may occur in the central retinal vein (CRV) at the lamina cribrosa[8] or at a variable distance in its journey within the optic nerve posterior to the lamina cribrosa. A more posterior occlusion with a greater number of tributaries of the CRV anterior to the occlusion may allow greater scope for collateral flow to bypass the occluded section of the CRV.[2] In BRVO, arterial compression of the vein at arteriovenous crossings is thought to incite thrombus formation by causing turbulent flow in combination with pre-existing vascular endothelial damage secondary to systemic cardiovascular risk factors.

In trying to determine etiology or associated risk factors for RVO, comparison is naturally made to factors involved in the occurrence of systemic venous thrombosis (such as deep vein thrombosis). Whilst these two entities may share some common cardiovascular and systemic risk factors, it is also important to understand that they are otherwise quite separate entities requiring different management strategies and leading to different complications.[2]

Systemic vascular/atherosclerotic risk factors in RVO

Study design, patient characteristics, and risk factor definitions are seldom standardized across the various published papers in the literature. However accounting for this it remains probable that systemic hypertension is the strongest independent risk factor associated with all types of RVO[9–13] especially in the older (over 50 years) age group. Uncontrolled or newly diagnosed hypertension is common in this group, and recurrence of RVO in the same or fellow eye is also noted when hypertension is poorly controlled. In their meta-analysis of 21 studies, O'Mahoney et al[12] report a significant association between hypertension and both CRVO (pooled odds ratio [OR = 3.8] and BRVO [pooled OR 3.0]. Accepting an inconsistent definition of hyperlipidemia across studies they also found hyperlipidemia to be twice as common in RVO cases (both CRVO and BRVO) compared to controls (pooled OR 2.5). Cheung et al[3] also report hypertension and hyperlipidemia as independent risk factors for RVO. The association of diabetes mellitus with RVO is weaker and has not been found to be consistent across all studies.[12] Its association with CRVO may be stronger than with BRVO.[9,12,13]

Hematological disorders and other systemic conditions

Conditions that lead to increased blood viscosity such as myeloproliferative disorders are uncommon but known to be associated with CRVO. Similarly, a number of rare systemic inflammatory disorders causing systemic vasculitis (such as Behçet's disease and polyarteritis nodosa) also cause retinal vasculitis leading to RVO, especially in the younger age group. The cause and management of the RVO here is closely linked to the underlying systemic disease and its management.

Over recent years there has been great interest in the potential role of thrombophilia in the development of RVO and in particular CRVO. Thrombophilia refers to the propensity to develop thrombosis (usually venous) due to an abnormality in the coagulation system. This can be congenital (eg, Factor V Leiden, hyperhomocysteinemia or protein C, protein S and antithrombin deficiencies) or acquired (eg, antiphospholipid syndrome), and its importance is potentially greater in the younger age group. However Fegan's review on CRVO and thrombophilia[14] suggested that there was a lack of consistency between studies in showing a valid association between CRVO and protein C, protein S and antithrombin III deficiency, and factor V Leiden/activated protein C resistance. These natural anticoagulants are very labile with fluctuating physiological levels. It is recommended that they should be measured on at least two separate samples and if found abnormal confirmed with a third estimation. Most studies used single measurements and varying types of assays. The studies also lacked the statistical power to show a true difference either due to small sample size or lack of a suitable control group.

In the antiphospholipid syndrome (APS) antibodies to phospholipid activate the coagulation cascade leading to both arterial and venous thrombosis. Tests can be done to either detect the antibody (using the anticardiolipin antibody assay) or its effect on coagulation using a test for lupus anticoagulant. Up to 8% of patients with APS have ocular manifestations and 4 of 8 studies reviewed by Fegan[14] showed a significant association of APS in CRVO. Further studies are required to determine the strength of association between APS and RVO.

Homocysteine is a naturally occurring amino acid not found in protein. There are many causes for hyperhomocysteinemia (including rare enzyme deficiencies leading to homocystinuria) which predisposes to both arterial and venous thrombosis.[14] Several studies have questioned the validity of carrying out exhaustive tests for thrombophilia in RVO in the absence of a suggestive medical history. However their results have shown notable evidence of an association of hyperhomocysteinemia with CRVO sufficient to recommend the benefit of checking for hyperhomocysteinemia, which is correctable with folic acid and vitamins B6 and B12 supplements.[14–17]

On current evidence it would be reasonable to not recommend general thrombophilia screening for all patients with RVO, but to reserve it for older patients with a past history of thromboembolic events and in young patients without any other general risk factors.

Glaucoma/ocular hypertension

The association between RVO (CRVO in particular) and glaucoma/ocular hypertension has been widely reported[2,9,11,13,18] with the Eye Disease Case-Control Study[9] reporting an adjusted OR of 5.4 in CRVO for a history of glaucoma. The pathophysiology of this association is unclear, although deformation of the lamina cribrosa in glaucoma may distort the central retinal vein as it exits the eye.

Familial RVO

Familial clustering of RVO (CRVO in particular) has been reported[19,20] but these reports have been few in number. It is interesting that such cases are more often bilateral, with a younger age at onset than sporadic cases. More data from existing and future familial clusters is required to establish if there is a genetic cause in these cases.

Pathophysiology of RVO

It is the occurrence of macular edema in retinal vein occlusion that most frequently leads to visual loss. A working understanding of the pathogenesis of the macular edema may in turn allow an understanding of the mechanism of action of some of the therapies more recently advocated in retinal vein occlusion.

Thrombosis within a retinal vein as described earlier will lead to a partial obstruction of blood flow within the vein and from the eye. The subsequent increased intraluminal pressure, if sufficiently high, will cause transudation of blood products into the retina according to Starling's law. This will result in increased interstitial (retinal) fluid and protein. The latter will increase the interstitial oncotic pressure, perpetuating tissue edema, which will impede capillary perfusion and lead to ischemia. As stated by Campochiaro et al[21] this ischemia is not an all or none dichotomy, as those patients classified as nonischemic will still have varying degrees of retinal ischemia.

It is well recognized that inflammation affects the progression and outcome of vitreoretinal disease including retinal vein occlusion.[22] Yoshimura et al[22] have found significantly elevated vitreous levels of the soluble cytokines interleukin (IL) 6 and 8, monocyte chemoattractant protein-1, and vascular endothelial growth factor (VEGF) in RVO, and especially in CRVO. Funk et al[23] have also demonstrated elevated aqueous levels of these same factors in patients with CRVO when compared with control samples. The exact interaction of these factors remains speculative but an understanding of the roles that VEGF fulfils is increasing. It is induced by tissue hypoxia such as retinal ischemia and acts as an angiogenic and vasopermeable factor on endothelial cell membrane bound receptors with tyrosine kinase activity.[24] Ozaki et al[25] have demonstrated that the implantation of slow release pellets of human recombinant VEGF into the vitreous cavity of rabbits and primates leads to retinal vessel dilatation, breakdown of the blood retinal barrier and retinal new vessel formation. Noma et al have reported elevated aqueous and vitreous levels of VEGF and IL-6 in patients with BRVO[26,27] and CRVO,[28,29] compared to controls. The levels of VEGF and IL-6 correlated with both the severity of macular edema and extent of retinal ischemia (capillary nonperfusion).

It is likely that the sudden retinal ischemia that occurs in BRVO and more so in CRVO will induce excessive VEGF production. VEGF is produced by the retina from retinal pigment epithelial cells, endothelial cells, and Muller cells, as well as other types of ocular tissue.[22] Boyd et al found a close correlation between aqueous VEGF levels and the course of iris neovascularization and vascular permeability in patients with ischemic CRVO.[30] The excessive vascular permeability induced by VEGF will likely contribute to the

macular edema that also occurs according to Starling's law as described above. It is tempting to theorize that even if the primary venous obstruction was overcome (eg, via collateral formation), the macular edema can persist for much longer due to a self perpetuating cycle of VEGF-induced vascular permeability leading to macular edema, capillary damage, and retinal ischemia, stimulating further release of VEGF and other inflammatory cytokines leading to chronic macula edema.

Treatment

The Branch Retinal Vein Occlusion Study (BRVOS)[31,32] and the Central Retinal Vein Occlusion Study (CRVOS)[33,34] have established a standard of care by providing both an understanding of the natural history and treatment algorithms for BRVO and CRVO in managing neovascular complications and reducing visual loss. The studies were designed to answer specific questions and so have inherent limitations. Whilst many aspects of these studies may now arguably seem dated, some remain pertinent. In their review of studies evaluating the natural history of CRVO Rogers et al[35] confirm that eyes with CRVO had generally poor vision at presentation which declined further with time. They found that over a quarter of nonischemic CRVO converted to ischemic CRVO, of which a quarter developed neovascular glaucoma within 15 months. Similarly they reviewed studies evaluating the natural history of BRVO and reported a general improvement in vision over time without treatment, although improvement beyond 20/40 was uncommon.

Therapeutic options for CRVO

Mohamed et al[36] carried out a systematic review of randomized clinical trials (RCTs) evaluating interventions for the treatment of CRVO. Only results from the CRVOS[33,34] met the criteria for level 1 evidence. In patients with macular edema secondary to nonischemic CRVO with a vision of 20/50 or worse, macular grid laser photocoagulation does not improve visual acuity although the edema may improve. Additionally prophylactic pan retinal photocoagulation (PRP) in ischemic CRVO does not prevent iris or angle neovascularization and is therefore not recommended. PRP is recommended when anterior segment, disc or retinal neovascularization develop.

Mohamed et al[36] also evaluated studies reporting on hemodilution, medical treatment with troxerutin and ticlopidine (inhibitors of platelet aggregation) and intravenous thrombolysis, and various surgical procedures to improve vision in CRVO. By lowering the hematocrit, and thus the plasma viscosity, hemodilution is thought to improve the retinal microcirculation. However the variations in study protocols and the use of multiple agents in combination have prevented any conclusions to be drawn for this treatment modality. Similarly there is limited evidence to recommend the routine use of troxerutin or ticlopidine as well for intravenous thrombolysis, which carries the potential for serious adverse effects such as stroke. The reviews by Squizzato et al[37] and Lazo-Langner et al[38] suggest that antithrombotic therapy, with low molecular weight heparin (LMWH) in particular, may be efficacious in the treatment of acute RVO with superiority over antiplatelet agents such as aspirin. LMWH appear to have additional properties such as anti-angiogenic effects, which may explain their additional benefits compared to other agents. However the limited evidence available precludes any recommendations about the use of LMWH.

Following a vitrectomy approach, several surgical procedures including internal limiting membrane peel,[39] radial optic neurotomy,[40,41] and direct retinal vein cannulation with injection of fibrinolytics,[42,43] have all been advocated for the management of macular edema in CRVO. However the mechanism of action of these interventions remains contentious and their safety and efficacy have not been evaluated in RCTs. Furthermore carrying out a vitrectomy in itself is thought to improve retinal oxygenation, so confounding the possible effects of the other procedures. Mohamed et al therefore conclude that the routine use of these procedures cannot be recommended.

McAllister et al[44] have reported the outcome of the first prospective randomized multicenter trial comparing laser-induced chorioretinal venous anastomosis (L-CRA) with conventional treatment (observation) for CRVO. This technique utilized a high power (argon or Nd:YAG) laser spot to rupture Bruch's membrane and a second spot to rupture a major branch of the retinal vein next to the first laser spot, the intention being to enable an anastomosis to form between the retinal and choroidal circulation. They were able to create a L-CRA in 76.4% of patients in whom an attempt was made, leading to a significant reduction in the mean retinal fluorescein transit time at 18 months in the treatment group compared to the controls. A mean improvement of 3.6 letters was seen in the treatment group that developed a L-CRA at 18 months compared to a loss of 8.1 letters from baseline in the control group. Although fewer eyes converted to ischemic CRVO in the treatment group compared to controls, 18.2% of treated eyes developed choroidal neovascularization (CNV) at the treatment site necessitating sector PRP. It remains to be seen whether

L-CRA becomes widely employed as a treatment option for CRVO. Although the technique is relatively noninvasive and readily accessible it does have a significant learning curve and a high potential rate of complication from CNV.

Therapeutic options for BRVO

The BRVOS[31] evaluated whether grid macular laser photocoagulation improved visual acuity (VA) in patients with VA of 20/40 or worse resulting from macular edema secondary to BRVO following at least 3 months of observation. McIntosh et al[45] conducted a literature search to identify all relevant RCTs evaluating interventions for BRVO. They concluded that only the results of the BRVOS[31] met criteria for level 1 evidence – patients treated with grid macular laser gained an average of 1.33 lines at the third year study visit from baseline compared with 0.23 lines in the control group. The grid laser group had statistically significant improvements in VA compared to controls over consecutive visits. Arnarsson and Stefansson[46] have postulated that destruction of photoreceptors by grid laser leads to increased oxygen flux to the inner retina. An autoregulatory arteriolar constriction and increased resistance then leads to reduced hydrostatic pressure in capillaries and venules, leading to reduced edema with vessel constriction and shortening.

Accepting methodological limitations (such as small sample sizes with insufficient power, short follow up, and lack of a control group), McIntosh et al[45] also evaluated studies reporting other interventions including hemodilution, surgery involving pars plana vitrectomy and adventitial sheathotomy, and medical treatment with ticlopidine and troxerutin. They found that these studies lacked sufficient evidence to support the routine use of these other treatment modalities. Muqit et al[47] recently reported on the long term vascular perfusion following arteriovenous sheathotomy for BRVO. In their small series they found that long-term epiretinal gliosis and subfoveal photoreceptor atrophy limited the visual recovery.

Intravitreal corticosteroids

With increasing awareness of the role of VEGF and other inflammatory mediators, the use of off label intravitreal corticosteroids (triamcinolone acetonide in particular) has become routine in the management of RVO in spite of a paucity of RCTs. Small scale studies have reported a positive short/intermediate term efficacy of intravitreal triamcinolone (IVT)[48,49] but Patel et al[50] found that whilst IVT was effective in the short term in treating macular edema secondary to all types of RVO, its effectiveness was not maintained after 1 year despite repeated injections.

The exact mechanism of action of corticosteroids in the resolution of macular edema remains speculative. Miyamoto et al[51] describe cases where macular edema from RVO or diabetic maculopathy had begun to resolve within 1–6 hours of injecting IVT. They proposed that in addition to the recognized genomic pathway whereby receptor-glucocorticoid interaction is translocated to the nucleus leading to regulation of gene expression and taking many hours or days, there is also a nongenomic pathway. Here the receptor-glucocorticoid complex may act within the cytoplasm to destabilize mRNA, such as VEGF messengers, with rapid effects.

The Standard Care vs Corticosteroid for Retinal Vein Occlusion (SCORE) studies[52,53] reported RCT data on the efficacy of 1mg and 4 mg of a preservative free, nondispersive formulation of triamcinolone injected intravitreally. This was compared to the standard of care – observation for macular edema in CRVO[52] and grid laser photocoagulation for macular edema in BRVO.[53] Whilst the SCORE studies have several methodological limitations, as discussed by Apte in his editorial,[54] they provide important information that modifies the standard of care established by the BRVOS[31,32] and CRVOS.[33,34] The SCORE-BRVO study[53] reported that at the 12 month end point there were no significant differences in visual acuity between the laser treatment, 1 mg and 4 mg groups. The SCORE-CRVO study[52] however found that subjects in the 1 mg and 4 mg arms were five times more likely to show a gain in visual acuity of 15 letters or more at the 12 month end point compared to observation. Conversely, the study also showed that over three quarters of the eyes that received IVT did not show a gain in vision by 15 letters or more at 12 months and a quarter of treated eyes had a loss of vision of a similar magnitude. The studies also demonstrated a 3–4 times greater rate of intraocular pressure elevation in the IVT (especially 4mg) arms compared to standard of care, and this together with a lack of definitive data to 2 years follow up beckons further studies on IVT and other agents, to search for improved outcomes and better side effect profiles.

Ozurdex (Allergan, Irvine, CA),[6] a biodegradable intravitreal 700 µg dexamethasone implant, received FDA approval in June 2009 for the treatment of macular edema secondary to BRVO or CRVO. Phase III results presented[55] showed that significantly more patients gained 15 letters or more in the treatment group compared to sham up to 90 days following injection, but this effect waned at 180 days to become nonsignificant. The effects of a repeat injection at 6 months were less pronounced when assessed at 12 months. Although designed to cause less intraocular pressure problems than

triamcinolone, 25% of those treated with Ozurdex showed an intraocular pressure rise which peaked at day 60 and returned to baseline by day 180. The incidence of cataract progression was noted at 4% in the treatment group, but this increased to 26% after 1 year where a second injection of Ozurdex had been carried out.

Anti-VEGF treatment

The anti-VEGF bevacizumab (Avastin, Genetech), a humanized monoclonal antibody binding to all isoforms of VEGF-A, was first reported to show short term efficacy in the resolution of macular edema secondary to CRVO by Rosenfeld in 2005[56] and has since been widely used as an off label treatment in RVO. Prager et al[57] have reported a prospective case series of patients with macular edema due to RVO and treated with bevacizumab, showing a mean increase in visual acuity of 16 letters at the 12-month follow up. Subgroup analysis showed a better response in patients with BRVO rather than CRVO, although the reduction in central retinal thickness (CRT) on optical coherence tomography was comparable in both subgroups. This incongruence between functional and anatomical effects was also reported in the SCORE-CRVO study,[52] where the observation and IVT groups had a comparable reduction in CRT at the 12 month point although visual outcomes were significantly better in the IVT groups.

Ranibizumab (Lucentis; Genentech, San Francisco, CA), approved for the treatment of neovascular age related macular degeneration (n-AMD), is a monoclonal antibody fragment derived from the same parent murine antibody as bevacizumab. The six-month data from two phase III Genentech-sponsored studies (BRAVO studying the effects of BRVO and CRUISE studying the effects of CRVO) evaluating the safety and efficacy of Lucentis, compared to sham, for the treatment of macular edema in RVO, were presented at the Retina Congress 2009.[58,59] BRAVO reported a 7.6 and 7.4 mean letter gain in the 0.3 mg and 0.5 mg study arms of Lucentis respectively, compared to 1.9 letters gained in the sham injection arm. CRUISE reported an 8.8 and 9.3 mean letter gain in the 0.3 mg and 0.5 mg study arms of Lucentis, respectively, compared with 1.1 letters gained in the sham treatment arm. Both studies showed a safety profile consistent with data from previous phase III Lucentis trials for n-AMD. Horizon RVO, an extension trial, will provide much needed longer term data upon completion of BRAVO and CRUISE.

Conclusion

Studies on n-AMD show that intravitreal treatment is accepted and well tolerated by patients. Corticosteroids and anti-VEGF medication currently seem to be at the forefront of treatment options for RVO, but RCTs have yet to compare these directly. Corticosteroids can be given as a depot with activity over several months, but the high incidence of intraocular pressure rise and cataract make them less attractive. Intravitreal anti-VEGFs have a low incidence of adverse side effects but are currently short acting requiring frequent injections. Both these agents are used as symptomatic treatments with no defined treatment end points and show high rates of regression and tachyphylaxis with loss of efficacy after repeated injections. There may also be a rebound phenomenon as observed by Matsumoto et al[60] with macular edema becoming more pronounced compared to pre-treatment levels.

Until a definitive treatment becomes available for RVO it is currently a case of using the various treatment options available to keep the macular dry (to prevent the irreversible damage caused by chronic macular edema) and titrating this to allow a sufficient collateral circulation to develop.

Disclosure

The author reports no conflicts of interest in this work.

References

1. Cugati S, Wang JJ, Rochtchina E, et al. Ten-year incidence of retinal vein occlusion in an older population: the Blue Mountains Eye Study. *Arch Ophthalmol*. 2006;124:726–732.
2. Hayreh S. Prevalent misconceptions about acute retinal vascular occlusive disorders. *Prog Retinal Eye Res*. 2005;24:493–519.
3. Cheung N, Klein R, Wang J, et al. Traditional and novel cardiovascular risk factors for retinal vein occlusion: the multiethnic study of atherosclerosis. *Invest Ophthalmol Vis Sci*. 2008;49:4297–4302.
4. Klein R, Moss SE, Meuer SM, Klein BE. The 15-year cumulative incidence of retinal vein occlusion: the Beaver Dam Eye Study. *Arch Ophthalmol*. 2008;126:513–518.
5. Dexamethasone 700 µg intravitreal implant in applicator for retinal vein occlusion. Allergan advance notification document. 2009 Oct.
6. Dexamethasone Intravitreal Implant Approved for Retinal Vein Occlusion. Medscape Medical News. Available from: http://www.medscape.com/viewarticle/704854. Accessed on March 1, 2010.
7. Rogers S, McIntosh RL, Cheung N, et al. The prevalence of retinal vein occlusion: pooled data from population studies from the United States, Europe, Asia, and Australia. *Ophthalmology*. 2010;117:313–319.
8. Green WR, Chan CC, Hutchins GM, et al. Central retinal vein occlusions: A prospective histopathologic study of 29 eyes in 28 cases. *Retina*. 1981;1:27–55.
9. The Eye Disease Case-Control Study Group. Risk factors for central retinal vein occlusion. *Arch Ophthalmol*. 1996;114:545–554.
10. Sperduto RD, Hiller R, Chew E, et al. Risk factors for hemiretinal vein occlusion: comparison with risk factors for central and branch retinal vein occlusion: the eye disease case-control study. *Ophthalmology*. 1999;106:439–440.
11. The Eye Disease Case-Control Study Group. Risk factors for branch retinal vein occlusion. *Am J Ophthalmol*. 1993;116:286–296.
12. O'Mahoney P, Wong T, Ray J. Retinal vein occlusion and traditional risk factors for atherosclerosis. *Arch Ophthalmol*. 2008;126:692–699.
13. Koizumi H, Ferrara D, Brue C, et al. Central retinal vein occlusion case-control study. *Am J Ophthalmol*. 2007;144:858–863.

14. Fegan CD. Central retinal vein occlusion and thrombophilia. *Eye*. 2002;16:98–106.
15. Janssen MC, den Heijer M, Cruysberg JR, Wollersheim H, Bredie SJ. Retinal vein occlusion: a form of venous thrombosis or a complication of atherosclerosis? A meta-analysis of thrombophilic factors. *Thromb Haemost*. 2005;93:1021–1026.
16. Salaun N, Delyfer MN, Rougier MB, Korobelnik JF. Assessment of risk factors for retinal vein occlusions in patients under 60 years of age. *J Fr Ophtalmol*. 2007;30:918–923.
17. Turello M, Pasca S, Daminato R, et al. Retinal vein occlusion: evaluation of "classic" and "emerging" risk factors and treatment. *J Thromb Thrombolysis*. 2010;29:459–464.
18. Klein BE, Meuer SM, Knudtson MD, Klein R. The relationship of optic disk cupping to retinal vein occlusion: the Beaver Dam Eye Study. *Am J Ophthalmol*. 2006;141:859–862.
19. Girmens JF, Scheer S, Heron E, et al. Familial central retinal vein occlusion. *Eye*. 2008;22:308–310.
20. Bhagat N, Goldberg MF, Gascon W, et al. Central retinal vein occlusion: report of two familial cases. *Eur J Ophthalmol*. 1999;9:181–195.
21. Campochiaro PA, Hafiz G, Shah SM, et al. Ranibizumab for macular edema due to retinal vein occlusion: implication of VEGF as a critical stimulator. *Mol Ther*. 2008;16:791–799.
22. Yoshimura T, Sonoda KH, Sugahara M, et al. Comprehensive analysis of inflammatory immune mediators in vitreoretinal diseases. *PLoS ONE* 2009;4(12):e8158.
23. Funk M, Kriechbaum K, Prager F, et al. Intraocular concentrations of growth factors and cytokines in retinal vein occlusion and the effect of therapy with bevacizumab. *Invest Ophthalmol Vis Sci*. 2009;50:1025–1032.
24. Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med*. 1994;331:1480–1487.
25. Ozaki H, Hayashi H, Vinores SA, Moromizato Y, Campochiaro PA, Oshima K. Intravitreal sustained release of VEGF causes retinal neovascularization in rabbits and breakdown of the blood-retinal barrier in rabbits and primates. *Exp Eye Res*. 1997;64:505–517.
26. Noma H, Funatsu H, Yamasaki M, et al. Pathogenesis of macular edema with branch retinal vein occlusion and intraocular levels of vascular endothelial growth factor and interleukin-6. *Am J Ophthalmol*. 2005;140:256–261.
27. Noma H, Funatsu H, Yamasaki M, et al. Aqueous humour levels of cytokines are correlated to vitreous levels and severity of macular edema in branch retinal vein occlusion. *Eye*. 2008;22:42–48.
28. Noma H, Funatsu H, Mimura T, Harino S, Sone T, Hori S. Increase of vascular endothelial growth factor and interleukin-6 in the aqueous humour of patients with macular edema and central retinal vein occlusion. *Acta Ophthalmol*. Epub 2009 Jun 26.
29. Noma H, Funatsu H, Mimura T, Harino S, Hori S. Vitreous levels of interleukin-6 and vascular endothelial growth factor in macular edema with central retinal vein occlusion. *Ophthalmology*. 2009;116:87–93.
30. Boyd SR, Zachary I, Chakravarthy U, et al. Correlation of increased vascular endothelial growth factor with neovascularization and permeability in ischemic central vein occlusion. *Arch Ophthalmol*. 2002;120:1644–1650.
31. The Branch Vein Occlusion Study Group: Argon laser photocoagulation for macula edema in branch vein occlusion. *Am J Ophthalmol*. 1984;98:271–282.
32. The Branch Vein Occlusion Study Group: Argon laser scatter photocoagulation for prevention of neovascularization and vitreous hemorrhage in branch vein occlusion. *Arch Ophthalmol*. 1986;104:34–41.
33. The Central Vein Occlusion Study Group: Evaluation of grid pattern photocoagulation for macular edema in central vein occlusion. *Ophthalmology*. 1995;102:1425–1433.
34. The Central Vein Occlusion Study Group: A randomized clinical trial of early panretinal photocoagulation for ischemic central vein occlusion. *Ophthalmology*. 1995;102:1434–1444.
35. Rogers SL, McIntosh RL, Lim L, et al. Natural history of branch retinal vein occlusion: An evidence-based systematic review. *Ophthalmology*. 2010;117:1094–1101.e5.
36. Mohamed Q, McIntosh RL, Saw SM, Wong TY. Interventions for central retinal vein occlusion: an evidence-based systematic review. *Ophthalmology*. 2007;114:507–519.
37. Squizzato A, Manfredi E, Bozzato S, et al. Antithrombotic and fibrinolytic drugs for retinal vein occlusion: A systematic review and a call for action. *Thromb Haemost*. 2010;103:271–276.
38. Lazo-Langner A, Hawel J, Ageno W, Kovacs MJ. Low molecular weight heparin for the treatment of retinal vein occlusion: a systematic review and meta-analysis of randomized trials. *Haematologica*. 2010; Mar 19 [Epub ahead of print].
39. Mandelcorn MS, Nrusimhadevara RK. Internal limiting membrane peeling for decompression of macular edema in retinal vein occlusion: a report of 14 cases. *Retina*. 2004;24:348–355.
40. Opremcak EM, Bruce RA, Lomeo MD, et al. Radial optic neurotomy for central retinal vein occlusion: a retrospective pilot study of 11 consecutive cases. *Retina*. 2001;21:408–415.
41. Weizer JS, Stinnett SS, Fekrat S. Radial optic neurotomy as treatment for central retinal vein occlusion. *Am J Ophthalmol*. 2003;136:814–819.
42. Weiss JN. Treatment of central retinal vein occlusion by injection of tissue plasminogen activator into a retinal vein. *Am J Ophthalmol*. 1998;126:142–144.
43. Bynoe LA, Hutchins RK, Lazarus HS, Friedberg MA. Retinal endovascular surgery for central retinal vein occlusion: initial experience of four surgeons. *Retina*. 2005;25:625–632.
44. McAllister IL, Gillies M, Smithies LA, et al. The central retinal vein bypass study: A trial of laser-induced chorioretinal venous anastomosis for central retinal vein occlusion. *Opthalmology*. 2010;117:954–965.
45. McIntosh RL, Mohamed Q, Saw SM, Wong TY. Interventions for branch retinal vein occlusion: an evidence-based systematic review. *Ophthalmology*. 2007;114:835–854.
46. Arnarsson A, Stefansson E. Laser treatment and the mechanism of edema reduction in branch retinal vein occlusion. *Invest Opthalmol Vis Sci*. 2000;41:877–879.
47. Muqit MM, Saidkasimova S, Keating D, Murdoch JR. Long-term study of vascular perfusion effects following arteriovenous sheathotomy for branch retinal vein occlusion. *Acta Ophthalmol*. 2010;88:e57–e65.
48. Park CH, Jaffe GJ, Fekrat S. Intravitreal triamcinolone acetonide in eyes with cystoid macular edema associated with central retinal vein occlusion. *Am J Ophthalmol*. 2003;136:419–425.
49. Ip MS, Gottlieb JL, Kahana A, et al. Intravitreal triamcinolone for the treatment of macular edema associated with central retinal vein occlusion. *Arch Ophthalmol*. 2004;122:1131–1136.
50. Patel PJ, Zaheer I, Karia N. Intravitreal triamcinolone acetonide for macular edema owing to retinal vein occlusion. *Eye*. 2008;22:60–64.
51. Miyamoto N, Iossifov D, Metge F, Behar-Cohen F. Early effects of intravitreal triamcinolone on macular edema. *Ophthalmology*. 2006;113:2048–2053.
52. The SCORE Study Research Group. A randomized trial comparing the efficacy and safety of intravitreal triamcinolone with observation to treat vision loss associated with macular edema secondary to central retinal vein occlusion: the standard care vs corticosteroid for retinal vein occlusion (SCORE) Study Report 5. *Arch Ophthalmol*. 2009;127:1101–1114.
53. The SCORE Study Research Group. A randomized trial comparing the efficacy and safety of intravitreal triamcinolone with standard care to treat vision loss associated with macular edema secondary to branch retinal vein occlusion: the standard care vs corticosteroid for retinal vein occlusion (SCORE) Study Report 6. *Arch Ophthalmol*. 2009;127:1115–1128.
54. Apte RS. SCOREing in retinal venous occlusive disease. *Arch Ophthalmol*. 2009;127:1203–1204.
55. Loewenstein A, Haller J, Bandello F, et al. 12-month evaluation of an applicator-delivered dexamethasone intravitreal implant in patients with macular edema due to branch or central retinal vein occlusion. Paper presented at: the 33rd Annual Macula Society Meeting; 2010 Feb 26; Tuscon, AZ.

56. Rosenfeld PJ, Fung AE, Puliafito CA. Optical coherence tomography findings after an intravitreal injection of bevacizumab (avastin) for macular edema from central vein occlusion. *Ophthalmic Surg Lasers Imaging*. 2005;36:336–339.
57. Prager F, Michels S, Kriechbaum K, et al. Intravitreal bevacizumab (Avastin) for macular edema secondary to retinal vein occlusion: 12-month results of a prospective clinical trial. *Br J Ophthalmol*. 2009; 93:452–456.
58. Campochiaro PA. Safety and efficacy of intravitreal ranibizumab (Lucentis) in patients with macular edema secondary to branch retinal vein occlusion: The BRAVO Study. Paper presented at: Retina Congress 2009; Oct 4; New York.
59. Brown DM. Safety and efficacy of intravitreal ranibizumab (Lucentis) in patients with macular edema secondary to central retinal vein occlusion: The CRUISE Study. Paper presented at: Retina Congress 2009 Oct 4; New York.
60. Matsumoto Y, Freund KB, Peiretti E, Cooney MJ, Ferrara DC, Yannuzzi LA. Rebound macular edema following bevacizumab (Avastin) therapy for retinal venous occlusive disease. *Retina*. 2007; 27:426–431.

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	16/055,847
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				First Named Inventor	YANCOPOULOS, GEORGE D.
				Art Unit	1647
				Examiner Name	Jon McClelland Lockard
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U.S. PATENT DOCUMENTS

Examiner Initial*	Cite No.	Patent Number Number-Kind Code (*if known*)	Issue Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1	7303746	2007-12-04	Wiegand	
	2	7303748	2007-12-04	Wiegand	
	3	7306799	2007-12-11	Wiegand	
	4	9254338	2016-02-09	Yancopoulos	
	5	9669069	2017-06-06	Yancopoulos	
	6	10130681	2018-11-20	Yancopoulos	

U.S. PATENT APPLICATION PUBLICATIONS

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FOREIGN PATENT DOCUMENTS

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	1	WO 2006/047325	2006-03-04	Genentech, Inc.		
	2	WO 2012/097019	2012-07-19	Regeneron Pharmaceuticals, Inc.		

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
	1	BROWNING et al. "Aflibercept for age-related macular degeneration: a game-changer or quiet addition?" American Journal of Ophthalmology, Vol. 154(2):222-226 (08/01/2012)	
	2	CAMPOCHIARO et al. "Ranibizumab for Macular Edema Due to Retinal Vein Occlusions Implication of VEGF as a Critical Stimulator" 16(4):791-799 (2008)	
	3	CAO, "A Subretinal Matrigel Rat Choroidal Neovascularization (CNV) Model and Inhibition of CNV and Associated Inflammation and Fibrosis by VEGF Trap" Investigative Ophthalmology & Visual Science, 51(11):6009- 6017 (11/2010)	
	4	EICHTEN, "Rapid decrease in tumor perfusion following VEGF blockade predicts long-term tumor growth inhibition in preclinical tumor models" Angiogenesis, 16:429-441 (2013)	
	5	HO, "VEGF Trap-Eye in Wet AMD - CLEAR-IT 2: One-Year OCT and FA Outcomes" CLEAR-IT 2 Study Group, pp 1-24 (09/28/2008)	
	6	HOLASH, "VEGF-Trap: A VEGF blocker with potent antitumor effects" PNAS 99(17)11393-11398 (8/20/2002)	
	7	HOLASH, "Inhibitors of growth factor receptors, signaling pathways and angiogenesis as therapeutic molecular agents." Cancer Metastasis 25:243-252 (2006)	
	8	KUO, "Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer" PNAS 98(8):4605-4610 (04/10/2001)	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	16/055,847
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	9	OHR, "Aflibercept in wet age-related macular degeneration: a perspective review" Ther. Adv. Chronic Dis., 3(4):153-161 (2012)	
	10	PAPADPPOULOS, "Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab" Angiogenesis, 15:171-185 (2012)	
	11	Regeneron Press Release "Enrollment Completed in Regeneron and Bayer HealthCare Phase 3 Studies of VEGF Trap-Eye in Neovascular Age-Related Macular Degeneration (Wet AMD)" September 14, 2009	
	12	REGILLO et al., "Randomized, Double-Masked, Sham-Controlled Trial of Ranibizumab for Neovascular Age-related Macular Degeneration: OIER Study Year 1" American Journal of Ophthalmology, 145(2):239-248 (2008)	
	13	SCHNICHELS, "Comparative toxicity and proliferation testing of aflibercept, bevacizumab and ranibizumab on different ocular cells." Br. J. Opthalmol. (05/17/2013)	
	14	SHARMA and S. AND KAISER, P. K., Update on VEGF TRAP-Eye Clinical Trials and Retinal. Physician, 2010, Nov/Dec, p.1-6, <URL: https://www.retinalphysician.com/issues/2010/nov-dec/update-on-vegf-trap-eye-clinical-trials>	
	15	STEWART et al., "Predicted biological activity of intravitreal VEGF Trap" British Journal of Ophthalmology, 2008, vol.92, no.5, p.667-668	
	16	STEWART, "Aflibercept" Nature Reviews: Drug Discovery 11:269-270 (04/01/2012)	
	17	THURSTON, "Vascular endothelial growth factor and other signaling pathways in developmental and pathologic angiogenesis." International Journal of Hematology 80:7-20 (2004)	
	18	WACHSBERGER, "VEGF trap in combination with radiotherapy improves tumor control in u87 glioblastoma." Int. J. Radiation Oncology Biol Phys. 67(5):1526-1537 (2007)	
	19	ADSIS R&D Profile "Aflibercept: AVE 0005, AVE 005, AVE0005, VEGF Trap - Regeneron, VEGF Trap (R1R2), VEGF Trap-Eye." Drugs R D, 9(4):261-269 (2008)	
	20	N/A "Materials from June 2011 FDA Committee Mtg" (06/17/2011)	
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(71) Applicant *(for all designated States except US)*: **GENENTECH, INC.** [US/US]; 1 Dna Way, South San Francisco, CA 94080-4990 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **SHAMS, Naveed** [US/US]; 216 Dove Creek Lane, Danville, CA 94506 (US).

(74) Agent: **CUI, Steven X.**; GENENTECH, INC., 1 Dna Way, South San Francisco, CA 94080-4990, (US).

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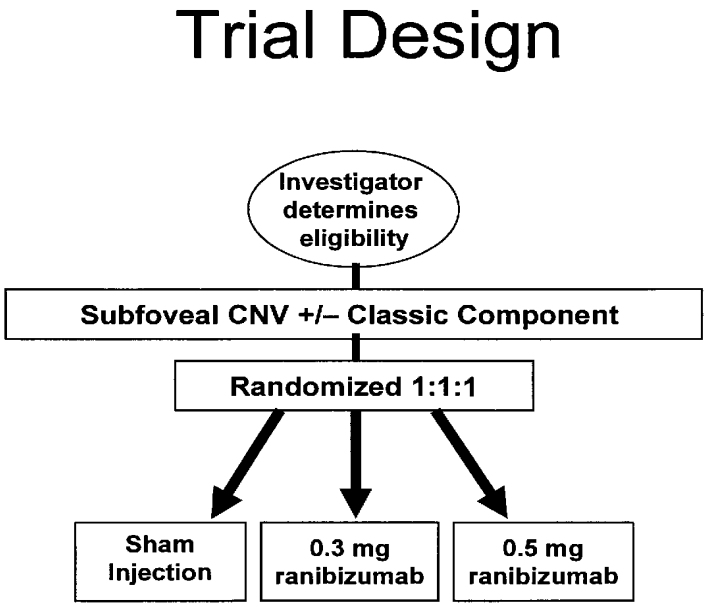
(54) **Title:** METHOD FOR TREATING INTRAOCULAR NEOVASCULAR DISEASES



(57) **Abstract:** A method is provided for administering to a mammal suffering from, or at risk for, an intraocular neovascular disorder with regular dosing of a therapeutically effective amount of VEGF antagonist, followed by less frequent dosing of a therapeutically effective amount of VEGF antagonist.

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METHOD FOR TREATING INTRAOCULAR NEOVASCULAR DISEASES

FIELD OF THE INVENTION

This invention relates to methods for treating an intraocular neovascular disorder with a VEGF antagonist. Methods for administering to a mammal suffering from, or at risk for, an intraocular neovascular disorder include monthly dosing of a therapeutically effective amount of VEGF antagonist, followed by less frequent dosing of a therapeutically effective amount of VEGF antagonist.

BACKGROUND OF THE INVENTION

Angiogenesis is implicated in the pathogenesis of intraocular neovascular diseases, e.g., proliferative retinopathies, age-related macular degeneration (AMD), etc., as well as a variety of other disorders. These include solid tumors, rheumatoid arthritis, and psoriasis (Folkman *et al. J. Biol. Chem.* 267:10931-10934 (1992); Klagsbrun *et al. Annu. Rev. Physiol.* 53:217-239 (1991); and Garner A, *Vascular diseases. In: Pathobiology of ocular disease. A dynamic approach.* Garner A, Klintworth GK, Eds. 2nd Edition Marcel Dekker, NY, pp 1625-1710 (1994)).

The search for positive regulators of angiogenesis has yielded many candidates, including aFGF, bFGF, TGF-α, TGF-β HGF, TNF-α, angiogenin, IL-8, etc. (Folkman *et al.* and Klagsbrun *et al*). The negative regulators so far identified include thrombospondin (Good *et al. Proc. Natl. Acad. Sci. USA.* 87:6624-6628 (1990)), the 16-kilodalton N-terminal fragment of prolactin (Clapp *et al. Endocrinology*, 133:1292-1299 (1993)), angiostatin (O'Reilly *et al. Cell*, 79:315-328 (1994)) and endostatin (O'Reilly *et al. Cell*, 88:277-285 (1996)).

Work done over the last several years has established the key role of vascular endothelial growth factor (VEGF) in the regulation of normal and abnormal angiogenesis (Ferrara *et al. Endocr. Rev.* 18:4-25 (1997)). The finding that the loss of even a single VEGF allele results in embryonic lethality points to an irreplaceable role played by this factor in the development and differentiation of the vascular system (Ferrara *et al.*).

Human VEGF exists as at least six isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$) that arise from alternative splicing of mRNA of a single gene (Ferrara N, Davis Smyth T. *Endocr Rev* 18:1–22 (1997)). $VEGF_{165}$, the most abundant isoform, is a basic, heparin binding, dimeric glycoprotein with a molecular mass of ~45,000 daltons (*Id*). Two VEGF receptor tyrosine kinases, VEGFR1and VEGFR2, have been identified (Shibuya et al. *Oncogene* 5:519–24 (1990); Matthews et al., *Proc Natl Acad Sci U S A* 88:9026–30 (1991); Terman et al., *Oncogene* 6:1677–83 (1991); Terman et al. *Biochem Biophys Res Commun* 187:1579–86 (1992); de Vries et al., *Science* 255:989–91 (1992); Millauer et al. *Cell* 72:835–46 (1993); and, Quinn et al. *Proc Natl Acad Sci USA* 90:7533–7 (1993)). VEGFR1 has the highest affinity for VEGF, with a Kd of ~10–20 pM (de Vries et al., *Science* 255:989–91 (1992)), and VEGFR2 has a somewhat lower affinity for VEGF, with a Kd of ~75–125 pM (Terman et al., *Oncogene* 6:1677–83 (1991); Millauer et al. *Cell* 72:835–46 (1993); and, Quinn et al. *Proc Natl Acad Sci USA* 90:7533–7 (1993)).

VEGF has several biologic functions, including regulation of VEGF gene expression under hypoxic conditions (Ferrara N, Davis Smyth T. *Endocr Rev* 18:1–22 (1997)), mitogenic activity for micro and macrovascular endothelial cells (Ferrara N, Henzel WJ. *Biochem Biophys Res Commun* 161:851–8 (1989); Leung et al., *Science* 246:1306–9 (1989); Connolly et al. *J Clin Invest* 84:1470–8 (1989a); Keck et al. *Science* 246:1309–12 (1989); Plouet et al., *EMBO J* 8:3801–6 (1989); Conn et al. *Proc Natl Acad Sci USA* 87:2628–32 (1990); and, Pepper et al., *Exp Cell Res* 210:298–305 (1994)), and induction of expression of plasminogen activators and collagenase (Pepper et al., *Biochem Biophys Res Commun* 181:902–6 (1991)).

Furthermore, VEGF has been shown to be a key mediator of neovascularization associated with tumors and intraocular disorders (Ferrara *et al.*). The VEGF mRNA is overexpressed by the majority of human tumors examined. Berkman *et al. J Clin Invest* 91:153-159 (1993); Brown *et al. Human Pathol* 26:86-91 (1995); Brown *et al. Cancer Res* 53:4727-4735 (1993); Mattern *et al. Brit J Cancer.* 73:931-934 (1996); and Dvorak *et al. Am J Pathol* 146:1029-1039 (1995). Also, the concentration of VEGF in eye fluids are highly correlated to the presence of active proliferation of blood vessels in patients with diabetic and other ischemia-related retinopathies. Aiello *et al., N. Engl. J. Med.* 331:1480-1487 (1994). Furthermore, recent studies have demonstrated the localization of VEGF in choroidal neovascular membranes in patients affected by AMD. Lopez et al., *Invest. Ophtalmo. Vis. Sci.* 37:855-868 (1996); Kvanta et al., *Invest Ophthalmol Vis Sci* 37:1929–34 (1996).

Age related macular degeneration (AMD) is a leading cause of severe, irreversible vision loss among the elderly. Bressler, *JAMA* 291:1900-1 (2004). It is characterized by a broad spectrum of clinical and pathologic findings, such as pale yellow spots known as drusen, disruption of the retinal pigment epithelium (RPE), choroidal neovascularization (CNV), and disciform macular degeneration. The manifestations of the disease are classified into two forms: non exudative (dry) and exudative (wet or neovascular). Drusen are the characteristic lesions of the dry form, and neovascularization characterizes the wet form. Disciform AMD is the fibrotic stage of the neovascular lesion.

There is a dramatic increase in the prevalence of AMD with advancing age. *See, e.g.,* Leibowitz et al., *Surv Ophthalmol* 24(Suppl):335–610 (1980) and Klein et al., *Ophthalmology* 99:933–43 (1992). Although the wet form of AMD is much less common, it is responsible for 80%–90% of the severe visual loss associated with AMD (Ferris et al., *Arch Ophthamol* 102:1640–2 (1984)). There is an estimated 1–1.2 million prevalent cases of wet AMD. The cause of AMD is unknown; however, it is clear that the risk of developing AMD increases with advancing age. Other known risk factors include family history and cigarette smoking. Postulated risk factors also include oxidative stress, diabetes, alcohol intake, and sunlight exposure. D'Amico, *N Engl J Med* 331:95–106 (1994) and Christen et al., *JAMA* 276:1147–51 (1996).

Dry AMD is characterized by changes in the RPE and Bruch's membrane. It is thought that the RPE, compromised by age and other risk factors, deposits lipofuscin and cellular debris on Bruch's membrane. These changes may be seen ophthalmoscopically as drusen, which are scattered throughout the macula and posterior retinal pole. There are also variable degrees of atrophy and pigmentation of the RPE. Dry AMD may be asymptomatic or accompanied by variable and usually minimal visual loss and is considered to be a prelude to development of wet AMD.

Wet AMD is typically characterized by CNV of the macular region. The choroidal capillaries proliferate and penetrate Bruch's membrane to reach the RPE and may extend into the subretinal space. The increased permeability of the newly formed capillaries leads to accumulation of serous fluid or blood under the RPE and/or the neurosensory retina or within the neurosensory retina. When the fovea becomes swollen or detached, decreases in vision

occur. Fibrous metaplasia and organization may ensue, resulting in an elevated subretinal mass called a disciform scar that constitutes end-stage AMD and is associated with permanent vision loss (D'Amico DJ. *N Engl J Med* 331:95–106 (1994)).

The neovascularization in AMD can be classified into different patterns based on fluorescein angiography of subfoveal chorodial neovascular lesions. TAP and VIP Study Groups, *Arch Ophthalmol* 121:1253-68 (2003). The major angiographic patterns are termed classic and occult and are associated with different degrees of aggressiveness, vision losses, and response to different treatment options.

The diffusible nature of VEGF and its specificity of action for endothelial cells support a key role in the process of abnormal blood vessel growth and vascular leakage. Increased expression of VEGF in retinal photoreceptors or RPE of transgenic mice stimulates neovascularization within the retina, and VEGF antagonists partially inhibit retinal neovascularization in animal models (Okamoto et al. *Am J Pathol* 151:281–91 (1997); Schwesinger et al., *AM J Pathol.* Mar;158(3):1161-72 (2001)). Anti-VEGF neutralizing antibodies inhibit intraocular angiogenesis in models of ischemic retinal disorders (Adamis *et al. Arch. Ophthalmol.* 114:66-71 (1996)), and also suppress the growth of a variety of human tumor cell lines in nude mice (Kim *et al. Nature* 362:841-844 (1993); Warren *et al. J. Clin. Invest.* 95:1789-1797 (1995); Borgström *et al. Cancer Res.* 56:4032-4039 (1996); and Melnyk *et al. Cancer Res.* 56:921-924 (1996)). Therefore, anti-VEGF monoclonal antibodies or other VEGF antagonists are promising candidates for use in treatments of intraocular neovascular disorders, and new methods of administering therapeutic compounds, which increases the effectiveness of the therapeutic compound, are needed.

SUMMARY OF THE INVENTION

One object of the present invention is to provide an improved method of administering a therapeutic compound. This and other objects will become apparent from the following description.

Methods for treating intraocular neovascular disease are provided. For example, methods include administering to a mammal a number of first individual doses of a VEGF antagonist, followed by administering to the mammal a number of second individual doses of the

antagonist, wherein the second individual doses are administered less frequently than the first individual doses.

In one embodiment of the invention, a method for treating wet form age-related macular degeneration is provided, which comprises administering to a mammal a number of first individual doses of an VEGF antagonist, followed by administering to the mammal a number of second individual doses of the antagonist, wherein the second individual doses are administered less frequently than the first individual doses.

In one embodiment, the mammal is in need of treatment. Typically, the mammal is a human.

In one embodiment, the administration of the VEGF antagonist is ocular. In one aspect, the administration is intraocular. In another aspect, the administration is intravitreal.

A VEGF antagonist is administered in the methods of the invention. In one aspect, the VEGF antagonist is an anti-VEGF antibody, e.g., a full length anti-VEGF antibody or an antibody fragment. In one embodiment, the anti-VEGF antibody is a Fab antibody fragment. In one embodiment, the antibody fragment is Y0317.

In one embodiment of the invention, the first individual doses are administered at one month intervals (e.g., about 3 individual doses). Typically, there is more than one first individual dose. In another embodiment, the second individual doses are administered at three month intervals (e.g., about 6 individual doses). In one aspect of the invention, the second individual doses are administered beginning three months after the number of first individual doses. In one embodiment, a number of second individual doses are administered to the mammal during a period of at least 22 months following the number of first individual doses.

In one embodiment of the invention, the number of first individual doses and the number of second individual doses are administered over a time period of about 2 years. In one aspect, the first individual dose is administered at month 0, 1 and 2. In another aspect, the second individual dose is administered at month 5, 8, 11, 14, 17, 20 and 23. For example, the first individual dose is administered at month 0, 1, and 2 and the second individual dose is administered at month 5, 8, 11, 14, 17, 20 and 23. In one embodiment, the VEGF antagonist is administered over less than 2 years, or optionally, administered over greater than 2 years.

Other aspects of the invention will become apparent from the following description of the embodiments which are not intended to be limiting of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 schematically illustrates the study in Example 1.

Figure 2 schematically illustrates a dosing regimen for treating, e.g., age-related macular degeneration (AMD) with a VEGF antagonist.

DETAILED DESCRIPTION

Definitions

Before describing the present invention in detail, it is to be understood that this invention is not limited to particular compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a molecule" optionally includes a combination of two or more such molecules, and the like.

The term "human VEGF" as used herein refers to the 165-amino acid human vascular endothelial cell growth factor, and related 121-, 189-, and 206-, (and other isoforms) amino acid vascular endothelial cell growth factors, as described by Leung *et al.*, *Science* 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.* 5:1806 (1991) together with the naturally occurring allelic and processed forms of those growth factors.

A "VEGF antagonist" refers to a molecule capable of neutralizing, blocking, inhibiting, abrogating, reducing or interfering with VEGF activities including its binding to one or more VEGF receptors. VEGF antagonists include anti-VEGF antibodies and antigen-binding fragments thereof, receptor molecules and derivatives which bind specifically to VEGF thereby sequestering its binding to one or more receptors, anti-VEGF receptor antibodies and VEGF receptor antagonists such as small molecule inhibitors of the VEGFR tyrosine kinases, and fusions proteins, e.g., VEGF-Trap (Regeneron), $VEGF_{121}$-gelonin (Peregrine). VEGF antagonists also include antagonist variants of VEGF, antisense molecules directed to VEGF,

RNA aptamers specific to VEGF, and ribozymes against VEGF or VEGF receptors. Antagonists of VEGF act by interfering with the binding of VEGF to a cellular receptor, by incapacitating or killing cells which have been activated by VEGF, or by interfering with vascular endothelial cell activation after VEGF binding to a cellular receptor. All such points of intervention by a VEGF antagonist shall be considered equivalent for purposes of this invention. Preferred VEGF antagonists are anti-VEGF antagonistic antibodies capable of inhibiting one or more of the biological activities of VEGF, for example, its mitogenic, angiogenic or vascular permeability activity. Anti-VEGF antagonistic antibodies include, but not limited to, antibodies A4.6.1, rhuMab VEGF (bevacizumab), Y0317 (ranibizumab), G6, B20, 2C3, and others as described in, for example, WO98/45331, US2003/0190317, U.S. Patents 6,582,959 and 6,703,020; WO98/45332; WO 96/30046; WO94/10202; WO2005/044853; EP 0666868B1; and Popkov et al., *Journal of Immunological Methods* 288:149-164 (2004). More preferably, the anti-VEGF antagonistic antibody of the invention is ranibizumab, which is a humanized, affinity matured anti-human VEGF antibody Fab fragment having the light and heavy chain variable domain sequences of Y0317 as described in WO98/45331 and Chen et al *J Mol Biol* 293:865-881 (1999).

The antibody is appropriately from any source, including chicken and mammalian such as rodent, goat, primate, and human. Typically, the antibody is from the same species as the species to be treated, and more preferably the antibody is human or humanized and the host is human. While the antibody can be a polyclonal or monoclonal antibody, typically it is a monoclonal antibody, which can be prepared by conventional technology. The antibody is an IgG-1, -2, -3, or -4, IgE, IgA, IgM, IgD, or an intraclass chimera in which Fv or a CDR from one class is substituted into another class. The antibody may have an Fc domain capable of an effector function or may not be capable of binding complement or participating in ADCC.

The term "VEGF receptor" or "VEGFr" as used herein refers to a cellular receptor for VEGF, ordinarily a cell-surface receptor found on vascular endothelial cells, as well as variants thereof which retain the ability to bind hVEGF. One example of a VEGF receptor is the *fms*-like tyrosine kinase (*flt*), a transmembrane receptor in the tyrosine kinase family. DeVries *et al.*, *Science* 255:989 (1992); Shibuya *et al.*, *Oncogene* 5:519 (1990). The *flt* receptor comprises an extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity. The extracellular domain is involved in the binding of VEGF, whereas the intracellular domain is involved in signal transduction. Another example of a

VEGF receptor is the *flk-1* receptor (also referred to as KDR). Matthews *et al.*, *Proc. Nat. Acad. Sci.* 88:9026 (1991); Terman *et al.*, *Oncogene* 6:1677 (1991); Terman *et al.*, *Biochem. Biophys. Res. Commun.* 187:1579 (1992). Binding of VEGF to the *flt* receptor results in the formation of at least two high molecular weight complexes, having apparent molecular weight of 205,000 and 300,000 Daltons. The 300,000 Dalton complex is believed to be a dimer comprising two receptor molecules bound to a single molecule of VEGF.

The term "epitope A4.6.1" when used herein, unless indicated otherwise, refers to the region of human VEGF to which the A4.6.1 antibody disclosed in Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al. Nature* 362:841 (1993), binds.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Typically, the mammal is human.

The term "antibody" is used in the broadest sense and includes monoclonal antibodies (including full length or intact monoclonal antibodies), polyclonal antibodies, multivalent antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments (see below) so long as they exhibit the desired biological activity.

Unless indicated otherwise, the expression "multivalent antibody" is used throughout this specification to denote an antibody comprising three or more antigen binding sites. The multivalent antibody is typically engineered to have the three or more antigen binding sites and is generally not a native sequence IgM or IgA antibody.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges.

Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light- chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework region (FR). The variable domains of native heavy and light chains each comprise four FRs (FR1, FR2, FR3 and FR4, respectively), largely adopting a β-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the β-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), pages 647-669). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity (ADCC).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)) and/or those residues from a "hypervariable loop" (*i.e.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.*

196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteine(s) from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain

constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

"Antibody fragments" comprise only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. Examples of antibody fragments encompassed by the present definition include: (i) the Fab fragment, having VL, CL, VH and CH1 domains; (ii) the Fab' fragment, which is a Fab fragment having one or more cysteine residues at the C-terminus of the CH1 domain; (iii) the Fd fragment having VH and CH1 domains; (iv) the Fd' fragment having VH and CH1 domains and one or more cysteine residues at the C-terminus of the CH1 domain; (v) the Fv fragment having the VL and VH domains of a single arm of an antibody; (vi) the dAb fragment (Ward et al., *Nature* 341, 544-546 (1989)) which consists of a VH domain; (vii) isolated CDR regions; (viii) F(ab')2 fragments, a bivalent fragment including two Fab' fragments linked by a disulphide bridge at the hinge region; (ix) single chain antibody molecules (e.g. single chain Fv; scFv) (Bird et al., *Science* 242:423-426 (1988); and Huston et al., *PNAS (USA)* 85:5879-5883 (1988)); (x) "diabodies" with two antigen binding sites, comprising a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (see, e.g., EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993)); (xi) "linear antibodies" comprising a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata et al. *Protein Eng.* 8(10):1057 1062 (1995); and US Patent No. 5,641,870).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the

monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature* 352:624-628 (1991) and Marks *et al., J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al., Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (*e.g.,* murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al., Nature* 321:522-525 (1986); Reichmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al. *Nature Biotechnology* 14:309-314 (1996): Sheets et al. *PNAS (USA)* 95:6157-6162 (1998)); Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581 (1991)). Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild et al., *Nature Biotechnology* 14: 845-51 (1996); Neuberger, *Nature Biotechnology* 14: 826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized *in vitro*). *See, e.g.,* Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner et al., *J. Immunol.*, 147 (1):86-95 (1991); and US Pat No. 5,750,373.

The term "Fc region" is used to define the C-terminal region of an immunoglobulin heavy chain which may be generated by papain digestion of an intact antibody. The Fc region may be a native sequence Fc region or a variant Fc region. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at about position Cys226, or from about position Pro230, to the carboxyl-terminus of the Fc region. The Fc region of an immunoglobulin generally comprises two constant domains, a CH2 domain and a CH3 domain, and optionally comprises a CH4 domain.

By "Fc region chain" herein is meant one of the two polypeptide chains of an Fc region.

The "CH2 domain" of a human IgG Fc region (also referred to as "Cg2" domain) usually extends from an amino acid residue at about position 231 to an amino acid residue at about position 340. The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It has been speculated that the carbohydrate may provide a substitute for the domain-domain pairing and help stabilize the CH2 domain. Burton, *Molec. Immunol.*22:161-206 (1985). The CH2 domain herein may be a native sequence CH2 domain or variant CH2 domain.

The "CH3 domain" comprises the stretch of residues C-terminal to a CH2 domain in an Fc region (i.e. from an amino acid residue at about position 341 to an amino acid residue at about position 447 of an IgG). The CH3 region herein may be a native sequence CH3 domain or a variant CH3 domain (e.g. a CH3 domain with an introduced "protroberance" in one chain thereof and a corresponding introduced "cavity" in the other chain thereof; see US Patent No. 5,821,333, expressly incorporated herein by reference). Such variant CH3 domains may be used to make multispecific (e.g. bispecific) antibodies as herein described.

"Hinge region" is generally defined as stretching from about Glu216, or about Cys226, to about Pro230 of human IgG1 (Burton, *Molec. Immunol.*22:161-206 (1985)). Hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by placing the first and last cysteine residues forming inter-heavy chain S-S bonds in the same positions. The hinge region herein may be a native sequence hinge region or a variant hinge region. The two polypeptide chains of a variant hinge region generally retain at least one cysteine residue per polypeptide chain, so that the two polypeptide chains of the variant hinge region can form a disulfide bond between the two chains. The preferred hinge region herein is a native sequence human hinge region, e.g. a native sequence human IgG1 hinge region.

A "functional Fc region" possesses at least one "effector function" of a native sequence Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding

domain (e.g. an antibody variable domain) and can be assessed using various assays known in the art for evaluating such antibody effector functions.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature.

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification. Preferably, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will typically possess, e.g., at least about 80% sequence identity with a native sequence Fc region and/or with an Fc region of a parent polypeptide, or at least about 90% sequence identity therewith, or at least about 95% sequence or more identity therewith.

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS (USA)* 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Typically, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with

PBMCs and NK cells being generally preferred. The effector cells may be isolated from a native source thereof, e.g. from blood or PBMCs as described herein.

The terms "Fc receptor" and "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain (reviewed in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976); and Kim et al., *J. Immunol.* 24:249 (1994)).

"Complement dependent cytotoxicity" and "CDC" refer to the lysing of a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule (e.g. an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. *Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et

al. *Proc Nat. Acad. Sci, USA* 91:3809-3813 (1994); Schier et al. *Gene* 169:147-155 (1995); Yelton et al. *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al, *J. Mol. Biol.* 226:889-896 (1992).

A "flexible linker" herein refers to a peptide comprising two or more amino acid residues joined by peptide bond(s), and provides more rotational freedom for two polypeptides (such as two Fd regions) linked thereby. Such rotational freedom allows two or more antigen binding sites joined by the flexible linker to each access target antigen(s) more efficiently. Examples of suitable flexible linker peptide sequences include gly-ser, gly-ser-gly-ser, ala-ser, and gly-gly-gly-ser.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata *et al. Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

A "variant" anti-VEGF antibody, refers herein to a molecule which differs in amino acid sequence from a "parent" anti-VEGF antibody amino acid sequence by virtue of addition,

deletion and/or substitution of one or more amino acid residue(s) in the parent antibody sequence. In the preferred embodiment, the variant comprises one or more amino acid substitution(s) in one or more hypervariable region(s) of the parent antibody. For example, the variant may comprise at least one, *e.g.* from about one to about ten, and preferably from about two to about five, substitutions in one or more hypervariable regions of the parent antibody. Ordinarily, the variant will have an amino acid sequence having at least 75% amino acid sequence identity with the parent antibody heavy or light chain variable domain sequences, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the parent antibody residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence shall be construed as affecting sequence identity or homology. The variant retains the ability to bind human VEGF and preferably has properties which are superior to those of the parent antibody. For example, the variant may have a stronger binding affinity, enhanced ability to inhibit VEGF-induced proliferation of endothelial cells and/or increased ability to inhibit VEGF-induced angiogenesis *in vivo*. To analyze such properties, one should compare a Fab form of the variant to a Fab form of the parent antibody or a full length form of the variant to a full length form of the parent antibody, for example, since it has been found that the format of the anti-VEGF antibody impacts its activity in the biological activity assays disclosed, e.g., in WO98/45331 and US2003/0190317. In one embodiment, the variant antibody is one which displays at least about 10 fold, preferably at least about 20 fold, and most preferably at least about 50 fold, enhancement in biological activity when compared to the parent antibody.

The "parent" antibody herein is one which is encoded by an amino acid sequence used for the preparation of the variant. Preferably, the parent antibody has a human framework region and, if present, has human antibody constant region(s). For example, the parent antibody may be a humanized or human antibody.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In

preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The term "epitope tagged" when used herein refers to the anti-VEGF antibody fused to an "epitope tag." The epitope tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the VEGF antibody. The epitope tag preferably is sufficiently unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field *et al. Mol. Cell. Biol.* 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al., Mol. Cell. Biol.* 5(12):3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al., Protein Engineering* 3(6):547-553 (1990)). In certain embodiments, the epitope tag is a "salvage receptor binding epitope". As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.,* IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

An "angiogenic factor or agent" is a growth factor which stimulates the development of blood vessels, e.g., promotes angiogenesis, endothelial cell growth, stability of blood vessels, and/or vasculogenesis, etc. For example, angiogenic factors, include, but are not limited to, e.g., VEGF and members of the VEGF family, PlGF, PDGF family, fibroblast growth factor family (FGFs), TIE ligands (Angiopoietins), ephrins, ANGPTL3, ANGPTL4, etc. It would also include factors that accelerate wound healing, such as growth hormone, insulin-like growth factor-I (IGF-I), VIGF, epidermal growth factor (EGF), CTGF and members of its family, and TGF-α and TGF-β. *See, e.g.,* Klagsbrun and D'Amore, *Annu. Rev. Physiol.*, 53:217-39

(1991); Streit and Detmar, *Oncogene*, 22:3172-3179 (2003); Ferrara & Alitalo, *Nature Medicine* 5(12):1359-1364 (1999); Tonini et al., *Oncogene*, 22:6549-6556 (2003) (e.g., Table 1 listing angiogenic factors); and, Sato *Int. J. Clin. Oncol.*, 8:200-206 (2003).

An "anti-angiogenesis agent" or "angiogenesis inhibitor" refers to a small molecular weight substance, a polynucleotide, a polypeptide, an isolated protein, a recombinant protein, an antibody, or conjugates or fusion proteins thereof, that inhibits angiogenesis, vasculogenesis, or undesirable vascular permeability, either directly or indirectly. For example, an anti-angiogenesis agent is an antibody or other antagonist to an angiogenic agent as defined above, e.g., antibodies to VEGF, antibodies to VEGF receptors, small molecules that block VEGF receptor signaling (e.g., PTK787/ZK2284, SU6668). Anti-angiogensis agents also include native angiogenesis inhibitors, e.g., angiostatin, endostatin, etc. *See, e.g.,* Klagsbrun and D'Amore, *Annu. Rev. Physiol.*, 53:217-39 (1991); Streit and Detmar, *Oncogene*, 22:3172-3179 (2003) (e.g., Table 3 listing anti-angiogenic therapy in malignant melanoma); Ferrara & Alitalo, *Nature Medicine* 5(12):1359-1364 (1999); Tonini et al., *Oncogene*, 22:6549-6556 (2003) (e.g., Table 2 listing antiangiogenic factors); and, Sato *Int. J. Clin. Oncol.*, 8:200-206 (2003) (e.g., Table 1 lists Anti-angiogenic agents used in clinical trials).

The term "effective amount" or "therapeutically effective amount" refers to an amount of a drug effective to treat a disease or disorder in a mammal. In the case of age-related macular degeneration (AMD), the effective amount of the drug can reduce or prevent vision loss. For AMD therapy, efficacy in vivo can, for example, be measured by one or more of the following: assessing the mean change in the best corrected visual acuity (BCVA) from baseline to a desired time, assessing the proportion of subjects who lose fewer than 15 letters in visual acuity at a desired time compared with baseline, assessing the proportion of subjects who gain greater than or equal to 15 letters in visual acuity at a desired time compared with baseline, assessing the proportion of subjects with a visual-acuity Snellen equivalent of 20/2000 or worse at desired time, assessing the NEI Visual Functioning Questionnaire, assessing the size of CNV and amount of leakage of CNV at a desired time , as assessed by fluorescein angiography, etc.

A therapeutic dose is a dose which exhibits a therapeutic effect on the patient and a sub-therapeutic dose is a dose which does not exhibit a therapeutic effect on the patient treated.

An "intraocular neovascular disease" is a disease characterized by ocular neovascularization. Examples of intraocular neovascular diseases include, but are not limited to, e.g., proliferative retinopathies, choroidal neovascularization (CNV), age-related macular degeneration (AMD), diabetic and other ischemia-related retinopathies, diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, Central Retinal Vein Occlusion (CRVO), corneal neovascularization, retinal neovascularization, etc.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody. The label may itself be detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

By "solid phase" is meant a non-aqueous matrix to which the antibody of the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (*e.g.* controlled pore glass), polysaccharides (*e.g.*, agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (*e.g.* an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, such as those described in U.S. Patent No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the anti-VEGF antibodies) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

MODES OF THE INVENTION

It has been discovered that the treatment effects of a VEGF antagonist, e.g., Ranibizumab, are maintained for an extended period of time, such as more than one month. Treatment with the VEGF antagonist was also found to be well tolerated for up to 2 years. The present invention describes a treatment schedule comprising an initial interval of administration of a therapeutic compound, followed by a subsequent, less frequent interval of administration of the therapeutic compound. The methods of the present invention allow one to decrease subsequent doses of the therapeutic compound, while at the same time maintaining the therapeutic efficacy.

The therapeutic compound which is administered using the treatment schedule of the present invention is a VEGF antagonist, preferably an anti-VEGF antibody (e.g., Ranibizumab). VEGF is a secreted homodimeric protein that is a potent vascular endothelial cells mitogen (Ferrara N, Davis Smyth T. *Endocr Rev* 18:1–22 (1997). VEGF stimulates vascular endothelial cell growth, functions as a survival factor for newly formed vessels, and induces vascular permeability. VEGF expression is upregulated by hypoxia as well as by a number of other stimuli.

In methods of the invention, therapeutic effects of a VEGF antagonist are provided by administering to a mammal a number of first individual doses of an VEGF antagonist; followed by, administering to the mammal a number of second individual doses of the antagonist, where the second individual doses are administered less frequently than the first individual doses.

The term "therapeutic" in this context means that the compounds binds to the ligand, VEGF, and produce a change in the symptoms or conditions associated with the disease or condition which is being treated. It is sufficient that a therapeutic dose produce an incremental change in the symptoms or conditions associated with the disease; a cure or complete remission of symptoms is not required. One having ordinary skill in this art can easily determine whether a dose is therapeutic by establishing criteria for measuring changes in symptoms or conditions of the disease being treated and then monitoring changes in these criteria according to known methods. External physical conditions, histologic examination of affected tissues in patients or the presence or absence of specific cells or compounds, associated with a disease may provide objective criteria for evaluating therapeutic effect. In one example, methods of the invention may be used to treat AMD where therapeutic effect is assessed by changes in preventing vision loss. Other indicators of therapeutic effect will be readily apparent to one having ordinary skill in the art and may be used to establish efficacy of the dose. See also section entitled herein, "Efficacy of the Treatment."

The doses may be administered according to any time schedule which is appropriate for treatment of the disease or condition. For example, the dosages may be administered on a daily, weekly, biweekly or monthly basis in order to achieve the desired therapeutic effect and reduction in adverse effects. The dosages can be administered before, during or after the development of the disorder. The specific time schedule can be readily determined by a

physician having ordinary skill in administering the therapeutic compound by routine adjustments of the dosing schedule within the method of the present invention. The time of administration of the number of first individual and second individual doses as well as subsequent dosages is adjusted to minimize adverse effects while maintaining a maximum therapeutic effect. The occurrence of adverse effects can be monitored by routine patient interviews and adjusted to minimize the occurrence of side effects by adjusting the time of the dosing. Any dosing time is to be considered to be within the scope of the present invention so long as the number of first individual doses of the VEGF antagonist is administered followed by a number of second individual doses, which are less frequently administered. For example, doses may be administered on a monthly schedule followed by subsequent quarterly or more dose schedule. Maintenance doses are also contemplated by the invention.

In a further embodiment, the first individual dose may be repeated one or more times before the second individual dose is administered. The first dose may be administered, for example, one, two or three times, typically three times before the less frequent administration dose(s) is (are) administered. In one embodiment of the invention, the first individual doses are administered at one month intervals (e.g., about 3 individual doses). The second dose is administered less frequently, e.g., at three month intervals (e.g., about 6 individual doses). In one aspect of the invention, the second individual doses are administered beginning three months after the number of first individual doses.

In one embodiment of the invention, the number of first individual doses and the number of second individual doses are administered over a time period of about 2 years. Shorter and longer time periods of 2 years are also included in the invention. In one aspect, the first individual dose is administered at month 0, 1 and 2. In another aspect, the second individual dose is administered at month 5, 8, 11, 14, 17, 20 and 23. In one example, the first individual dose is administered at month 0, 1, and 2 and the second individual dose is administered at month 5, 8, 11, 14, 17, 20 and 23.

Another aspect of the invention is the treatment of an intraocular neovascular disease, e.g., wet form AMD, by administering to a mammal, preferably a human patient, a number of first individual doses of a compound, e.g., a VEGF antagonist, followed by administering a number of second individual doses of the compound, where the number of second individual doses are administered less frequently than the number of first individual doses. This aspect of the

invention is different than previous dosing methods for the treatment of such diseases which generally treat with regularly spaced, even doses of a therapeutic compound. For example, the Ranibizumab (rhuFab V2), which is an antihuman VEGF, affinity-matured Fab has been administered in equal monthly (about 28 days) doses of 0.3 mg or 0.5 mg. In contrast, the method of the invention provides a number of first individual doses which are typically evenly spaced follow by a number of second individual doses that are less frequently administered.

The patient receives an initial dose of the VEGF antagonist. Since the VEGF antagonist treatment effects are maintained for more than a month, the patient can receive less frequent doses of the therapeutic compound in subsequent doses. However, it is possible to give more frequent doses, within the scope of the invention, to patients who do not experience effects on first administration.

The dosage amount depends on the specific disease or condition which is treated and can be readily determined using known dosage adjustment techniques by a physician having ordinary skill in treatment of the disease or condition. The dosage amount will generally lie with an established therapeutic window for the therapeutic compound which will provide a therapeutic effect while minimizing additional morbidity and mortality. Typically, therapeutic compounds are administered in a dosage ranging from 0.001 mg to about 100 mg per dose, preferably 0.1-20 mg.

Also within the scope of the present invention are additional doses, which may be administered after the number of first individual doses and after the number of second individual doses. For example, an additional, third set of doses can be administered.

Typically, the therapeutic compound used in the methods of this invention is formulated by mixing it at ambient temperature at the appropriate pH, and at the desired degree of purity, with physiologically acceptable carriers, i.e., carriers that are non-toxic to recipients at the dosages and concentrations employed. The pH of the formulation depends mainly on the particular use and the concentration of antagonist, but preferably ranges anywhere from about 3 to about 8. Where the therapeutic compound is an anti-VEGF antibody (e.g., ranibizumab), a suitable embodiment is a formulation at about pH 5.5.

The therapeutic compound, e.g. an anti-VEGF antibody, for use herein is preferably sterile. Sterility can be readily accomplished by sterile filtration through (0.2 micron) membranes. Preferably, therapeutic peptides and proteins are stored as aqueous solutions, although lyophilized formulations for reconstitution are acceptable.

The therapeutic compound may be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the time scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" of the therapeutic compound to be administered is governed by such considerations, and is the minimum amount necessary to prevent, ameliorate, or treat an intraocular neovascular disease.

The therapeutic compound for treatment of an intraocular neovascular disease is typically administered by ocular, intraocular, and/or intravitreal injection. Other methods administration by also be used, which includes but is not limited to, topical, parenteral, subcutaneous, intraperitoneal, intrapulmonary, intranasal, and intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. As described herein, the therapeutic compound for treatment of an intraocular neovascular syndrome may be formulated, dosed, and administered in a fashion consistent with good medical practice.

The efficacy of the treatment of the invention can be measured by various endpoints commonly used in evaluating intraocular neovascular diseases. For example, vision loss can be assessed. Vision loss can be evaluated by, but not limited to, e.g., measuring by the mean change in best correction visual acuity (BCVA) from baseline to a desired time point (e.g., where the BCVA is based on Early Treatment Diabetic Retinopathy Study (ETDRS) visual acuity chart and assessment at a test distance of 4 meters), measuring the proportion of subjects who lose fewer than 15 letters in visual acuity at a desired time point compared to baseline, measuring the proportion of subjects who gain greater than or equal to 15 letters in visual acuity at a desired time point compared to baseline, measuring the proportion of subjects with a visual-acuity Snellen equivalent of 20/2000 or worse at a desired time point, measuring the NEI Visual Functioning Questionnaire, measuring the size of CNV and amount

of leakage of CNV at a desired time point, e.g., by fluorescein angiography, etc. Ocular assessments can be done, e.g., which include, but are not limited to, e.g., performing eye exam, measuring intraocular pressure, assessing visual acuity, measuring slitlamp pressure, assessing intraocular inflammation, etc.

Any compound which binds to VEGF or a VEGF receptor and reduces the severity of symptoms or conditions associated with an intraocular neovascular disease may be used in this embodiment of the invention. Preferred compounds are peptide or protein compounds, more preferably are compounds which are or which contain an antibody or fragment thereof or which are fusions to an antibody fragment such as an immunoadhesin. Particularly preferred compounds are anti-VEGF antibodies or compounds containing fragments thereof.

VEGF is expressed in a variety of cells in the normal human retina. Co-localization of VEGF mRNA and protein is observed in the ganglion cell, inner nuclear and outer plexiform layers, the walls of the blood vessels, and photoreceptors (Gerhardinger et al., *Am J Pathol* 152:1453–62 (1998)). Retinal pigment epithelium, Muller cells, pericytes, vascular endothelium, and ganglion cells all produce VEGF (Miller et al., *Diabetes Metab Rev* 13:37–50 (1997); and, Kim et al. *Invest Ophthalmol Vis Sci* 40:2115–21 (1999)).

Studies have documented the immunohistochemical localization of VEGF in surgically resected CNV membranes from AMD patients. Kvanta et al. (1996) demonstrated the presence of VEGF mRNA and protein in RPE cells and fibroblast like cells. *See* Kvanta et al., *Invest Ophthalmol Vis Sci* 37:1929–34 (1996). Lopez et al. (1996) noted that the RPE cells that were strongly immunoreactive for VEGF were present primarily in the highly vascularized regions of CNV membranes, whereas the RPE cells found in fibrotic regions of CNV membranes showed little VEGF reactivity. *See* Lopez et al., *Invest Ophthalmol Vis Sci* 37:855–68 (1996). Kliffen et al. (1997) also demonstrated increased VEGF expression in RPE cells and choroidal blood vessels in maculae from patients with wet AMD compared with controls. *See* Kliffen et al., *Br J Ophthalmol* 81:154–62 (1997).

An increase in VEGF expression has been noted in experimental models of CNV in rats and in non human primates (Husain et al., *Ophthalmology* 104:1242̃50 (1997); and, Yi et al. Vascular endothelial growth factor expression in choroidal neovascularization in rats. *Graefes Arch Clin Exp Ophthalmol* 235:313–9 (1997)). In addition, transgenic mice with increased VEGF

expression in photoreceptors (Okamoto et al. 1997, *supra*) or retinal pigment epithelium (Schwesinger et al., *AM J Pathol.* 158(3):1161-72 (2001)) developed neovacularization reminiscent of CNV seen in humans with neovascular AMD. This further supports the involvement of VEGF in ocular neovascularization.

Of particular relevance to wet AMD are the angiogenic properties of VEGF, which have been demonstrated in a variety of in vivo models, including the chick chorioallantoic membrane (Leung et al., *Science* 246:1306–9 (1989); and, Plouet J, Schilling J, Gospodarowicz D. *EMBO J* 8:3801–6 (1989)), rabbit cornea (Phillips et al., *In Vivo* 8:961–5 (1994)), and rabbit bone (Connolly et al. *J Clin Invest* 84:1470–8 (1989a)). VEGF also functions as a survival factor for newly formed endothelial cells (Dvorak HF. *N Engl J Med* 315:1650–9 (1986); and, Connolly et al. *J Biol Chem* 264:20017–24 (1989b)). Consistent with pro survival activity, VEGF induces expression of the anti apoptotic proteins Bcl 2 and A1 in human endothelial cells (Connolly et al. *J Biol Chem* 264:20017–24 (1989b)).

VEGF has been shown to induce vascular leakage in guinea pig skin (Connolly et al. *J Biol Chem* 264:20017–24 (1989b)). Dvorak (1986) and colleagues (1987) proposed that an increase in microvascular permeability is a crucial step in angiogenesis associated with tumors and wound healing. Dvorak HF. *N Engl J Med* 315:1650–9 (1986); and, Dvorak et al., *Lab Invest* 57:673–86 (1987). A major function of VEGF in the angiogenic process can be the induction of plasma protein leakage. This effect would result in the formation of an extravascular fibrin gel, which serves as a substrate for endothelial cells. This activity can have relevance for AMD, as it is well established that permeability of the CNV membranes results in transudation of serum components beneath and into the retina, leading to serous macular detachment, macular edema and vision loss.

Thus, VEGF antagonists are good therapeutic compounds for treating intraocular neovascular diseases.

Many therapeutic compounds are well known to exert a therapeutic effect by binding to a selective cell surface marker or receptor or ligand. These known therapeutic compounds, e.g., anti-angiogenesis agents, are apparent to one having ordinary skill in the art and may be used in the method of the present invention. Suitable therapeutic compounds include non-peptidic organic compounds, preferably having a molecular weight less than about 1,000 g/mol, more

preferably less than about 600 g/mol; peptide therapeutic compounds, generally containing 8 to about 200, preferably about 15 to about 150, more preferably about 20 to about 100 amino acid residues; and protein therapeutic compounds, generally having secondary, tertiary and possibly quaternary structure. Suitable peptides compounds can be prepared by known solid-phase synthesis or recombinant DNA technology which are well known in the art.

A particularly preferred method of selecting a peptide compound is through the use of phage display technology. Using known phage display methods, libraries of peptides or proteins are prepared in which one or more copies of individual peptides or proteins are displayed on the surface of a bacteriophage particle. DNA encoding the particular peptide or protein is within the phage particle. The surface-displayed peptides or proteins are available for interaction and binding to target molecules which are generally immobilized on a solid support such as a 96-well plate or chromatography column support material. Binding and/or interaction of the display peptide or protein with a target molecule under selected screening conditions allows one to select members of the library which bind or react with the target molecule under the selected conditions. For example, peptides which bind under particular pH or ionic conditions may be selected. Alternatively, a target cell population can be immobilized on a solid surface using known techniques and the peptide or protein phage library can be panned against the immobilized cells to select peptides or proteins which bind to cell surface receptors on the target cell population. Phage display techniques are disclosed, for example, in U.S. Pat. Nos. 5,750,373; 5,821,047; 5,780,279; 5,403,484; 5,223,407; 5,571,698; and others.

One category of polypeptide compounds, are compounds containing an antibody or a fragment thereof which immunologically recognize and bind to cell surface receptors or ligands. Methods of preparing antibodies are well known in the art and have been practiced for many years. Suitable antibodies may be prepared using conventional hybridoma technology or by recombinant DNA methods. Preferred antibodies are humanized forms of non-human antibodies. Alternatively, antibodies may be prepared from antibody phage libraries using methods described, for example, in U.S. Pat. Nos. 5,565,332; 5,837,242; 5,858,657; 5,871,907; 5,872,215; 5,733,743, and others. Suitable compounds include full-length antibodies as well as antibody fragments such as Fv, Fab, Fab' and F $(ab')_2$ fragments which can be prepared by reformatting the full length antibodies using known methods.

Additional preferred polypeptide therapeutic compounds are immunoadhesin molecules also known as hybrid immunoglobulins. These polypeptides are useful as cell adhesion molecules and ligands and also useful in therapeutic or diagnostic compositions and methods. An immunoadhesin typically contains an amino acid sequence of a ligand binding partner protein fused at its C-terminus to the N-terminus of an immunoglobulin constant region sequence. Immunoadhesins and methods of preparing the same are described in U.S. Pat. Nos. 5,428,130; 5,714,147; 4,428,130; 5,225,538; 5,116,964; 5,098,833; 5,336,603; 5,565,335; etc.

Pharmaceutical Compositions

Therapeutic compounds of the invention used in accordance with the present invention are prepared for storage by mixing a polypeptide(s) having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in

macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. In one embodiment of the invention, an intraocular implant can be used for providing the VEGF antagonist. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing a polypeptide of the invention, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

EXAMPLES

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Example 1: Dosing Regiment

This study assesses the efficacy and safety of intravitreal injections of VEGF antagonist (e.g., ranibizumab) administered monthly for 3 doses followed by doses every 3 months compared with sham injections administered at the same schedule in subjects with primary or recurrent subfoveal choroidal neovascularization (CNV) with or without a classic CNV component secondary to AMD.

In this study, two treatment groups receive multiple intravitreal doses of VEGF antagonist from 0.3 mg to 0.5 mg for 24 months. See **Figure 1**. Each dose of VEGF antagonist is administered every month for 3 doses (Day 0, Month 1 and Month 2) followed by doses every 3 months (Months 5, 8, 11, 14, 17, 20, and 23) until study termination. See **Figure 2**. Subjects randomized to sham injections follow the same schedule as subjects receiving ranibizumab. During the 24 month study period, a total of 10 ranibizumab or 10 sham injections can be administered. Typically, the dosing does not occur earlier than 14 days after the previous treatment. If a dose is withheld or is missed, it may be optionally administered within 14 days following the previous treatment during the monthly injection period or within 45 days after the previous treatment during the 3-month dosing period. A maximum of 10 doses of study drug is administered during this study. Ranibizumab is administered in one eye only (study eye) during this study.

An example of a VEGF antagonist is ranibizumab (LUCENTIS™). Ranibizumab (rhuFab V2) is a humanized, affinity-matured anti-human VEGF Fab fragment. Ranibizumab is produced by standard recombinant technology methods in Escherichia coli expression vector and bacterial fermentation. Ranibizumab is not glycosylated and has a molecular mass of ~48,000 daltons. See WO98/45331 and US20030190317.

Ranibizumab Injection: For intravitreal administration, the study drug, ranibizumab, is supplied in a liquid-filled vial of ranibizumab. Each vial contains 0.7 mL of either 6 mg/mL (0.3 mg dose level) or 10 mg/mL (0.5-mg dose level) of ranibizumab aqueous solution (pH 5.5) with 10 mM of histidine, 100 mg/mL of trehalose, and 0.01% polysorbate 20. All study drug is stored at 2°C–8°C (36°F–46°F), and should not be frozen. Drug should be protected vials from direct sunlight.

Procedures are implemented to minimize the risk of potential adverse events associated with serial intraocular injections (e.g., endophthalmitis). Aseptic technique is observed for the injection tray assembly, anesthetic preparation and administration, and study drug preparation and administration.

Intravitreal injections are performed by the injecting physician(s) following the slitlamp examination. After thorough cleansings of the lid, lashes, and periorbital area with an antiseptic, local anesthesia and antimicrobials can administered prior to study drug injection.

A 30 gauge, ½-inch needle attached to a low volume (e.g., tuberculin) syringe containing 50 µL of study drug solution is inserted through the pre anesthetized conjunctiva and sclera, approximately 3.5–4.0 mm posterior to the limbus, avoiding the horizontal meridian and aiming toward the center of the globe. The injection volume should be delivered slowly. The needle is then be removed slowly to ensure that all drug solution is in the eye. Immediately following the intraocular injection, antimicrobial drops can be administered and the subject is instructed to self-administer antimicrobial drops four times daily for 3 days following each intraocular injection of ranibizumab. The scleral site for subsequent intravitreal injections should be rotated.

Sham Injection: The injecting physician(s) performs the same pre-injection cleansing and anesthetizing procedures (including subconjunctival injection of anesthesia) outlined above for subjects receiving ranibizumab. An empty syringe without a needle is used in the sham injection. The injecting physician(s) mimics an intraocular injection by making contact with the conjunctiva and applying pressure without the needle. Immediately following the sham injection, the injecting physician(s) performs the same post-injection procedures as those performed on subjects receiving ranibizumab.

Pre-Injection Procedures for All Subjects (Raninizumab or Sham Injection): The following procedures can be implemented to minimize the risk of potential adverse events associated with serial intravitreal injections (e.g., endophthalmitis). Aseptic technique is observed for injection tray assembly, anesthetic preparation, and study drug preparation and administration. The following procedures (except where noted) can be conducted by the physician performing the intravitreal injection of ranibizumab or sham injection. Subjects receive antimicrobials

(e.g., ofloxacin ophthalmic solution or trimethoprim polymyxin B ophthalmic solution) for self-administration four times daily for 3 days prior to treatment.

- The supplies are assembled and and a sterile field is prepared. Supplies can include 10% povidone iodine swabs, sterile surgical gloves, 4X4 sterile pads, pack of sterile cotton tipped applicators, eyelid speculum, sterile ophthalmic drape, 0.5% proparacaine hydrochloride, 5% povidone iodine ophthalmic solution, 1% lidocaine for injection, ophthalmic antimicrobial solution (e.g., ofloxacin ophthalmic solution or trimethoprim polymyxin B ophthalmic solution, single-use vial), and injection supplies.
- 2 drops of 0.5% proparacaine hydrochloride are instilled into the study eye, followed by 2 drops of a broad spectrum antimicrobial solution (e.g., ofloxacin ophthalmic solution or trimethoprim polymyxin B ophthalmic solution, single-use vial).
- The periocular skin and eyelid of the study eye are disinfected in preparation for injection. The eyelid, lashes, and periorbital skin are scrubbed with 10% povidone iodine swabs, starting with the eyelid and lashes and continuing with the surrounding periocular skin. The eyelid margins and lashes are swabbed, e.g., in a systematic fashion, from medial to temporal aspects.
- A sterile ophthalmic drape can be placed to isolate the field, and the speculum can be placed underneath the eyelid of the study eye.
- 2 drops of 5% povidone iodine ophthalmic solution are instilled in the study eye, making sure the drops cover the planned injection site on the conjunctiva.
- Wait 90 seconds.
- A sterile cotton-tipped applicator is saturated with 0.5% proparacaine hydrochloride drops and the swab is held against the planned intravitreal injection site for 10 seconds in preparation for the subconjunctival injection of 1% lidocaine hydrochloride ophthalmic solution for injection (without epinephrine).
- 1% lidocaine (without epinephrine) is injected subconjunctivally.
- A sterile 4X4 pad in a single wipe can be used to absorb excess liquid and to dry the periocular skin.
- The subject is instructed to direct gaze away from syringe prior to ranibizumab or sham injection.

Ranibizumab Preparation and Administration Instructions: The ranibizumab injection can be prepared as herein. Dose solutions are typically prepared immediately before dosing. Dose solutions are typically for single use only.

After preparing the study eye as outlined above, 0.2 mL ranibizumab dose solution is withdrawn through a 5-μm filter needle. The filter needle is removed and replaced with a 30-gauge, ½ inch Precision Glide® needle, and excess ranibizumab is expelled so that the syringe contains 0.05 mL ranibizumab solution. The syringe is inserted through an area 3.5–4.0 mm posterior to the limbus, avoiding the horizontal meridian and aiming toward the center of the globe. The injection volume should be delivered slowly. The needle is then removed slowly to ensure about all drug solution is in the eye. The scleral site for subsequent intravitreal injections should be rotated. Refer to next section for detailed post injection procedures.

The subject can be monitored with a finger count test for the study eye within, e.g., 15 minutes of the ranibizumab injection. A measurement of intraocular pressure of the study eye can be obtained, e.g., 60 minutes (±10 minutes) following the ranibizumab injection.

Post-Injection Procedures for All Subjects: Immediately following the ranibizumab or sham injection, 2 drops of antimicrobial drops (e.g., ofloxacin ophthalmic solution or trimethoprim polymyxin B ophthalmic solution, single-use vial) are instilled in the study eye. The subject is instructed to self-administer antimicrobial drops (e.g., ofloxacin ophthalmic solution or trimethoprim polymyxin B ophthalmic solution, single-use vial) four times daily for 3 days following each injection (ranibizumab or sham).

Preparation and Administration of the Sham Injection: See above for detailed instructions for pre-injection procedures.

Subjects receiving sham injections do not receive an actual injection of study drug. The physician follows the procedures for cleansing and anesthetizing the study eye as outlined above. The subject should be instructed to direct his or her gaze away from the syringe prior to administration of the sham injection. The tuberculin syringe plunger is withdrawn to the 0.05 mL mark on the syringe, the hub of the syringe—without the needle—is then placed against the pre-anesthetized conjunctival surface. The syringe hub is pressed firmly against the globe and then the plunger is slowly depressed, mimicking the action of an intravitreal injection.

For subsequent sham injections, the procedure of rotating the location of the injection site, as is done with ranibizumab injections is followed. See above for detailed post-injection procedures.

The subject can be monitored using a finger count test within, e.g., 15 minutes of the sham injection. A measurement of intraocular pressure can be obtained, e.g., 60 minutes (±10 minutes) following the sham injection.

Safety is assessed by the incidence of ocular and non-ocular adverse events, including but not limited to, serious adverse events, ocular assessments, deaths, laboratory test results, vital signs, antibodies to Raninizumab, intraocular inflammation, visual acuity, intraocular pressure, slitlamp pressure, indirect ophthalmoscopy, fluorescein angiography, fundus photography, vitreous hemorrhage, sensory rhegmatogenous retinal break or detachment (including macular hole), subfoveal hemorrhage, local or systemic infection, intraocular surgery, etc. In one embodiment, if verteporfin PDT was given within the last 28 days, the ranibizumab/sham injection is withheld. Efficacy is assessed by changes in preventing vision loss, e.g., measured by the mean change in best correction visual acuity (BCVA) from baseline to 12 months or 24 months (where the BCVA is based on the Early Treatment Diabetic Retinopathy Study (ETDRS) visual acuity chart and assessment at a test distance of 4 meters), other means include but are not limited to measuring the proportion of subjects who lose fewer than 15 letters in visual acuity at 12 months or 24 months compared to baseline, measuring the proportion of subjects who gain greater than or equal to 15 letters in visual acuity at 12 months or 24 months compared to baseline, measuring the proportion of subjects with a visual-acuity Snellen equivalent of 20/2000 or worse at 12 months or 24 months, measuring the NEI Visual Functioning Questionnaire, measuring the size of CNV and amount of leakage of CNV at 12 months or 24 months, e.g., by fluorescein angiography.

The specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

CLAIMS

We claim:

1. A method for treating wet form age-related macular degeneration in a mammal, comprising the steps of:

a) administering to the mammal a number of first individual doses of an VEGF antagonist; and

b) administering to the mammal a number of second individual doses of the VEGF antagonist, wherein the second individual doses are administered less frequently than the first individual doses.

2. The method of claim 1, wherein the mammal is a human.

3. The method of claim 1, wherein the administration is ocular.

4. The method of claim 3, wherein the administration is intraocular.

5. The method of claim 4, wherein the administration is intravitreal.

6. The method of claim 1, wherein the VEGF antagonist is an anti-VEGF antibody.

7. The method of claim 6, wherein the anti-VEGF antibody is a full length anti-VEGF antibody.

8. The method of claim 6, wherein the anti-VEGF antibody is an antibody fragment.

9. The method of claim 6, wherein the anti-VEGF antibody is a Fab antibody fragment.

10. The method of claim 8, wherein the antibody fragment is Y0317.

11. The method of claim 1, wherein the first individual doses are administered at one month intervals.

12. The method of claim 1, wherein the second individual doses are administered at three month intervals.

13. The method of claim 1, wherein the second individual doses are administered beginning three months after the number of first individual doses.

14. The method of claim 1, wherein the number of second individual doses are administered to the mammal during a period of at least 22 months following the number of first individual doses.

15. The method of claim 1, wherein the number of the first individual doses comprises about 3 individual doses.

16. The method of claim 1, wherein the number of the second individual doses comprises about 6 individual doses.

17. The method of claim 1, wherein the number of first individual doses and the number of second individual doses are administered over a time period of about 2 years.

18. The method of claim 1, wherein the first individual dose is administered at month 0, 1 and 2.

19. The method of claim 1, wherein the second individual dose is administered at month 5, 8, 11, 14, 17, 20 and 23.

20. The method of claim 1, wherein the first individual dose is administered at month 0, 1, and 2 and the second individual dose is administered at month 5, 8, 11, 14, 17, 20 and 23.

21. A method for treating intraocular neovascular disease, comprising:
administering to a mammal a number of first individual doses of an VEGF antagonist;
followed by,
administering to the mammal a number of second individual doses of the antagonist, wherein the second individual doses are administered less frequently than the first individual doses.

Trial Design

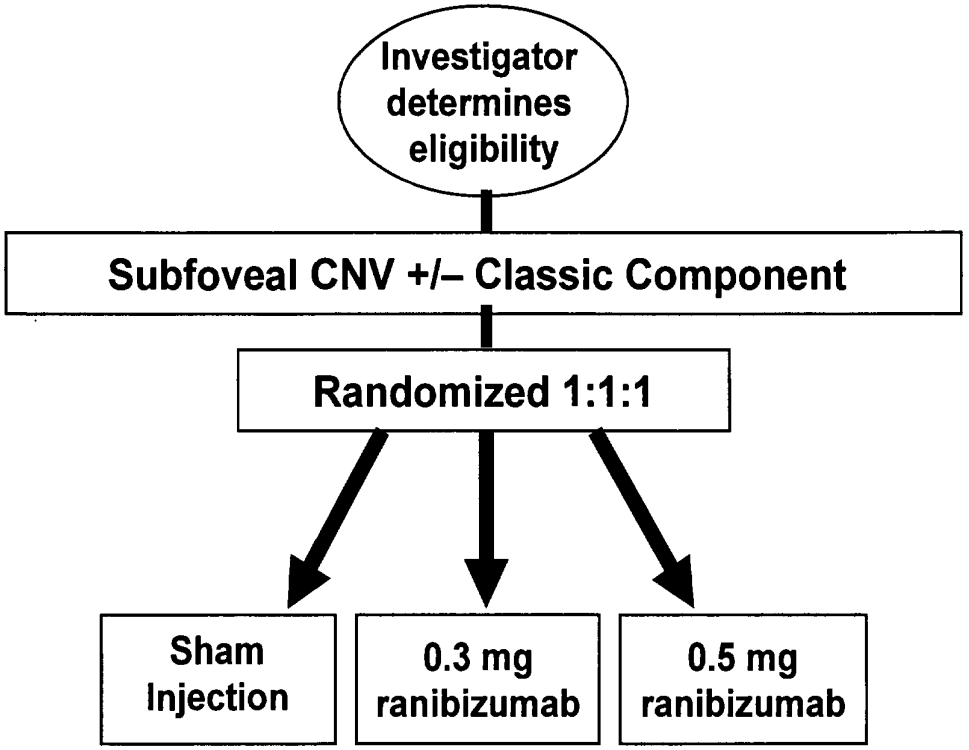

Figure 1

Treatment Schema

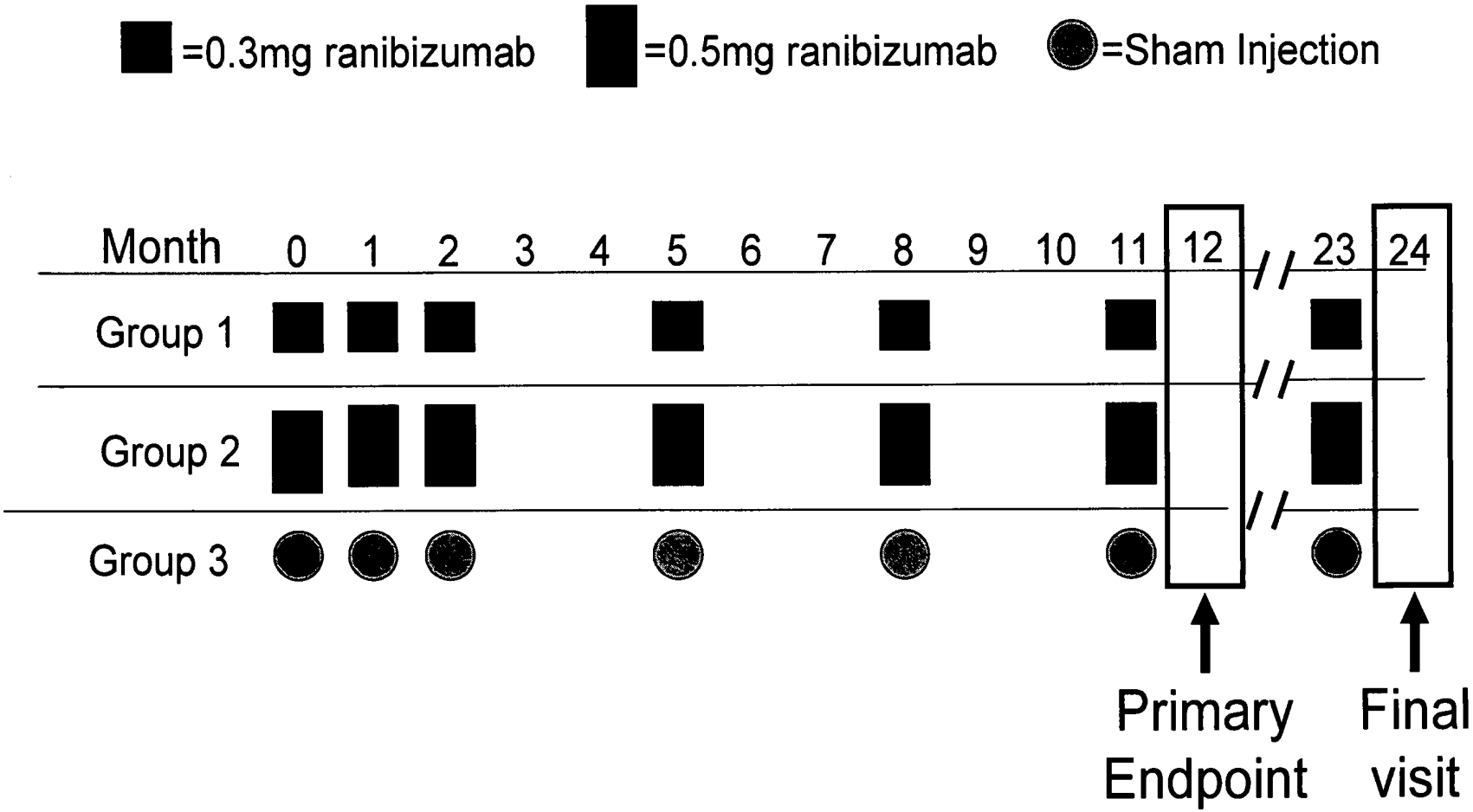

Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2005/038006

A. CLASSIFICATION OF SUBJECT MATTER
A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KRZYSTOLIK MAGDALENA G ET AL: "Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment" ARCHIVES OF OPHTHALMOLOGY, vol. 120, no. 3, March 2002 (2002-03), pages 338-346, XP009061383 ISSN: 0003-9950 page 339; figures 4,5; tables 1,4 -/--	1-21

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 February 2006	17/03/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Trommsdorff, M

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2005/038006

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EARL R. NICOLS: "AAO:Ranibizumab(rhuFab) May Improve Vision in Age-Related Macular Degeneration" INTERNET ARTICLE, 'Online! 24 November 2003 (2003-11-24), XP002366839 Retrieved from the Internet: URL:http://www.pslgroup.com/dg/23f2aa.htm> 'retrieved on 2006-02-08! the whole document ----	1-21
Y	WO 98/45331 A (GENENTECH, INC; BACA, MANUEL; WELLS, JAMES, A; PRESTA, LEONARD, G; LOW) 15 October 1998 (1998-10-15) page 45 - page 46; table 14 ----	1-21
Y	WO 96/30046 A (GENENTECH, INC) 3 October 1996 (1996-10-03) page 14 - page 15; claims 1-5 ----	1-21
Y	THE EYETECH STUDY GROUP ET AL: "Anti-vascular endothelial growth factor therapy for subfoveal choroidal neovascularization secondary to age-related macular degeneration: Phase II study results." OPHTHALMOLOGY, vol. 110, no. 5, May 2003 (2003-05), pages 979-986, XP002366840 ISSN: 0161-6420 table 1A tables 1A,2,3 ----	1,2,4, 11-15, 18,21
P,Y	ANONYMOUS: "EINE MULTIZENTRISCHE,RANDOMISIERTE,DOPPELT MASKIERTE,WIRKSTOFF-KONTROLIERTE PHASE III STUDIE ZUR WIRKSAMHEIT UND SICHERHEIT VON RHUFAB V2 (RHANIBIZUMAB) IM VERGLEICH MIT EINER PHOTODYNAMISCHEN VERTEPORFIN-THERAPIE (VISUDYNE) AN PATIENTEN MIT VORWIEGEND KLASSISCHER SUBFOVEALER NEOVASKULÄRER ..." INTERNET ARTICLE - ANCHOR-STUDIE, 'Online! XP002366841 Retrieved from the Internet: URL:http://www.medizin.uni-koeln.de/kliniken/augenklinik/angiolab/klinischestudien/anchor.shtml?print> 'retrieved on 2006-02-09! the whole document ----	1-21

-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2005/038006

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CAMPOCHIARO PETER ANTHONY ET AL: "Ocular neovascularization: A valuable model system." ONCOGENE, vol. 22, no. 42, 29 September 2003 (2003-09-29), pages 6537-6548, XP002366842 ISSN: 0950-9232 the whole document	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2005/038006

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: –
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 1-21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.2

The present claims 1-5, 11-21 encompass compounds defined only by their desired function, contrary to the requirements of clarity of Article 6 PCT, because the result-to-be-achieved type of definition does not allow the scope of the claim to be ascertained. The fact that any compound could be screened does not overcome this objection, as the skilled person would not have knowledge beforehand as to whether it would fall within the scope claimed, except for the compounds disclosed in the description which are also structurally defined, see p.6-7. Undue experimentation would be required to screen compounds randomly. This non-compliance with the substantive provisions is to such an extent, that the search was performed taking into consideration the non-compliance in determining the extent of the search for claims 1-5, 11-21.
The search of said claims was consequently restricted to antibodies against VEGF or structurally well defined other antagonists (such as e.g., aptamers disclosed in the art).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2005/038006

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9845331 A	15-10-1998	AT 293640 T	15-05-2005
		AU 743758 B2	07-02-2002
		AU 7100798 A	30-10-1998
		BR 9809387 A	11-09-2001
		CA 2286330 A1	15-10-1998
		CN 1259962 A	12-07-2000
		DE 69829891 D1	25-05-2005
		DE 69829891 T2	06-10-2005
		DE 122005000026 I1	04-08-2005
		DE 122005000050 I1	29-12-2005
		EP 0973804 A2	26-01-2000
		ES 2236634 T3	16-07-2005
		JP 2001509817 T	24-07-2001
		NO 994869 A	06-12-1999
		NZ 500078 A	26-10-2001
		PT 1325932 T	30-06-2005
		TR 9903123 T2	22-05-2000
WO 9630046 A	03-10-1996	AT 311902 T	15-12-2005
		AT 285251 T	15-01-2005
		AU 696487 B2	10-09-1998
		AU 5378796 A	16-10-1996
		CA 2213833 A1	03-10-1996
		DE 69634079 D1	27-01-2005
		DE 69634079 T2	19-01-2006
		EP 0817648 A1	14-01-1998
		ES 2233967 T3	16-06-2005
		IL 117645 A	31-08-2005
		JP 11502853 T	09-03-1999
		NZ 305699 A	28-02-2000
		PT 817648 T	29-04-2005

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61/434,836 21 January 2011 (21.01.2011) US
61/561,957 21 November 2011 (21.11.2011) US

(71) Applicant *(for all designated States except US)*: **REGENERON PHARMACEUTICALS, INC.** [US/US]; 777 Old Saw Mill River Road, Tarrytown, NY 10591 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **YANCOPOULOS, George D.** [US/US]; 1519 Baptist Church Road, Yorktown Heights, NY 10598 (US).

(74) Agent: **COTTINGHAM, Frank**; Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS

(57) **Abstract**: The present invention provides methods for treating angiogenic eye disorders by sequentially administering multiple doses of a VEGF antagonist to a patient. The methods of the present invention include the administration of multiple doses of a VEGF antagonist to a patient at a frequency of once every 8 or more weeks. The methods of the present invention are useful for the treatment of angiogenic eye disorders such as age related macular degeneration, diabetic retinopathy, diabetic macular edema, central retinal vein occlusion and corneal neovascularization.

WO 2012/097019 A1

USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS

FIELD OF THE INVENTION

[0001] The present invention relates to the field of therapeutic treatments of eye disorders. More specifically, the invention relates to the administration of VEGF antagonists to treat eye disorders caused by or associated with angiogenesis.

BACKGROUND

[0002] Several eye disorders are associated with pathological angiogenesis. For example, the development of age-related macular degeneration (AMD) is associated with a process called choroidal neovascularization (CNV). Leakage from the CNV causes macular edema and collection of fluid beneath the macula resulting in vision loss. Diabetic macular edema (DME) is another eye disorder with an angiogenic component. DME is the most prevalent cause of moderate vision loss in patients with diabetes and is a common complication of diabetic retinopathy, a disease affecting the blood vessels of the retina. Clinically significant DME occurs when fluid leaks into the center of the macula, the light-sensitive part of the retina responsible for sharp, direct vision. Fluid in the macula can cause severe vision loss or blindness. Yet another eye disorder associated with abnormal angiogenesis is central retinal vein occlusion (CRVO). CRVO is caused by obstruction of the central retinal vein that leads to a back-up of blood and fluid in the retina. The retina can also become ischemic, resulting in the growth of new, inappropriate blood vessels that can cause further vision loss and more serious complications. Release of vascular endothelial growth factor (VEGF) contributes to increased vascular permeability in the eye and inappropriate new vessel growth. Thus, inhibiting the angiogenic-promoting properties of VEGF appears to be an effective strategy for treating angiogenic eye disorders.

[0003] FDA-approved treatments of angiogenic eye disorders such as AMD and CRVO include the administration of an anti-VEGF antibody called ranibizumab (Lucentis®, Genentech, Inc.) on a monthly basis by intravitreal injection.

[0004] Methods for treating eye disorders using VEGF antagonists are mentioned in, *e.g.*, US 7,303,746; US 7,306,799; US 7,300,563; US 7,303,748; and US 2007/0190058. Nonetheless, there remains a need in the art for new administration regimens for angiogenic eye disorders, especially those which allow for less frequent dosing while maintaining a high level of efficacy.

BRIEF SUMMARY OF THE INVENTION

[0005] The present invention provides methods for treating angiogenic eye disorders. The methods of the invention comprise sequentially administering multiple doses of a VEGF antagonist to a patient over time. In particular, the methods of the invention comprise sequentially administering to the patient a single initial dose of a VEGF antagonist, followed by

one or more secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonists. The present inventors have surprisingly discovered that beneficial therapeutic effects can be achieved in patients suffering from angiogenic eye disorders by administering a VEGF antagonist to a patient at a frequency of once every 8 or more weeks, especially when such doses are preceded by about three doses administered to the patient at a frequency of about 2 to 4 weeks. Thus, according to the methods of the present invention, each secondary dose of VEGF antagonist is administered 2 to 4 weeks after the immediately preceding dose, and each tertiary dose is administered at least 8 weeks after the immediately preceding dose. An example of a dosing regimen of the present invention is shown in Figure 1. One advantage of such a dosing regimen is that, for most of the course of treatment (*i.e.*, the tertiary doses), it allows for less frequent dosing (*e.g.*, once every 8 weeks) compared to prior administration regimens for angiogenic eye disorders which require monthly administrations throughout the entire course of treatment. (*See, e.g.*, prescribing information for Lucentis® [ranibizumab], Genentech, Inc.).

[0006] The methods of the present invention can be used to treat any angiogenic eye disorder, including, *e.g.*, age related macular degeneration, diabetic retinopathy, diabetic macular edema, central retinal vein occlusion, corneal neovascularization, etc.

[0007] The methods of the present invention comprise administering any VEGF antagonist to the patient. In one embodiment, the VEGF antagonist comprises one or more VEGF receptor-based chimeric molecule(s), (also referred to herein as a "VEGF-Trap" or "VEGFT"). An exemplary VEGF antagonist that can be used in the context of the present invention is a multimeric VEGF-binding protein comprising two or more VEGF receptor-based chimeric molecules referred to herein as "VEGFR1R2-FcΔC1(a)" or "aflibercept."

[0008] Various administration routes are contemplated for use in the methods of the present invention, including, *e.g.*, topical administration or intraocular administration (*e.g.*, intravitreal administration).

[0009] Aflibercept (EYLEA™, Regeneron Pharmaceuticals, Inc) was approved by the FDA in November 2011, for the treatment of patients with neovascular (wet) age-related macular degeneration, with a recommended dose of 2 mg administered by intravitreal injection every 4 weeks for the first three months, followed by 2 mg administered by intravitreal injection once every 8 weeks.

[0010] Other embodiments of the present invention will become apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE FIGURE

[0011] Figure 1 shows an exemplary dosing regimen of the present invention. In this regimen, a single "initial dose" of VEGF antagonist ("VEGFT") is administered at the beginning of the treatment regimen (*i.e.* at "week 0"), two "secondary doses" are administered at weeks 4 and 8,

respectively, and at least six "tertiary doses" are administered once every 8 weeks thereafter, *i.e.*, at weeks 16, 24, 32, 40, 48, 56, etc.).

DETAILED DESCRIPTION

[0012] Before the present invention is described, it is to be understood that this invention is not limited to particular methods and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0013] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. As used herein, the term "about," when used in reference to a particular recited numerical value, means that the value may vary from the recited value by no more than 1%. For example, as used herein, the expression "about 100" includes 99 and 101 and all values in between (*e.g.*, 99.1, 99.2, 99.3, 99.4, etc.).

[0014] Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

DOSING REGIMENS

[0015] The present invention provides methods for treating angiogenic eye disorders. The methods of the invention comprise sequentially administering to a patient multiple doses of a VEGF antagonist. As used herein, "sequentially administering" means that each dose of VEGF antagonist is administered to the patient at a different point in time, *e.g.*, on different days separated by a predetermined interval (*e.g.*, hours, days, weeks or months). The present invention includes methods which comprise sequentially administering to the patient a single initial dose of a VEGF antagonist, followed by one or more secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonist.

[0016] The terms "initial dose," "secondary doses," and "tertiary doses," refer to the temporal sequence of administration of the VEGF antagonist. Thus, the "initial dose" is the dose which is administered at the beginning of the treatment regimen (also referred to as the "baseline dose"); the "secondary doses" are the doses which are administered after the initial dose; and the "tertiary doses" are the doses which are administered after the secondary doses. The initial, secondary, and tertiary doses may all contain the same amount of VEGF antagonist, but will generally differ from one another in terms of frequency of administration. In certain embodiments, however, the amount of VEGF antagonist contained in the initial, secondary and/or tertiary doses will vary from one another (*e.g.*, adjusted up or down as appropriate) during the course of treatment.

[0017] In one exemplary embodiment of the present invention, each secondary dose is administered 2 to 4 (*e.g.*, 2, 2½, 3, 3½, or 4) weeks after the immediately preceding dose, and each tertiary dose is administered at least 8 (*e.g.*, 8, 8½, 9, 9½, 10, 10½, 11, 11½, 12, 12½, 13, 13½, 14, 14½, or more) weeks after the immediately preceding dose. The phrase "the immediately preceding dose," as used herein, means, in a sequence of multiple administrations, the dose of VEGF antagonist which is administered to a patient prior to the administration of the very next dose in the sequence with no intervening doses.

[0018] In one exemplary embodiment of the present invention, a single initial dose of a VEGF antagonist is administered to a patient on the first day of the treatment regimen (*i.e.*, at week 0), followed by two secondary doses, each administered four weeks after the immediately preceding dose (*i.e.*, at week 4 and at week 8), followed by at least 5 tertiary doses, each administered eight weeks after the immediately preceding dose (*i.e.*, at weeks 16, 24, 32, 40 and 48). The tertiary doses may continue (at intervals of 8 or more weeks) indefinitely during the course of the treatment regimen. This exemplary administration regimen is depicted graphically in Figure 1.

[0019] The methods of the invention may comprise administering to a patient any number of secondary and/or tertiary doses of a VEGF antagonist. For example, in certain embodiments, only a single secondary dose is administered to the patient. In other embodiments, two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, or more) secondary doses are administered to the patient. Likewise, in certain embodiments, only a single tertiary dose is administered to the patient. In other embodiments, two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, or more) tertiary doses are administered to the patient.

[0020] In embodiments involving multiple secondary doses, each secondary dose may be administered at the same frequency as the other secondary doses. For example, each secondary dose may be administered to the patient 4 weeks after the immediately preceding dose. Similarly, in embodiments involving multiple tertiary doses, each tertiary dose may be administered at the same frequency as the other tertiary doses. For example, each tertiary dose may be administered to the patient 8 weeks after the immediately preceding dose. Alternatively, the frequency at which the secondary and/or tertiary doses are administered to a patient can vary over the course of the treatment regimen. For example, the present invention includes methods which comprise administering to the patient a single initial dose of a VEGF antagonist, followed by one or more secondary doses of the VEGF antagonist, followed by at least 5 tertiary doses of the VEGF antagonist, wherein the first four tertiary doses are administered 8 weeks after the immediately preceding dose, and wherein each subsequent tertiary dose is administered from 8 to 12 (*e.g.*, 8, 8½, 9, 9½, 10, 10½, 11, 11½, 12) weeks after the immediately preceding dose. The frequency of administration may also be adjusted during the course of treatment by a physician depending on the needs of the individual patient following clinical examination.

VEGF ANTAGONISTS

[0021] The methods of the present invention comprise administering to a patient a VEGF antagonist according to specified dosing regimens. As used herein, the expression "VEGF antagonist" means any molecule that blocks, reduces or interferes with the normal biological activity of VEGF.

[0022] VEGF antagonists include molecules which interfere with the interaction between VEGF and a natural VEGF receptor, *e.g.*, molecules which bind to VEGF or a VEGF receptor and prevent or otherwise hinder the interaction between VEGF and a VEGF receptor. Specific exemplary VEGF antagonists include anti-VEGF antibodies, anti-VEGF receptor antibodies, and VEGF receptor-based chimeric molecules (also referred to herein as "VEGF-Traps").

[0023] VEGF receptor-based chimeric molecules include chimeric polypeptides which comprise two or more immunoglobulin (Ig)-like domains of a VEGF receptor such as VEGFR1 (also referred to as Flt1) and/or VEGFR2 (also referred to as Flk1 or KDR), and may also contain a multimerizing domain (*e.g.*, an Fc domain which facilitates the multimerization [*e.g.*, dimerization] of two or more chimeric polypeptides). An exemplary VEGF receptor-based chimeric molecule is a molecule referred to as VEGFR1R2-FcΔC1(a) which is encoded by the nucleic acid sequence of SEQ ID NO:1. VEGFR1R2-FcΔC1(a) comprises three components: (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130 to 231 of SEQ ID NO:2; and (3) a multimerization component ("FcΔC1(a)") comprising amino acids 232 to 457 of SEQ ID NO:2 (the C-terminal amino acid of SEQ ID NO:2 [*i.e.*, K458] may or may not be included in the VEGF antagonist used in the methods of the invention; *see e.g.*, US Patent 7,396,664). Amino acids 1-26 of SEQ ID NO:2 are the signal sequence.

[0024] The VEGF antagonist used in the Examples set forth herein below is a dimeric molecule comprising two VEGFR1R2-FcΔC1(a) molecules and is referred to herein as "VEGFT." Additional VEGF receptor-based chimeric molecules which can be used in the context of the present invention are disclosed in US 7,396,664, 7,303,746 and WO 00/75319.

ANGIOGENIC EYE DISORDERS

[0025] The methods of the present invention can be used to treat any angiogenic eye disorder. The expression "angiogenic eye disorder," as used herein, means any disease of the eye which is caused by or associated with the growth or proliferation of blood vessels or by blood vessel leakage. Non-limiting examples of angiogenic eye disorders that are treatable using the methods of the present invention include choroidal neovascularization, age-related macular degeneration (AMD), diabetic retinopathies, diabetic macular edema (DME), central retinal vein occlusion (CRVO), corneal neovascularization, and retinal neovascularization.

PHARMACEUTICAL FORMULATIONS

[0026] The present invention includes methods in which the VEGF antagonist that is administered to the patient is contained within a pharmaceutical formulation. The pharmaceutical formulation may comprise the VEGF antagonist along with at least one inactive ingredient such as, *e.g.*, a pharmaceutically acceptable carrier. Other agents may be incorporated into the pharmaceutical composition to provide improved transfer, delivery, tolerance, and the like. The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly, in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the antibody is administered. A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences (15th ed, Mack Publishing Company, Easton, Pa., 1975), particularly Chapter 87 by Blaug, Seymour, therein. These formulations include, for example, powders, pastes, ointments, jellies, waxes, oils, lipids, lipid (cationic or anionic) containing vesicles (such as LIPOFECTIN™), DNA conjugates, anhydrous absorption pastes, oil-in-water and water-in-oil emulsions, emulsions carbowax (polyethylene glycols of various molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax. Any of the foregoing mixtures may be appropriate in the context of the methods of the present invention, provided that the VEGF antagonist is not inactivated by the formulation and the formulation is physiologically compatible and tolerable with the route of administration. See also Powell et al. PDA (1998) J Pharm Sci Technol. 52:238-311 and the citations therein for additional information related to excipients and carriers well known to pharmaceutical chemists.

[0027] Pharmaceutical formulations useful for administration by injection in the context of the present invention may be prepared by dissolving, suspending or emulsifying a VEGF antagonist in a sterile aqueous medium or an oily medium conventionally used for injections. As the aqueous medium for injections, there are, for example, physiological saline, an isotonic solution containing glucose and other auxiliary agents, etc., which may be used in combination with an appropriate solubilizing agent such as an alcohol (e.g., ethanol), a polyalcohol (e.g., propylene glycol, polyethylene glycol), a nonionic surfactant [e.g., polysorbate 80, HCO-50 (polyoxyethylene (50 mol) adduct of hydrogenated castor oil)], etc. As the oily medium, there may be employed, e.g., sesame oil, soybean oil, etc., which may be used in combination with a solubilizing agent such as benzyl benzoate, benzyl alcohol, etc. The injection thus prepared can be filled in an appropriate ampoule if desired.

MODES OF ADMINISTRATION

[0028] The VEGF antagonist (or pharmaceutical formulation comprising the VEGF antagonist) may be administered to the patient by any known delivery system and/or administration method.

In certain embodiments, the VEGF antagonist is administered to the patient by ocular, intraocular, intravitreal or subconjunctival injection. In other embodiments, the VEGF antagonist can be administered to the patient by topical administration, *e.g.*, via eye drops or other liquid, gel, ointment or fluid which contains the VEGF antagonist and can be applied directly to the eye. Other possible routes of administration include, *e.g.*, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral.

AMOUNT OF VEGF ANTAGONIST ADMINISTERED

[0029] Each dose of VEGF antagonist administered to the patient over the course of the treatment regimen may contain the same, or substantially the same, amount of VEGF antagonist. Alternatively, the quantity of VEGF antagonist contained within the individual doses may vary over the course of the treatment regimen. For example, in certain embodiments, a first quantity of VEGF antagonist is administered in the initial dose, a second quantity of VEGF antagonist is administered in the secondary doses, and a third quantity of VEGF antagonist is administered in the tertiary doses. The present invention contemplates dosing schemes in which the quantity of VEGF antagonist contained within the individual doses increases over time (*e.g.*, each subsequent dose contains more VEGF antagonist than the last), decreases over time (*e.g.*, each subsequent dose contains less VEGF antagonist than the last), initially increases then decreases, initially decreases then increases, or remains the same throughout the course of the administration regimen.

[0030] The amount of VEGF antagonist administered to the patient in each dose is, in most cases, a therapeutically effective amount. As used herein, the phrase "therapeutically effective amount" means a dose of VEGF antagonist that results in a detectable improvement in one or more symptoms or indicia of an angiogenic eye disorder, or a dose of VEGF antagonist that inhibits, prevents, lessens, or delays the progression of an angiogenic eye disorder. In the case of an anti-VEGF antibody or a VEGF receptor-based chimeric molecule such as VEGFR1R2-FcΔC1(a), a therapeutically effective amount can be from about 0.05 mg to about 5 mg, *e.g.*, about 0.05 mg, about 0.1 mg, about 0.15 mg, about 0.2 mg, about 0.25 mg, about 0.3 mg, about 0.35 mg, about 0.4 mg, about 0.45 mg, about 0.5 mg, about 0.55 mg, about 0.6 mg, about 0.65 mg, about 0.7 mg, about 0.75 mg, about 0.8 mg, about 0.85 mg, about 0.9 mg, about 1.0 mg, about 1.05 mg, about 1.1 mg, about 1.15 mg, about 1.2 mg, about 1.25 mg, about 1.3 mg, about 1.35 mg, about 1.4 mg, about 1.45 mg, about 1.5 mg, about 1.55 mg, about 1.6 mg, about 1.65 mg, about 1.7 mg, about 1.75 mg, about 1.8 mg, about 1.85 mg, about 1.9 mg, about 2.0 mg, about 2.05 mg, about 2.1 mg, about 2.15 mg, about 2.2 mg, about 2.25 mg, about 2.3 mg, about 2.35 mg, about 2.4 mg, about 2.45 mg, about 2.5 mg, about 2.55 mg, about 2.6 mg, about 2.65 mg, about 2.7 mg, about 2.75 mg, about 2.8 mg, about 2.85 mg, about 2.9 mg, about 3.0 mg, about 3.5 mg, about 4.0 mg, about 4.5 mg, or about 5.0 mg of the antibody or receptor-based chimeric molecule.

[0031] The amount of VEGF antagonist contained within the individual doses may be expressed in terms of milligrams of antibody per kilogram of patient body weight (*i.e.*, mg/kg). For example, the VEGF antagonist may be administered to a patient at a dose of about 0.0001 to about 10 mg/kg of patient body weight.

TREATMENT POPULATION AND EFFICACY

[0032] The methods of the present invention are useful for treating angiogenic eye disorders in patients that have been diagnosed with or are at risk of being afflicted with an angiogenic eye disorder. Generally, the methods of the present invention demonstrate efficacy within 104 weeks of the initiation of the treatment regimen (with the initial dose administered at "week 0"), *e.g.*, by the end of week 16, by the end of week 24, by the end of week 32, by the end of week 40, by the end of week 48, by the end of week 56, etc. In the context of methods for treating angiogenic eye disorders such as AMD, CRVO, and DME, "efficacy" means that, from the initiation of treatment, the patient exhibits a loss of 15 or fewer letters on the Early Treatment Diabetic Retinopathy Study (ETDRS) visual acuity chart. In certain embodiments, "efficacy" means a gain of one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or more) letters on the ETDRS chart from the time of initiation of treatment.

EXAMPLES

[0033] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

[0034] The exemplary VEGF antagonist used in all Examples set forth below is a dimeric molecule having two functional VEGF binding units. Each functional binding unit is comprised of Ig domain 2 from VEGFR1 fused to Ig domain 3 from VEGFR2, which in turn is fused to the hinge region of a human IgG1 Fc domain (VEGFR1R2-FcΔC1(a); encoded by SEQ ID NO:1). This VEGF antagonist is referred to in the examples below as "VEGFT". For purposes of the following Examples, "monthly" dosing is equivalent to dosing once every four weeks.

Example 1: Phase I Clinical Trial of Intravitreally Administered VEGF Receptor-Based Chimeric Molecule (VEGFT) in Subjects with Neovascular AMD

[0035] In this Phase I study, 21 subjects with neovascular AMD received a single intravitreal (IVT) dose of VEGFT. Five groups of three subjects each received either 0.05, 0.15, 0.5, 2 or 4

mg of VEGFT, and a sixth group of six subjects received 1 mg. No serious adverse events related to the study drug, and no identifiable intraocular inflammation was reported. Preliminary results showed that, following injection of VEGFT, a rapid decrease in foveal thickness and macular volume was observed that was maintained through 6 weeks. At Day 43 across all dose groups, mean excess retinal thickness [excess retinal thickness = (retinal thickness – 179μ)] on optical coherence tomography (OCT) was reduced from 119μ to 27μ as assessed by Fast Macular Scan and from 194μ to 60μ as assessed using a single Posterior Pole scan. The mean increase in best corrected visual acuity (BCVA) was 4.75 letters, and BCVA was stable or improved in 95% of subjects. In the 2 highest dose groups (2 and 4 mg), the mean increase in BCVA was 13.5 letters, with 3 of 6 subjects demonstrating improvement of ≥ 3 lines.

Example 2: Phase II Clinical Trial of Repeated Doses of Intravitreally Administered VEGF Receptor-Based Chimeric Molecule (VEGFT) in Subjects with Neovascular AMD

[0036] This study was a double-masked, randomized study of 3 doses (0.5, 2, and 4 mg) of VEGFT tested at 4-week and/or 12-week dosing intervals. There were 5 treatment arms in this study, as follows: 1) 0.5 mg every 4 weeks, 2) 0.5 mg every 12 weeks, 3) 2 mg every 4 weeks, 4) 2 mg every 12 weeks and 5) 4 mg every 12 weeks. Subjects were dosed at a fixed interval for the first 12 weeks, after which they were evaluated every 4 weeks for 9 months, during which additional doses were administered based on pre-specified criteria. All subjects were then followed for one year after their last dose of VEGFT. Preliminary data from a pre-planned interim analysis indicated that VEGFT met its primary endpoint of a statistically significant reduction in retinal thickness after 12 weeks compared with baseline (all groups combined, decrease of 135μ, $p < 0.0001$). Mean change from baseline in visual acuity, a key secondary endpoint of the study, also demonstrated statistically significant improvement (all groups combined, increase of 5.9 letters, $p < 0.0001$). Moreover, patients in the dose groups that received only a single dose, on average, demonstrated a decrease in excess retinal thickness ($p < 0.0001$) and an increase in visual acuity ($p = 0.012$) at 12 weeks. There were no drug-related serious adverse events, and treatment with the VEGF antagonists was generally well-tolerated. The most common adverse events were those typically associated with intravitreal injections.

Example 3: Phase I Clinical Trial of Systemically Administered VEGF Receptor-Based Chimeric Molecule (VEGFT) in Subjects with Neovascular AMD

[0037] This study was a placebo-controlled, sequential-group, dose-escalating safety, tolerability and bioeffect study of VEGFT by IV infusion in subjects with neovascular AMD. Groups of 8 subjects meeting eligibility criteria for subfoveal choroidal neovascularization (CNV) related to AMD were assigned to receive 4 IV injections of VEGFT or placebo at dose levels of 0.3, 1, or 3 mg/kg over an 8-week period.

[0038] Most adverse events that were attributed to VEGFT were mild to moderate in severity, but 2 of 5 subjects treated with 3 mg/kg experienced dose-limiting toxicity (DLT) (one with Grade 4 hypertension and one with Grade 2 proteinuria); therefore, all subjects in the 3 mg/kg dose group did not enter the study. The mean percent changes in excess retinal thickness were: -12%, -10%, -66%, and -60% for the placebo, 0.3, 1, and 3 mg/kg dose groups at day 15 (ANOVA $p < 0.02$), and -5.6%, +47.1%, and -63.3% for the placebo, 0.3, and 1 mg/kg dose groups at day 71 (ANOVA $p < 0.02$). There was a numerical improvement in BCVA in the subjects treated with VEGFT. As would be expected in such a small study, the results were not statistically significant.

Example 4: Phase III Clinical Trials of the Efficacy, Safety, and Tolerability of Repeated Doses of Intravitreal VEGFT in Subjects with Neovascular Age-Related Macular Degeneration

A. Objectives, Hypotheses and Endpoints

[0039] Two parallel Phase III clinical trials were carried out to investigate the use of VEGFT to treat patients with the neovascular form of age-related macular degeneration (Study 1 and Study 2). The primary objective of these studies was to assess the efficacy of IVT administered VEGFT compared to ranibizumab (Lucentis®, Genentech, Inc.), in a non-inferiority paradigm, in preventing moderate vision loss in subjects with all subtypes of neovascular AMD.

[0040] The secondary objectives were (a) to assess the safety and tolerability of repeated IVT administration of VEGFT in subjects with all sub-types of neovascular AMD for periods up to 2 years; and (b) to assess the effect of repeated IVT administration of VEGFT on Vision-Related Quality of Life (QOL) in subjects with all sub-types of neovascular AMD.

[0041] The primary hypothesis of these studies was that the proportion of subjects treated with VEGFT with stable or improved BCVA (<15 letters lost) is similar to the proportion treated with ranibizumab who have stable or improved BCVA, thereby demonstrating non-inferiority.

[0042] The primary endpoint for these studies was the prevention of vision loss of greater than or equal to 15 letters on the ETDRS chart, compared to baseline, at 52 weeks. Secondary endpoints were as follows: (a) change from baseline to Week 52 in letter score on the ETDRS chart; (b) gain from baseline to Week 52 of 15 letters or more on the ETDRS chart; (c) change from baseline to Week 52 in total NEI VFQ-25 score; and (d) change from baseline to Week 52 in CNV area.

B. Study Design

[0043] For each study, subjects were randomly assigned in a 1:1:1:1 ratio to 1 of 4 dosing regimens: (1) 2 mg VEGFT administered every 4 weeks (2Q4); (2) 0.5 mg VEGFT administered every 4 weeks (0.5Q4); (3) 2 mg VEGFT administered every 4 weeks to week 8 and then every 8 weeks (with sham injection at the interim 4-week visits when study drug was not administered

(2Q8); and (4) 0.5 mg ranibizumab administered every 4 weeks (RQ4). Subjects assigned to (2Q8) received the 2 mg injection every 4 weeks to week 8 and then a sham injection at interim 4-week visits (when study drug is not to be administered) during the first 52 weeks of the studies. (No sham injection were given at Week 52).

[0044] The study duration for each subject was scheduled to be 96 weeks plus the recruitment period. For the first 52 weeks (Year 1), subjects received an IVT or sham injection in the study eye every 4 weeks. (No sham injections were given at Week 52). During the second year of the study, subjects will be evaluated every 4 weeks and will receive IVT injection of study drug at intervals determined by specific dosing criteria, but at least every 12 weeks. (During the second year of the study, sham injections will not be given.) During this period, injections may be given as frequently as every 4 weeks, but no less frequently than every 12 weeks, according to the following criteria: (i) increase in central retinal thickness of ≥100 μm compared to the lowest previous value as measured by optical coherence tomography (OCT); or (ii) a loss from the best previous letter score of at least 5 ETDRS letters in conjunction with recurrent fluid as indicated by OCT; or (iii) new or persistent fluid as indicated by OCT; or (iv) new onset classic neovascularization, or new or persistent leak on fluorescein angiography (FA); or (v) new macular hemorrhage; or (vi) 12 weeks have elapsed since the previous injection. According to the present protocol, subjects must receive an injection at least every 12 weeks.

[0045] Subjects were evaluated at 4 weeks intervals for safety and best corrected visual acuity (BCVA) using the 4 meter ETDRS protocol. Quality of Life (QOL) was evaluated using the NEI VFQ-25 questionnaire. OCT and FA examinations were conducted periodically.

[0046] Approximately 1200 subjects were enrolled, with a target enrollment of 300 subjects per treatment arm.

[0047] To be eligible for this study, subjects were required to have subfoveal choroidal neovascularization (CNV) secondary to AMD. "Subfoveal" CNV was defined as the presence of subfoveal neovascularization, documented by FA, or presence of a lesion that is juxtafoveal in location angiographically but affects the fovea. Subject eligibility was confirmed based on angiographic criteria prior to randomization.

[0048] Only one eye was designated as the study eye. For subjects who met eligibility criteria in both eyes, the eye with the worse VA was selected as the study eye. If both eyes had equal VA, the eye with the clearest lens and ocular media and least amount of subfoveal scar or geographic atrophy was selected. If there was no objective basis for selecting the study eye, factors such as ocular dominance, other ocular pathology and subject preference were considered in making the selection.

[0049] Inclusion criteria for both studies were as follows: (i) signed informed consent; (ii) at least 50 years of age; (iii) active primary subfoveal CNV lesions secondary to AMD, including juxtafoveal lesions that affect the fovea as evidenced by FA in the study eye; (iv) CNV at least 50% of total lesion size; (v) early treatment diabetic retinopathy study (ETDRS) best-corrected

visual acuity of: 20/40 to 20/320 (letter score of 73 to 25) in the study eye; (vi) willing, committed, and able to return for all clinic visits and complete all study-related procedures; and (vii) able to read, understand and willing to sign the informed consent form (or, if unable to read due to visual impairment, be read to verbatim by the person administering the informed consent or a family member).

[0050] Exclusion criteria for both studies were as follows: 1. Any prior ocular (in the study eye) or systemic treatment or surgery for neovascular AMD except dietary supplements or vitamins. 2. Any prior or concomitant therapy with another investigational agent to treat neovascular AMD in the study eye, except dietary supplements or vitamins. 3. Prior treatment with anti-VEGF agents as follows: (a) Prior treatment with anti-VEGF therapy in the study eye was not allowed; (b) Prior treatment with anti-VEGF therapy in the fellow eye with an investigational agent (not FDA approved, e.g. bevacizumab) was allowed up to 3 months prior to first dose in the study, and such treatments were not allowed during the study. Prior treatment with an approved anti-VEGF therapy in the fellow eye was allowed; (c) Prior systemic anti-VEGF therapy, investigational or FDA/Health Canada approved, was only allowed up to 3 months prior to first dose, and was not allowed during the study. 4. Total lesion size > 12 disc areas (30.5 mm2, including blood, scars and neovascularization) as assessed by FA in the study eye. 5. Subretinal hemorrhage that is either 50% or more of the total lesion area, or if the blood is under the fovea and is 1 or more disc areas in size in the study eye. (If the blood is under the fovea, then the fovea must be surrounded 270 degrees by visible CNV.) 6. Scar or fibrosis, making up > 50% of total lesion in the study eye. 7. Scar, fibrosis, or atrophy involving the center of the fovea. 8. Presence of retinal pigment epithelial tears or rips involving the macula in the study eye. 9. History of any vitreous hemorrhage within 4 weeks prior to Visit 1 in the study eye. 10. Presence of other causes of CNV, including pathologic myopia (spherical equivalent of –8 diopters or more negative, or axial length of 25 mm or more), ocular histoplasmosis syndrome, angioid streaks, choroidal rupture, or multifocal choroiditis in the study eye. 11. History or clinical evidence of diabetic retinopathy, diabetic macular edema or any other vascular disease affecting the retina, other than AMD, in either eye. 12. Prior vitrectomy in the study eye. 13. History of retinal detachment or treatment or surgery for retinal detachment in the study eye. 14. Any history of macular hole of stage 2 and above in the study eye. 15. Any intraocular or periocular surgery within 3 months of Day 1 on the study eye, except lid surgery, which may not have taken place within 1 month of day 1, as long as it was unlikely to interfere with the injection. 16. Prior trabeculectomy or other filtration surgery in the study eye. 17. Uncontrolled glaucoma (defined as intraocular pressure greater than or equal to 25 mm Hg despite treatment with anti-glaucoma medication) in the study eye. 18. Active intraocular inflammation in either eye. 19. Active ocular or periocular infection in either eye. 20. Any ocular or periocular infection within the last 2 weeks prior to Screening in either eye. 21. Any history of uveitis in either eye. 22. Active scleritis or episcleritis in either eye. 23. Presence

or history of scleromalacia in either eye. 24. Aphakia or pseudophakia with absence of posterior capsule (unless it occurred as a result of a yttrium aluminum garnet [YAG] posterior capsulotomy) in the study eye. 25. Previous therapeutic radiation in the region of the study eye. 26. History of corneal transplant or corneal dystrophy in the study eye. 27. Significant media opacities, including cataract, in the study eye which might interfere with visual acuity, assessment of safety, or fundus photography. 28. Any concurrent intraocular condition in the study eye (e.g. cataract) that, in the opinion of the investigator, could require either medical or surgical intervention during the 96 week study period. 29. Any concurrent ocular condition in the study eye which, in the opinion of the investigator, could either increase the risk to the subject beyond what is to be expected from standard procedures of intraocular injection, or which otherwise may interfere with the injection procedure or with evaluation of efficacy or safety. 30. History of other disease, metabolic dysfunction, physical examination finding, or clinical laboratory finding giving reasonable suspicion of a disease or condition that contraindicates the use of an investigational drug or that might affect interpretation of the results of the study or render the subject at high risk for treatment complications. 31. Participation as a subject in any clinical study within the 12 weeks prior to Day 1. 32. Any systemic or ocular treatment with an investigational agent in the past 3 months prior to Day 1. 33. The use of long acting steroids, either systemically or intraocularly, in the 6 months prior to day 1. 34. Any history of allergy to povidone iodine. 35. Known serious allergy to the fluorescein sodium for injection in angiography. 36. Presence of any contraindications indicated in the FDA Approved label for ranibizumab (Lucentis®). 37. Females who were pregnant, breastfeeding, or of childbearing potential, unwilling to practice adequate contraception throughout the study. Adequate contraceptive measures include oral contraceptives (stable use for 2 or more cycles prior to screening); IUD; Depo-Provera®; Norplant® System implants; bilateral tubal ligation; vasectomy; condom or diaphragm plus either contraceptive sponge, foam or jelly.

[0051] Subjects were not allowed to receive any standard or investigational agents for treatment of their AMD in the study eye other than their assigned study treatment with VEGFT or ranibizumab as specified in the protocol until they completed the Completion/Early Termination visit assessments. This includes medications administered locally (e.g., IVT, topical, juxtascleral or periorbital routes), as well as those administered systemically with the intent of treating the study and/or fellow eye.

[0052] The study procedures are summarized as follows:

[0053] Best Corrected Visual Acuity: Visual function of the study eye and the fellow eye were assessed using the ETDRS protocol (The Early Treatment Diabetic Retinopathy Study Group) at 4 meters. Visual Acuity examiners were certified to ensure consistent measurement of BCVA. The VA examiners were required to remain masked to treatment assignment.

[0054] Optical Coherence Tomography: Retinal and lesion characteristics were evaluated using OCT on the study eye. At the Screen Visit (Visit 1) images were captured and transmitted

for both eyes. All OCT images were captured using the Zeiss Stratus OCT™ with software Version 3 or greater. OCT images were sent to an independent reading center where images were read by masked readers at visits where OCTs were required. All OCTs were electronically archived at the site as part of the source documentation. A subset of OCT images were read. OCT technicians were required to be certified by the reading center to ensure consistency and quality in image acquisition. Adequate efforts were made to ensure that OCT technicians at the site remained masked to treatment assignment.

[0055] Fundus Photography and Fluorescein Angiography (FA): The anatomical state of the retinal vasculature of the study eye was evaluated by funduscopic examination, fundus photography and FA. At the Screen Visit (Visit 1) funduscopic examination, fundus photography and FA were captured and transmitted for both eyes. Fundus and angiographic images were sent to an independent reading center where images were read by masked readers. The reading center confirmed subject eligibility based on angiographic criteria prior to randomization. All FAs and fundus photographs were archived at the site as part of the source documentation. Photographers were required to be certified by the reading center to ensure consistency and quality in image acquisition. Adequate efforts were made to ensure that all photographers at the site remain masked to treatment assignment.

[0056] Vision-Related Quality of Life: Vision-related QOL was assessed using the National Eye Institute 25-Item Visual Function Questionnaire (NEI VFQ-25) in the interviewer-administered format. NEI VFQ-25 was administered by certified personnel at a contracted call center. At the screening visit, the sites assisted the subject and initiated the first call to the call center to collect all of the subject's contact information and to complete the first NEI VFQ-25 on the phone prior to randomization and IVT injection. For all subsequent visits, the call center called the subject on the phone, prior to IVT injection, to complete the questionnaire.

[0057] Intraocular Pressure: Intraocular pressure (IOP) of the study eye was measured using applanation tonometry or Tonopen. The same method of IOP measurement was used in each subject throughout the study.

[0058]

C. Results Summary (52 Week Data)

[0059] The primary endpoint (prevention of moderate or severe vision loss as defined above) was met for all three VEGFT groups (2Q4, 0.5Q4 and 2Q8) in this study. The results from both studies are summarized in Table 1.

Table 1

	Ranibizumab 0.5 mg monthly (RQ4)	VEGFT 0.5 mg monthly (0.5Q4)	VEGFT 2 mg monthly (2Q4)	VEGFT 2 mg every 8 weeks[a] (2Q8)
Maintenance of vision* (% patients losing <15 letters) at week 52 versus baseline				
Study 1	94.4%	95.9%**	95.1%**	95.1%**
Study 2	94.4%	96.3%**	95.6%**	95.6%**
Mean improvement in vision* (letters) at 52 weeks versus baseline (p-value vs RQ4)***				
Study 1	8.1	6.9 (NS)	10.9 (p<0.01)	7.9 (NS)
Study 2	9.4	9.7 (NS)	7.6 (NS)	8.9 (NS

[a] Following three initial monthly doses
* Visual acuity was measured as the total number of letters read correctly on the Early Treatment Diabetic Retinopathy Study (ETDRS) eye chart.
** Statistically non-inferior based on a non-inferiority margin of 10%, using confidence interval approach (95.1% and 95% for Study 1 and Study 2, respectively)
*** Test for superiority
NS = non-significant

[0060] In Study 1, patients receiving VEGFT 2mg monthly (2Q4) achieved a statistically significant greater mean improvement in visual acuity at week 52 versus baseline (secondary endpoint), compared to ranibizumab 0.5mg monthly (RQ4); patients receiving VEGFT 2mg monthly on average gained 10.9 letters, compared to a mean 8.1 letter gain with ranibizumab 0.5mg dosed every month ($p<0.01$). All other dose groups of VEGFT in Study 1 and all dose groups in Study 2 were not statistically different from ranibizumab in this secondary endpoint.

[0061] A generally favorable safety profile was observed for both VEGFT and ranibizumab. The incidence of ocular treatment emergent adverse events was balanced across all four treatment groups in both studies, with the most frequent events associated with the injection procedure, the underlying disease, and/or the aging process. The most frequent ocular adverse events were conjunctival hemorrhage, macular degeneration, eye pain, retinal hemorrhage, and vitreous floaters. The most frequent serious non-ocular adverse events were typical of those reported in this elderly population who receive intravitreal treatment for wet AMD; the most frequently reported events were falls, pneumonia, myocardial infarction, atrial fibrillation, breast cancer, and acute coronary syndrome. There were no notable differences among the study arms.

Example 5: Phase II Clinical Trial of VEGFT in Subjects with Diabetic Macular Edema (DME)

[0062] In this study, 221 patients with clinically significant DME with central macular involvement were randomized, and 219 patients were treated with balanced distribution over five groups. The control group received macular laser therapy at baseline, and patients were eligible for repeat laser treatments, but no more frequently than at 16 week intervals. The

remaining four groups received VEGFT by intravitreal injection as follows: Two groups received 0.5 or 2 mg of VEGFT once every four weeks throughout the 12-month dosing period (0.5Q4 and 2Q4, respectively). Two groups received three initial doses of 2 mg VEGFT once every four weeks (*i.e.*, at baseline, and weeks 4 and 8), followed through week 52 by either once every 8 weeks dosing (2Q8) or as needed dosing with very strict repeat dosing criteria (PRN). Mean gains in visual acuity versus baseline were as shown in Table 2:

Table 2

	n	Mean change in visual acuity at week 24 versus baseline (letters)	Mean change in visual acuity at week 52 versus baseline (letters)
Laser	44	2.5	-1.3
VEGFT 0.5 mg monthly (0.5Q4)	44	8.6**	11.0**
VEGFT 2 mg monthly (2Q4)	44	11.4**	13.1**
VEGFT 2 mg every 8 weeks[a] (2Q8)	42	8.5**	9.7**
VEGFT 2 mg as needed[a] (PRN)	45	10.3**	12.0**

[a] Following three initial monthly doses
** $p < 0.01$ versus laser

[0063] In this study, the visual acuity gains achieved with VEGFT administration at week 24 were maintained or numerically improved up to completion of the study at week 52 in all VEGFT study groups, including 2 mg dosed every other month

[0064] As demonstrated in the foregoing Examples, the administration of VEGFT to patients suffering from angiogenic eye disorders (*e.g.*, AMD and DME) at a frequency of once every 8 weeks, following a single initial dose and two secondary doses administered four weeks apart, resulted in significant prevention of moderate or severe vision loss or improvements in visual acuity.

Example 6: A Randomized, Multicenter, Double-Masked Trial in Treatment Naïve Patients with Macular Edema Secondary to CRVO

[0065] In this randomized, double-masked, Phase 3 study, patients received 6 monthly injections of either 2 mg intravitreal VEGFT (114 patients) or sham injections (73 patients). From Week 24 to Week 52, all patients received 2 mg VEGFT as-needed (PRN) according to retreatment criteria. Thus, "sham-treated patients" means patients who received sham injections once every four weeks from Week 0 through Week 20, followed by intravitreal VEGFT as needed from Week 24 through Week 52. "VEGFT-treated patients" means patients who received VEGFT intravitreal injections once every four weeks from Week 0 through Week 20, followed by intravitreal VEGFT as needed from Week 24 through Week 52. The primary

endpoint was the proportion of patients who gained ≥15 ETDRS letters from baseline at Week 24. Secondary visual, anatomic, and Quality of Life NEI VFQ-25 outcomes at Weeks 24 and 52 were also evaluated.

[0066] At Week 24, 56.1% of VEGFT-treated patients gained ≥15 ETDRS letters from baseline vs 12.3% of sham-treated patients (P<0.0001). Similarly, at Week 52, 55.3% of VEGFT-treated patients gained ≥15 letters vs 30.1% of sham-treated patients (P<0.01). At Week 52, VEGFT-treated patients gained a mean of 16.2 letters vs 3.8 letters for sham-treated patients (P<0.001). Mean number of injections was 2.7 for VEGFT-treated patients vs 3.9 for sham-treated patients. Mean change in central retinal thickness was -413.0 µm for VEGFT-treated patients vs -381.8 µm for sham-treated patients. The proportion of patients with ocular neovascularization at Week 24 were 0% for VEGFT-treated patients and 6.8% for sham-treated patients, respectively; at Week 52 after receiving VEGFT PRN, proportions were 0% and 6.8% for VEGFT-treated and sham-treated. At Week 24, the mean change from baseline in the VFQ-25 total score was 7.2 vs 0.7 for the VEGFT-treated and sham-treated groups; at Week 52, the scores were 7.5 vs 5.1 for the VEGFT-treated and sham-treated groups.

[0067] This Example confirms that dosing monthly with 2 mg intravitreal VEGFT injection resulted in a statistically significant improvement in visual acuity at Week 24 that was maintained through Week 52 with PRN dosing compared with sham PRN treatment. VEGFT was generally well tolerated and had a generally favorable safety profile.

SEQUENCES

[0068] SEQ ID NO:1 (DNA sequence having 1377 nucleotides):

ATGGTCAGCTACTGGGACACCGGGGTCCTGCTGTGCGCGCTGCTCAGCTGTCTGCTTCTC
ACAGGATCTAGTTCCGGAAGTGATACCGGTAGACCTTTCGTAGAGATGTACAGTGAAATCC
CCGAAATTATACACATGACTGAAGGAAGGGAGCTCGTCATTCCCTGCCGGGTTACGTCAC
CTAACATCACTGTTACTTTAAAAAAGTTTCCACTTGACACTTTGATCCCTGATGGAAAACGC
ATAATCTGGGACAGTAGAAAGGGCTTCATCATATCAAATGCAACGTACAAAGAAATAGGGC
TTCTGACCTGTGAAGCAACAGTCAATGGGCATTTGTATAAGACAAACTATCTCACACATCGA
CAAACCAATACAATCATAGATGTGGTTCTGAGTCCGTCTCATGGAATTGAACTATCTGTTGG
AGAAAAGCTTGTCTTAAATTGTACAGCAAGAACTGAACTAAATGTGGGGATTGACTTCAACT
GGGAATACCCTTCTTCGAAGCATCAGCATAAGAAACTTGTAAACCGAGACCTAAAAACCCA
GTCTGGGAGTGAGATGAAGAAATTTTTGAGCACCTTAACTATAGATGGTGTAACCCGGAGT
GACCAAGGATTGTACACCTGTGCAGCATCCAGTGGGCTGATGACCAAGAAGAACAGCACA
TTTGTCAGGGTCCATGAAAAGGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAA
CTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATC
TCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGT
CAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGG
AGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACT

GGCTGAATGGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCG
AGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCC
CATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCT
ATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAG
ACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTG
GACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTG
CACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGA

[0069] SEQ ID NO:2 (polypeptide sequence having 458 amino acids):

MVSYWDTGVLLCALLSCLLLTGSSSGSDTGRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITV
TLKKFPLDTLIPDGKRIIWDSRKGFIISNATYKEIGLLTCEATVNGHLYKTNYLTHRQTNTIIDVVLS
PSHGIELSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNRDLKTQSGSEMKKFLSTLT
IDGVTRSDQGLYTCAASSGLMTKKNSTFVRVHEKDKTHTCPPCPAPELLGGPSVFLFPPKPKD
TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQD
WLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSD
IAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ
KSLSLSPGK

[0070] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. A method for treating an angiogenic eye disorder in a patient, said method comprising sequentially administering to the patient a single initial dose of a VEGF antagonist, followed by one or more secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonist;

wherein each secondary dose is administered 2 to 4 weeks after the immediately preceding dose; and

wherein each tertiary dose is administered at least 8 weeks after the immediately preceding dose.

2. The method of claim 1, wherein only a single secondary dose is administered to the patient, and wherein the single secondary dose is administered 4 weeks after the initial dose of the VEGF antagonist.

3. The method of claim 1, wherein only two secondary doses are administered to the patient, and wherein each secondary dose is administered 4 weeks after the immediately preceding dose.

4. The method of claim 3, wherein each tertiary dose is administered 8 weeks after the immediately preceding dose.

5. The method of claim 1, wherein at least 5 tertiary doses of the VEGF antagonist are administered to the patient, and wherein the first four tertiary doses are administered 8 weeks after the immediately preceding dose, and wherein each subsequent tertiary dose is administered 8 or 12 weeks after the immediately preceding dose.

6. The method of claim 1, wherein the angiogenic eye disorder is selected from the group consisting of: age related macular degeneration, diabetic retinopathy, diabetic macular edema, central retinal vein occlusion and corneal neovascularization.

7. The method of claim 6, wherein the angiogenic eye disorder is age related macular degeneration.

8. The method of claim 1, wherein the VEGF antagonist is an anti-VEGF antibody or fragment thereof, an anti-VEGF receptor antibody or fragment thereof, or a VEGF receptor-based chimeric molecule.

9. The method of claim 8, wherein the VEGF antagonist is a VEGF receptor-based chimeric molecule.

10. The method of claim 9, wherein the VEGF receptor-based chimeric molecule comprises VEGFR1R2-FcΔC1(a) encoded by the nucleic acid sequence of SEQ ID NO:1.

11. The method of claim 9, wherein the VEGF receptor-based chimeric molecule comprises (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130-231 of SEQ ID NO:2; and (3) a multimerization component comprising amino acids 232-457 of SEQ ID NO:2.

12. The method of claim 1, wherein all doses of the VEGF antagonist are administered to the patient by topical administration or by intraocular administration.

13. The method of claim 12, wherein all doses of the VEGF antagonist are administered to the patient by intraocular administration.

14. The method of claim 13, wherein the intraocular administration is intravitreal administration.

15. The method of claim 11, wherein all doses of the VEGF antagonist are administered to the patient by topical administration or by intraocular administration.

16. The method of claim 15, wherein all doses of the VEGF antagonist are administered to the patient by intraocular administration.

17. The method of claim 16, wherein the intraocular administration is intravitreal administration.

18. The method of claim 17, wherein all doses of the VEGF antagonist comprise from about 0.5 mg to about 2 mg of the VEGF antagonist.

19. The method of claim 18, wherein all doses of the VEGF antagonist comprise 0.5 mg of the VEGF antagonist.

20. The method of claim 18, wherein all doses of the VEGF antagonist comprise 2 mg of the VEGF antagonist.

21. A VEGF antagonist for use in a method of treating an angiogenic eye disorder in a patient, wherein the method comprises sequentially administering to the patient a single initial dose of a VEGF antagonist, followed by one or more secondary doses of the VEGF antagonist, followed by one or more tertiary doses of the VEGF antagonist;

wherein each secondary dose is administered 2 to 4 weeks after the immediately preceding dose; and

wherein each tertiary dose is administered at least 8 weeks after the immediately preceding dose.

22. The VEGF antagonist of claim 21, wherein only a single secondary dose is administered to the patient, and wherein the single secondary dose is administered 4 weeks after the initial dose of the VEGF antagonist.

23. The VEGF antagonist of claim 21, wherein only two secondary doses are administered to the patient, and wherein each secondary dose is administered 4 weeks after the immediately preceding dose.

24. The VEGF antagonist of any one of claims 21 to 23, wherein each tertiary dose is administered 8 weeks after the immediately preceding dose.

25. The VEGF antagonist of any one of claims 21 to 23, wherein at least 5 tertiary doses of the VEGF antagonist are administered to the patient, and wherein the first four tertiary doses are administered 8 weeks after the immediately preceding dose, and wherein each subsequent tertiary dose is administered 8 or 12 weeks after the immediately preceding dose.

26. The VEGF antagonist of any one of claims 21 to 25, wherein the angiogenic eye disorder is selected from the group consisting of: age related macular degeneration, diabetic retinopathy, diabetic macular edema, central retinal vein occlusion and corneal neovascularization.

27. The VEGF antagonist of claim 26, wherein the angiogenic eye disorder is age related macular degeneration.

28. The VEGF antagonist of any one of claims 21 to 27, wherein the VEGF antagonist is an anti-VEGF antibody or fragment thereof, an anti-VEGF receptor antibody or fragment thereof, or a VEGF receptor-based chimeric molecule.

29. The VEGF antagonist of claim 28, wherein the VEGF antagonist is a VEGF receptor-based chimeric molecule.

30. The VEGF antagonist of claim 29, wherein the VEGF receptor-based chimeric molecule comprises VEGFR1R2-FcΔC1(a) encoded by the nucleic acid sequence of SEQ ID NO:1.

31. The VEGF antagonist of claim 29, wherein the VEGF receptor-based chimeric molecule comprises (1) a VEGFR1 component comprising amino acids 27 to 129 of SEQ ID NO:2; (2) a VEGFR2 component comprising amino acids 130-231 of SEQ ID NO:2; and (3) a multimerization component comprising amino acids 232-457 of SEQ ID NO:2.

32. The VEGF antagonist of any one of claims 21 to 31, wherein all doses of the VEGF antagonist are administered to the patient by topical administration or by intraocular administration.

33. The VEGF antagonist of claim 32, wherein all doses of the VEGF antagonist are administered to the patient by intraocular administration.

34. The VEGF antagonist of claim 33, wherein the intraocular administration is intravitreal administration.

35. The VEGF antagonist of claim 34, wherein all doses of the VEGF antagonist comprise from about 0.5 mg to about 2 mg of the VEGF antagonist.

36. The VEGF antagonist of claim 35, wherein all doses of the VEGF antagonist comprise 0.5 mg of the VEGF antagonist.

37. The VEGF antagonist of claim 35, wherein all doses of the VEGF antagonist comprise 2 mg of the VEGF antagonist.

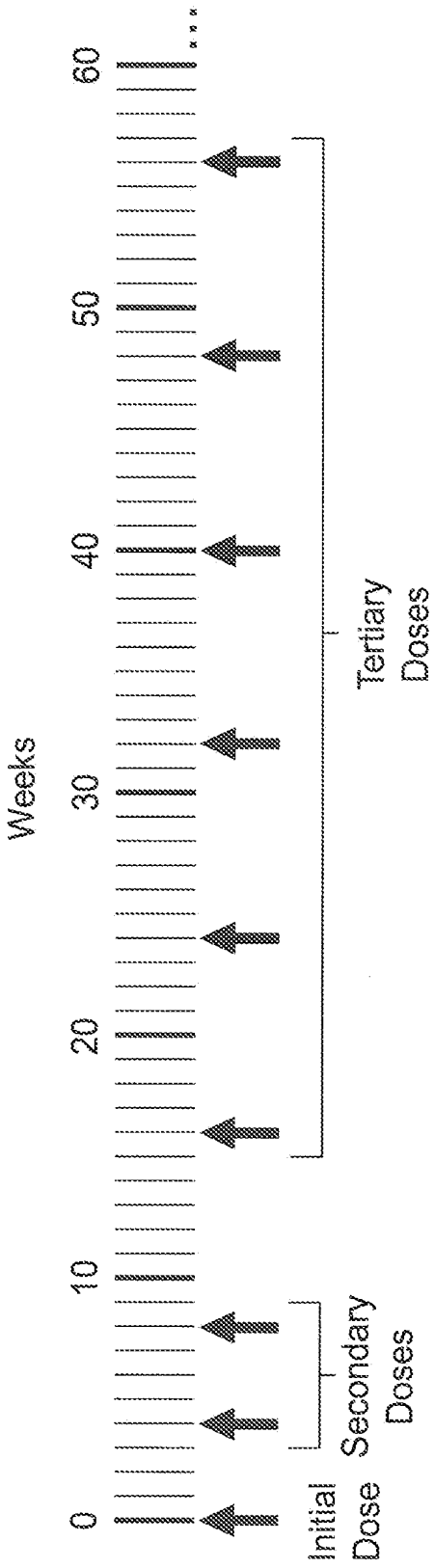

Figure 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/020855

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/18 A61P27/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Thomson Reuters Integrity: "VEGF Trap-Eye final phase II results in age-related macular degeneration presented at 2008 Retina Society Meeting", , 28 September 2008 (2008-09-28), pages 1-1, XP002674126, Retrieved from the Internet: URL:https://integrity.thomson-pharma.com/integrity/xmlxsl/pk_ref_list.xml_show_llist at_refs?p_session_id=1868065&p_orig=&p_count=354&p_num_dailys=42&p_qry_save=&p_subtitol=Biomedical%20Literature%20List&p_dailys=N&p_tsearch=A&p_text=N&p_whatToShow=REF&p_prousQuantity=5&p_page=10#link [retrieved on 2012-04-18] the whole document ----- -/--	1-37

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 April 2012	22/05/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Rodrigo-Simón, Ana

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NGUYEN Q D ET AL: "A Phase I Study of Intravitreal Vascular Endothelial Growth Factor Trap-Eye in Patients with Neovascular Age-Related Macular Degeneration", OPHTHALMOLOGY, J. B. LIPPINCOTT CO., PHILADELPHIA, PA, US, vol. 116, no. 11, 1 November 2009 (2009-11-01), pages 2141-2148.E1, XP026732998, ISSN: 0161-6420, DOI: 10.1016/J.OPHTHA.2009.04.030 [retrieved on 2009-08-22] abstract page 2142, column 1 - paragraph 3 page 2147, column 1, paragraph 2	1-37
A	US 2007/190058 A1 (SHAMS NAVEED [US]) 16 August 2007 (2007-08-16) claim 1 paragraph [0112] page 12, paragraph 111	1-37
A	US 2006/172944 A1 (WIEGAND STANLEY J [US] ET AL) 3 August 2006 (2006-08-03) paragraphs [0008], [0017] claims 1,2	1-37
A	US 2005/163798 A1 (PAPADOPOULOS NICHOLAS J [US] ET AL) 28 July 2005 (2005-07-28) claim 65 paragraph [0122]	1-37
A	WO 00/75319 A1 (REGENERON PHARMA [US]; PAPADOPOULOS NICHOLAS J [US]; DAVIS SAMUEL [US]) 14 December 2000 (2000-12-14) page 87, line 14 - line 88 page 14, line 7 - line 12	1-37
A	US 2006/058234 A1 (DALY THOMAS J [US] ET AL) 16 March 2006 (2006-03-16) paragraph [0067] claim 23 page 2, paragraph 15	1-37
A	US 2005/260203 A1 (WIEGAND STANLEY J [US] ET AL) 24 November 2005 (2005-11-24) page 7; example 8 page 8; example 9 page 8; example 11 page 9; example 2	1-37

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1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DO DV ET AL.: "An exploratory study of the safety, tolerability and bioactivity of a single intravitreal injection of vascular endothelial growth factor Trap-Eye in patients with diabetic macular oedema.", BR J OPHTHALMOL, vol. 93, no. 2, February 2009 (2009-02), pages 144-1449, XP009158490, abstract page 149, paragraph 2	1-37
A	ANANT PAI ET AL.: "Current concepts in intravitreal drug therapy for diabetic retinopathy", SAUDI JOURNAL OF OPHTHALMOLOGY, vol. 24, no. 4, 30 June 2010 (2010-06-30), pages 143-149, XP002674122, page 147, paragraph 4	1-37
X,P	DO DIANA V ET AL: "The DA VINCI Study: phase 2 primary results of VEGF Trap-Eye in patients with diabetic macular edema.", OPHTHALMOLOGY SEP 2011 LNKD-PUBMED:21546089, vol. 118, no. 9, September 2011 (2011-09), pages 1819-1826, XP002674123, ISSN: 1549-4713 the whole document	1-37
T	STEWART MW.: "The expanding role of vascular endothelial growth factor inhibitors in ophthalmology", MAYO CLIN PROC, vol. 87, no. 1, January 2012 (2012-01), pages 77-88, XP002674124, the whole document	
A	anonymous: "LUCENTIS® (ranibizumab injection) Intravitreal Injection", , June 2006 (2006-06), pages 1-3, XP002674125, Retrieved from the Internet: URL:http://www.gene.com/gene/products/information/pdf/lucentis-prescribing.pdf [retrieved on 2012-04-18] page 1, column 1, line 20 - page 1, column 2, line 9	1-37

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2012/020855

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2007190058 A1	16-08-2007	NONE	
US 2006172944 A1	03-08-2006	US 2006172944 A1	03-08-2006
		US 2008085276 A1	10-04-2008
US 2005163798 A1	28-07-2005	US 7070959 B1	04-07-2006
		US 2005163798 A1	28-07-2005
		US 2005175610 A1	11-08-2005
		US 2005245447 A1	03-11-2005
		US 2008194460 A1	14-08-2008
		US 2008194799 A1	14-08-2008
		US 2008220004 A1	11-09-2008
		US 2009081217 A1	26-03-2009
		US 2009155899 A1	18-06-2009
		US 2010221782 A1	02-09-2010
		US 2011028698 A1	03-02-2011
		US 2012064621 A1	15-03-2012
WO 0075319 A1	14-12-2000	AT 293164 T	15-04-2005
		AT 417928 T	15-01-2009
		AU 779303 B2	13-01-2005
		AU 5040400 A	28-12-2000
		BR 0011407 A	02-04-2002
		CA 2376379 A1	14-12-2000
		CN 1369009 A	11-09-2002
		CN 101433715 A	20-05-2009
		CZ 20014387 A3	16-10-2002
		DE 60019415 D1	19-05-2005
		DE 60019415 T2	09-03-2006
		DK 1544299 T3	23-03-2009
		EP 1183353 A1	06-03-2002
		EP 1544299 A1	22-06-2005
		ES 2237429 T3	01-08-2005
		ES 2319305 T3	06-05-2009
		HK 1043388 A1	10-06-2005
		HR 20010908 A2	30-04-2005
		HU 0201515 A2	28-08-2002
		IL 146890 A	08-07-2008
		JP 4723140 B2	13-07-2011
		JP 2003501089 A	14-01-2003
		JP 2011024595 A	10-02-2011
		MX PA01012630 A	22-07-2002
		NO 20016036 A	08-02-2002
		NZ 515913 A	30-01-2004
		PL 352246 A1	11-08-2003
		PT 1183353 E	30-06-2005
		PT 1544299 E	18-03-2009
		SK 17522001 A3	01-04-2003
		WO 0075319 A1	14-12-2000
		ZA 200110068 A	06-12-2002
US 2006058234 A1	16-03-2006	NONE	
US 2005260203 A1	24-11-2005	NONE	

PERSPECTIVE

Aflibercept for Age-Related Macular Degeneration: A Game-Changer or Quiet Addition?

DAVID J. BROWNING, PETER K. KAISER, PHILIP J. ROSENFELD, AND MICHAEL W. STEWART

• PURPOSE: To describe the pharmacokinetics, preclinical studies, and clinical trials of the newly approved anti–vascular endothelial growth factor (VEGF) drug aflibercept (Eylea (VEGF Trap-Eye); Regeneron; and Bayer).

• DESIGN: Review with editorial commentary.

• METHODS: A review of the medical literature and pertinent Internet postings combined with analysis of key studies with expert opinion regarding the use of aflibercept for the treatment of exudative age-related macular degeneration.

• RESULTS: Aflibercept, a fusion protein with binding domains from native VEGF receptors, binds VEGF-A, VEGF-B, and placental growth factors 1 and 2 with high affinity. Preclinical ophthalmologic studies demonstrated that aflibercept suppresses choroidal neovascularization in several animal models. The results of phase 1 and 2 trials showed excellent short-term suppression of choroidal neovascularization in patients with exudative age-related macular degeneration and suggested a longer durability of aflibercept compared with other anti-VEGF drugs. The pivotal phase 3 Vascular Endothelial Growth Factor (VEGF) Trap-Eye: Investigation of Efficacy and Safety in Wet Age-Related Macular Degeneration 1 and 2 trials showed that monthly and bimonthly aflibercept were noninferior to monthly ranibizumab at preventing vision loss (< 15-letter loss) with comparable vision gains and safety. Year 2 treatment involved monthly pro re nata injections with required injections every 3 months and maintained vision gains from the first year, with an average of 4.2 injections of aflibercept and 4.7 injections of ranibizumab.

• CONCLUSIONS: Aflibercept promises to deliver excellent visual outcomes for exudative age-related macular degeneration patients while undergoing fewer injections compared with ranibizumab. With a wholesale cost of $1850 per dose, the cost per patient with aflibercept treatment promises to be lower than with ranibizumab. (Am J Ophthalmol 2012;154:222–226. © 2012 by Elsevier Inc. All rights reserved.)

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From the Charlotte Eye, Ear, Nose and Throat Associates, Charlotte, North Carolina (D.J.B.); the Cole Eye Institute, Cleveland Clinic, Cleveland, Ohio (P.K.K.); the Bascom Palmer Eye Institute, Miller School of Medicine, Miami, Florida (P.J.R.); and the College of Medicine, Mayo Clinic, Jacksonville, Florida (M.W.S.).

Inquiries to Michael W. Stewart, Department of Ophthalmology, College of Medicine, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224; e-mail: stewart.michael@mayo.edu

THE RECENT INTRODUCTION OF DRUGS THAT INHIBIT the actions of vascular endothelial growth factor (VEGF) has revolutionized the treatment of exudative age-related macular degeneration (AMD). Current anti-VEGF drugs bind to VEGF in the extracellular space and prevent its activation of transmembrane VEGF receptors. Between 2004 and 2006, 3 anti-VEGF drugs were introduced to ophthalmology either after receiving regulatory approval for the treatment of AMD or being used in an off-label manner. They exhibit important differences in their sites of activity, formulation methods, binding affinities, and biologic activities. Pegaptanib (Macugen; Eyetech, New York, New York, USA) is a ribonucleic acid aptamer that blocks the main pathologic isoform of VEGF ($VEGF_{165}$) by attaching to its heparin binding domain, whereas ranibizumab (Lucentis; Novartis, Basel, Switzerland; Genentech, South San Francisco, California, USA; and Roche, Basel, Switzerland) and bevacizumab (Avastin; Genentech and Roche) are, respectively, an affinity-matured, humanized, monoclonal antibody fragment and a full-length, humanized, monoclonal antibody to VEGF. Both work by blocking the receptor binding domain of all isoforms of VEGF-A.[1]

Now, more than 5 years later, the next anti-VEGF drug, aflibercept (Eylea; Regeneron, Tarrytown, New York, USA, and Bayer, Berlin, Germany) has been approved by the United States Food and Drug Administration. This soluble decoy receptor is produced by fusing all-human DNA sequences of the second immunoglobulin (Ig) domain of human VEGF receptor (VEGFR) 1 to the third Ig domain of human VEGFR-2, which then is fused to the Fc region of human IgG-1.[2] Aflibercept binds to all VEGF-A and VEGF-B isoforms, as well as the highly related placental growth factor. Aflibercept is produced in a Chinese hamster ovary cell line, and then is specially purified and formulated exclusively for intraocular injection.

When developing aflibercept, investigators noted that most of the vasoproliferative and hyperpermeability effects of VEGF are mediated by activation of VEGFR-2, yet

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VEGFR-1 actually binds to VEGF-A with a much higher affinity (K_d = 10 to 20 pM vs K_d = 100 to 300 pM; binding affinity is inversely proportional to K_d). Therefore, investigators initially created a parent VEGF Trap with 3 binding domains from the high-affinity VEGFR-1 fused to the Fc region of IgG1. Although this molecule bound VEGF tightly, it exhibited unfavorable pharmacokinetic characteristics as a result of rapid sequestration within the intercellular matrix. By substituting the more basic binding sequences from VEGFR-2, Regeneron developed a high-affinity (K_d = 0.5 pM) soluble receptor molecule that exhibited minimal binding to the extracellular matrix.[2] This gives aflibercept a significantly higher binding affinity to $VEGF_{165}$ than either bevacizumab (K_d = 58 pM) or ranibizumab (K_d = 46 pM).[3] The intravitreal half-life of aflibercept is 4.7 days in rabbits (Regeneron, data on file), comparable with that of bevacizumab (4.32 days), but longer than that of ranibizumab (2.88 days). Although pharmacokinetic studies have not been performed in human eyes, the molecular size of aflibercept (115 kDa) would suggest that its intraocular half-life would more closely resemble bevacizumab (149 kDa; human half-life, 8.24 days) than the smaller ranibizumab (48 kDa).[4]

The predicted biological activity of a therapeutic macromolecule depends to a large degree on both its intraocular half-life and its VEGF binding affinity.[5] The binding affinity of aflibercept to VEGF-A is substantially greater than any of the other 3 anti-VEGF drugs, but recent biological assays with aflibercept, ranibizumab, and bevacizumab report that the relative inhibition of both endothelial cell proliferation and migration ranges from 1× to 100×.[3,6] The reason for this wide variation in inhibitory effects is unknown, but the use of different reagent concentrations has been suggested.[3] Based on these data and estimated intraocular drug half-lives, mathematical modeling predicts that a single intravitreal injection of aflibercept 2 mg would last between 48 and 83 days (compared with 30 days for ranibizumab).[5]

PRECLINICAL AND ONCOLOGY TRIALS

THE DEVELOPMENT OF OUR CURRENTLY USED ANTI-VEGF drugs was based on a different long-term therapeutic strategy. Whereas bevacizumab was developed with a long systemic residence time exclusively for the systemic treatment of advanced cancers, ranibizumab was designed to have a short systemic clearance half-life by removing the Fc fragment from the parent IgG molecule, and its affinity for VEGF was enhanced by changing 5 of its amino acids, thereby optimizing it for the intraocular treatment of exudative ocular diseases. Additionally, creating a molecule smaller than the retinal exclusion limit (76 kDa) was believed to be necessary to penetrate the inner retina. Aflibercept, however, was developed to treat both advanced solid tumors and ophthalmic vascular conditions.

Aflibercept has decreased tumor growth successfully in several orthotopic mouse models: ovarian carcinoma, hepatoblastoma, cholangiocarcinoma, pancreatic ductal carcinoma, Wilms tumor, renal cell carcinoma, and glioblastoma.[7] Suppression of experimental neuroblastoma growth by anti-VEGF agents was achieved with the following relative efficacies: aflibercept > monoclonal antibody > aptamer to $VEGF_{165}$.[8]

Phase 1 and 2 oncologic studies evaluated escalating doses of aflibercept in patients with chemotherapy-resistant renal cell carcinoma, Hodgkin lymphoma, glioblastoma, and anaplastic glioma. In a phase 3 trial of patients with advanced adenocarcinoma of the colon, intravenously administered aflibercept (Zaltrap; Regeneron and Sanofi Aventis, Bridgewater, New Jersey, USA) extended progression-free survival from 4.7 to 6.9 months (P = .00007) and overall survival from 12.1 to 13.5 months (P = .0032).[9]

In ocular preclinical studies, aflibercept demonstrated in vivo activity against several murine models of choroidal neovascularization (CNV). It prevented the development of CNV after intense laser photocoagulation to the retinal pigment epithelium, inhibited the development of CNV in VEGF-secreting transgenic mice, and prevented the development of CNV in mice receiving exogenous VEGF.[10] This favorable response in part was the result of decreases in both intercellular adhesion molecule-1 and endothelial nitric oxide synthetase synthesis within the CNV.[11] After matrigel-induced CNV in rats, aflibercept injections at 2 and 6 days prevented the development of CNV, whereas injections at 10 days decreased collagen synthesis and leukocyte infiltration.[12] Prolonged, high-dose aflibercept therapy causes loss of both endothelial cells and pericytes, thereby reducing vessels to basement membrane ghosts.[13] These successful preclinical studies justified the development of aflibercept trials for the treatment of exudative AMD in humans.

OPHTHALMOLOGY TRIALS

BASED ON A PLAUSIBLE BIOLOGIC RATIONALE FOR SUSpected efficacy and an acceptable safety profile in preclinical animal studies, aflibercept first was administered intravenously to patients with neovascular AMD in a placebo-controlled clinical trial.[14] In 2 of the 5 patients receiving 3.0 mg/kg aflibercept, systemic toxicity developed (1 patient had grade 2 proteinuria and 1 patient had grade 4 hypertension). A subsequent phase 1 study of intravitreal aflibercept showed that up to 4-mg dosing decreased macular edema and subretinal fluid for at least 6 weeks and was tolerated well with no ocular inflammation.[15] A phase 2 clinical trial of 159 patients explored 5 different aflibercept dosing regimens with a primary outcome at 12 weeks: 0.5 mg every 4 weeks, 2 mg every 4 weeks, 0.5 mg every 12 weeks, 2 mg every 12 weeks (thus,

TABLE. Primary and Secondary Outcomes of 2 Phase 3 Clinical Trials of Aflibercept at 52 Weeks of Follow-up

Study/End Point	Aflibercept 0.5 mg Monthly	Aflibercept 2 mg Monthly	Aflibercept 2 mg Bimonthly after 3 Monthly Injections	Ranibizumab 0.5 mg Monthly
VIEW 1/maintenance of VA (%)	95.9	95.1	95.1	94.4
VIEW 2/maintenance of VA (%)	96.3	95.6	95.6	94.4
VIEW 1/mean VA improvement (letters)	6.9	10.9[a]	7.9	8.1
VIEW 2/mean VA improvement (letters)	9.7	7.6	8.9	9.4

VIEW = Vascular Endothelial Growth Factor (VEGF) Trap-Eye: Investigation of Efficacy and Safety in Wet Age-Related Macular Degeneration Study; VA = visual acuity.

[a]$P < .01$ compared with the ranibizumab group. All other comparisons of aflibercept groups with the ranibizumab group were not statistically significantly different. Maintenance of VA = lost fewer than 3 lines on the Early Treatment Diabetic Retinopathy Study chart.

only 1 treatment before the primary outcome), and 4 mg every 12 weeks. At 12 weeks, quarterly dosing reduced macular thickening and improved best-corrected visual acuity, but monthly dosing was more effective.[16] The same group of patients subsequently was followed up monthly and was retreated pro re nata with their assigned dose based on the following criteria: central retinal or lesion thickness increased 100 μm or more from best previous reading or best-corrected vision dropped 5 or more letters with recurrent fluid on optical coherence tomography if persistent fluid was seen on optical coherence tomography or if there was new CNV, persistent leakage on fluorescein angiography, or a new macular hemorrhage.[17] Pro re nata dosing maintained the efficacy established in each of the 5 groups during the first part of the trial. At 52 weeks of follow-up, patients in the initial monthly dosing regimens tended to have improved visual acuity outcomes compared with those in the quarterly dosing groups, regardless of the aflibercept dose, indicating the importance of loading dose before the less frequent dosing.

Based on the results from these phase 1 and 2 studies, 2 parallel phase 3 pivotal clinical trials were designed and conducted to establish the noninferiority of 3 regimens of aflibercept compared with monthly ranibizumab 0.5 mg. These trials were the Vascular Endothelial Growth Factor (VEGF) Trap-Eye: Investigation of Efficacy and Safety in Wet Age-Related Macular Degeneration Study 1, a study of 1217 patients in the United States and Canada, and the Vascular Endothelial Growth Factor (VEGF) Trap-Eye: Investigation of Efficacy and Safety in Wet Age-Related Macular Degeneration Study 2, a study of 1240 patients in Europe, Asia, Japan, and Latin America.[18] In both trials, patients were randomized 1:1:1:1 to 1 of 4 groups: ranibizumab 0.5 mg given monthly, aflibercept 0.5 mg given monthly, aflibercept 2 mg given monthly, and aflibercept 2 mg given monthly for 3 injections followed by continued injections every 2 months. The end point for both trials was at 52 weeks. The primary outcome measure, termed maintenance of vision, was the percentage of eyes losing fewer than 3 lines of visual acuity on the Early Treatment Diabetic Retinopathy chart. A secondary visual acuity outcome was mean improvement in visual acuity measured in number of additional letters read on the Early Treatment Diabetic Retinopathy chart compared with baseline.

Both studies showed that all 3 regimens of aflibercept were noninferior to the ranibizumab monthly regimen (Table). Of greatest interest to clinicians was the aflibercept arm featuring bimonthly injections after 3 monthly loading doses. This regimen offers fewer injections for the patient, less risk of endophthalmitis, and no loss of efficacy at least through 52 weeks.

The secondary outcomes were concordant with the primary outcomes (Table). Both studies showed that all 3 aflibercept regimens were associated with mean visual acuity improvements and were noninferior to the ranibizumab monthly regimen.[18] Ocular adverse events in both studies were balanced across the 4 treatment groups and were those commonly associated with intravitreal injections for exudative AMD: conjunctival hemorrhages, eye pain, and vitreous floaters. Systemic adverse events also were balanced across the groups and were those commonly found in elderly patients with exudative AMD: falls, pneumonia, cancer, and cardiovascular disease. There was no evidence to suggest that any arm was associated with an increased risk of thromboembolic events such as stroke or myocardial infarction.[18]

In year 2 of both trials, patients in all 4 groups were continued on the same drug and dosage to which they were originally randomized, but with a modified frequency. Repeat injections were administered as frequently as every month if patients met prespecified optical coherence tomography or visual acuity retreatment criteria, or both, but all patients were required to receive an injection once every 3 months. In an integrated analysis of both trials, patients initially receiving aflibercept 2 mg every 8 weeks, and those receiving ranibizumab 0.5 mg every 4 weeks maintained the vision gains reported at week 52 through week 96 (+8.4 letters to +7.6 letters vs +8.7 letters to +7.9 letters, respectively). On average, patients in both groups required relatively few injections during the second year (aflibercept, 4.2 injections; ranibizumab, 4.7 injections). The proportion of patients that required 6 or more injections in the second year was higher in

the ranibizumab group (26.5%) compared with the aflibercept group (15.9%).[19]

DRUG AND TREATMENT REGIMENS

IN 2008, BEVACIZUMAB WAS USED FOR 60% OF THE ANTI-VEGF injections in Medicare-fee-for-service beneficiaries with exudative AMD.[20] In 2011, the Patterns and Trends Survey by the American Society of Retina Specialists reported that 70% of retina specialists used bevacizumab compared with ranibizumab for exudative AMD. The preference for off-label bevacizumab was influenced by its low cost and the perception that its efficacy and safety were similar to ranibizumab, which was confirmed by the 1-year results of the Comparison of Age-Related Macular Degeneration Treatment Trials.[21] This is in contrast to the use of ranibizumab, which was influenced by the clinical efficacy demonstrated in its extensive phase 3 testing and its on-label status. In addition to the difference in cost between the 2 drugs, there are other financial incentives, such as volume discounts to high-using physicians and the accumulation of frequent flyer miles for credit card purchases, and disincentives that can influence the use of one drug over the other in clinical practice.[22]

Now that aflibercept is approved in the United States for exudative AMD, how will clinicians decide between these available anti-VEGF drugs and the different treatment regimens? Most likely, those clinicians using a given anti-VEGF therapy because of the financial incentives will continue using the same drug as their first-line treatment, at least in the short term. For those clinicians using ranibizumab because it is the approved on-label treatment for exudative AMD, the use of aflibercept will begin cautiously for 2 reasons. First, clinicians need to be assured that Medicare will reimburse aflibercept in a timely fashion, and second, retina specialists will want to gain some experience with aflibercept with selected patients, particularly patients who have required frequent retreatment with ranibizumab. If postapproval experience confirms clinical trial evidence that aflibercept has a longer duration of effect than ranibizumab and Medicare reliably reimburses aflibercept claims, then the use of aflibercept in this population of patients should accelerate. For those clinicians using bevacizumab because of its low cost, the transition to aflibercept will be slowed by its cost, which is only $100 less than ranibizumab but still $1800 more than bevacizumab. However, when cost is denominated by time between injections, the total cost of aflibercept treatment is not so near to that of ranibizumab as the per-vial cost would suggest, and therefore adoption by cost-conscious clinicians may be faster than expected. Moreover, if aflibercept proves to be more effective in reducing the need for retreatment, this will provide further impetus driving conversion of bevacizumab patients to aflibercept, because monthly bevacizumab was the only regimen shown to be equivalent to ranibizumab in the Comparison of Age-Related Macular Degeneration Treatment Trials. Aflibercept already has received its Medicare J-code, and if we assume that its durability proves to be superior to current anti-VEGF therapies in treating exudative AMD patients, then we should assume that aflibercept would become the dominant on-label drug for the treatment of exudative AMD.

It is unlikely that clinicians will adopt a treatment regimen that is significantly different from the current regimens used in clinical practice. The 2011 Patterns and Trends Survey showed that most clinicians used a treat-and-extend (60%) or a treat-and-observe (32%) strategy, rather than the fixed treatment intervals used in the phase 3 trials and Comparison of Age-Related Macular Degeneration Treatment Trials. Although the Vascular Endothelial Growth Factor (VEGF) Trap-Eye: Investigation of Efficacy and Safety in Wet Age-Related Macular Degeneration trials demonstrated noninferiority compared with monthly ranibizumab when aflibercept was given monthly for 3 doses followed by doses every 2 months, most clinicians likely will continue to customize their management strategy to reduce the number of visits and injections without compromising visual acuity outcomes.

Aflibercept is a useful addition to our treatment armamentarium. Although visual acuity outcomes should be the same as monthly ranibizumab or bevacizumab, the patients could benefit by receiving fewer injections, and clinicians will benefit by seeing these patients less often. However, this decrease in the total number of visits and injections may be short lived. As new patients are diagnosed with exudative AMD and require treatment, the overall number of injections will once again increase. For the individual patient who requires fewer visits to the physician's office and fewer injections, aflibercept is definitely a game changer. For the clinician, it is only a temporary reprieve until even longer-acting therapies arrive.

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REFERENCES

1. Ferrara N, Damico L, Shams N, et al. Development of ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration. *Retina* 2006;26(8):859–870.
2. Holash J, Davis S, Papadopoulos N, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci U S A* 2002;99(17):11393–11398.
3. Papadopoulos N, Martin J, Run Q, et al. Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab. *Angiogenesis* 2012. Forthcoming.
4. Stewart MW. What are the half-lives of ranibizumab and aflibercept (VEGF Trap-eye) in human eyes? Calculations with a mathematical model. *Eye Reports* 2011;1(1):e5.
5. Stewart MW, Rosenfeld PJ. Predicted biological activity of intravitreal VEGF Trap. *Br J Ophthalmol* 2008;92(5):667–668.
6. Yu L, Liang XH, Ferrara N. Comparing protein VEGF inhibitors: *in vitro* biological studies. *Biochem Biophys Res Commun* 2011;408(2):276–281.
7. Stewart MW. Aflibercept (VEGF-Trap): the next anti-VEGF drug. *Inflamm Allergy Drug Targets* 2011;10(6):497–508.
8. Kim ES, Serur A, Huang J, et al. Potent VEGF blockade causes regression of co-opted vessels in a model of neuroblastoma. *Proc Natl Acad Sci U S A* 2002;99(17):11399–11404.
9. Wang T-F, Lockhart AC. Aflibercept in the treatment of metastatic colorectal cancer. *Clin Med Insights Oncol* 2012; 6:19–30.
10. Saichin Y, Saichin Y, Takahashi K, et al. VEGF-trap$_{R1R2}$ suppresses choroidal neovascularization and VEGF-induced breakdown of the blood-retinal barrier. *J Cell Physiol* 2003; 195(2):241–248.
11. Joussen AM, Poulaki V, Qin W, et al. Retinal vascular endothelial growth factor induces intercellular adhesion molecule-1 and endothelial nitric oxide synthase expression and initiates early diabetic retinal leukocyte adhesion in vivo. *Am J Pathol* 2002;160(2):501–509.
12. Cao J, Zhao L, Li Y, et al. A subretinal matrigel rat choroidal neovascularization (CNV) model and inhibition of CNV and associated inflammation and fibrosis by VEGF trap. *Invest Ophthalmol Vis Sci* 2010;51(11):6009–6017.
13. Inai T, Mancuso M, Hashizume H, et al. Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. *Am J Pathol* 2004;165(1):35–52.
14. Nguyen QD, Shah SM, Hafiz G, et al. CLEAR-AMD 1 Study Group. A phase I trial of an IV-administered vascular endothelial growth factor trap for treatment in patients with choroidal neovascularization due to age-related macular degeneration. *Ophthalmology* 2006;113(9):1522–1532.
15. Nguyen QD, Shah SM, Browning DJ, et al. A phase I study of intravitreal vascular endothelial growth factor trap-eye in patients with neovascular age-related macular degeneration. *Ophthalmology* 2009;116(11):2141–2148.
16. Brown DM, Heier JS, Ciulla T, et al. CLEAR-IT 2 Investigators. Primary endpoint results of a phase II study of vascular endothelial growth factor trap-eye in wet age-related macular degeneration. *Ophthalmology* 2011;118(6):1089–1097.
17. Heier JS, Boyer D, Nguyen QD, et al. CLEAR-IT 2 Investigators. The 1-year results of CLEAR-IT 2, a phase 2 study of vascular endothelial growth factor trap-eye dosed as-needed after 12-week fixed dosing. *Ophthalmology* 2011; 118(6):1098–1106.
18. Regeneron Pharmaceuticals, Inc. Bayer and Regeneron report positive top-line results of two phase 3 studies with VEGF Trap-Eye in wet age-related macular degeneration. Available at: http://investor.regeneron.com/releasedetail.cfm?ReleaseID=552099. Accessed April 13, 2012.
19. Regeneron Pharmaceuticals, Inc. Two year results of phase 3 studies with EYLEA (aflibercept) injection in wet AMD show sustained improvement in visual acuity. Available at: http://investor.regeneron.com/releasedetail.cfm?ReleaseID=629800. Accessed April 13, 2012.
20. Brechner RJ, Rosenfeld PJ, Babish JD, Caplan S. Pharmacotherapy for neovascular age-related macular degeneration: an analysis of the 100% 2008 medicare fee-for-service part B claims file. *Am J Ophthalmol* 2011;151(5):887–895e1.
21. Martin DF, Maguire MG, Ying G-S, et al. Ranibizumab and bevacizumab for neovascular age-related macular degeneration. *N Engl J Med* 2011;364(20):1897–1908.
22. United States Special Committee on Aging. A prescription for savings: reducing drug costs to medicare. Testimony before the Senate Special Committee on Aging by Philip J. Rosenfeld, MD, PhD. Available at: http://aging.senate.gov/events/hr236pr.pdf. Accessed April 13, 2011.

A Subretinal Matrigel Rat Choroidal Neovascularization (CNV) Model and Inhibition of CNV and Associated Inflammation and Fibrosis by VEGF Trap

Jingtai Cao,[1,2] *Lian Zhao,*[2,3] *Yiwen Li,*[4] *Yang Liu,*[1] *Weihong Xiao,*[1] *Ying Song,*[3] *Lingyu Luo,*[4] *Deqiang Huang,*[4] *George D. Yancopoulos,*[1] *Stanley J. Wiegand,*[1] *and Rong Wen*[4]

Purpose. The exudative, or the wet form of age-related macular degeneration (AMD) is characterized by choroidal neovascularization (CNV). A subretinal Matrigel (BD Biosciences, Bedford MA) model of CNV is described here, along with the effects of vascular endothelial growth factor (VEGF) neutralization on the development of CNV and associated inflammation and fibrosis.

Methods. CNV was induced in adult Sprague-Dawley rats by subretinal injection of Matrigel. CNV growth and associated leukocyte infiltration and collagen deposition were examined. VEGF Trap (Regeneron Pharmaceuticals, Tarrytown, NY), a recombinant protein that comprises portions of the extracellular domains of VEGF receptors 1 and 2 and that binds all isoforms of VEGF-A as well as placental growth factor with high affinity, was administered subcutaneously.

Results. Initiation of CNV was detected 4 days after Matrigel injection and then increased progressively in size. Systemic administration of VEGF Trap beginning on day 2 and 6 completely prevented development of CNV. When CNV was allowed to develop for 10 days before treatment was initiated, VEGF Trap not only prevented its further progression, but also induced substantial regression of existing lesions. In addition, VEGF Trap treatment reduced the total lesion volume and largely prevented the progressive leukocyte infiltration and fibrosis associated with CNV.

Conclusions. The subretinal Matrigel CNV model provides a convenient tool for the study of the diverse components of complex CNV lesions. The data not only confirm the critical roles of VEGF in the development and maintenance of CNV, but further demonstrate that VEGF and other VEGF receptor 1 ligands promote CNV-associated inflammation and fibrosis. (*Invest Ophthalmol Vis Sci.* 2010;51:6009–6017) DOI:10.1167/iovs.09-4956

Age-related macular degeneration (AMD) is the leading cause of irreversible visual disability and blindness in the population over 65 years of age in North America and Europe. It affects 11% of the population between 65 and 74 years of age and 28% of the population older than 74 years.[1] A more rapid and severe visual loss occurs in those with exudative, or wet, AMD in which new blood vessel invasion from the choroid results in severe retinal edema and can ultimately destroy the architecture of the retina due to hemorrhage, retinal detachment, and disciform scar formation, leading to irreversible loss of central vision.

Although the pathogenesis of choroidal neovascularization (CNV) in AMD is not entirely clear, it is highly associated with accumulation of abnormal extracellular deposits in the space between the retinal pigment epithelium (RPE) and Bruch's membrane,[2–5] suggesting a role of these deposits in the development of CNV. In addition, it is believed that the deposits provide a favorable microenvironment and space for new vessels to grow and ramify, as they create a plane of least resistance.[5] Supporting this notion, recent studies have shown that artificially created sub-RPE deposits are sufficient to induce the development of CNV in rodents (Wen R, et al. *IOVS* 2002;43: ARVO E-Abstract 1297)[6] and in rabbits.[7]

The critical role of VEGF, the major regulator of vasculogenesis and angiogenesis,[8] in CNV is now clear.[9–11] VEGF immunoreactivity is found in CNV membranes removed from AMD patients.[12,13] Blocking VEGF signaling by a variety of pharmacologic agents has been shown to effectively inhibit laser-induced CNV in experimental animals,[14,15] and neutralization of VEGF has become standard in treating wet AMD.[16–20]

In the present work, we further characterized the features of the Matrigel (BD Biosciences, Franklin Lakes, NJ) model of CNV and show that it replicates several of the cardinal features of human AMD. The progressive nature of the CNV in this model offers several advantages with respect to understanding the etiology of CNV, as well as evaluating potential treatments. In addition, we confirmed that, as in the human disease, endogenous VEGF plays a critical role in the development and maintenance of CNV in the Matrigel model.

From [1]Regeneron Pharmaceuticals, Inc., Tarrytown, New York; the [3]Department of Ophthalmology, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania; and the [4]Bascom Palmer Eye Institute, University of Miami, Miller School of Medicine, Miami, Florida.

[2]These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Corresponding author: Rong Wen, Bascom Palmer Eye Institute, University of Miami, Miller School of Medicine, 506 McKnight Building, 1638 NW 10th Avenue, Miami, Fl. 33136; rwen@med.miami.edu.

Materials and Methods

Animals and Subretinal Injections

All animal procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult Sprague-Dawley rats (2–3 months old) were purchased from Harlan Laboratories (Indianapolis, IN) or Taconic Farm (Germantown, NY). Subretinal injections of Matrigel (growth factor–reduced synthetic matrix; BD Biosciences) were performed on the temporal side, as described.[6] Briefly, rats were anesthetized with ketamine (40 mg/kg, IP) and xylazine (6 mg/kg, IP). A 33-gauge needle was inserted between the limbus and the equator to reach the subretinal space. A blunt 33-gauge needle attached to a 10-μL microsyringe (Hamilton, Reno, NV) was then introduced into the subretinal space, to inject 1.2 μL of Matrigel, diluted 3:1 with phosphate-buffered saline (PBS; 75% gel).

Visualization of Blood Vessels

Blood vessels were labeled with DiI, (1,1′-dioctadecyi-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Sigma-Aldrich, St. Louis, MO), as described.[21] The animals were euthanatized by CO_2 inhalation and perfused with PBS, followed by the DiI solution and 4% paraformaldehyde (in 0.1 M phosphate buffer; pH 7.4). The eye cups were embedded in 5% agarose. Thick (100 μm) serial sections were cut on a soft tissue microtome (Vibratome model VT1000S; Leica Microsystems, Bannockburn, IL) and examined by confocal microscopy. Three-dimensional (3-D) reconstruction was performed using software (AutoVisualize 3-D; AutoQuant Image, Inc, Watervliet, NY). Alternatively, serial frozen sections (50 μm) were cut through the area of the Matrigel deposit and examined by fluorescence microscopy.

Measurement of CNV

CNV area was calculated through the entire Matrigel area (Fig. 1). The CNV area of a section was calculated by multiplying the width (W_i), the maximum measurement of CNV along the sclera, by the thickness of the section T_i ($C_i = T_i W_i$). The height of CNV, the maximum distance between Bruch's membrane and the front edge of CNV, was not included, since its variation was negligible. The thickness of each section (100 μm), T_i, was the same for all sections as T. The entire CNV area of each eye (C) was calculated according to the equation:

$$C = T\sum_{i=1}^{n} W_i$$

Histologic Evaluation of Lesion Volume, Inflammation, and Fibrosis

To study lesion volume, we fixed the eye cups in 4% paraformaldehyde and embedded them in optimal cutting temperature (OCT) compound (Miles Inc., Elkhart, IN). Serial cryosections of 50 μm were cut through the entire Matrigel area. The area of the lesion in every third section was measured as the area between photoreceptors and the choriocapillaris. The total lesion volume was calculated using the Cavalieri method.[22] Briefly, the lesion area in each section was measured, and the total volume (V) of the CNV lesion in each eye was calculated by multiplying the sum of the areas in all sections (A) by the sum of the stepped thicknesses of the sections (T).

For immunofluorescence staining, cryosections of 10 μm were fixed in acetone at −20°C for 10 minutes, then incubated with Cy5-conjugated monoclonal anti-mouse CD45 antibody (BD PharMingen, San Diego, CA), Cy3-conjugated monoclonal anti-vimentin antibody (Sigma-Aldrich), Cy3-conjugated monoclonal anti-GFAP antibody (Sigma-Aldrich), or Cy3-conjugated anti-α smooth muscle actin (αSMA) antibody (Sigma-Aldrich) for 1 hour at room temperature. Cell nuclei were counterstained with 4′, 6′-diamino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA) and examined by fluorescence microscopy.

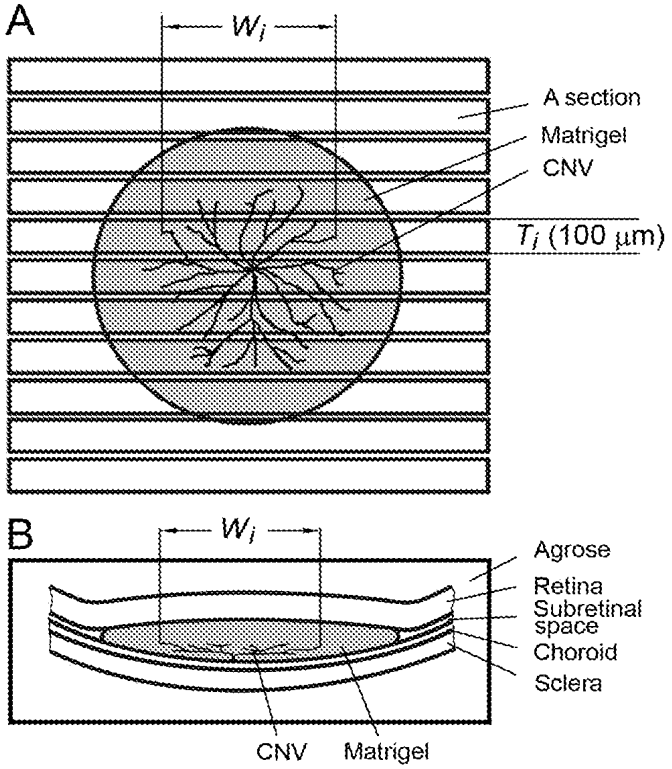

Figure 1. Schematic illustration of measurement and calculation of CNV area. (**A**) Serial microtome sections (thickness T_i = 100 μm) that cover the Matrigel area (*shaded*); (**B**) an individual section. The width of CNV area in a section, W_i, was the maximum measurement of CNV along the sclera (**A**, **B**). The CNV area of the section (C_i) was calculated according to the equation: $C_i = T_i W_i$. The entire CNV area of each eye (C) was calculated as the sum of all sections containing CNV.

To examine cell infiltration and collagen deposition, we stained alternate series of cryosections of 10 μm with hematoxylin and eosin or Masson's trichrome.

Semiquantitative scoring was used to analyze differences between experimental groups in immunofluorescence and Masson's trichrome-stained sections. The intensity and extent of specific staining in the Matrigel lesion was assessed by two masked observers and scored on a semiquantitative scale, with score of 0 for no visible staining and 1 for weak, 2 for mild, 3 for moderate, 4 for strong, 5 for very strong, and 6 for most intense staining.

Statistical Analysis

The results were analyzed by Student's t-test, one-way ANOVA, or Kruskal-Wallis test, followed by the Tukey or Dunn post hoc multiple analyses for comparisons between different groups (GraphPad Prism; ver. 5.0a; GraphPad Software Inc., San Diego, CA). Data are expressed as the mean ± SD.

Results

Inhibition of CNV Development by VEGF Trap

Angiogenic sprouting was detected as early as 4 days after Matrigel injection (data not shown). Extensive neovascular networks from the choriocapillaris had developed in the Matrigel area by 10 days in all eyes (Fig. 2A). In contrast, subretinal injection of sodium hyaluronate (1.2 μL, 10 mg/mL) resulted in local retinal detachment, but only rarely in the appearance of small CNV, confined to the area immediately adjacent to the break in Bruch's membrane (Fig. 2B). These results indicate that the deposition of ECM, in conjunction with the disruption

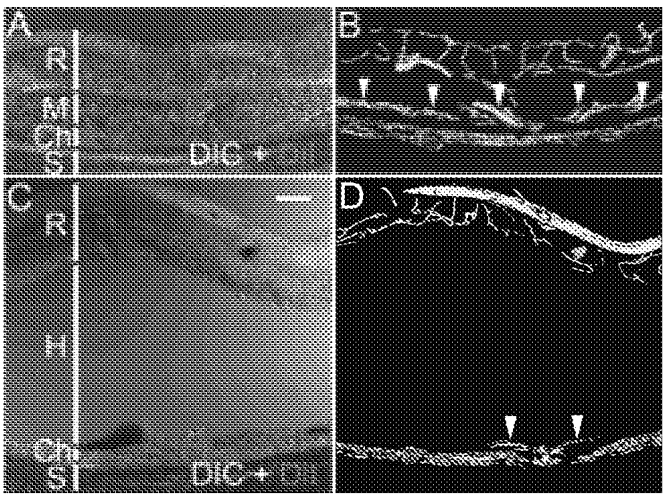

FIGURE 2. Induction of CNV by subretinal Matrigel. A confocal image showing blood vessels (DiI, *red*) of a section (100-μm-thick) from an eye injected with Matrigel and harvested 10 days later is superimposed on a DIC (differential interference contrast) image of the same section (**A**) to show CNV in the Matrigel area (M), as well as choroidal and retinal vasculature relative to the structure of the eye, including sclera (S), choroids (Ch), and retina (R). (**B**) 3-D reconstructed image of blood vessels in this section (*arrowheads*, CNV). In the eye injected with sodium hyaluronate (H in **C**), CNV developed infrequently and only within a small area immediately adjacent to the break in Bruch's membrane (**C**). (**D**) A 3-D reconstructed image of blood vessels in the same section (*arrowheads*, CNV). An extensive retinal detachment was induced by sodium hyaluronate (H) (**C**, **D**). Scale bar, 100 μm.

of Bruch's membrane, greatly facilitates the growth and spread of CNV.

To determine whether pharmacologic inhibition of VEGF could prevent CNV development in this model, we administered VEGF Trap (12.5 mg/kg, SC) on days 2 and 6 after Matrigel injection. Control animals were injected with an equimolar amount of the control protein, human Fc (hFc, 6.25 mg/kg SC). VEGF Trap is a potent inhibitor of VEGF-A, and the related VEGF receptor 1 (VEGFR1) ligands placental growth factor (PlGF) and VEGF-B. It comprises ligand-binding portions of the extracellular domains of human VEGFR1 and -2, which are expressed in sequence with the Fc domain of human IgG.[15,23] At a dose of 12.5 mg/kg, VEGF Trap has been shown to effectively suppress pathologic angiogenesis in several disease models.[24,25] Substantial development of CNV was seen in every eye in the control group (CNV area: $211.35 \pm 146.00 \times 10^3$ μm², $n = 12$; Figs. 3A, 3C), but was completely absent in the eyes of VEGF Trap–treated animals (CNV area: $0 \pm 0 \times 10^3$ μm², $n = 12$; $P < 0.001$, *t*-test; Figs. 3B, 3C). These results confirm that, as in wet AMD in humans, VEGF plays a vital role in induction and development of CNV in the Matrigel model.

CNV Regression Induced by VEGF Trap

To evaluate the effect of VEGF Trap on newly formed CNV, we allowed CNV to develop for 10 days, at which time we collected eyes from one group of animals (10-day [10-D] control) and established the pretreatment baseline. The remaining animals received either VEGF Trap (12.5 mg/kg, SC) or hFc (6.25 mg/kg SC) on days 10, 13, and 16. The eyes were collected on day 20 (Fig. 4A).

CNV was well developed in the 10-D control group (CNV area: $244.50 \pm 225.21 \times 10^3$ μm², $n = 16$) and increased in size by day 20 in the hFc-treated group (CNV area: $1274.27 \pm 807.18 \times 10^3$ μm², $n = 14$; Fig. 4B). In contrast, the CNV area in the group treated with VEGF Trap ($37.10 \pm 45.87 \times 10^3$ μm², $n = 14$) was only 15% of that in the 10-D control group (Fig. 4B). A replicate experiment produced similar results (data not shown). Representative confocal images of sections from a 10-D control, a 20-day [20-D] VEGF Trap–treated, and a 20-D hFc-treated control eye are presented in Figures 4C, 4D, and 4E, respectively. It is worth noting that CNV was completely absent in 6 of the 14 eyes in the VEGF Trap–treated group. These results indicate that VEGF Trap not only prevented the substantial growth of CNV from 10 to 20 days, but also induced significant regression of the existing CNV.

Effects of VEGF Trap on the Total Lesion Volume and Cellularity

In addition to suppressing CNV, VEGF Trap appeared to substantially reduce the overall volume of the Matrigel lesion. In a separate experiment, we measured the total lesion volume, defined as the area lying between the photoreceptors and the choriocapillaris. The total lesion volume in the 10-D control eyes was $6.7 \pm 1.2 \times 10^8$ μm³ ($n = 10$) and $7.5 \pm 0.9 \times 10^8$ μm³ ($n = 8$) in the 20-D Fc-treated control eyes. In contrast, the total lesion volume in the VEGF Trap–treated eyes was $2.0 \pm 0.2 \times 10^8$ μm³ ($n = 10$), one third of that in the 10-D control group (Fig. 5D). Representative images from 10-D control, 20-D hFc-treated control and 20-D VEGF Trap-treated retinas are shown in Figures 5A, 5B, and 5C, respectively.

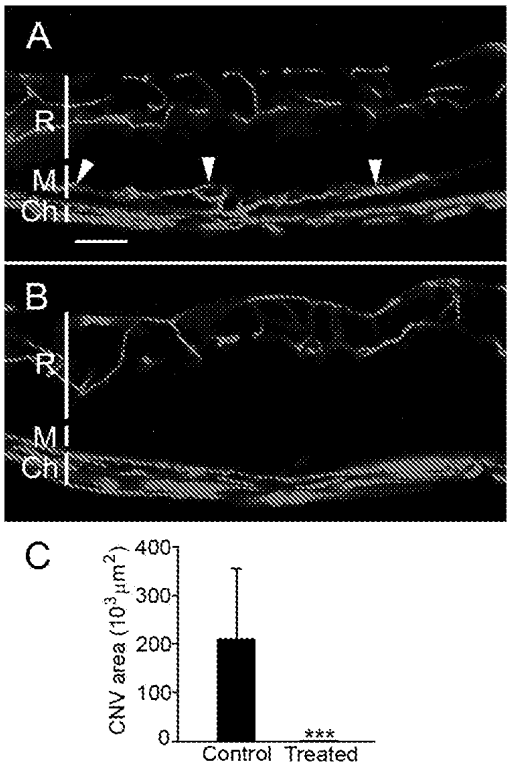

FIGURE 3. Inhibition of CNV development by VEGF Trap. VEGF Trap (12.5 mg/kg, SC) was given at days 2 and 6 after Matrigel injection. Control eyes received an equimolar concentration of hFc (6.25 mg/kg, SC). The eyes were harvested 10 days after Matrigel injection. CNV (*arrowheads*) was detected in every eye in the control group (CNV area: $211.35 \pm 146.00 \times 10^3$ μm², $n = 12$) (**A**), but was completely absent in all eyes of the VEGF Trap–treated group (**B**) (CNV area: $0 \pm 0 \times 10^3$ μm², $n = 12$). (**C**) Quantitative analysis shows that the CNV area in the treated group was significantly less than in the control eyes ($P < 0.001$, *t*-test). R, retina; M, Matrigel; Ch, choroid. Scale bar, 100 μm.

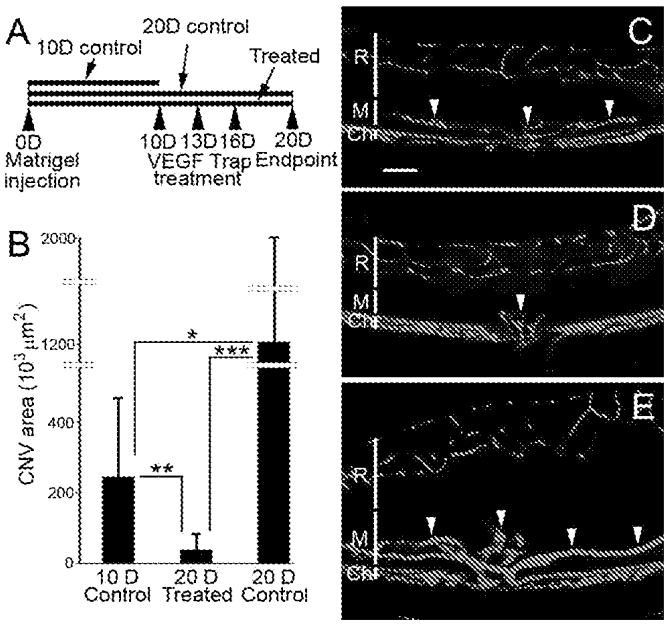

FIGURE 4. CNV regression induced by VEGF Trap. (**A**) Experimental design. CNV was allowed to develop for 10 days in all three groups: untreated 10-D control, 20-D control treated with an inactive protein (hFc), and 20-D group treated with VEGF Trap, at which time the pretreatment baseline CNV area was established by measuring the CNV in the 10-D control group. (**B**) Extensive CNV had developed in all eyes in the 10-D control group ($244.50 \pm 225.21 \times 10^3$ μm^2, $n = 16$) and was increased further in size in the 20-D control group ($1274.26 \pm 807.18 \times 10^3$ μm^2, $n = 14$). In contrast, the CNV area in the VEGF Trap–treated group ($37.10 \pm 45.87 \times 10^3$ μm^2, $n = 14$) was reduced to approximately 15% of the pretreatment baseline. Representative images of the 10-D control, VEGF Trap–treated, and 20-D control groups are presented in (**C**), (**D**), and (**E**), respectively. *Arrowheads*, CNV. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respectively (Kruskal-Wallis test and Dunn test). Ch, choroid; M, Matrigel layer; R, retina. Scale bar, 100 μm.

The number of cells present in the Matrigel deposit increased markedly between days 10 and 20 in the hFc-treated control group, whereas the reduction in lesion volume in the VEGF Trap–treated group was associated with a near complete inhibition of the progressive increase in cellular density, accompanied by an ongoing clearance of the Matrigel (Fig. 6).

Inhibition of Progressive Leukocyte Infiltration and Fibrosis by VEGF Trap

CD45, a pan-leukocyte marker, was used to characterize leukocyte infiltration into the lesion area. Infiltration was evident in the subretinal space and to a lesser degree within the Matrigel deposit in the 10-D control eyes (Fig. 7A). An increase was seen in the 20-D control group, particularly within the Matrigel lesion (Fig. 7B). This progressive increase in leukocyte infiltration was abrogated by VEGF Trap treatment (Fig. 7C). Semiquantitative analysis confirmed the progression in leukocyte infiltration between 10- and 20-D control eyes, and the suppression of the infiltration by VEGF Trap treatment (Table 1). Moreover, CD45-negative cells, visualized by the nuclear DAPI counterstain seemed to be similarly affected (Fig. 7C).

Fibroblasts and other cells of mesenchymal origin were identified by using vimentin as a marker. In normal eyes (no Matrigel injection), Müller glial cells and fibroblasts in the sclera were well stained, but there was no vimentin staining in the subretinal space (Fig. 8A). In the 10-D group, many vimentin-positive cells were present in and around the Matrigel (Fig. 8B). Vimentin staining was even more prominent in the 20-D control group with a similar staining pattern to that of the 10-D group (Fig. 8C). In contrast, the density of vimentin-positive cells in the VEGF Trap–treated eyes was similar to that in the 10-D control eyes (Fig. 8D). Particularly strong vimentin staining was seen at the border between the retina and the lesion in all groups. This appeared to reflect both the accumulation of vimentin-positive cells at the photoreceptor–Matrigel interface and also increased vimentin staining in the Müller cells. Vimentin staining was strongest in this area in the 20-D control eyes, in which vimentin-positive cells also were dispersed throughout the lesion. Semiquantitative analysis demonstrated that vimentin staining was significantly increased in the 20-D hFc-treated controls when compared with 10-D controls, and VEGF Trap treatment inhibited this increase (Table 1).

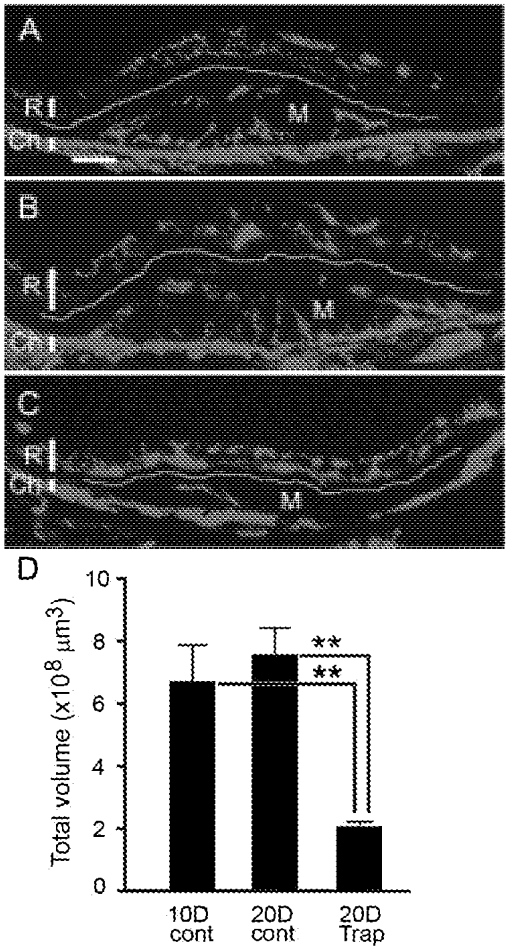

FIGURE 5. VEGF Trap–induced decrease in total lesion volume. Treatment was as described in Figure 4A. Total lesion volume was measured as the area between photoreceptors and the choriocapillaris. Representative images of sections from the 10-D control, 20-D control, and 20-D VEGF Trap–treated groups are shown in (**A**), (**B**), and (**C**), respectively. In addition to reducing the CNV area, the total lesion volume, defined as the entire mass lying between the photoreceptor layer (*line*) and the choroid, was reduced by VEGF Trap treatment. (**D**) The total lesion volume was $6.7 \pm 1.2 \times 10^8$ μm^3 ($n = 10$) in the 10-D control and $7.5 \pm 0.9 \times 10^8$ μm^3 ($n = 8$) in the 20-D control. In comparison, the total volume in the VEGF Trap–treated group was significantly reduced to $2.0 \pm 0.2 \times 10^8$ μm^3 ($n = 10$), one third of that in the 10-D control, and 27% of that in the 20-D hFc controls. $^{**}P < 0.01$ (ANOVA and Tukey test) Ch, choroid; M, Matrigel layer; R, retina. Scale bar, 100 μm.

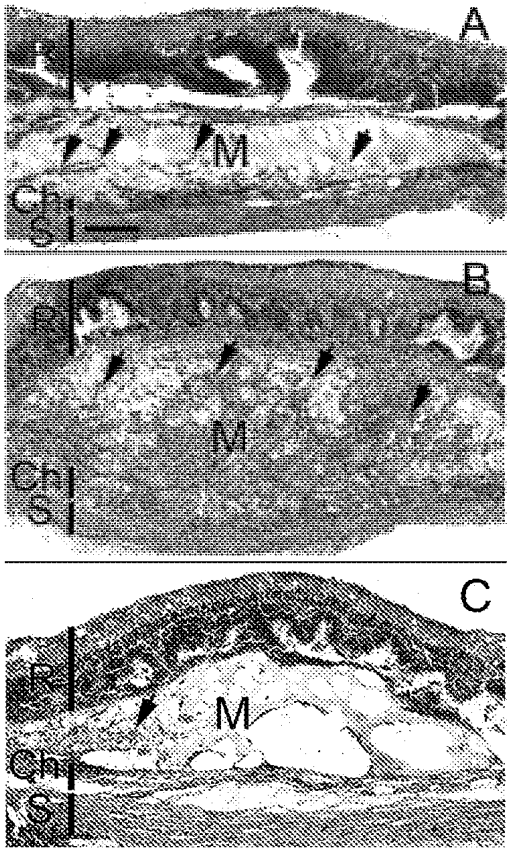

Figure 6. Increased cellularity in the Matrigel lesion. Treatment was as described in Figure 4A. Retinal sections were stained with hematoxylin and eosin to show the presence of cells within the Matrigel deposits (*arrows*). There was a marked increase in cellular density within the lesions of the control animals between days 10 (**A**, untreated) and 20 (**B**, treated with hFc). This increase in cellular density was inhibited by treatment with VEGF Trap (**C**). Scale bar, 50 μm.

We next used αSMA to identify smooth muscle cells and myofibroblasts. In the normal retina, αSMA-positive cells were present only in blood vessels at the retinal surface and the inner retina, as well as in blood vessels in the choroid and sclera (Fig. 9A). Ten days after Matrigel injection, αSMA-positive cells were present at the interface between the retina and the Matrigel. These cells were not associated with blood vessels (Fig. 9B). The number of αSMA-stained cells was further increased at 20 days in the hFc-treated controls (Fig. 9C). Thus, within the lesion, the distribution of αSMA-positive cells was similar to that of vimentin-positive cells, particularly at the boundary between the Matrigel deposit and neuronal retina. Treatment with VEGF Trap not only prevented this increase but significantly reduced the extent of αSMA-positive staining, when compared to the 10- and 20-D control groups (Fig. 9D, Table 1). Therefore, in addition to inhibiting the accumulation of mesenchymal cells in and around the Matrigel deposit, VEGF Trap appeared to inhibit the expression of the myofibroblast phenotype by these cells.

Given the progressive accumulation of a large number of αSMA- and vimentin-positive cells in the lesions, we next evaluated the pattern of collagen deposition in the Matrigel area by using Masson's trichrome stain. With this stain, collagen stains dark blue, whereas the Matrigel itself stains pale blue (Fig. 10A). Collagen staining was very strong in the sclera, but was minimal in and around the Matrigel deposits in the 10-D control eyes. However, focal deposition of collagen was clearly evident in and around the lesions in the 20-D control group (Fig. 10B), especially at the border between the retina and the Matrigel, where vimentin and αSMA-positive cells were most densely and consistently aggregated. In contrast, less collagen staining was visible in animals treated with VEGF Trap than in the 20-D controls (Fig. 10C, Table 1).

Using RPE65 as a marker, we confirmed that subretinal injection of Matrigel induced a rapid translocation of RPE cells from their original position next to Bruch's membrane, to the opposite side of the Matrigel mass immediately subjacent to the photoreceptors, consistent with earlier findings.[6] This migratory response of the RPE was not inhibited by treatment with VEGF Trap (data not shown).

Discussion

We have developed a Matrigel CNV model in rats, to study the mechanisms underlying CNV development and to evaluate

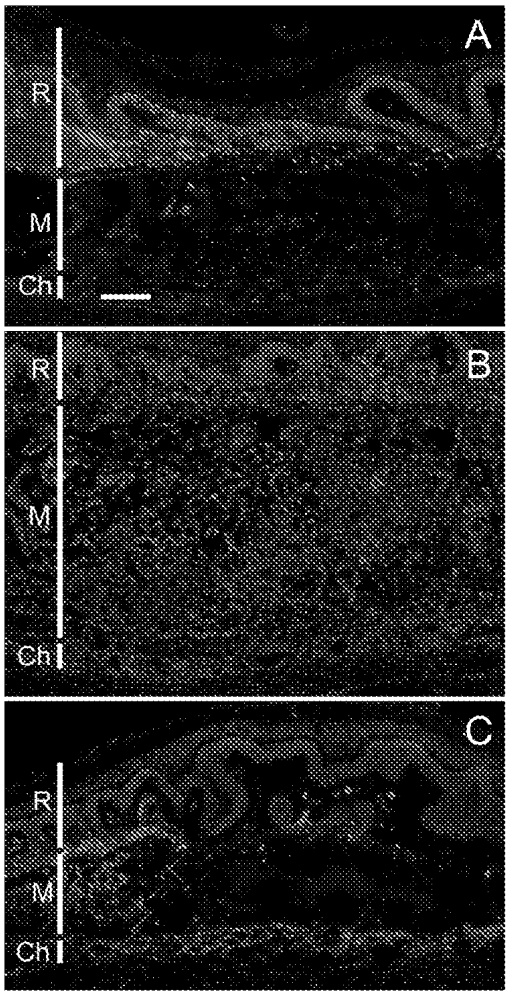

Figure 7. Inhibition of leukocyte infiltration by VEGF Trap. Treatment was as described in Figure 4A. Retinal cryosections were stained with anti-CD45 antibodies to identify leukocytes (*red*). Cell nuclei were stained with DAPI (*blue*). Many leukocytes were detected in the Matrigel area (M) 10 days after Matrigel injection (**A**). The number of leukocytes increased substantially in the 20-D control (treated with hFc, **B**) as did the number of CD45 negative cells identified by DAPI staining. In contrast, there was no appreciable increase in CD45-positive leukocytes or other cells in the VEGF Trap–treated group (**C**). Subretinal injection of Matrigel sometimes induced disorganization of the overlying retina, particularly folding or rosette formation in the photoreceptor layer, as shown in the retinal sections in (**A**), (**B**), and (**C**). Ch, choroid; M, Matrigel layer; R, retina. Scale bar, 100 μm.

TABLE 1. Semiquantitative Analyses of CD45, Vimentin, aSMA, and Trichrome Stains in CNV

Stain Type	10-D Matrigel*	20-D Matrigel + hFC*	20-D Matrigel + VEGF Trap*	*P*† 10-D vs. 20-D hFc	*P*† 20-D hFc vs. 20-D VEGF Trap
CD45	2.25 ± 0.42	5.5 ± 0.55	2.58 ± 0.49	<0.01	<0.05
Vimentin	3.75 ± 0.76	5.33 ± 0.82	3.50 ± 0.45	<0.05	<0.05
αSMA	3.42 ± 0.49	4.75 ± 0.88	1.5 ± 0.35	>0.05	<0.001
Trichrome	2.50 ± 0.45	5.67 ± 0.52	2.96 ± 0.33	<0.01	<0.05

* Mean ± SD (n = 6).
† Kruskal-Wallis test and Dunn test.

potential anti-CNV treatments. Matrigel, an extract of extracellular matrix proteins from the murine EHS (Engelbreth-Holm-Swarm) tumor,[26,27] has been widely used as reconstituted basement membrane in cell culture, as well as in the Matrigel plug assay, to assess angiogenic or anti-angiogenic agents in vivo.[28] Matrigel also promotes tubulogenic behavior of endothelial cells in vitro.[29] The subretinal Matrigel model is unique in several ways. First, CNV is initiated by subretinal deposition of ECM. Sub-RPE accumulation of ECM is associated with CNV in wet AMD, and it has been thought to play a role in the induction of neovascularization.[2–5] The present work provides further evidence to support a role for ECM in facilitating CNV development. In addition, the CNV lesions were shown to increase progressively in size in this model (Fig. 4). Finally, this model is characterized by a progressive infiltration of leukocytes and myofibroblasts into the developing CNV.

We have reported that subretinal Matrigel deposition induces RPE translocation and CNV in rodents (Wen R, et al. *IOVS* 2002;43:ARVO E-abstract 1297).[6] In rabbits, subretinal injection of Matrigel was also reported to be associated with fluorescein leakage,[7] which was sustained for weeks in some animals, indicating that the blood vessels newly formed by this method are highly permeable. In the present work, we have further characterized the progression and histopathologic characteristics of these lesions in rats. By directly measuring the size of the CNV network, we documented a progressive increase in CNV size for up to at least 20 days after Matrigel injection (Fig. 4). In addition, the entire lesion mass was shown to progress in size (Fig. 5). This increase was associated with a progressive infiltration of nonvascular cells into the developing CNV lesions, notably leukocytes and myofibroblasts, accompanied by deposition of collagen. These features of the subretinal Matrigel model resemble the inflammatory reaction and fibrosis that are well recognized elements of CNV lesions in wet AMD.[30,31] Thus, the rat Matrigel model not only provides a tool for the study of mechanisms involved in the induction and early progression of CNV, but also to evaluate the effects of potential therapies on diverse aspects of CNV development.

Several animal models of experimental CNV have been developed. The most widely used employs laser photocoagulation to disrupt Bruch's membrane.[32] First characterized in primates,[33] it has been used to induce CNV in other species, including rabbits,[34] rats,[35,36] and mice.[37] Photocoagulation, however, also directly damages the choroid, RPE and overlying retina. In contrast, selective damage to Bruch's membrane, either mechanical or enzymatic, has met with only limited success in inducing subretinal neovascularization in primates.[38] Subretinal delivery of adenovirus or adenoassociated virus carrying a VEGF transgene has been used successfully to induce subretinal neovascularization in rats.[39–41] Subretinal injection of FGF2-impregnated gelatin microspheres also has been reported to induce CNV in more

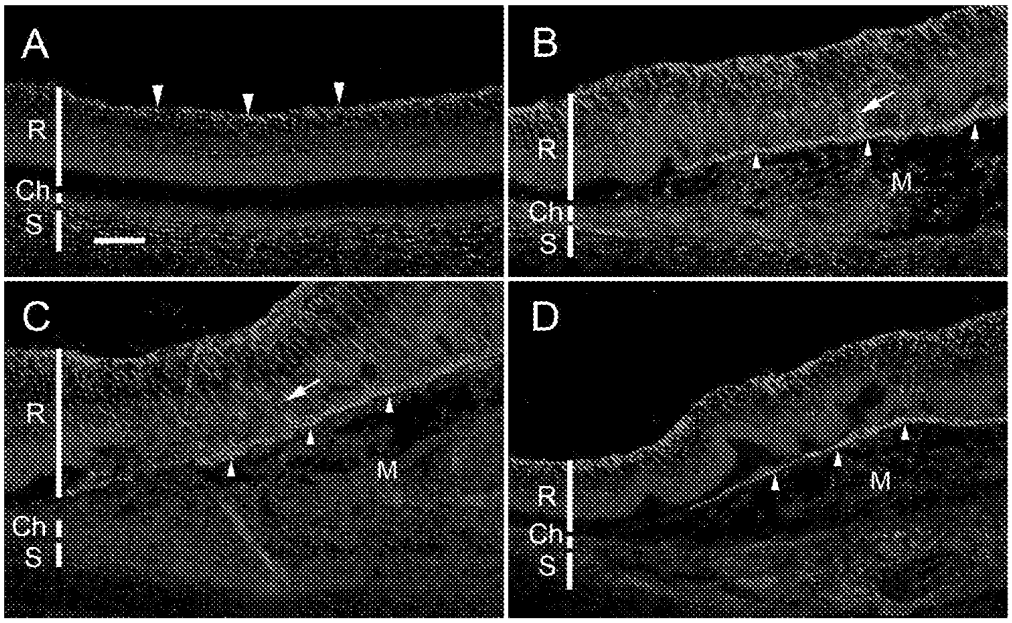

FIGURE 8. Vimentin-immunoreactive cells. Treatment was as described in Figure 4A. Retinal cryosections were stained with anti-vimentin antibodies to identify fibroblasts and other cells of mesenchymal origin (*red*). Cell nuclei were stained with DAPI (*blue*). In normal control eyes without Matrigel injection (**A**), vimentin immunoreactivity was found in Müller cells of the retina (R), especially in the end feet at the inner limiting membrane (*arrowheads*), as well as in fibroblasts in the choroid (Ch) and sclera (S). Ten days after Matrigel injection (**B**), many vimentin-positive cells were dispersed within the Matrigel (M). Increased vimentin staining was evident in Müller cells, including apical as well as the basal end feet, and also in the processes in the outer nuclear layer (*arrow*). Particularly intense vimentin staining also was found at the boundary between Matrigel and photoreceptors (*arrowheads*). The staining pattern of vimentin in the 20-D control (C) was similar to that in the 10-D control (**B**), with intense staining in Müller cell fibers (*arrow*) and between Matrigel and the photoreceptors (*arrowheads*), and many more vimentin-positive cells also were found dispersed throughout the lesion (**C**). In VEGF Trap–treated animals (**D**), intense staining was still evident at the border between the Matrigel deposit and photoreceptors (*arrowheads*), but fewer vimentin-positive cells were present in the Matrigel than in the 10- or 20-D control eyes. Ch, choroid; M, Matrigel layer; R, retina; S, sclera. Scale bar, 50 μm.

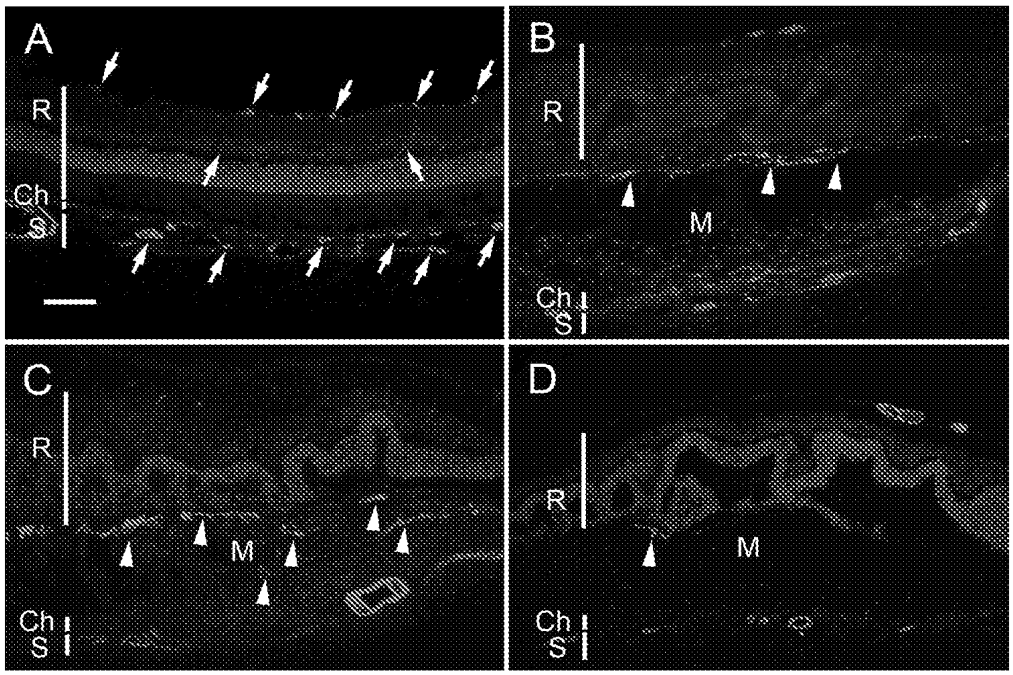

FIGURE 9. αSMA immunoreactive cells. Treatment was as described in Figure 4A. Retinal sections were stained with anti-αSMA antibodies (*red*) to identify smooth muscle cells and myofibroblasts. Cell nuclei were counterstained with DAPI (*blue*). In normal control eyes without Matrigel injection (**A**), αSMA immunoreactivity was restricted to blood vessels (*arrows*) in the retina (R), choroid (Ch), and sclera (S) (*arrows*). Ten days after Matrigel injection (**B**), many αSMA-positive cells were present in the Matrigel (M), with particularly intense αSMA staining in cells present at the border between the Matrigel and photoreceptors (*arrowheads*), an area that was devoid of blood vessels. The staining of αSMA in the 20-D control (**C**) was similar to that in the 10-D control (**B**), although more intense at the retina–Matrigel boundary and with more αSMA-positive cells dispersed throughout the Matrigel (*arrowheads*). In VEGF Trap–treated animals (**D**), fewer αSMA-positive cells were found in the Matrigel area (*arrowhead*) than in the 10- or 20-D control eyes. Ch, choroid; M, Matrigel layer; R, retina; S, sclera. Scale bar, 50 μm.

than 80% of injected eyes in rabbits,[42] and VEGF-impregnated gelatin microspheres induces CNV in more than 90% of injected eyes in primates.[43]

Other CNV models have been developed in genetically altered animals. For example, mice with a spontaneous, autosomal semidominant mutation in the *Bst* locus were reported to exhibit CNV and retinal detachment by 7 months or older, but no basal deposits were found. These animals also exhibit many developmental abnormalities in the retina and eye.[44] Mice lacking monocyte chemoattractant protein-1 (MCP-1; also known as Ccl-2) or its cognate C-C chemokine receptor-2 (Ccr-2) have been reported to develop pathologic features resembling human AMD, including accumulation of lipofuscin in RPE cells, drusen-like deposits, photoreceptor atrophy, and CNV.[45] However, Luhmann et al.[46] recently showed that the lesions in the Ccr-2$^{-/-}$ mice are not drusen, but rather are accumulations of swollen CD88^{+}, F4/80^{-} macrophages in the subretinal space. They also failed to detect spontaneous CNV development.[46]

AMD-like lesions also have been induced in mice immunized with mouse serum albumin adducted with carboxyethylpyrrole. Immunized animals develop antibodies to this hapten, fixed complement component-3 in Bruch's membrane, accumulate drusen during aging, and develop lesions that mimic geographic atrophy characteristic of the dry form of AMD,[47] but there is no CNV development.

In comparison, the rat Matrigel model offers a highly reproducible, convenient, and inexpensive means by which complex, progressive CNV lesions having many of the features of neovascular AMD can be induced within a well-defined time frame. The unique features of this model allowed us to demonstrate that VEGF Trap not only arrests the growth of CNV, but induces regression of recently established CNV, and that VEGF Trap also suppresses leukocyte infiltration and fibrosis associated with CNV progression, resulting in a significant reduction in overall lesion volume.

In addition to being a critically important angiogenic factor, VEGF-A is known to promote vascular permeability and inflammation. For example, infusion of exogenous VEGF into the brain can induce vascular leak and influx of inflammatory cells at doses that are insufficient to induce angiogenesis. At higher doses, leukocyte infiltration precedes the initiation of angiogenesis.[48] Similarly, after corneal injury VEGFR1-mediated leukocyte influx precedes and amplifies the subsequent VEGF-dependent neovascularization.[24] The findings in the present work are consistent with a central role for VEGF in mediating the inflammatory reaction associated with CNV formation. However, the inhibitory effects of VEGF Trap on inflammation may not be due solely to its ability to bind all VEGF-A isoforms with high affinity,[23] as this receptor-based agent also binds and neutralizes the VEGFR1 ligands PlGF and VEGF-B.

In contrast to VEGFR2, which is expressed predominantly on vascular endothelial cells, VEGFR1 is also expressed by a variety of nonendothelial cell types, including subpopulations of smooth muscle cells, leukocytes, and their progenitors.[49] PlGF, in particular, is known not only to synergize with VEGF to promote pathologic angiogenesis, but also to act as a chemoattractant for monocytes and macrophages.[49,50] Like VEGF, PlGF is present in human CNV membranes, and animal studies have shown that PlGF contributes to the development of experimental CNV.[51]

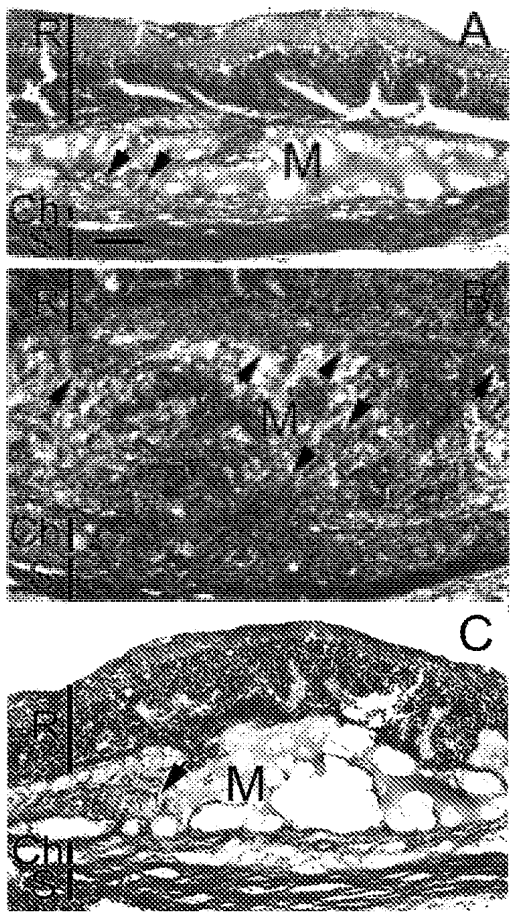

FIGURE 10. Collagen deposition. Treatment was as described in Figure 4A. Collagen was visualized by Masson's trichrome stain: collagen components are stained *dark blue*, and the Matrigel is *pale blue*. Minimal collagen deposition (*arrows*) was seen in the 10-D control (**A**), whereas collagen deposition (*arrows*) was clearly evident in the 20-D control (**B**), most notably at the boundaries between the Matrigel deposit and the retina and to a somewhat lesser extent at the boundary with the choroid. In VEGF Trap–treated retinas (**C**), there was minimal collagen deposition (*arrow*), similar to that in 10-D control eyes. S, sclera; Ch, choroid; M, Matrigel layer; R, retina. Scale bar, 50 μm.

In summary, we have developed a CNV model in the rat by subretinal deposition of Matrigel, which exhibits many features of human wet AMD. Using this model, we have shown that VEGF Trap, a potent receptor-based inhibitor of VEGF-A and PlGF, not only arrests the growth of experimental CNV, but also the associated inflammatory and fibrotic responses and can induce regression of recently established lesions. VEGF Trap-Eye (Regeneron Pharmaceuticals), an iso-osmotic, ultrapurified formulation of VEGF Trap for intravitreal injection, is currently in phase III clinical trials for the treatment of wet AMD and central retinal vein occlusion.

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References

1. Klein R, Klein BE, Linton KL. Prevalence of age-related maculopathy. The Beaver Dam Eye Study. *Ophthalmology.* 1992;99:933–943.
2. Sarks SH. New vessel formation beneath the retinal pigment epithelium in senile eyes. *Br J Ophthalmol.* 1973;57:951–965.
3. Sarks SH. Ageing and degeneration in the macular region: a clinico-pathological study. *Br J Ophthalmol.* 1976;60:324–341.
4. Green WR, Enger C. Age-related macular degeneration histopathologic studies. The 1992 Lorenz E. Zimmerman Lecture. *Ophthalmology.* 1993;100:1519–1535.
5. Sarks JP, Sarks SH, Killingsworth MC. Morphology of early choroidal neovascularisation in age-related macular degeneration: correlation with activity. *Eye.* 1997;11:515–522.
6. Zhao L, Wang Z, Liu Y, et al. Translocation of the retinal pigment epithelium and formation of sub-retinal pigment epithelium deposit induced by subretinal deposit. *Mol Vis.* 2007;13:873–880.
7. Qiu G, Stewart JM, Sadda S, et al. A new model of experimental subretinal neovascularization in the rabbit. *Exp Eye Res.* 2006;83:141–152.
8. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. *Endocr Rev.* 1997;18:4–25.
9. Das A, McGuire PG. Retinal and choroidal angiogenesis: pathophysiology and strategies for inhibition. *Prog Retin Eye Res.* 2003;22:721–748.
10. Grisanti S, Tatar O. The role of vascular endothelial growth factor and other endogenous interplayers in age-related macular degeneration. *Prog Retin Eye Res.* 2008;27:372–390.
11. Penn JS, Madan A, Caldwell RB, Bartoli M, Caldwell RW, Hartnett ME. Vascular endothelial growth factor in eye disease. *Prog Retin Eye Res.* 2008;27:331–371.
12. Kvanta A, Algvere PV, Berglin L, Seregard S. Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. *Invest Ophthalmol Vis Sci.* 1996;37:1929–1934.
13. Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. *Invest Ophthalmol Vis Sci.* 1996;37:855–868.
14. Kwak N, Okamoto N, Wood JM, Campochiaro PA. VEGF is major stimulator in model of choroidal neovascularization. *Invest Ophthalmol Vis Sci.* 2000;41:3158–3164.
15. Saishin Y, Takahashi K, Lima e Silva R, et al. VEGF-TRAP(R1R2) suppresses choroidal neovascularization and VEGF-induced breakdown of the blood-retinal barrier. *J Cell Physiol.* 2003;195:241–248.
16. Emerson MV, Lauer AK. Emerging therapies for the treatment of neovascular age-related macular degeneration and diabetic macular edema. *Biodrugs.* 2007;21:245–257.
17. Chappelow AV, Kaiser PK. Neovascular age-related macular degeneration: potential therapies. *Drugs.* 2008;68:1029–1036.
18. Rosenfeld PJ, Schwartz SD, Blumenkranz MS, et al. Maximum tolerated dose of a humanized anti-vascular endothelial growth factor antibody fragment for treating neovascular age-related macular degeneration. *Ophthalmology.* 2005;112:1048–1053.
19. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med.* 2006;355:1419–1431.
20. Lin RC, Rosenfeld PJ. Antiangiogenic therapy in neovascular age-related macular degeneration. *Int Ophthalmol Clin.* 2007;47:117–137.
21. Li Y, Song Y, Zhao L, Gaidosh G, Laties AM, Wen R. Direct labeling and visualization of blood vessels with lipophilic carbocyanine dye DiI. *Nat Protoc.* 2008;3:1703–1708.
22. Howard CV, Reed MG. *Unbiased Stereology: Three-Dimensional Measurements in Microscopy.* Oxford, UK: Garland Science/Bios Scientific Publishers; 1998.
23. Holash J, Davis S, Papadopoulos N, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci U S A.* 2002;99:11393–11398.
24. Cursiefen C, Chen L, Borges LP, et al. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Investig.* 2004;113:1040–1050.
25. Rudge JS, Holash J, Hylton D, et al. Inaugural article: VEGF Trap complex formation measures production rates of VEGF, providing

a biomarker for predicting efficacious angiogenic blockade. *Proc Natl Acad Sci U S A.* 2007;104:18363–18370.

26. Orkin RW, Gehron P, McGoodwin EB, Martin GR, Valentine T, Swarm R. A murine tumor producing a matrix of basement membrane. *J Exp Med.* 1977;145:204–220.
27. Kleinman HK, McGarvey ML, Liotta LA, Robey PG, Tryggvason K, Martin GR. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry.* 1982;21:6188–6193.
28. Passaniti A, Taylor RM, Pili R, et al. A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. *Lab Invest.* 1992;67:519–528.
29. Grant DS, Tashiro K, Segui-Real B, Yamada Y, Martin GR, Kleinman HK. Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. *Cell.* 1989;58:933–943.
30. Killingsworth MC, Sarks JP, Sarks SH. Macrophages related to Bruch's membrane in age-related macular degeneration. *Eye.* 1990; 4:613–621, 1990.
31. Dastgheib K, Green WR. Granulomatous reaction to Bruch's membrane in age-related macular degeneration. *Arch Ophthalmol.* 1994;112:813–818.
32. Campochiaro PA. Retinal and choroidal neovascularization. *J Cell Physiol.* 2000;184:301–310.
33. Ryan SJ. Subretinal neovascularization: natural history of an experimental model. *Arch Ophthalmol.* 1982;100:1804–1809.
34. elDirini AA, Ogden TE, Ryan SJ. Subretinal endophotocoagulation: a new model of subretinal neovascularization in the rabbit. *Retina.* 1991;11:244–249.
35. Frank RN, Das A, Weber ML. A model of subretinal neovascularization in the pigmented rat. *Curr Eye Res.* 1989;8:239–247.
36. Dobi ET, Puliafito CA, Destro M. A new model of experimental choroidal neovascularization in the rat. *Arch Ophthalmol.* 1989; 107:264–269.
37. Tobe T, Ortega S, Luna JD, et al. Targeted disruption of the FGF2 gene does not prevent choroidal neovascularization in a murine model. *Am J Pathol.* 1998;153:1641–1646.
38. Ryan SJ, Mittl RN, Maumenee AE. Enzymatic and mechanically induced subretinal neovascularization. *Albrecht Von Graefes Arch Klin Exp Ophthalmol.* 1980;215:21–27.
39. Spilsbury K, Garrett KL, Shen WY, Constable IJ, Rakoczy PE. Overexpression of vascular endothelial growth factor (VEGF) in the retinal pigment epithelium leads to the development of choroidal neovascularization. *Am J Pathol.* 2000;157:135–144.
40. Baffi J, Byrnes G, Chan CC, Csaky KG. Choroidal neovascularization in the rat induced by adenovirus mediated expression of vascular endothelial growth factor. *Invest Ophthalmol Vis Sci.* 2000;41:3582–3589.
41. Wang F, Rendahl KG, Manning WC, Quiroz D, Coyne M, Miller SS. AAV-mediated expression of vascular endothelial growth factor induces choroidal neovascularization in rat. *Invest Ophthalmol Vis Sci.* 2003;44:781–790.
42. Kimura H, Sakamoto T, Hinton DR, et al. A new model of subretinal neovascularization in the rabbit. *Invest Ophthalmol Vis Sci.* 1995;36:2110–2119.
43. Cui JZ, Kimura H, Spee C, Thumann G, Hinton DR, Ryan SJ. Natural history of choroidal neovascularization induced by vascular endothelial growth factor in the primate. *Graefes Arch Clin Exp Ophthalmol.* 2000;238:326–333.
44. Smith RS, John SW, Zabeleta A, Davisson MT, Hawes NL, Chang B. The Bst locus on mouse chromosome 16 is associated with age-related subretinal neovascularization. *Proc Natl Acad Sci U S A.* 2000;97:2191–2195.
45. Ambati J, Anand A, Fernandez S, et al. An animal model of age-related macular degeneration in senescent Ccl-2- or Ccr-2-deficient mice. *Nat Med.* 2003;9:1390–1397.
46. Luhmann UF, Robbie S, Munro PM, et al. The drusenlike phenotype in aging Ccl2-knockout mice is caused by an accelerated accumulation of swollen autofluorescent subretinal macrophages. *Invest Ophthalmol Vis Sci.* 2009;50:5934–5943.
47. Hollyfield JG, Bonilha VL, Rayborn ME, et al. Oxidative damage-induced inflammation initiates age-related macular degeneration. *Nat Med.* 2008;14:194–198.
48. Croll SD, Ransohoff RM, Cai N, et al. VEGF-mediated inflammation precedes angiogenesis in adult brain. *Exp Neurol.* 2004;187:388–402.
49. Cao Y. Positive and negative modulation of angiogenesis by VEGFR1 ligands. *Science Signal.* 2009;2:re1.
50. Autiero M, Luttun A, Tjwa M, Carmeliet P. Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. *J Thromb Haemost.* 2003;1:1356–1370.
51. Rakic JM, Lambert V, Devy L, et al. Placental growth factor, a member of the VEGF family, contributes to the development of choroidal neovascularization. *Invest Ophthalmol Vis Sci.* 2003;44: 3186–3193.

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ORIGINAL PAPER

Rapid decrease in tumor perfusion following VEGF blockade predicts long-term tumor growth inhibition in preclinical tumor models

Alexandra Eichten · Alexander P. Adler · Blerta Cooper · Jennifer Griffith · Yi Wei · George D. Yancopoulos · Hsin Chieh Lin · Gavin Thurston

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Abstract Vascular endothelial growth factor (VEGF) is a key upstream mediator of tumor angiogenesis, and blockade of VEGF can inhibit tumor angiogenesis and decrease tumor growth. However, not all tumors respond well to anti-VEGF therapy. Despite much effort, identification of early response biomarkers that correlate with long-term efficacy of anti-VEGF therapy has been difficult. These difficulties arise in part because the functional effects of VEGF inhibition on tumor vessels are still unclear. We therefore assessed rapid molecular, morphologic and functional vascular responses following treatment with aflibercept (also known as VEGF Trap or ziv-aflibercept in the United States) in preclinical tumor models with a range of responses to anti-VEGF therapy, including Colo205 human colorectal carcinoma (highly sensitive), C6 rat glioblastoma (moderately sensitive), and HT1080 human fibrosarcoma (resistant), and correlated these changes to long-term tumor growth inhibition. We found that an overall decrease in tumor vessel perfusion, assessed by dynamic contrast-enhanced ultrasound (DCE-US), and increases in tumor hypoxia correlated well with long-term tumor growth inhibition, whereas changes in vascular gene expression and microvessel density did not. Our findings support previous clinical studies showing that decreased tumor perfusion after anti-VEGF therapy (measured by DCE-US) correlated with response. Thus, measuring tumor perfusion changes shortly after treatment with VEGF inhibitors, or possibly other anti-angiogenic therapies, may be useful to predict treatment efficacy.

A. Eichten · A. P. Adler · B. Cooper · J. Griffith · Y. Wei · G. D. Yancopoulos · H. C. Lin · G. Thurston (✉)
Regeneron Pharmaceuticals Inc, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA
e-mail: gavin.thurston@regeneron.com

Keywords VEGF blockade · Tumor perfusion · Tumor growth response · Preclinical model · Response biomarker

Introduction

Vascular endothelial growth factor (VEGF) plays a key role in physiological and pathological angiogenesis, including tumor angiogenesis [1]. Therefore, a number of agents that inhibit VEGF signaling have been developed and tested in clinical trials [2, 3]. Bevacizumab, a VEGF specific antibody that prevents receptor binding and activation, slowed tumor progression and provided survival benefits in several human tumor types when used in combination with chemotherapy. In addition, several small molecule inhibitors of VEGF receptor tyrosine kinase activity provided benefit in various cancers [4–6]. In preclinical models, VEGF inhibition results in reduced tumor growth, decreased microvessel density (MVD) and normalization of tumor vessel morphology in a wide range of tumor types [7, 8]. Similar MVD reductions were also reported in clinical studies of colorectal tumors sampled shortly after bevacizumab treatment [9].

Despite clear evidence for tumor vessel loss following VEGF inhibition, the functional consequences on tumor blood flow and oxygenation are not entirely clear. Naïvely, one might expect that vessel loss would result in decreased tumor perfusion. However, more detailed considerations suggested the opposite, namely, that tumor vessel pruning and "normalization" may lead to decreased intra-tumoral pressure, increased tumor perfusion, and consequently decreased tumor hypoxia [10]. Indeed, some preclinical studies indicate increased tumor perfusion after VEGF blockade [11, 12]. In contrast, other studies have reported increased tumor hypoxia and decreased perfusion in

preclinical models and non-small cell lung cancer (NSCLC) patients [13–15]. Thus, the functional consequences of anti-VEGF therapy are not clear, even in preclinical tumor models.

To add to the complexity, not all tumors within a given tumor type respond equally well to anti-VEGF therapy. For example, in glioblastoma patients treated with a small molecule kinase inhibitor (cediranib), approximately 60 % of tumors displayed changes in dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) signals indicative of a response to anti-VEGF therapy, whereas the remaining 40 % did not [16]. Despite much effort, predicting which tumors will respond to anti-VEGF therapy, or how long-term tumor growth response is related to vascular changes, has been difficult. For instance, it is unknown whether tumors with the largest MVD reduction show the greatest tumor growth inhibition (TGI). Further, tumor vessel features rendering them sensitive, or resistant, to VEGF inhibition are not well understood. Ultimately, predictive biomarkers based on mechanistic differences in tumor cells and tumor blood vessels are needed.

To begin to address these issues, we characterized initial responses of tumor vessels to VEGF blockade in preclinical tumors with a range of responses to anti-VEGF therapy (sensitive, moderately responsive, and resistant). For these studies, we used aflibercept (also known as VEGF Trap or ziv-aflibercept in the United States), a recombinant fusion protein that potently binds all isoforms of human and murine VEGF-A, VEGF-B and Placental Growth Factor (PlGF). Tumor bearing mice were treated with aflibercept, and tumors were analyzed for rapid (within 3 days) changes in molecular (gene expression), morphologic (MVD) and functional (vascular perfusion, tumor hypoxia) tumor vessel properties. These changes were then compared to aflibercept-mediated longer-term tumor growth effects. Using this approach, we observed that functional changes correlated well with the overall level of TGI, whereas molecular or morphological changes showed a poor correlation. These findings suggest that changes in functional parameters, such as tumor perfusion and hypoxia, may be good predictors of long term growth inhibition.

Materials and methods

In vivo tumor studies

Animal studies were performed in accordance with Regeneron's Institutional Animal Care and Use Committee guidelines. Tumor cells were obtained from the American Type Culture Collection (ATCC), except for the PC3 M line, which was obtained from the NCI, DCT Tumor Repository, NCI-FCRF, Frederick, MD. 1×10^6 Colo205 human colon carcinoma, 1×10^6 C6 rat glioblastoma, 2×10^6 HT1080 human fibosarcoma, 1×10^6 A431 human squamous cell carcinoma, 1×10^6 786-0 human renal cell carcinoma, 5×10^5 MMT murine mammary carcinoma, 1×10^6 PC-3 M metastasis-derived variant of human prostate adenocarcinoma PC-3 and 1×10^6 LLC murine Lewis lung carcinoma cells were grown s.c. in male CB.17/SCID mice (Taconic). When tumors reached approximately 100 mm^3, mice were treated by s.c. injection with hFc (control protein, 25 mg/kg) or a maximally effective anti-tumor dose of aflibercept [17] (VEGF Trap, ziv-aflibercept, 25 mg/kg) (# mice per treatment group: n = 5–7 tumor growth; n = 4–5 IHC; n = 3–4 TaqMan; n = 8–24 micro-ultrasound; n = 5–10 FITC-lectin flow cytometry). For long-term studies treatments occurred 2 × per week. Mice were monitored for tumor growth and overall health. HypoxyProbe-1 (Chemicon; 60 mg/kg) was injected i.p. 1 h prior to sacrifice. Tumors were harvested: ~½ tumor in 4 % paraformaldehyde, a cross-section in OCT, ~½ tumor in RNAlater. % Tumor Growth Inhibition (TGI) was calculated as follows: $[1 - ((T_{final} - T_{initial})/(C_{final} - C_{initial}))]*100$, where T = aflibercept-treated tumor volumes and C = control-treated tumor volumes at treatment start and after 10-14 day treatment (10 days: LLC, MMT; 14 days: HT1080, Colo205, C6, A431, 786-0, PC-3 M). Tumor growth curves are presented as mean ± standard error of the mean (SEM).

Immunohistochemistry and image analysis

IHC on gelatin embedded tissue sections: Tissues were fixed in 4 % paraformaldehyde for 72 h and embedded in a 4 % gelatin/PBS solution. Gelatin blocks were fixed in 4 % paraformaldehyde overnight at 4 °C, then transferred into a 30 % sucrose/PBS solution at 4 °C until the blocks sunk (~72 h). Tissue was cut into 80 μm sections, which were stored in cryoprotectant (1 % Polyvinylpyrrolidone, 30 % glycerol, and 30 % sucrose in NaPBS) at −20 °C until further use. For IHC detection of CD31 and HypoxyProbe (pimonidazole), sections were treated as follows: 30 min in 0.3 % H_2O_2 at 4 °C, 2 h in blocking solution (CD31: 0.3 % Triton X100/4 % normal rabbit serum/1 % BSA/PBS; HypoxyProbe: 0.3 % Triton X100/4 % normal horse serum/1 % BSA/PBS) at RT followed by an overnight incubation at 4 °C with rat anti-murine CD31 Ab (1:150; BD; MEC13.3) or a mouse anti-HypoxyProbe-1 antibody (1:1,000; Chemicon) diluted in the respective blocking solution containing 1 % serum. After five 3 min washes in PBS, CD31 was detected with a biotinylated mouse-adsorbed rabbit anti-rat antibody (1:150; Vector Laboratories;) and HypoxyProbe was detect with a biotinylated horse anti-mouse antibody (1:500; Vector Laboratories;) in a 2 h incubation at RT. Sections were subjected to an ABC

reaction according to the manufacturers recommendations (Vector Laboratories; ABC VectaStain Elite) for 1 h at RT diluted in 1 % BSA in 50 mM PBS. After five 3 min washes in PBS, antigens were revealed with 3,3'-diaminobenzidine (DAB, Sigma).

OCT embedded tumors were cut into 30 μm frozen sections. Tissue was air dried, 10 min fixed in acetone (−20 °C), avidin–biotin blocked (Vector), blocked in 2.5 % normal goat serum/1 % BSA/PBS for 30–45 min (RT), incubated for 16 h at 4 °C with rat anti-murine CD31 Ab (1:50; BD) diluted in 0.5 × block followed by a 45 min (RT) incubation with a biotinylated anti-rat antibody (1:150; Vector). Antigens were revealed with 3,3'-diaminobenzidine (DAB, Sigma).

For analysis photomicrographs were acquired at 2.5 × magnification. Vessel density and hypoxia area were determined using NIH image software as previously described [18].

RNA preparation and TaqMan analysis

Total RNA was purified using RNeasy (Qiagen). RNA quality and concentration were evaluated using a spectophotometer (NanoDrop ND-1000). cDNA was synthesized using 1 μg of total RNA and High Capacity RNA to cDNA Mastermix Kit (ABI). Expression of various genes was normalized to cyclophilin expression. TaqMan primer and probe sequences are as follows:

Gene	Forward primer	Reverse primer	Probe sequence
mKcne3	AGACCTGGTACATGAGCCTCCAT	CAAGTGACTGTGAAGGGTTGTGTT	TGGGCAGTCTCATCCT
mNid2	CCGCTGTGGCCCTAATTCT	TGCGGCATTCACACCTGTA	TGTGTGTCAACTTGGTGGG
mCdh5	AATCGGGAGCATGCCAAGT	TGGGCACCCCGTTGTC	CCCGTGCTCATCTC
mTie1	AGCCTGAGCCCTTGAGTTACC	AAAGTTGCCCTCCCCTATGAG	TGGGAGGACATCACC
mRobo4	GCTAGGCGCTTTCCATCCA	GCGGCTGCAGAGACTATCTGA	TTGGCTGGAACCTC
mEsm1	TCTGGACTTTCCCTTCTTCCAG	CTGTGTGGGAGGCAGAGGTC	TGCAGCAGCCAAATCTCCCAGCA
mVegfA	GTATGGCTGGCTGGGTCACT	GTTTGATCCGCATGATCTGTAGAG	ACCACTGTGATCTGC
mCyclophilin	CGTGGGCTCCGTCGTC	CCCTTCTTCTTATCGTTGGCC	TTGCTGCCCGGACCCTCCG

Flow cytometry

Tumor bearing mice (C6 or HT1080 tumors, 100 mm^3), or non-tumor control mice, were treated s.c. with hFc (control protein, 25 mg/kg) or aflibercept (VEGF Trap, 25 mg/kg) 24 h prior to tissue harvest. To label endothelial cells of functional vessels, mice were i.v. injected with FITC-conjugated *Lycopersicon esculentum* (tomato) lectin (2.0 mg/ml; Vector) 3 min prior to tissue harvest. Single cell suspensions were prepared from normal skin (n = 4, n = 2 no FITC-lectin), C6 tumors (n = 7 control or aflibercept, n = 4 no FITC-lectin) or HT1080 tumors (n = 7 control or aflibercept, n = 3 no FITC-lectin) as described previously [19] and endothelial cells were detected using a PE-conjugated anti-CD31 Ab (1:200; BD). DAPI (1 μg/ml; Invitrogen) was used to exclude dead cells. Data acquisition: Beckman-Coulter MoFlo Legacy; data analysis: FlowJo software (Tree Star). Data shown represent mean ± standard error of the mean (SEM).

Dynamic contrast-enhanced micro-ultrasound (DCE-micro US)

Animals were anaesthetized (isofluorane (3.0 %)/medical air mixture), secured to heated platform and dehaired. Ultrasound gel (Aquasonic, Parker Laboratories) provided coupling interface between ultrasound probe and animal. Image acquisition: Vevo2100 micro-ultrasound imaging system (VisualSonics); contrast agent: MicroMarker™ (microbubbles, VisualSonics). Contrast agent was prepared with a final concentration of 2×10^9 microbubbles/ml saline and a 50 μl bolus was delivered via tail vein catheter during image acquisition. Quantification of relative blood volume, which represents tumor perfusion, was determined by analysis of a 2D area representing the largest tumor cross-section (Vevo2100 analysis software).

Statistical analyses

Statistical analyses were performed using Prism software. Specific test include 2-way ANOVA with Bonferroni post hoc test (tumor growth curves), 1-way ANOVA with Bonferroni post hoc test (vessel density, gene expression changes, hypoxia analysis) and Mann–Whitney test (micro-ultrasound analysis). p values <0.05 were considered statistically significant.

Results

Vessel morphology changes in tumors with a range of responses to aflibercept

Based on studies with a wide variety of murine tumor models, three tumors that display a range of responses to

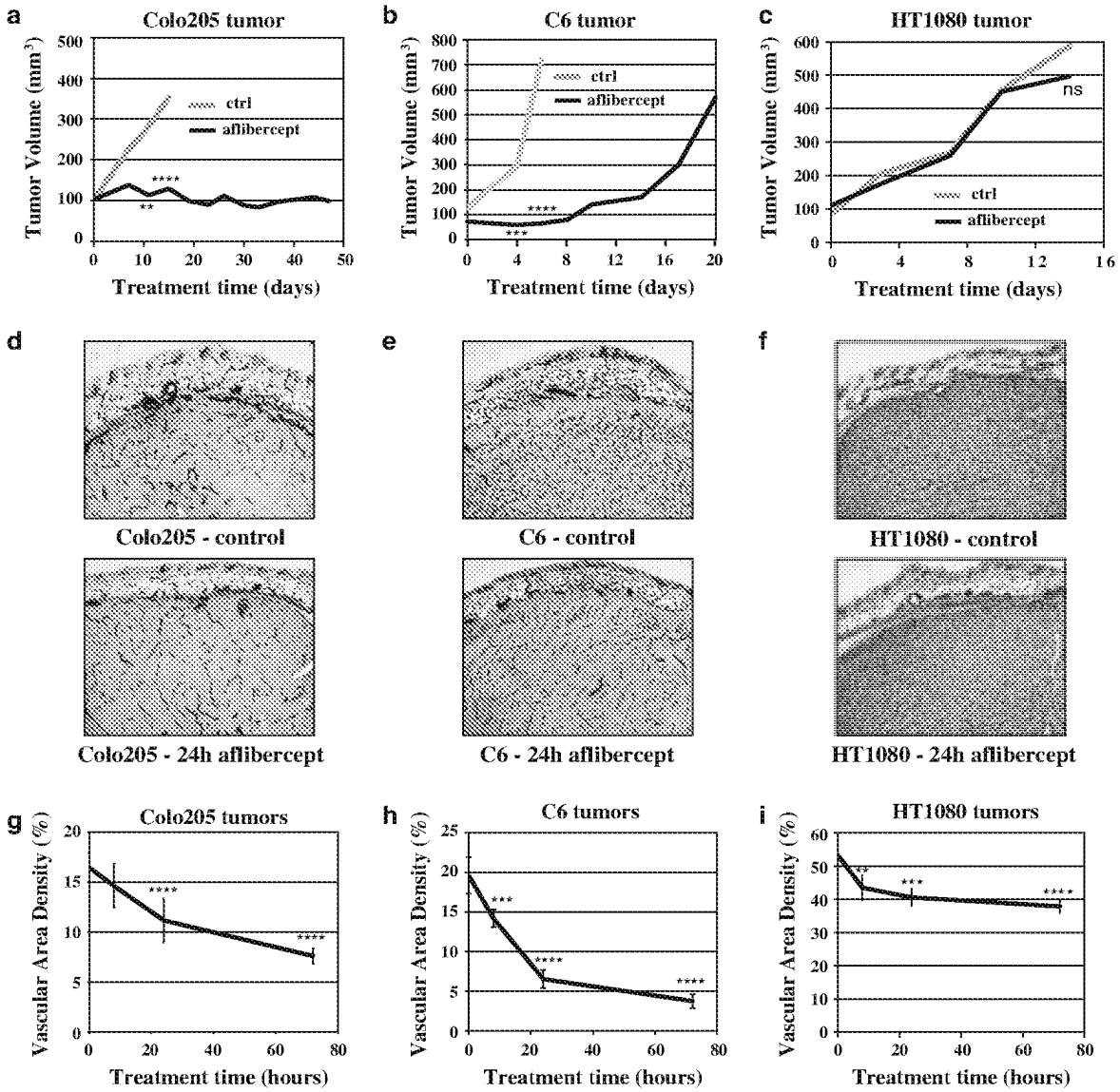

Fig. 1 Tumor growth and vascular response to aflibercept in Colo205, C6 and HT1080 tumors. **a–c** Colo205, C6 and HT1080 xenografts (n = 5–7 each treatment group/tumor type) show different levels of TGI in response to aflibercept treatment (*black*) compared to control-treated tumors (*grey*): sensitive Colo205, moderately responsive C6 and resistant HT1080. **d–f** Representative images of MVD assessed by CD31 IHC in control and 24 h aflibercept-treated Colo205, C6 and HT1080 tumors (80 µm gelatin sections). **g–i** Quantitative analysis of vessel area density (%) in control and 8, 24 and 72 h aflibercept-treated Colo205, C6 and HT1080 tumors (n = 4–5 each time point/tumor type). All experiments were repeated at least twice; shown is an example experiment (n = 5–7 for each treatment group (tumor growth data) or n = 4–5 for each time point (MVD data)). Results shown represent means for tumor growth data and mean ± standard deviation (SD) for MVD analysis. $P < 0.05$*, <0.01**, <0.001***, <0.0001**** by 2 way-ANOVA with Bonferroni post hoc test (tumor growth data compared to control treated tumor growth) and by 1 way-ANOVA with Bonferroni post hoc test (MVD, each time point compared to control (0 time point))

aflibercept were chosen for more detailed study. Colo205 tumors were potently growth inhibited (Fig. 1a), C6 tumors showed an intermediate growth inhibition in response to aflibercept treatment, with an initial growth delay followed by restrained tumor growth (Fig. 1b). In contrast, HT1080 tumors showed no growth inhibition upon aflibercept treatment (Fig. 1c). These differences in tumor response were observed at a saturating dose of aflibercept (25 mg/kg twice per week), thus the differences reflect inherent responses to aflibercept and not merely different dose responses.

We investigated the rapid effects of VEGF blockade on the vasculature of these 3 tumor types. As revealed by immunohistochemistry (IHC) for the vessel specific marker CD31 in thick sections, untreated Colo205 and C6 tumors have a significantly lower MVD (17 and 20 %, respectively)

than HT1080 tumors (55 %) (Fig. 1d–f upper images; g–i time point 0). Following aflibercept treatment, MVD rapidly decreased in all tumor types, albeit to varying degrees (Fig. 1d–f lower images; g–i). Quantitative analysis of relative MVD after aflibercept administration in comparison to control-treated tumors revealed that Colo205 tumors lost 11, 32 and 54 % of their vasculature at 8, 24 and 72 h after treatment, respectively. C6 tumors lost even more vessels (28, 67 and 81 % at 8, 24 and 72 h after treatment, respectively). Aflibercept resistant HT1080 tumors progressively lost vessels after aflibercept treatment, albeit to a much lesser degree (up to 29 % by 72 h), suggesting that the HT1080 tumor vasculature is only partially dependent on VEGF. These results show that blockade of VEGF can cause rapid loss of tumor vascularity, and further, that the vasculature in different xenograft tumors varies in its dependence on ongoing VEGF signaling.

Identification of two phases of gene expression changes in tumor vessels following aflibercept treatment

To determine how morphological tumor vessel changes manifest as molecular changes in gene expression, microarray analysis was performed on RNA from whole tumors treated with aflibercept for 8, 24 and 72 h. Mouse and human genes were assessed separately using mouse and human specific gene chips (custom Agilent microarray). Microarray analysis of mouse (host) genes in different tumors implied a rapid and consistent decrease in expression of a number of genes specific to endothelial cells [20–22] following aflibercept treatment. To confirm and extend the microarray findings, six genes were analyzed for expression changes in Colo205, C6 and HT1080 tumors treated with aflibercept for 8, 24 and 72 h by TaqMan, using primer pairs specific for murine mRNA. Gene expression was normalized to cyclophilin expression (similar results were obtained using GAPDH as a normalization gene; data not shown). Close inspection of these gene expression changes revealed two distinct temporal patterns: 'acute' and 'delayed' response genes. The 'acute' set of genes decreased in expression rapidly after aflibercept treatment (8 h) and remained decreased (Fig. 2a–c, black lines). Further, these 'acute' genes showed a large absolute decrease in expression levels, dropping up to 85 %. Among the 'acute' genes were potassium voltage-gated channel Isk-related subfamily gene 3 (Kcne3), endothelial cell-specific molecule 1 (Esm1) and nidogen2 (Nid2). Because of their rapid decrease after VEGF blockade (Fig. 2a–c, black lines), these 'acute' genes are likely direct targets of VEGF signaling.

A second set of genes decreased in expression at 24 and 72 h after aflibercept treatment, but were not yet significantly affected at the 8 h time point, thus showing a 'delayed' response (Fig. 2a–c, grey lines). Examples of genes that displayed robust 'delayed' changes were roundabout homolog 4 (Robo4), cadherin 5 (Cdh5) and tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie1). Other genes in this category included platelet/endothelial cell adhesion molecule 1 (Pecam1 or CD31) and intercellular adhesion molecule 2 (Icam2) (data not shown), two commonly used IHC endothelial cell markers [23, 24]. Thus, these 'delayed' genes may reflect a decrease in overall tumor vascularity or endothelial cell number. Changes in 'delayed' gene expression and MVD (Fig. 1d–i) appeared to have similar trends in terms of both timing and magnitude of decrease in different tumor types. When the combined expression of the 'delayed' gene changes was overlayed with MVD changes, comparable patterns emerged for each tumor (Fig. 2d–f), suggesting that 'delayed' gene changes can be used as markers for changed MVD in tumors treated with VEGF inhibitors.

Decreased tumor perfusion following treatment with aflibercept

To determine whether VEGF blockade also affected vessel functionality, we assessed tumor vessel perfusion 24 h after aflibercept administration using contrast-enhanced micro-ultrasound. Analysis of 2-dimensional (2D) ultrasound data revealed that perfusion of Colo205 and C6 tumors decreased by 32 and 59 %, respectively (Fig. 3a, b, d, e). In comparison, HT1080 tumor perfusion was not decreased at 24 h after aflibercept treatment (Fig. 3c, f). Interestingly, although HT1080 tumors have a dramatically higher baseline MVD (55 %; Fig. 1i) than C6 (20 %; Fig. 1h) or Colo205 tumors (17 %; Fig. 1g), baseline perfusion in the three tumor types was comparable (relative contrast intensity values of 8–10; Fig. 3d–f, control), as was previously shown for other tumor types [25]. These data suggest that a smaller fraction of vessels are well perfused in HT1080 tumors compared to C6 or Colo205 tumors.

To further compare the relative amounts of perfused vessels in C6 and HT1080 tumors, vessel perfusion was assessed by another method, namely i.v. injection of FITC-conjugated *Lycopersicon esculentum* tomato lectin (FITC-lectin), which binds to the luminal surface of blood endothelial cells (BECs, defined as CD31 positive) in functionally perfused vessels. Following in vivo labeling, the proportion of endothelial cells in the tumor and normal skin, and the fraction of endothelial cells labeled by FITC-lectin were both assessed by flow cytometry. For reference, BECs from normal skin comprise 1.9 % of all skin cells, and 96 % of the BECs in normal skin were labeled by FITC-lectin (Fig. 3g, skin). As a further control, the same proportion of BECs were found in skin and tumors of mice that were injected with FITC-lectin versus those that were not injected,

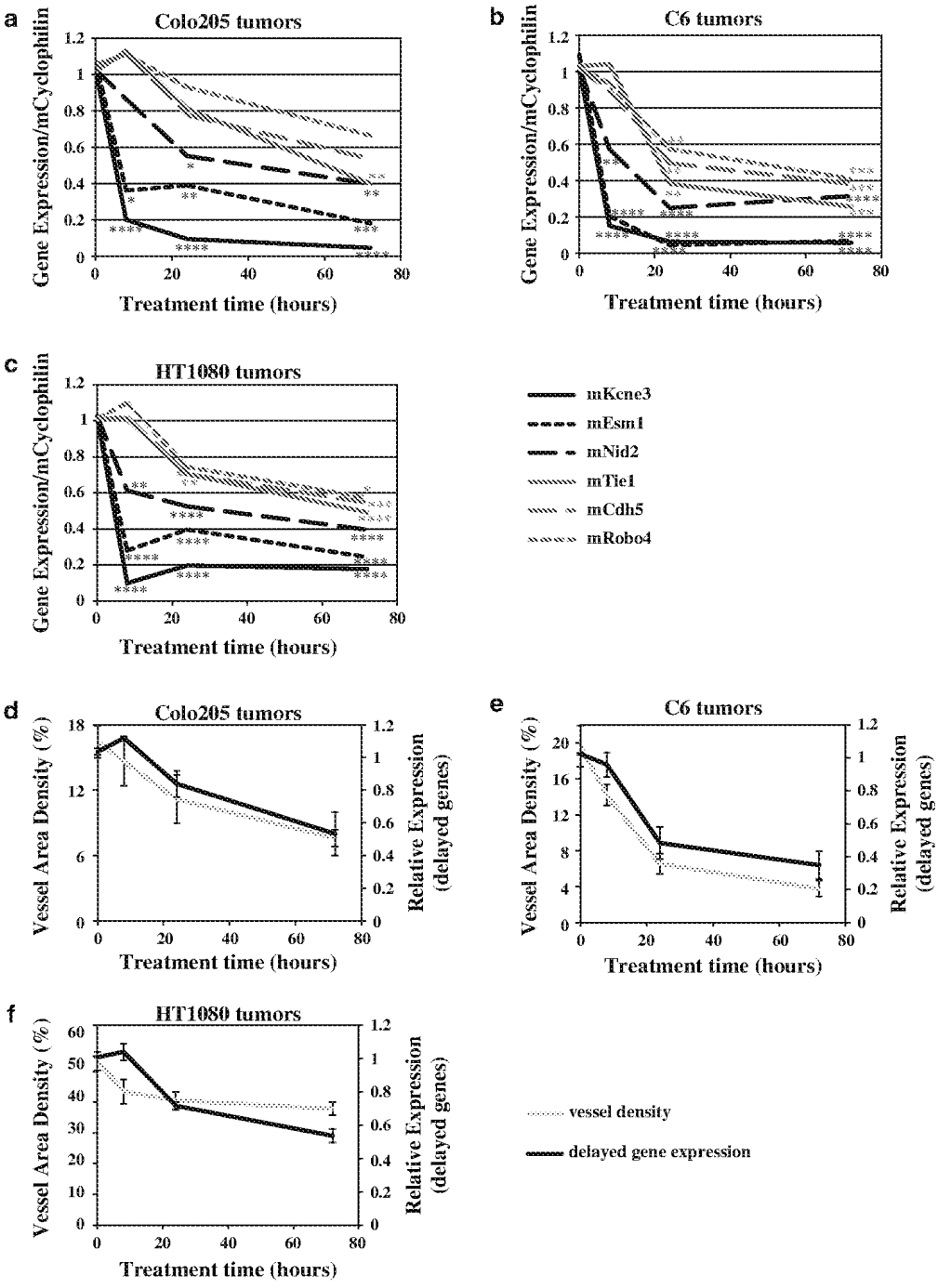

Fig. 2 Gene expression analysis revealed two phases of gene expression changes in tumor vessels following aflibercept treatment. **a–c** Gene changes (TaqMan) upon VEGF blockade occur in two distinct patterns: 'acute' gene changes occur in Kcne3, Esm1 and Nid2 (*black*), while 'delayed' gene changes occur in Tie1, Cdh5 and Robo4 (*grey*) in Colo205, C6 and HT1080 tumors after 8, 24 and 72 h aflibercept treatment (n = 3–4 each time point/tumor type) **d–f** Averages of MVD changes (*grey*) and averages of 'delayed' gene expression changes (*black*) show a corresponding pattern in Colo205, C6 and HT1080 tumors after 8, 24 and 72 h aflibercept treatment. All experiments were repeated at least twice; shown is an example experiment for TaqMan data (n = 3–4 for each time point). Results shown represent means or mean ± standard deviation (SD). $P < 0.05$*, <0.01**, <0.001***, <0.0001**** by 1 way-ANOVA with Bonferroni post hoc test (TaqMan data, each time point compared to control (0 time point))

but virtually no BECs were found to be positive for FITC-lectin in non-injected mice (Fig. 3g).

The number of BECs in untreated C6 tumors (0.8 % of total cells) was significantly less than in HT1080 tumors (2.2 %) (Fig. 3g). Of the BECs in untreated C6 tumors, approximately 55 % were perfused (i.e., positive for FITC-lectin). In contrast, only 18 % of the BECs in untreated HT1080 tumors were perfused (Fig. 3g; Table 1). Thus, despite more than a twofold difference in total BEC, the fraction of BECs labeled by intravascular lectin (FITC-positive BECs) was similar in C6 and HT1080 tumors (0.40 vs. 0.43 % of total cells, respectively). This finding corroborates our micro-ultrasound findings that untreated C6 and HT1080 tumors have similar levels of perfusion as measured by micro-ultrasound (Fig. 3e, f, control), despite dramatically different MVD (Fig. 1i, h, control).

Treatment with aflibercept (24 h) decreased the number of BECs in C6 tumors to 0.5 % of total cells (~37 % decrease) and to 1.6 % in HT1080 tumors (~28 % decrease) (Fig. 3g, h; Table 1). These data correspond with

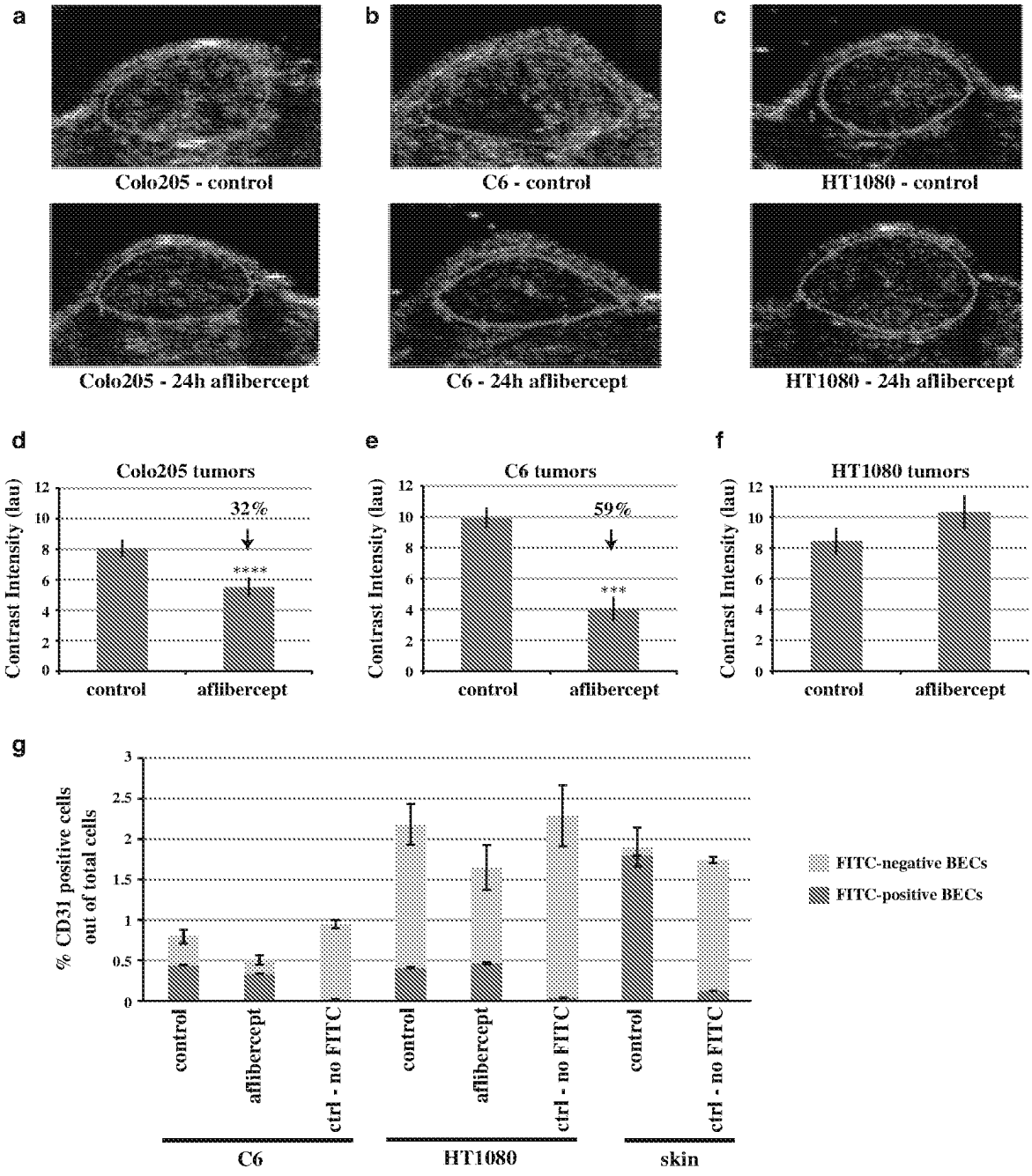

Fig. 3 Perfusion decreased in response to aflibercept treatment in Colo205 and C6 tumors, but remained unchanged in HT1080 tumors. **a–c** Representative images of vessel perfusion assessed by 2-dimensional (2D) DCE-US in control and 24 h aflibercept-treated Colo205, C6 and HT1080 tumors. Tumors are outlined in *red*. **d–f** Quantitative analysis of vessel perfusion in control and 24 h aflibercept-treated Colo205 (n = 18 and 24, respectively), C6 (n = 8 and 12, respectively) and HT1080 tumors (n = 12 and 9, respectively) **g** Flow cytometry analysis of CD31-positive blood vessel endothelial cells (BECs) in combination with the intravenously injected perfusion marker FITC-lectin in control and 24 h aflibercept-treated C6 (n = 10 each treatment group; n = 5 for 'no-FITC' group) and HT1080 tumors (n = 10 each treatment group; n = 5 for 'no-FITC' group) as well as control-treated skin tissue (n = 5 each group). Shown are perfusion and flow cytometry results combined from multiple experiments. Results shown represent mean ± standard error of the mean (SEM). $P < 0.05$*, <0.01**, <0.001***, <0.0001**** by Mann–Whitney test (tumor perfusion). Differences between FITC-lectin positive BECs in ctrl versus aflibercept treated C6 or HT1080 tumors and between ctrl treated C6 and ctrl treated HT1080 tumors were not statistically significant by Mann–Whitney test

the relative decrease in MVD after aflibercept treatment (Fig. 1h, i; Table 1). After 24 h of aflibercept treatment, the proportion of FITC-lectin positive BEC in C6 tumors increased slightly to 66 % of all BECs, although the total number of FITC-lectin positive BECs went down (to 0.33 % of total cells). In HT1080 tumors after aflibercept

Table 1 Flow cytometry analysis of all CD31-positive blood vessel endothelial cells (BECs) and perfused (FITC-lectin positive) BECs derived from control and 24 h aflibercept-treated C6 and HT1080 tumors

	% CD31 + (BEC) cells out of total cells		Relative change (%)	% FITC-lectin + BECs out of total cells		Relative change (%)	% FITC-lectin + BECs out of total BECs	
	Control	Aflibercept		Control	Aflibercept		Control	Aflibercept
C6	0.8	0.5	−37	0.43	0.33	−24	55	66
HT1080	2.2	1.6	−28	0.4	0.48	+20	18	31

treatment, the proportion of FITC-lectin positive BEC also increased slightly to 31 % of all BECs, whereas the total number of BECs increased slightly (to 0.48 % of all cells) (Fig. 3g; Table 1). Again, these findings are consistent with perfusion changes seen by micro-ultrasound following treatment of these tumors with aflibercept (Fig. 3e, f). Thus, this flow cytometry-based analysis of tumor vessel perfusion provides a powerful link between functional perfusion assays and immunohistochemistry of tumor blood vessels following anti-VEGF treatment.

Increased tumor hypoxia following treatment with aflibercept

To determine whether the decreased tumor perfusion following aflibercept treatment resulted in tumor oxygenation changes, we analyzed hypoxia in Colo205, C6, and HT1080 tumors at 8, 24 and 72 h after aflibercept treatment. Hypoxia was assessed by HypoxyProbe IHC (Fig. 4a–c) as well as by analyzing the expression of VEGF, a hypoxia regulated gene (Fig. 4g–i). Colo205 and C6 tumors have hypoxic regions even under baseline conditions, which become more pronounced upon aflibercept treatment starting at 8 h (Fig. 4a, b, d, e). The increase in HypoxyProbe staining observed in C6 and Colo205 tumors after 24 h aflibercept treatment (Fig. 4d, e) corresponded with decreased perfusion (Fig. 3d, e). In comparison, HT1080 tumors had little or no hypoxic regions at baseline, and no increase in hypoxia at 72 h of treatment with aflibercept (Fig. 4c, f), consistent with the unchanged tumor perfusion (Fig. 3c, f). Similarly, expression of VEGF progressively increased in C6 and Colo205 tumors, whereas VEGF expression was unchanged in HT1080 tumors (Fig. 4g). Taken together, increased tumor hypoxia correlated with decreased tumor perfusion.

Tumor perfusion changes correlated with long-term response to aflibercept

The results from our analysis of three tumor types suggested that rapid changes in tumor perfusion and/or hypoxia correlated better with long-term tumor growth response to aflibercept than did other parameters such as changes in microvessel density or vascular gene expression (Fig. 5a). To further assess whether tumor vascular perfusion changes at 24 h after aflibercept treatment correlated with long-term growth inhibition, we extended our analyses to several additional tumor types (A431, 786-0, MMT, LLC and PC-3 M) grown in immunocompromised SCID mice. We also included a syngeneic model, LLC tumors grown in C57Bl6 mice, to assess the effects of aflibercept on tumor perfusion and growth in immunocompetent mice. As expected, tumor growth inhibition in immunocompromised mice did not correlate well with changes in tumor vessel density (Fig. 5b, $R^2 = 0.09$). In comparison, in this larger sample including one syngeneic model, tumor growth inhibition showed a correlation with changes in tumor perfusion (Fig. 5c, $R^2 = 0.73$).

Discussion

The search for early response and predictive biomarkers of tumor response to anti-angiogenic agents has so far not provided definitive candidates. While clinical studies have sought such markers by sampling numerous growth factors and cytokines, preclinical studies may be able to provide more mechanism-based candidates and approaches. In this study, we analyzed several tumors with a wide range of long-term tumor growth responses to anti-VEGF therapy. Using subcutaneous tumor models, we correlated early morphologic and functional vascular changes following treatment with aflibercept to long-term tumor growth inhibition (TGI). We found that changes in tumor hypoxia and perfusion correlated with long-term TGI, whereas changes in vascular gene expression and MVD showed a poor correlation.

In early clinical analyses, MVD was proposed as a prognostic indicator for disease stage, likelihood of metastasis, recurrence, and survival in a range of tumor types [26–28]. To date, however, neither baseline values, nor treatment-related changes in MVD have proven useful for evaluating or guiding anti-angiogenic treatments [29]. To extend the analysis of MVD, we identified a set of endothelial cell marker genes, including Tie1, Pecam1, Cdh5, Icam2 and Robo4 [20–22], which decreased following treatment with aflibercept. The timing and

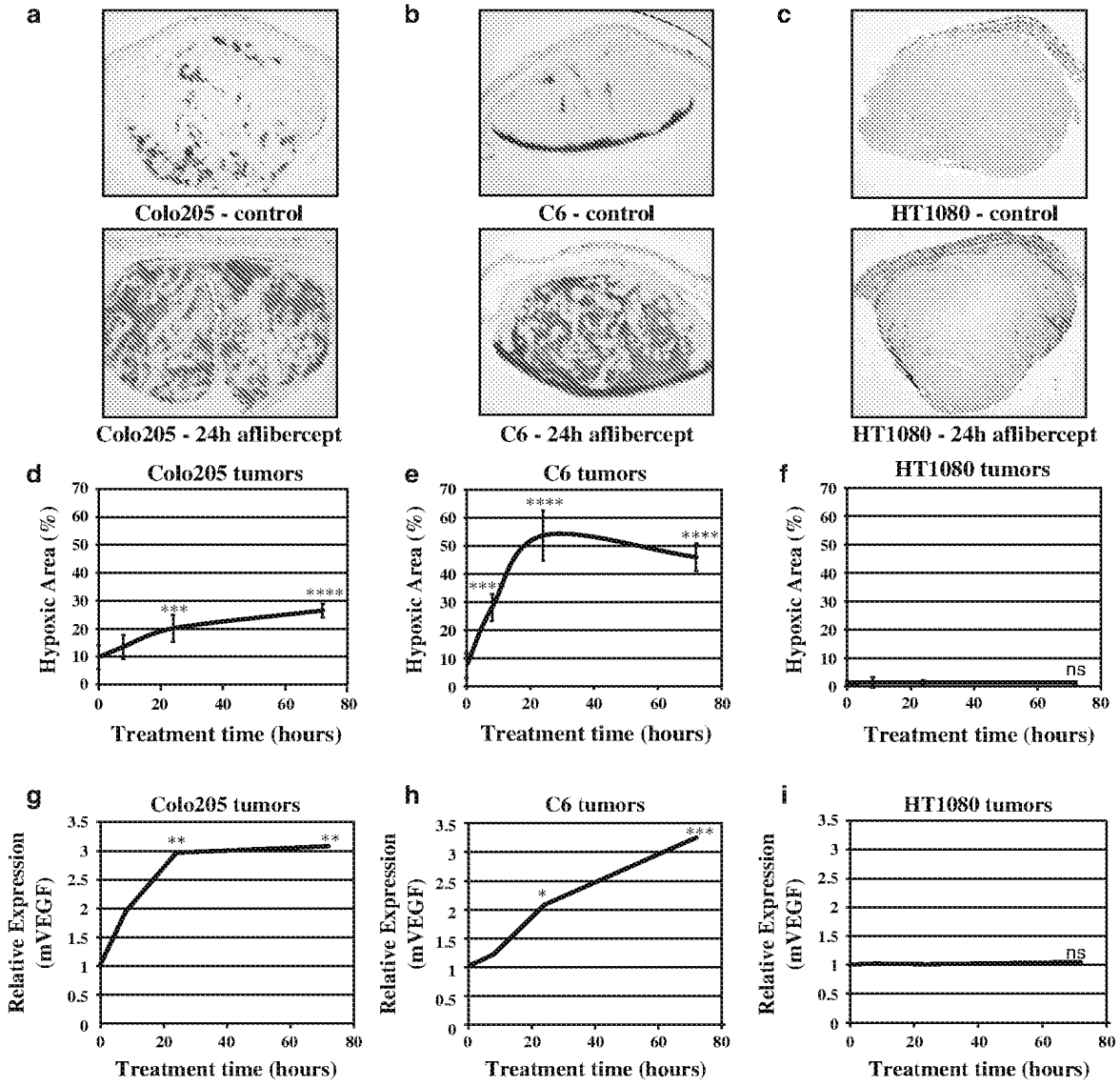

Fig. 4 Tissue oxygenation decreased in response to aflibercept treatment in Colo205 and C6, but not in HT1080 tumors. **a–c** Representative images of hypoxia assessed by HypoxyProbe IHC in control and 24 h aflibercept-treated Colo205, C6 and HT1080 tumors (80 μm gelatin sections). **d-f)** Quantitative analysis of hypoxia area (%) in control and 8, 24 and 72 h aflibercept-treated Colo205, C6 and HT1080 tumors (n = 4–5 each time point/tumor type). **g–i** TaqMan analysis of the hypoxia responsive gene VEGF in Colo205, C6 and HT1080 tumors after 8, 24 and 72 h aflibercept treatment (n = 3–4 each time point/tumor type). All experiments were repeated at least twice; shown is an example experiment (n = 4–5 (hypoxia data) or n = 3–4 (TaqMan data) for each time point). Results shown represent means or mean ± standard deviation (SD). $P < 0.05$*, <0.01**, <0.001***, <0.0001**** by 1 way-ANOVA with Bonferroni post hoc test (hypoxia IHC, each time point compared to control (0 time point); TaqMan data, each time point compared to control (0 time point))

magnitude of the decrease in these genes correlated well with changes in tumor MVD. However, these gene expression changes did not correlate with long-term TGI. Further, changes in 'acute' gene expression, such as Esm1 and Nid2, which appear to reflect direct VEGF target genes [30–32], similarly did not correlate with long-term TGI following treatment with aflibercept. This latter finding suggests that VEGF inhibition within a tumor is a necessary but not sufficient determinant of efficacy of anti-VEGF therapy.

Agents that target other angiogenic signaling pathways further confound the attempts to correlate MVD, vascular markers or indicators of VEGF signaling with anti-tumor effects. For example, in pre-clinical models, blockade of the angiogenic ligand Dll4 results in increased MVD [18, 33] and endothelial cell marker genes (data not shown), but inhibits tumor growth, thus clearly showing that MVD changes are not predictive of anti-angiogenic treatment efficacy. In the case of Dll4 inhibition, the newly formed tumor vascular structures are non-functional [18, 33].

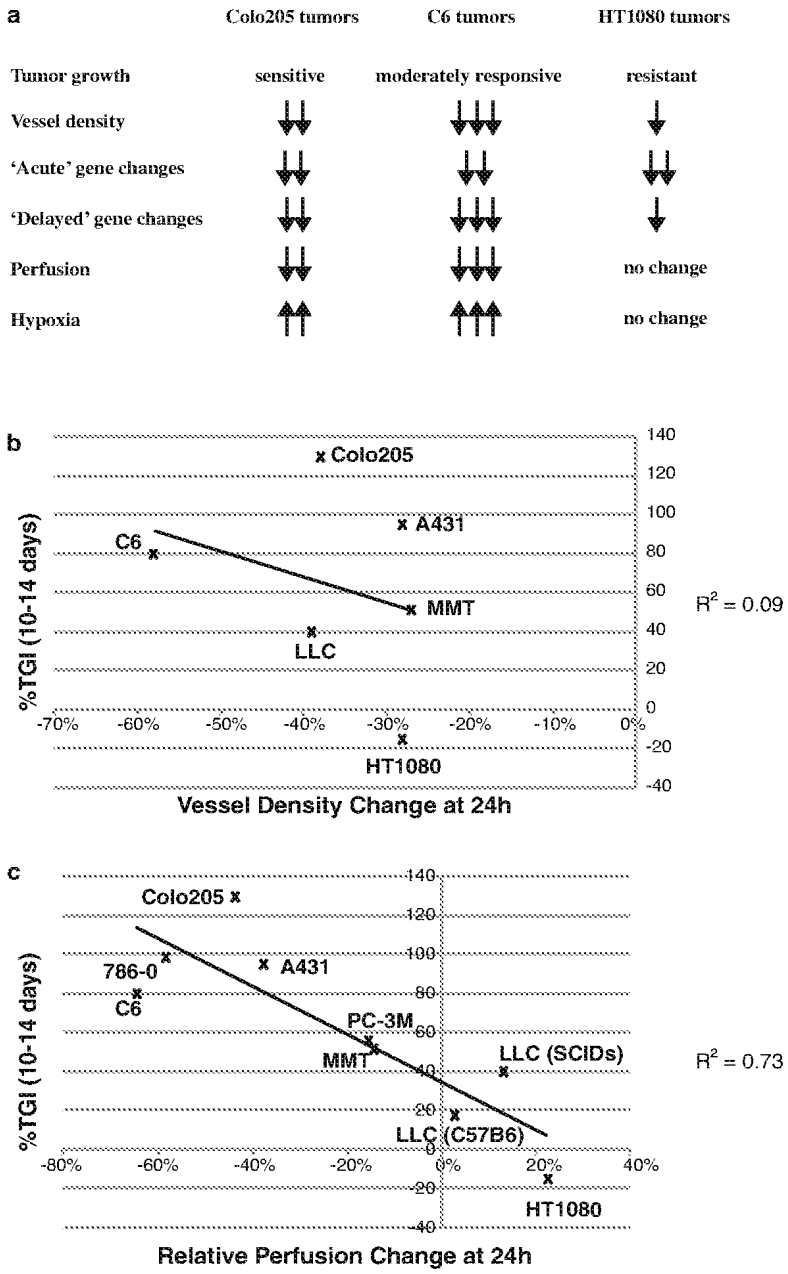

Fig. 5 Changes in tumor perfusion, but not in MVD, 24 h after aflibercept treatment are predictive of long-term tumor growth inhibition. **a** Summary of aflibercept effects on long-term tumor growth and short-term (up to 72 h) MVD, gene expression, tumor perfusion and hypoxia. **b** Poor correlation between MVD changes (30 μm OCT sections; n = 4–5 each treatment group/tumor type) and long-term TGI (n = 5–7 each treatment group/tumor type) in Colo205, C6, HT1080, MMT, A431 and LLC tumors. **c** Good correlation between tumor perfusion changes (n = 7–24 each treatment group/tumor type) and long-term TGI (n = 5–7 each treatment group/tumor type) in Colo205, C6, HT1080, MMT, A431, LLC, 786-0 and PC-3 M tumors. All experiments were repeated at least twice; shown is an example experiment for tumor growth (n = 5–7) and vessel density (n = 4–5) data along with combined data for tumor perfusion data (n = 7–24 each treatment group/tumor type)

These findings, as well as our current results, emphasize the concept that changes in tumor vessel functionality are much more important for predicting tumor growth response than changes in the number of vessels, their morphology or their signaling profiles.

In preclinical models, intravenous injection of dyes like FITC-lectin, Hoechst 33342 or DiOC7 prior to sacrifice has frequently been used to distinguish perfused/functional vessels from non-perfused vessels in tissue sections [7, 19, 34–36]. In addition, FITC-lectin or Hoechst 33342 have been used with flow cytometry to detect perfused endothelial cells or to assess the ratio of tumor cells close to perfused blood vessels versus those further away [37, 38]. Our studies further validate the use of i.v. FITC-lectin combined with CD31 flow cytometry, to distinguish endothelial cells from perfused versus non-perfused vessels.

In clinical studies of VEGF blockade, DCE-MRI has been used frequently to evaluate the functional microvasculature within tumors. In particular, decreases in K^{trans}, a volume transfer constant for contrast agent in blood/plasma and the extravascular extracellular space, was shown to be predictive of time to progression in liver cancer upon VEGF blockade [39]. Similarly, changes in K^{trans} allowed the prediction of responses in glioblastoma patients treated with bevacizumab and irinotecan [40]. In some preclinical tumor models, DCE-MRI has also revealed a decrease in

K^{trans} in response to VEGF blockade [41]. However, DCE-MRI was not predictive of treatment efficacy upon anti-angiogenic therapy in other cancers, such as NSCLC [42]. In an attempt to better predict anti-angiogenic efficacy, DCE-MRI was combined with assessment of MVD and plasma collagen IV levels shortly (24 h) after the start of treatment. This 'vascular normalization index', was predictive of responsiveness to anti-angiogenic therapy in glioma patients [43]. In a follow-up study, a prolonged increase in tumor perfusion, as evidenced by DCE-MRI, was associated with longer survival in glioma patients [44]. However, a recent positron emission tomography (PET) imaging study reported a decrease in perfusion and impaired docetaxel delivery after a single dose of bevacizumab in NSCLC patients [14], suggesting that vessel normalization after VEGF blockade does not occur in NSCLC. In another recent study, single-photon emission computed tomography (SPECT) imaging revealed that anti-VEGF treatment decreased tumor uptake of an anti-Her2 antibody (trastuzumab) in preclinical breast cancer models, thus further supporting that VEGF blockade results in decreased tumor perfusion rather than vessel normalization [15].

In addition to DCE-MRI, PET and SPECT, other imaging modalities have been used to predict efficacy of anti-angiogenic therapies. For example, dynamic contrast-enhanced ultrasound (DCE-US) imaging has been used to assess tumor perfusion before and after treatment of various cancers with anti-angiogenic agents [45–47]. DCE-US imaging typically uses gas-filled lipid-shell microbubbles several micrometers in diameter as contrast agent [48]. DCE-US differs from DCE-MRI in that it solely assesses changes in vascular perfusion, while DCE-MRI measures a combination of blood flow through the vasculature as well as tracer movement across the vessel wall [49]. Although DCE-MRI and DCE-US appear to have predictive potential in anti-angiogenic therapy, DCE-US may be less sensitive to changes in tumor vascular permeability, and thus be more robust for assessing changes in tumor perfusion.

Decreases in tumor perfusion can result in hypoxia, as was observed after aflibercept treatment of sensitive Colo205 and moderately responsive C6 tumors, but not in resistant HT1080 tumors. These findings can be compared to the vascular normalization hypothesis, which proposed that tumor vessels remaining after anti-VEGF therapy temporarily 'normalize' in terms of morphology and functionality, resulting in increased tumor blood flow and decreased hypoxia [10]. Other preclinical studies, however, have shown that the anti-angiogenic agents DC101 and AG-013736 induce decreased perfusion and increased hypoxia [13, 35, 50]. Although it is well established that human tumors are often hypoxic and poorly perfused [51], direct measurement of tumor oxygenation before and after VEGF blockade in patients is challenging. Instead, hypoxia changes have been assessed indirectly. For example, bevacizumab treatment of RCC patients resulted in increased tumor cell apoptosis, along with increased tumor cell proliferation, which were hypothesized to be at least partially due to increased blood flow and decreased hypoxia [52].

The current study used various tumor models grown subcutaneously in mice, a site that can be readily accessed for micro-ultrasound studies of tumor perfusion. While the vascular structures and responses to anti-angiogenic therapies of such tumors may not fully reflect those of primary and metastatic human tumors, the ability to directly measure tumor blood flow provides opportunities to identify potential early response biomarkers that can be further tested in orthotopic preclinical tumor models and in clinical settings.

Early response biomarkers that can predict long-term outcome to therapy would be powerful tools, and panels of such potential biomarkers for anti-angiogenic therapies have been explored. For example, changes in circulating VEGF or PlGF levels, as well as tumor VEGF levels, were thought to be predictive, but to date have not shown to be well correlated with outcome [53]. In the current preclinical study, decreases in tumor perfusion and increases in hypoxia following treatment of subcutaneous xenograft and syngeneic models with aflibercept correlated with long-term TGI. Our results suggest that perfusion changes, as measured by DCE-US, shortly after treatment with VEGF inhibitors or possibly other anti-angiogenic therapies, could potentially be used as an early response biomarker to assess treatment efficacy.

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References

1. Ferrara N (2009) VEGF-A: a critical regulator of blood vessel growth. Eur Cytokine Netw 20(4):158–163. doi:10.1684/ecn.2009.0170
2. Duda DG, Batchelor TT, Willett CG, Jain RK (2007) VEGF-targeted cancer therapy strategies: current progress, hurdles and

future prospects. Trends Mol Med 13(6):223–230. doi:10.1016/j.molmed.2007.04.001
3. Grepin R, Pages G (2010) Molecular mechanisms of resistance to tumour anti-angiogenic strategies. J Oncol 2010:835680. doi:10.1155/2010/835680
4. Motzer RJ, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, DePrimo SE, Li JZ, Bello CL, Theuer CP, George DJ, Rini BI (2006) Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. J Clin Oncol 24(1):16–24. doi:10.1200/JCO.2005.02.2574
5. Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, Negrier S, Chevreau C, Solska E, Desai AA, Rolland F, Demkow T, Hutson TE, Gore M, Freeman S, Schwartz B, Shan M, Simantov R, Bukowski RM (2007) Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med 356(2):125–134. doi:10.1056/NEJMoa060655
6. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Haussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J (2008) Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 359(4):378–390. doi:10.1056/NEJMoa0708857
7. Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos GD, McDonald DM (2004) Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. Am J Pathol 165(1):35–52
8. Benjamin LE, Keshet E (1997) Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. Proc Natl Acad Sci U S A 94(16):8761–8766
9. Willett CG, Boucher Y, Di Tomaso E, Duda DG, Munn LL, Tong RT, Chung DC, Sahani DV, Kalva SP, Kozin SV, Mino M, Cohen KS, Scadden DT, Hartford AC, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Chen HX, Shellito PC, Lauwers GY, Jain RK (2004) Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. Nat Med 10(2):145–147
10. Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307(5706):58–62
11. Dickson PV, Hamner JB, Sims TL, Fraga CH, Ng CY, Rajasekeran S, Hagedorn NL, McCarville MB, Stewart CF, Davidoff AM (2007) Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenografts results in improved delivery and efficacy of systemically administered chemotherapy. Clin Cancer Res 13(13):3942–3950. doi:10.1158/1078-0432.CCR-07-0278
12. Wildiers H, Guetens G, De Boeck G, Verbeken E, Landuyt B, Landuyt W, de Bruijn EA, van Oosterom AT (2003) Effect of antivascular endothelial growth factor treatment on the intratumoral uptake of CPT-11. Br J Cancer 88(12):1979–1986. doi:10.1038/sj.bjc.6601005
13. Franco M, Man S, Chen L, Emmenegger U, Shaked Y, Cheung AM, Brown AS, Hicklin DJ, Foster FS, Kerbel RS (2006) Targeted anti-vascular endothelial growth factor receptor-2 therapy leads to short-term and long-term impairment of vascular function and increase in tumor hypoxia. Cancer Res 66(7):3639–3648. doi:10.1158/0008-5472.CAN-05-3295
14. Van der Veldt AA, Lubberink M, Bahce I, Walraven M, de Boer MP, Greuter HN, Hendrikse NH, Eriksson J, Windhorst AD, Postmus PE, Verheul HM, Serne EH, Lammertsma AA, Smit EF (2012) Rapid decrease in delivery of chemotherapy to tumors after anti-VEGF therapy: implications for scheduling of anti-angiogenic drugs. Cancer Cell 21(1):82–91. doi:10.1016/j.ccr.2011.11.023
15. Pastuskovas CV, Mundo EE, Williams SP, Nayak TK, Ho J, Ulufatu S, Clark S, Ross S, Cheng E, Parsons-Reponte K, Cain G, Van Hoy M, Majidy N, Bheddah S, Dela Cruz Chuh J, Kozak KR, Lewin-Koh N, Nauka P, Bumbaca D, Sliwkowski M, Tibbitts J, Theil FP, Fielder PJ, Khawli LA, Boswell CA (2012) Effects of Anti-VEGF on pharmacokinetics, biodistribution, and tumor penetration of trastuzumab in a preclinical breast cancer model. Mol Cancer Ther 11(3):752–762. doi:10.1158/1535-7163.MCT-11-0742-T
16. Batchelor TT, Duda DG, di Tomaso E, Ancukiewicz M, Plotkin SR, Gerstner E, Eichler AF, Drappatz J, Hochberg FH, Benner T, Louis DN, Cohen KS, Chea H, Exarhopoulos A, Loeffler JS, Moses MA, Ivy P, Sorensen AG, Wen PY, Jain RK (2010) Phase II study of cediranib, an oral pan-vascular endothelial growth factor receptor tyrosine kinase inhibitor, in patients with recurrent glioblastoma. J Clin Oncol 28(17):2817–2823. doi:10.1200/JCO.2009.26.3988
17. Rudge JS, Holash J, Hylton D, Russell M, Jiang S, Leidich R, Papadopoulos N, Pyles EA, Torri A, Wiegand SJ, Thurston G, Stahl N, Yancopoulos GD (2007) VEGF Trap complex formation measures production rates of VEGF, providing a biomarker for predicting efficacious angiogenic blockade. Proc Natl Acad Sci USA 104(47):18363–18370. doi:10.1073/pnas.0708865104
18. Noguera-Troise I, Daly C, Papadopoulos NJ, Coetzee S, Boland P, Gale NW, Lin HC, Yancopoulos GD, Thurston G (2006) Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. Nature 444(7122):1032–1037. doi:10.1038/nature05355
19. Eichten A, Hyun WC, Coussens LM (2007) Distinctive features of angiogenesis and lymphangiogenesis determine their functionality during de novo tumor development. Cancer Res 67(11):5211–5220
20. Ho M, Yang E, Matcuk G, Deng D, Sampas N, Tsalenko A, Tabibiazar R, Zhang Y, Chen M, Talbi S, Ho YD, Wang J, Tsao PS, Ben-Dor A, Yakhini Z, Bruhn L, Quertermous T (2003) Identification of endothelial cell genes by combined database mining and microarray analysis. Physiol Genomics 13(3):249–262. doi:10.1152/physiolgenomics.00186.2002
21. Rodewald HR, Sato TN (1996) Tie1, a receptor tyrosine kinase essential for vascular endothelial cell integrity, is not critical for the development of hematopoietic cells. Oncogene 12(2):397–404
22. de Fougerolles AR, Stacker SA, Schwarting R, Springer TA (1991) Characterization of ICAM-2 and evidence for a third counter-receptor for LFA-1. J Exp Med 174(1):253–267
23. Woodfin A, Voisin MB, Nourshargh S (2007) PECAM-1: a multi-functional molecule in inflammation and vascular biology. Arterioscler Thromb Vasc Biol 27(12):2514–2523. doi:10.1161/ATVBAHA.107.151456
24. Garlanda C, Dejana E (1997) Heterogeneity of endothelial cells. Specific markers. Arterioscler Thromb Vasc Biol 17(7):1193–1202
25. Gullino PM, Grantham FH (1961) Studies on the exchange of fluids between host and tumor. II. The blood flow of hepatomas and other tumors in rats and mice. J Natl Cancer Inst 27:1465–1491
26. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J (1993) Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol 143(2):401–409
27. de Jong D, Boot H, Taal B (2000) Histological grading with clinical relevance in gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Recent Results Cancer Res 156:27–32
28. Borre M, Offersen BV, Nerstrom B, Overgaard J (1998) Microvessel density predicts survival in prostate cancer patients subjected to watchful waiting. Br J Cancer 78(7):940–944

29. Hlatky L, Hahnfeldt P, Folkman J (2002) Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. J Natl Cancer Inst 94(12):883–893
30. Shin JW, Huggenberger R, Detmar M (2008) Transcriptional profiling of VEGF-A and VEGF-C target genes in lymphatic endothelium reveals endothelial-specific molecule-1 as a novel mediator of lymphangiogenesis. Blood 112(6):2318–2326. doi:10.1182/blood-2008-05-156331
31. Rennel E, Mellberg S, Dimberg A, Petersson L, Botling J, Ameur A, Westholm JO, Komorowski J, Lassalle P, Cross MJ, Gerwins P (2007) Endocan is a VEGF-A and PI3 K regulated gene with increased expression in human renal cancer. Exp Cell Res 313(7):1285–1294. doi:10.1016/j.yexcr.2007.01.021
32. Su SC, Mendoza EA, Kwak HI, Bayless KJ (2008) Molecular profile of endothelial invasion of three-dimensional collagen matrices: insights into angiogenic sprout induction in wound healing. Am J Physiol Cell Physiol 295(5):C1215–C1229. doi:10.1152/ajpcell.00336.2008
33. Ridgway J, Zhang G, Wu Y, Stawicki S, Liang WC, Chanthery Y, Kowalski J, Watts RJ, Callahan C, Kasman I, Singh M, Chien M, Tan C, Hongo JA, de Sauvage F, Plowman G, Yan M (2006) Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature 444(7122):1083–1087. doi:10.1038/nature05313
34. Trotter MJ, Chaplin DJ, Durand RE, Olive PL (1989) The use of fluorescent probes to identify regions of transient perfusion in murine tumors. Int J Radiat Oncol Biol Phys 16(4):931–934
35. Fenton BM, Paoni SF (2007) The addition of AG-013736 to fractionated radiation improves tumor response without functionally normalizing the tumor vasculature. Cancer Res 67(20):9921–9928. doi:10.1158/0008-5472.CAN-07-1066
36. Eichten A, Shen H-CJ, Coussens LM (2005) Three-dimensional visualization of blood and lymphatic vasculature in tissue whole mounts using confocal microscopy. In: Robinson JP (ed) Current protocols in cytometry, vol Suppl. 32. John Wiley & Sons, Inc, New Jersey, pp 12.15.11–12.15.11
37. Sahagun G, Moore SA, Fabry Z, Schelper RL, Hart MN (1989) Purification of murine endothelial cell cultures by flow cytometry using fluorescein-labeled griffonia simplicifolia agglutinin. Am J Pathol 134(6):1227–1232
38. Chaplin DJ, Olive PL, Durand RE (1987) Intermittent blood flow in a murine tumor: radiobiological effects. Cancer Res 47(2):597–601
39. Yopp AC, Schwartz LH, Kemeny NE, Gultekin D, Gonen M, Bamboat Z, Shia J, D'Angelica MI, DeMatteo RP, Jarnagin WR (2010) Dynamic contrast enhanced MRI (DCE-MRI) to measure antiangiogenic therapy and predict treatment response in primary liver cancer (PLC). J Clin Oncol (ASCO meeting abstract) 28 (suppl; abstr e14537)
40. Desjardins A, Barboriak DP, Herndon JE, Reardon DA, Quinn JA, Rich JN, Sathornsumetee S, Gururangan S, Friedman HS, Vredenburgh JJ (2007) Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) evaluation in glioblastoma (GBM) patients treated with bevacizumab (BEV) and irinotecan (CPT-11). J Clin Oncol. ASCO Annual Meeting Proceedings Part I 25 (18S)
41. Checkley D, Tessier JJ, Wedge SR, Dukes M, Kendrew J, Curry B, Middleton B, Waterton JC (2003) Dynamic contrast-enhanced MRI of vascular changes induced by the VEGF-signaling inhibitor ZD4190 in human tumour xenografts. Magn Reson Imaging 21(5):475–482
42. Morgan B, Horsfield MA, Stattaus J, Khalil A, Gauler TC, Gounant V, Fischer B, Laurent D, Krissel H, Eberhardt WE (2007) Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) as a biomarker for the effect of PTK787/ZK 222584 (PTK/ZK) as second-line mono-therapy in patients with stage IIIB or stage IV non-small cell lung cancer (NSCLC). J Clin Oncol. ASCO Annual Meeting Proceedings Part I 25 (18S)
43. Sorensen AG, Batchelor TT, Zhang WT, Chen PJ, Yeo P, Wang M, Jennings D, Wen PY, Lahdenranta J, Ancukiewicz M, di Tomaso E, Duda DG, Jain RK (2009) A "vascular normalization index" as potential mechanistic biomarker to predict survival after a single dose of cediranib in recurrent glioblastoma patients. Cancer Res 69(13):5296–5300. doi:10.1158/0008-5472.CAN-09-0814
44. Sorensen AG, Emblem KE, Polaskova P, Jennings D, Kim H, Ancukiewicz M, Wang M, Wen PY, Ivy P, Batchelor TT, Jain RK (2012) Increased survival of glioblastoma patients who respond to antiangiogenic therapy with elevated blood perfusion. Cancer Res 72(2):402–407. doi:10.1158/0008-5472.CAN-11-2464
45. Lassau N, Koscielny S, Albiges L, Chami L, Benatsou B, Chebil M, Roche A, Escudier BJ (2010) Metastatic renal cell carcinoma treated with sunitinib: early evaluation of treatment response using dynamic contrast-enhanced ultrasonography. Clin Cancer Res 16(4):1216–1225. doi:10.1158/1078-0432.CCR-09-2175
46. Lassau N, Chami L, Koscielny S, Chebil M, Massard C, Benatsou B, Bidault S, Cioffi A, Blay JY, Le Cesne A (2010) Quantitative functional imaging by dynamic contrast enhanced ultrasonography (DCE-US) in GIST patients treated with masatinib. Invest New Drugs. doi:10.1007/s10637-010-9592-2
47. Lassau N, Chebil M, Chami L, Bidault S, Girard E, Roche A (2010) Dynamic contrast-enhanced ultrasonography (DCE-US): a new tool for the early evaluation of antiangiogenic treatment. Target Oncol 5(1):53–58. doi:10.1007/s11523-010-0136-7
48. Greis C (2009) Ultrasound contrast agents as markers of vascularity and microcirculation. Clin Hemorheol Microcirc 43(1):1–9. doi:10.3233/CH-2009-1216
49. Marcus CD, Ladam-Marcus V, Cucu C, Bouche O, Lucas L, Hoeffel C (2009) Imaging techniques to evaluate the response to treatment in oncology: current standards and perspectives. Crit Rev Oncol Hematol 72(3):217–238. doi:10.1016/j.critrevonc.2008.07.012
50. Nakahara T, Norberg SM, Shalinsky DR, Hu-Lowe DD, McDonald DM (2006) Effect of inhibition of vascular endothelial growth factor signaling on distribution of extravasated antibodies in tumors. Cancer Res 66(3):1434–1445. doi:10.1158/0008-5472.CAN-05-0923
51. Willett CG, Duda DG, di Tomaso E, Boucher Y, Ancukiewicz M, Sahani DV, Lahdenranta J, Chung DC, Fischman AJ, Lauwers GY, Shellito P, Czito BG, Wong TZ, Paulson E, Poleski M, Vujaskovic Z, Bentley R, Chen HX, Clark JW, Jain RK (2009) Efficacy, safety, and biomarkers of neoadjuvant bevacizumab, radiation therapy, and fluorouracil in rectal cancer: a multidisciplinary phase II study. J Clin Oncol 27(18):3020–3026. doi:10.1200/JCO.2008.21.1771
52. Willett CG, Boucher Y, Duda DG, di Tomaso E, Munn LL, Tong RT, Kozin SV, Petit L, Jain RK, Chung DC, Sahani DV, Kalva SP, Cohen KS, Scadden DT, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Shellito PC, Mino-Kenudson M, Lauwers GY (2005) Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. J Clin Oncol 23(31):8136–8139. doi:10.1200/JCO.2005.02.5635
53. Otrock ZK, Hatoum HA, Musallam KM, Awada AH, Shamseddine AI (2011) Is VEGF a predictive biomarker to anti-angiogenic therapy? Crit Rev Oncol Hematol 79(2):103–111. doi:10.1016/j.critrevonc.2010.07.008

VEGF-Trap: A VEGF blocker with potent antitumor effects

Jocelyn Holash*, Sam Davis, Nick Papadopoulos, Susan D. Croll, Lillian Ho, Michelle Russell, Patricia Boland, Ray Leidich, Donna Hylton, Elena Burova, Ella Ioffe, Tammy Huang, Czeslaw Radziejewski, Kevin Bailey, James P. Fandl, Tom Daly, Stanley J. Wiegand, George D. Yancopoulos, and John S. Rudge

Regeneron Pharmaceuticals, Incorporated, 777 Old Saw Mill River Road, Tarrytown, NY 10591

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Vascular endothelial growth factor (VEGF) plays a critical role during normal embryonic angiogenesis and also in the pathological angiogenesis that occurs in a number of diseases, including cancer. Initial attempts to block VEGF by using a humanized monoclonal antibody are beginning to show promise in human cancer patients, underscoring the importance of optimizing VEGF blockade. Previous studies have found that one of the most effective ways to block the VEGF-signaling pathway is to prevent VEGF from binding to its normal receptors by administering decoy-soluble receptors. The highest-affinity VEGF blocker described to date is a soluble decoy receptor created by fusing the first three Ig domains of VEGF receptor 1 to an Ig constant region; however, this fusion protein has very poor *in vivo* pharmacokinetic properties. By determining the requirements to maintain high affinity while extending *in vivo* half life, we were able to engineer a very potent high-affinity VEGF blocker that has markedly enhanced pharmacokinetic properties. This VEGF-Trap effectively suppresses tumor growth and vascularization *in vivo*, resulting in stunted and almost completely avascular tumors. VEGF-Trap-mediated blockade may be superior to that achieved by other agents, such as monoclonal antibodies targeted against the VEGF receptor.

The sprouting of new blood vessels, termed angiogenesis, is required to support growth in the embryo and young animal, as well as to allow for repair and remodeling processes in the adult. However, aberrant angiogenesis is also associated with a number of pathological conditions and diseases, including cancer (1, 2). Tumors, like many normal tissues, use the vasculature as a means to obtain oxygen and nutrients and to remove waste products. Although tumors can in part grow by coopting existing host vessels (3–6), most tumors also induce new vessel formation, suggesting that this neovascularization is required for their growth (1, 2, 7). Consequently, much effort has been directed toward discovering antiangiogenic agents and evaluating them as cancer therapeutics. Perhaps the best characterized and most highly validated antiangiogenic approach involves targeting the vascular endothelial growth factor (VEGF) pathway (1, 8–11). Based on numerous animal studies, the VEGF pathway is the only well-defined signaling pathway known to be required for normal development of the vasculature as well as for the pathologic angiogenesis that accompanies cancer and other disease states (8–10).

The VEGF pathway is initiated when VEGF binds to its receptors on endothelial cells. The two best characterized VEGF receptors are termed VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2). VEGFR1 and VEGFR2 are highly related transmembrane tyrosine kinases that use their ectodomains to bind VEGF; this binding in turn activates the intrinsic tyrosine kinase activity of their cytodomains, initiating intracellular signaling. Interestingly, although VEGFR1 binds to VEGF with substantially higher affinity, most of the biologic effects of VEGF seem to be mediated via VEGFR2. In animals, blockade of the VEGF pathway has been achieved by many different means, including blocking antibodies targeted against VEGF (12–14) or its receptors (15), soluble decoy receptors that prevent VEGF from binding to its normal receptors (16–20), as well as small molecule inhibitors of the tyrosine kinase activity of the VEGFRs (21–23). Recently, a study that compared the efficacy of VEGF blockade to other "antiangiogenic" strategies established that this approach is superior to many others (ref. 11). Consistent with predictions from animal studies, blockade of VEGF using a humanized monoclonal antibody has emerged as the first and thus far only antiangiogenesis approach reporting promising results in human cancer patients, based on preliminary reports from early clinical trials.† The hope is that anti-VEGF approaches can be generalized to many different types of cancer, as well as to other diseases in which pathologic angiogenesis contributes, such as diabetic retinopathy and psoriasis.

The clinical promise of initial anti-VEGF approaches highlights the need to optimize blockade of this pathway. Previous studies have found that one of the most effective ways to block the VEGF signaling pathway is to prevent VEGF from binding to its normal receptors by administering decoy VEGF receptors (11, 16, 17, 24). The highest-affinity VEGF blocker described to date is a soluble decoy receptor created by fusing the first three Ig domains of VEGFR1 to the constant region (Fc portion) of human IgG1, resulting in a forced homodimer that has picomolar binding affinity (16, 17). In tumor experiments, this VEGFR1-Fc reagent is efficacious at approximately 500-fold lower concentration than a similar VEGFR-2 construct (11). Despite its high affinity, the VEGFR1-Fc is not a feasible clinical candidate because of its poor pharmacokinetic profile; in rodent studies, this protein has to be administered frequently and at very high levels to achieve efficacious levels (16, 17, 24). In addition, the VEGFR1-Fc exhibits certain toxicological side effects that are not seen with the VEGFR2-Fc (11). These effects appear to be due to nonmechanism-based and nonspecific properties of this agent (see *Discussion*). By determining the requirements to maintain high affinity while extending *in vivo* half life, we were able to engineer a very potent high-affinity VEGF blocker that has prolonged *in vivo* pharmacokinetics and pharmacodynamics, lacks nonspecific toxicities, and can effectively suppress the growth and vascularization of a number of different types of tumors *in vivo*.

Materials and Methods

Engineering VEGF-Traps. The parental VEGF-Trap was created by fusing the first three Ig domains of VEGFR1 to the constant region (Fc) of human IgG1. VEGF-Trap$_{\Delta B1}$ was created by removing a highly basic 10-aa stretch from the third Ig domain of the parental VEGF-Trap. VEGF-Trap$_{\Delta B2}$ was created by removing the entire first Ig domain from VEGF-Trap$_{\Delta B1}$. VEGF-Trap$_{R1R2}$ was created by fusing the second Ig domain

Abbreviations: VEGF, vascular endothelial growth factor; VEGFR1, VEGF receptor 1; VEGFR2, VEGF receptor 2; AUC, area under the curve.

*To whom reprint requests should be addressed. E-mail: jocelyn.holash@regeneron.com.

†Yang, J., Haworth, L., Steinberg, S., Rosenberg, S. & Novotny, W. (2002) *Am. Soc. Clin. Oncol.* (abstr. 15).

of VEGFR1 with the third Ig domain of VEGFR2. All of the VEGF-Trap variants were produced and purified from Chinese hamster ovary cells.

Pharmacokinetic Analysis of VEGF-Traps. BALB/c mice (25–30 g) were injected s.c. with 4 mg/kg of the various Traps and bled at 1, 2, 4, 6, 24, 48, 72, and 144 hr after injection. Levels of all VEGF-Traps were measured by an ELISA by using human $VEGF_{165}$ to capture and an antibody to the human Fc region as the reporter.

Extracellular Matrix (ECM)-Binding Assay. ECM-coated plates (Becton Dickinson no. 35–4607) were incubated with varying concentrations of VEGF-Traps for 1 hr at room temperature. They were washed and incubated with alkaline phosphatase-conjugated anti-human Fc antibody (Promega, 1:4,000 in PBS + 10% BCS) for 1 hr at room temperature. Plates were washed four times with PBS + 0.1%Triton-X 100 and reagent buffer added for color development. Plates were read at 405–570 nm.

VEGF-Trap-Binding Assay. Binding affinities of VEGF-Traps were measured by using a specific and sensitive ELISA (R&D Systems kit no. DVE00) for detecting free (unbound) human VEGF in mixtures of the VEGF-Traps (ranging in concentration from 0.1 to 160 pM) with human $VEGF_{165}$ (at 10 pM), incubated overnight at room temperature.

Human Umbilical Vein Endothelial Cell Phosphorylation Assay. Confluent monolayers of human umbilical vein endothelial cells [Vec Technologies (Rensselaer, NY) passage no. 5] were serum-starved for 2 hr and then challenged for 5 min with vehicle or 40 ng/ml of human $VEGF_{165}$, alone or preincubated with VEGF-Traps at 1.5-fold molar excess. Cells were then lysed, immunoprecipitated by using a VEGFR2-specific antibody, and immunoblotted with a phosphotyrosine-specific antibody (Upstate Biotechnology, 4G10 mAb).

VEGF-Induced Proliferation Assay. Cells that proliferate in response to VEGF were generated by stably transfecting NIH 3T3 cells with a VEGFR2/TrkB chimeric receptor (in which the cytodomain of VEGFR2 was replaced with that of TrkB, a receptor for brain-derived neurotrophic factor that effectively drives proliferation in these cells). Five thousand cells were plated per well of a 96-well plate, allowed to settle for 2 hr, incubated for 1 hr with VEGF-Trap variants (titrated from 40 nM to 20 pM), then challenged for 72 hr with human $VEGF_{165}$ at a concentration of 1.56 nM, followed by addition of [3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, innersai and spectrophotometric analysis at 450/570 nm.

Acute Hypotension. Male Wistar–Kyoto rats (180–240 g) from Taconic Laboratories were maintained on a 12:12 light/dark cycle (lights on 0600) with food and water available *ad libitum*. Before challenge with VEGF, animals were pretreated with VEGF-Traps or PBS as indicated, anesthetized with 1.5–2% isoflurane in oxygen, and the left femoral artery catheterized for direct measurement of systolic blood pressure through a blood pressure transducer (ITC, Woodland Hills, CA) into a chart recorder (Linseis, Princeton Junction, NJ). Animals were then injected in the right jugular vein with a 200-μl bolus containing 10 μg of recombinant human $VEGF_{165}$. Systolic blood pressure was measured before VEGF injection and every minute thereafter for 20 min. Blood pressures were normalized to baseline preinjection and analyzed by using mixed factorial ANOVAs (see supporting information on the PNAS web site, www.pnas.org).

Tumor Growth Experiments. C6 glioma cells (1.0×10^6 cells/mouse) and A673 rhabdomyosarcoma cells (2.0×10^6 cells/mouse) were obtained from American Type Culture Collection, and B16F10.9 melanoma cells (5.0×10^5 cells/mouse) were a generous gift from Charles Lin (Duke University, Durham, NC). Cells were suspended in serum-free medium and implanted s.c. on the shaved right flank of male C.B-17 SCID mice at the indicated concentrations. After tumor cell implantation and twice weekly thereafter for the duration of the experiment, mice received a s.c. injection (at the nape of the neck) of vehicle (PBS + 0.5% glycerol), VEGF-Trap, or DC101 (from American Type Culture Collection). After 2–3.0 weeks, animals were killed and tumors were measured *ex vivo* with calipers (tumor volume = length × width × height). For immunohistochemistry studies, mice were perfused with 4% paraformaldehyde, and tissue was processed as previously described (25).

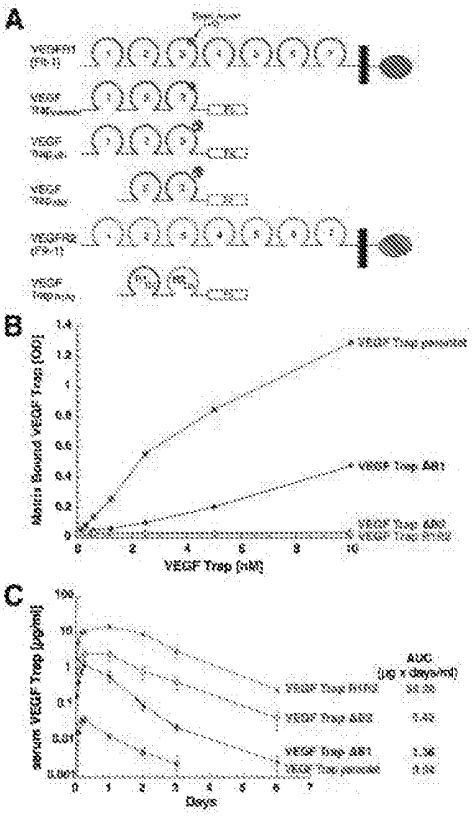

Fig. 1. Engineering of VEGF-Traps with improved pharmacokinetics. (*A*) Schematics of full-length VEGFR1 (red) and VEGFR2 (blue) are provided, indicating their seven Ig domains, transmembrane regions (black bars), and kinase domains (ovals). The parental VEGF-Trap contains the first three Ig domains of VEGFR1 (including the highly basic 10-aa stretch in Ig3, blue box) fused to the Fc portion of human IgG1. VEGF-$Trap_{\Delta B1}$ is identical to the parental VEGF-Trap, except that the basic stretch in Ig3 has been removed. VEGF-$Trap_{\Delta B2}$ is the same construct as ΔB1, except that the first Ig domain has been removed. VEGF-$Trap_{R1R2}$ possesses the second Ig domain of VEGFR1 and the third Ig domain of VEGFR2 fused to the Fc portion of human IgG1. (*B*) The four indicated VEGF-Traps were assayed *in vitro* for their capacity to bind to extracellular matrix, with only the parental VEGF-Trap and VEGF-$Trap_{\Delta B1}$ demonstrating binding. (*C*) Pharmacokinetic analysis of the VEGF-Traps reveals that the parental VEGF-Trap has the poorest profile, whereas VEGF-$Trap_{R1R2}$ showed the best profile.

Results

Reengineering Parental VEGF-Trap to Improve Its Pharmacokinetic Profile. On the basis of the previously reported high affinity of a soluble decoy receptor in which VEGFR1 is fused to the Fc portion of human IgG1 (16, 17), we produced this fusion protein to study its properties (see parental VEGF-Trap, Fig. 1*A*). Single s.c. injections of parental VEGF-Trap (4 mg/kg) into mice were

performed to confirm that it indeed displayed poor pharmacokinetic properties, with a maximal concentration (C_{max}) of only 0.05 μg/ml and total "area under the curve concentration" (AUC) of 0.04 μg $\times$ days/ml (Fig. 1*C*). We postulated that these poor pharmacokinetic properties might be due to the high positive charge of this protein (pI 9.4), which in turn may result in its deposition at the site of s.c. injection because of nonspecific adhesion to highly negatively charged proteoglycans that comprise the extracellular matrix. To test this hypothesis, we next engineered several variants of the parental VEGF-Trap with reduced positive charges. On review of the charge density in the parental molecule, we noted a highly basic stretch of 10 amino acids in the third Ig domain of VEGFR1 (see blue box in Fig. 1*A*). To reduce the charge, this region was excised, resulting in a decrease in the pI of this VEGF-Trap (termed VEGF-Trap$_{\Delta B1}$; see Fig. 1*A*) from 9.4 to 9.1. It was also noted that the first Ig domain of VEGFR1 had a basic pI, and we thus decided to test removal of this domain as well as the above-noted basic region, resulting in a protein termed VEGF-Trap$_{\Delta B2}$ (Fig. 1*A*), with a further reduced pI of 8.9. Finally, because the third Ig domain of VEGFR2 has a lower pI than the corresponding domain of VEGFR1, we simply switched these domains to make a Trap in which the second Ig domain of VEGFR1 is directly fused to the third Ig domain of VEGFR2; this trap was termed VEGF-Trap$_{R1R2}$ (Fig. 1*A*) and had a pI of 8.82. Previous structural analyses indicated that VEGFR1 might make greater use of its second Ig domain in contacting VEGF, whereas VEGFR2 instead makes greater use of its third Ig domain (26), raising the interesting and useful possibility that VEGF-Trap$_{R1R2}$ might actually bind more tightly to VEGF than the parental versions. Combining the distinct binding regions of two different receptors to create a higher-affinity interactor has previously been used in the creation of a series of interleukin and cytokine blockers also termed Traps (A. Economides, L. Rocco Carpenter, J.S.R., V. Wong, E. Koehler-Stec, C. Hartnett, E. Pyles, T.D., M. Young, J.P.F., Frank Lee, Scott Carver, Jennifer McNay, K.B., S. Ramakanth, R. Hatabarat, C.R., T.H., G.D.Y., and N. Stahl, unpublished results). Using a simple extracellular matrix-binding assay, we then confirmed the hypothesis that decreasing the positive charge of the VEGF-Traps would result in decreased adhesion to extracellular matrix (Fig. 1*B*). Binding to extracellular matrix in this assay was directly related to the pI of the Traps, with both VEGF-Trap$_{R1R2}$ and VEGF-Trap$_{\Delta B2}$ displaying negligible binding in this assay.

On the basis of the above results, we next tested these various VEGF-Traps *in vivo* for their pharmacokinetic behavior. Their *in vivo* behavior followed the theoretical charge predictions as well as the *in vitro* adhesion properties. Every reduction in pI was accompanied by a corresponding improvement in C_{max} and AUC: VEGF-Trap$_{\Delta B1}$ had a C_{max} of 1.3 μg/ml and an AUC of 1.36 μg $\times$ days/ml; VEGF-Trap$_{\Delta B2}$ had a C_{max} of 2.65 μg/ml and an AUC of 5.42 μg $\times$ days/ml; whereas VEGF-Trap$_{R1R2}$ revealed the best profile with a C_{max} of 16 μg/ml and an AUC of 36.28 μg $\times$ days/ml (Fig. 1*C*). Thus, VEGF-Trap$_{R1R2}$ had an AUC that was almost 1,000-fold higher than that of the parental VEGF-Trap, raising the possibility that it might be a far superior pharmacologic agent, assuming it retained its ability to bind and block VEGF.

Comparison of Parental VEGF-Trap with VEGF-Trap$_{R1R2}$ in Binding, Phosphorylation, and Cell Proliferation Assays *in Vitro*. Because of the superior pharmacokinetic properties of VEGF-Trap$_{R1R2}$, we next compared this Trap to its parent for its ability to bind and block VEGF *in vitro*. To determine binding affinity of the Traps for VEGF, equilibrium binding assays were performed in which different concentrations of the Traps were incubated with VEGF$_{165}$, and the amount of unbound VEGF$_{165}$ was measured, revealing that parental VEGF-Trap displays a kD of $\approx$5 pM, whereas VEGF-Trap$_{R1R2}$ has a binding affinity of about 1 pM (Fig. 2*A*). Preliminary

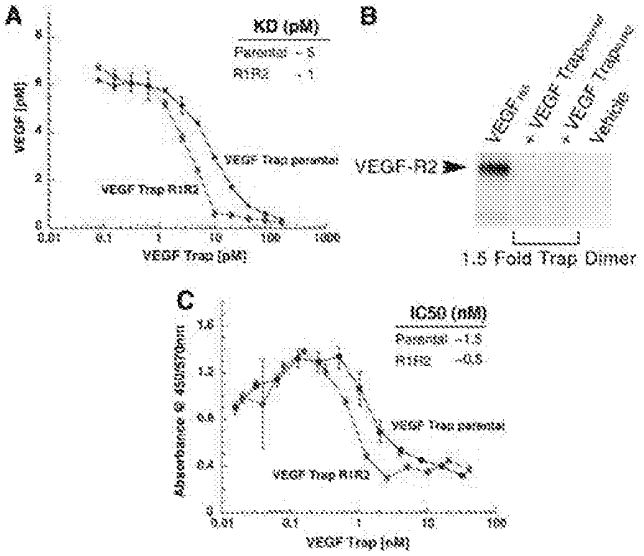

Fig. 2. Binding affinity and inhibitory properties of VEGF-Traps. (*A*) Affinities of indicated VEGF-Traps for VEGF, as determined by using a binding assay that measures unbound VEGF (ordinate) after incubation of 10 pM of human VEGF$_{165}$ with varying concentrations of VEGF-Traps (abscissa). (*B*) Inhibition of VEGF-induced phosphorylation of VEGFR2 in human umbilical vein endothelial cell phosphorylations using indicated VEGF-Traps at 1.5-fold molar excess, as revealed with immunoblotting assay. (*C*) Inhibition of VEGF-induced proliferation of fibroblasts containing a chimeric VEGFR2/TrkB receptor, using varying concentrations of VEGF-Traps in the presence of 1.56 nM of VEGF.

analyses show that VEGF-Trap$_{R1R2}$ has a kD of $\approx$1–10 pM for VEGF$_{121}$ and approximately 45 pM for placental growth factor 2 (not shown); other VEGF isoforms and relatives have not been analyzed.

To determine whether Trap binding of VEGF could potently and effectively block the ability of VEGF to activate its receptor, VEGF and Traps were added to cultured endothelial cells, and the effects on VEGFR2 phosphorylation were examined, revealing that both parental VEGF-Trap as well as VEGF-Trap$_{R1R2}$ can completely block VEGF-induced VEGFR2 phosphorylation when added at a 1.5-fold molar excess compared with the added VEGF, consistent with very high-affinity binding to VEGF (Fig. 2*B*). Finally, to assess whether these Traps would also be effective in cell-based proliferation assays, we engineered a cell line containing a chimeric VEGFR2 receptor that mediates a very strong proliferative response to VEGF and found that both parental VEGF-Trap and VEGF-Trap$_{R1R2}$ potently blocked VEGF-induced proliferation in 3-day growth assays in these cells, with an IC$_{50}$ at approximately an equimolar concentration of Trap with the added VEGF, once again consistent with very high-affinity binding of the Traps for VEGF (Fig. 2*C*).

VEGF-Trap$_{R1R2}$ Provides Long-Term Blockade of Exogenously Administered VEGF-Induced Acute Hypotension. The above studies indicated that VEGF-Trap$_{R1R2}$ was at least as impressive a blocker of VEGF as the parental version, but that it had far superior pharmacokinetic properties. To initially explore whether these attributes translated into superior pharmacodynamic performance, we compared these reagents by using an acute readout of VEGF responsiveness *in vivo*. Administration of a single bolus dose (10 μg) of recombinant VEGF$_{165}$ to rats results in acute hypotension, with a drop of about 40% from baseline systolic blood pressure; this drop is maximal at 5 min and slowly rectifies to normal by about 30 min (Fig. 3*A*). To compare the pharmacodynamic efficacy of the VEGF-Traps in blocking this acute response, we preadministered the parental VEGF-Trap or

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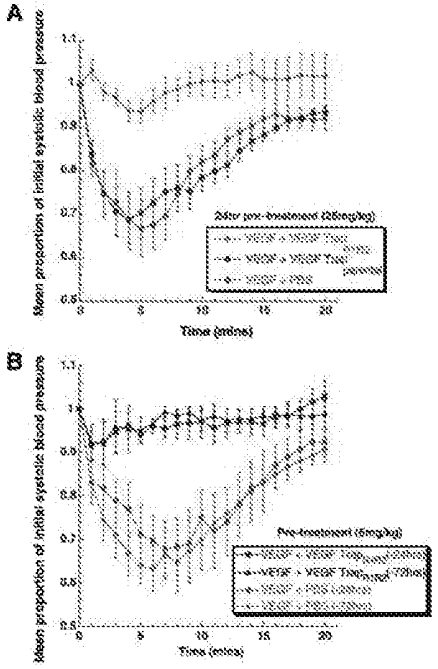

Fig. 3. Using blockade of VEGF-induced acute hypotension to pharmacodynamically compare VEGF-Traps. (*A*) When rats were treated with VEGF-Traps at 25 mg/kg at 1 day before VEGF challenge, VEGF-Trap$_{R1R2}$ (n = 8) completely blocked VEGF-induced hypotension, whereas PBS (n = 6) and parental VEGF-Trap (n = 6) were ineffective. ANOVA shows treatment effect, $P < 0.007$. (*B*) At a 5-fold lower dose (5 mg/kg), VEGF-Trap$_{R1R2}$ was still effective at 1 day (n = 4) or 3 days (n = 3) before the VEGF challenge. ANOVA shows treatment effect, $P < 0.03$.

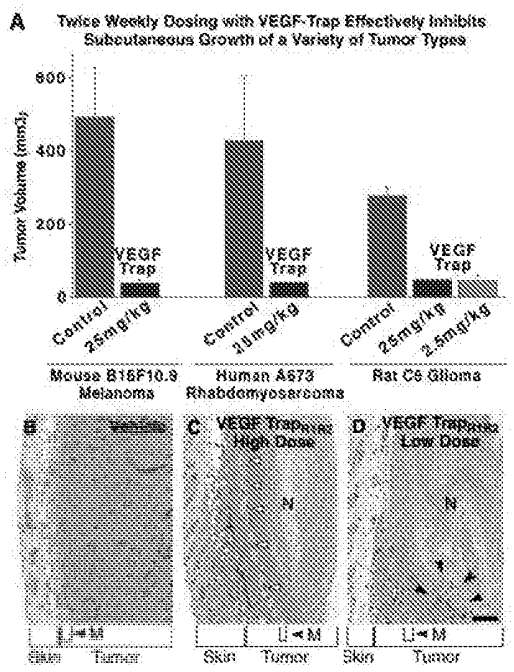

Fig. 4. VEGF-Trap$_{R1R2}$ dramatically inhibits the s.c. growth and vascularity of implanted tumors from diverse tissues and species. (*A*) VEGF-Trap $_{R1R2}$ substantially blocked the growth of the indicated s.c. implanted tumors, at the indicated doses twice weekly for 2 weeks (C6 and B16F10.9) or 3.0 weeks (A673). Error bars represent standard error of mean, n = five mice/treatment group. The differences between control tumor volumes and VEGF-Trap$_{R1R2}$–treated tumor volumes were analyzed by using Student's t tests and found to be significant at the following levels: B16F10 $P = 0.01$; A673 $P = 0.06$; C6 $P < 0.0001$. (*B–D*) Histological analysis reveals that VEGF-Trap$_{R1R2}$ can effectively block blood vessel growth in these implanted tumors. Sections of C6 tumors stained with antibodies to platelet–endothelial cell adhesion molecule reveal that vehicle-treated animals had large tumors that were highly vascularized (*B*), whereas animals treated with 25 mg/kg VEGF-Trap$_{R1R2}$ (*C*) had tumors that were largely avascular with large areas of necrosis (N). Viable tumor appeared to be vascularized because of cooption of preexisting host vessels (white arrowheads) associated with hypodermal musculature (M) and dermis. Treatment with 2.5 mg/kg VEGF-Trap$_{R1R2}$ greatly stunted tumor growth (*C*) and resulted in large necrotic regions (N), although small pockets of vessels were occasionally apparent (black arrows). (Bar = 100 μm.)

VEGF-Trap$_{R1R2}$ at 25 mg/kg, 24 hr before VEGF administration (Fig. 3*A*). Consistent with what would be expected from the above pharmacokinetic studies, this dose of VEGF-Trap$_{R1R2}$ completely blocked VEGF-induced hypotension, whereas the parental VEGF-Trap had no discernable effect. Thus, although the parental VEGF-Trap and its VEGF-Trap$_{R1R2}$ derivative are quite comparable *in vitro* (see above), the VEGF-Trap$_{R1R2}$ performs much better *in vivo*, presumably because of its dramatically enhanced pharmacokinetic profile.

To further characterize the length of time in which VEGF-Trap$_{R1R2}$ remained efficacious, we waited 1, 3, and 7 days after injection of the Trap at 5 mg/kg before inducing hypotension. At this dose, VEGF-Trap$_{R1R2}$ was completely effective in blocking VEGF-induced acute hypotension at 1 and 3 days after a single bolus (Fig. 3*B*) but was not significantly different from controls at 7 days (data not shown).

VEGF-Trap$_{RiR2}$ Dramatically Blocks Tumor Growth *in Vivo*. Altogether, the above pharmacokinetic and pharmacodynamic studies indicated that VEGF-Trap$_{R1R2}$ has the potential to be a long-term and potent pharmacologic blocker of VEGF-mediated activities *in vivo*, far superior to that of parental VEGF-Trap. To begin to explore the value of VEGF-Trap$_{R1R2}$ as an anticancer therapeutic and to compare it to other effective agents targeting the VEGF pathway, we evaluated its ability to block the growth of a variety of tumor cell lines in s.c. tumor models in mice. Tumor cells were derived from diverse tissue origins and different species (mouse B16F10.9 melanoma, human A673 rhabdomyosarcoma, and rat C6 glioma). After implantation of tumor cells, mice were allowed a brief recovery period and then received s.c. injections of VEGF-Trap$_{R1R2}$ (25 mg/kg) or vehicle twice weekly for the duration of the experiment (2–3.0 weeks), after which the animals were killed and tumors excised and measured. VEGF-Trap$_{R1R2}$ significantly inhibited the growth of all three types of tumors (Fig. 4*A*). In the study using C6 glioma cells, a 10-fold lower dose of VEGF-Trap$_{R1R2}$ (2.5 mg/kg) was tested and found to be equally effective at inhibiting tumor growth.

To evaluate the effects of VEGF-Trap$_{R1R2}$ on tumor-associated angiogenesis, the tumors from the above studies were sectioned and immunostained with antibodies to platelet–endothelial cell adhesion molecule, so that the vasculature could be visualized (Fig. 4 *B–D*). This analysis revealed that the higher dose of VEGF-Trap$_{R1R2}$ almost completely blocked tumor-associated angiogenesis, with the stunted tumors being largely avascular, save for regions in which preexisting host vessels appeared to be coopted by surrounding tumor (see open arrowheads, Fig. 4*C*). The lower dose of VEGF-Trap$_{R1R2}$, which was quite comparable at inhibiting tumor growth (see above), appeared to be slightly less effective at completely blocking tumor-associated angiogenesis, allowing for small pockets of tumor-associated vessels in otherwise avascular tumors (see black arrowheads in Fig. 4*D*). In contrast to the VEGF-Trap-treated tumors, control tumors in vehicle-treated mice not only were much larger (see above) but also had a very high vascular density (Fig. 4*B*).

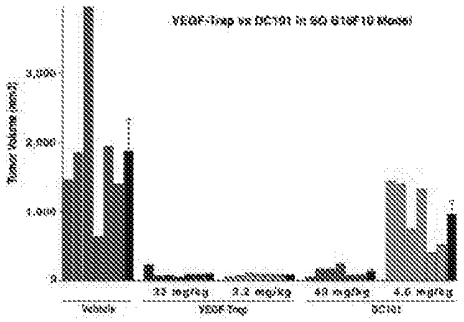

Fig. 5. VEGF-Trap$_{R1R2}$ blocks tumor growth (of subcutaneously implanted B16F10.9 cells) at far lower concentrations than DC101, a monoclonal antibody directed to VEGFR2. Mice were treated twice weekly with the indicated dose of VEGF-Trap$_{R1R2}$, DC101, or vehicle. After 2.5 weeks, mice were killed, and tumors were excised and measured. Individual tumor volumes are shown (colored bars), as are average tumor volumes for each treatment (black bars) ± SEM, n = six mice/treatment group. Differences between treatment groups were analyzed by using a one-way ANOVA followed by Fisher's protected least significant difference test. Average volume of tumors in all treatment groups is significantly smaller than control tumor volume ($P < 0.01$). Differences in tumor volume between the high-dose VEGF-Trap, low-dose VEGF-Trap, and high-dose DC101 treatment groups are not significantly different, but they are significantly different from those of the low-dose DC101 treatment group ($P < 0.02$).

VEGF-Trap$_{R1R2}$ Compares Favorably with Antibodies Targeting VEGFR2. After establishing that VEGF-Trap$_{R1R2}$ was effective at blocking s.c. tumor growth, we undertook studies to compare its efficacy with other known VEGF blockers. One particularly effective and well-characterized blocker is a monoclonal antibody, termed DC101, that targets VEGFR2 (15). When equimolar doses of VEGF-Trap$_{R1R2}$ and DC101 were compared in the B16F10 melanoma model, it was apparent that much higher doses of DC101 are required to inhibit tumor growth (Fig. 5). Furthermore, because antibodies have longer circulation times in mice than simple Fc fusion proteins, the highly efficacious dose of DC101 accumulates to approximately 60-fold higher serum levels than that of the equally efficacious low dose of VEGF-Trap: circulating levels of DC101 in animals treated with the 40-mg/kg dose were 2,442 ± 272 μg/ml, in contrast to the circulating levels of VEGF-Trap in animals treated with 3.2 mg/kg, which were 40 ± 8 μg/ml. Thus, circulating levels of VEGF-Trap that were approximately 60-fold lower than those of DC101 were equally efficacious in inhibiting tumor growth. Importantly, the favorable allometric scaling of Fc fusion proteins relative to antibodies (27, 28) suggests that in humans the circulation time for the VEGF-Trap will be much more comparable to that of antibodies, which in turn suggests that in humans the difference in efficacious doses would be further magnified and may be as great as 60-fold.

As described in an accompanying manuscript (29), when used at the same dose, VEGF-Trap shows efficacy equal to or better than a monoclonal antibody to VEGF (30). As noted above, because Fc fusion proteins have much shorter circulating half-lives than antibodies in mice, but comparable half-lives in humans, the finding that the VEGF-Trap$_{R1R2}$ is at least as potent as the monoclonal antibody in mice suggests that the efficacious dose of VEGF-Trap will be much lower than that of the monoclonal antibody in humans.

Discussion

Validation of VEGF as an important new target in the war against cancer comes from pioneering clinical studies using a humanized monoclonal antibody that binds and blocks VEGF.[†] Because anti-VEGF approaches act by blocking tumor-associated angiogenesis, which appears to be widely required by many different types of tumors, these approaches may prove to be generally useful against a wide assortment of cancers. In addition, pathological angiogenesis seems to contribute to a number of non-neoplastic diseases, such as diabetic retinopathy (31) and psoriasis (32), extending the potential utility of anti-VEGF therapeutics. All this promise highlights the need to optimize anti-VEGF approaches. Herein we describe the engineering of an anti-VEGF agent, termed VEGF-Trap$_{R1R2}$. VEGF-Trap$_{R1R2}$ is a derivative of perhaps the most potent VEGF binder known, VEGFR1. Soluble forms of VEGFR1 suffer from poor pharmacokinetic properties, which seem to correlate with their nonspecific interactions with extracellular matrix. VEGF-Trap$_{R1R2}$ was engineered to have minimal interactions with extracellular matrix, and this property apparently accounts for its satisfying pharmacokinetic profile. The combination of high-affinity and improved pharmacokinetics apparently contributes toward making VEGF-Trap$_{R1R2}$ one of the most, if not the most, potent and efficacious VEGF blocker available. An additional advantage is that VEGF-Trap$_{R1R2}$ is composed of entirely human sequences, hopefully minimizing the possibility that it might prove immunogenic in human patients. Despite its wholly human nature, VEGF-Trap$_{R1R2}$ binds all species of VEGF tested, from human to chicken VEGF (not shown), making it a very versatile reagent that can be used in almost any experimental animal models.

A recent study comparing numerous antiangiogenesis approaches concluded that anti-VEGF approaches were the most efficacious (11). The particular anti-VEGF agent used for these studies was essentially equivalent to our parental VEGF-Trap but was delivered in an adenoviral system in which it was highly expressed in the livers of infected animals. In contrast to other anti-VEGF approaches that seem to be well-tolerated, the adenovirally delivered VEGF-Trap caused severe liver toxicity and ascites, raising the possibility that it might have some unique mechanism-based side effects compared with other anti-VEGF approaches. To explore this possibility, we made adenoviral versions of both the parental VEGF-Trap as well as VEGF-Trap$_{R1R2}$ and found that, whereas adenoviral delivery of parental VEGF-Trap reproduces the previously reported toxicities (11), adenoviral delivery of VEGF-Trap$_{R1R2}$ did not cause these side effects even though much higher levels were achieved in the circulation. Our conclusion is that the nonspecific interactions of the parental VEGF-Trap with extracellular matrix contribute to its increased toxicity after adenoviral administration, and that comparable toxicity is not noted with adenoviral administration of the engineered VEGF-Trap$_{R1R2}$.

In addition to the anticancer findings reported here, recent studies have shown that various versions of the VEGF-Trap can efficaciously treat a cancer-associated condition in mice similar to liver peliosis (33), as well as noncancer-associated disease models, such as of diabetic retinopathy (34–36) and psoriasis (Y.-P. Xia, M. Detmar, G.D.Y., and J.S.R., unpublished results). The accompanying manuscript (29) compares the efficacy of the VEGF-Trap to that of several other VEGF blockers, including a humanized monoclonal antibody to VEGF, in a model of kidney cancer. Among the several VEGF blockers tested, the VEGF-Trap shows the best overall efficacy. In this manuscript, we compare the efficacy of the VEGF-Trap to that of a monoclonal antibody to VEGFR2 in cancer models and find that far lower circulating levels of VEGF-Trap$_{R1R2}$ are required for similar efficacy. Tumors treated with highest doses of the VEGF-Trap are not only stunted but also strikingly avascular. Our description of a VEGF blocker with such superior blocking and pharmacologic properties seems to demand that it be tested in human patients suffering from diseases involving neoangiogenesis. Toward this end, the safety of the VEGF-Trap has recently been confirmed in toxicological studies in cynomologus monkeys (data not shown). Consequently, the VEGF-Trap is currently in human clinical trials for several different types of cancer.

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1. Ferrara, N. & Alitalo, K. (1999) *Nat. Med.* **5,** 1359–1364.
2. Folkman, J. (1995) *Nat. Med.* **1,** 27–31.
3. Holash, J., Wiegand, S. J. & Yancopoulos, G. D. (1999) *Oncogene* **18,** 5356–5362.
4. Pezzella, F., Pastorino, U., Tagliabue, E., Andreola, S., Sozzi, G., Gasparini, G., Menard, S., Gatter, K. C., Harris, A. L., Fox, S., *et al.* (1997) *Am. J. Pathol.* **151,** 1417–1423.
5. Zagzag, D., Friedlander, D. R., Margolis, B., Grumet, M., Semenza, G. L., Zhong, H., Simons, J. W., Holash, J., Wiegand, S. J. & Yancopoulos, G. D. (2000) *Pediatr. Neurosurg.* **33,** 49–55.
6. Zagzag, D., Amirnovin, R., Greco, M. A., Yee, H., Holash, J., Wiegand, S. J., Zabski, S., Yancopoulos, G. D. & Grumet, M. (2000) *Lab. Invest.* **80,** 837–849.
7. van Hinsbergh, V. W., Collen, A. & Koolwijk, P. (1999) *Ann. Oncol.* **10,** 60–63.
8. Eriksson, U. & Alitalo, K. (1999) *Curr. Top. Microbiol. Immunol.* **237,** 41–57.
9. Ferrara, N. (1999) *Curr. Top. Microbiol. Immunol.* **237,** 1–30.
10. Yancopoulos, G. D., Davis, S., Gale, N. W., Rudge, J. S., Wiegand, S. J. & Holash, J. (2000) *Nature (London)* **407,** 242–248.
11. Kuo, C. J., Farnebo, F., Yu, E. Y., Christofferson, R., Swearingen, R. A., Carter, R., von Recum, H. A., Yuan, J., Kamihara, J., Flynn, E., *et al.* (2001) *Proc. Natl. Acad. Sci. USA* **98,** 4605–4610.
12. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S. & Ferrara, N. (1993) *Nature (London)* **362,** 841–844.
13. Asano, M., Yukita, A., Matsumoto, T., Hanatani, M. & Suzuki, H. (1998) *Hybridoma* **17,** 185–190.
14. Kamiya, K., Konno, H., Tanaka, T., Baba, M., Matsumoto, K., Sakaguchi, T., Yukita, A., Asano, M., Suzuki, H., Arai, T. & Nakamura, S. (1999) *Jpn. J. Cancer Res.* **90,** 794–800.
15. Prewett, M., Huber, J., Li, Y., Santiago, A., O'Connor, W., King, K., Overholser, J., Hooper, A., Pytowski, B., Witte, L., Bohlen, P. & Hicklin, D. J. (1999) *Cancer Res.* **59,** 5209–5218.
16. Ferrara, N., Chen, H., Davis-Smyth, T., Gerber, H. P., Nguyen, T. N., Peers, D., Chisholm, V., Hillan, K. J. & Schwall, R. H. (1998) *Nat. Med.* **4,** 336–340.
17. Gerber, H. P., Vu, T. H., Ryan, A. M., Kowalski, J., Werb, Z. & Ferrara, N. (1999) *Nat. Med.* **5,** 623–628.
18. Gerber, H. P., Hillan, K. J., Ryan, A. M., Kowalski, J., Keller, G. A., Rangell, L., Wright, B. D., Radtke, F., Aguet, M. & Ferrara, N. (1999) *Development (Cambridge, U.K.)* **126,** 1149–1159.
19. Goldman, C. K., Kendall, R. L., Cabrera, G., Soroceanu, L., Heike, Y., Gillespie, G. Y., Siegal, G. P., Mao, X., Bett, A. J., Huckle, W. R., *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95,** 8795–8800.
20. Millauer, B., Longhi, M. P., Plate, K. H., Shawver, L. K., Risau, W., Ullrich, A. & Strawn, L. M. (1996) *Cancer Res.* **56,** 1615–1620.
21. Fong, T. A., Shawver, L. K., Sun, L., Tang, C., App, H., Powell, T. J., Kim, Y. H., Schreck, R., Wang, X., Risau, W., *et al.* (1999) *Cancer Res.* **59,** 99–106.
22. Drevs, J., Hofmann, I., Hugenschmidt, H., Wittig, C., Madjar, H., Muller, M., Wood, J., Martiny-Baron, G., Unger, C. & Marme, D. (2000) *Cancer Res.* **60,** 4819–4824.
23. Wedge, S. R., Ogilvie, D. J., Dukes, M., Kendrew, J., Curwen, J. O., Hennequin, L. F., Thomas, A. P., Stokes, E. S., Curry, B., Richmond, G. H., *et al.* (2000) *Cancer Res.* **60,** 970–975.
24. Gerber, H. P., Kowalski, J., Sherman, D., Eberhard, D. A. & Ferrara, N. (2000) *Cancer Res.* **60,** 6253–6258.
25. Holash, J., Maisonpierre, P. C., Compton, D., Boland, P., Alexander, C. R., Zagzag, D., Yancopoulos, G. D. & Wiegand, S. J. (1999) *Science* **284,** 1994–1998.
26. Lu, D., Kussie, P., Pytowski, B., Persaud, K., Bohlen, P., Witte, L. & Zhu, Z. (2000) *J. Biol. Chem.* **275,** 14321–14330.
27. Mordenti, J. & Green, J. D. (1991) in *New Trends in Pharmacokinetics*, eds. Rescigno, A. & Thakur, A. K. (Plenum, New York), pp. 411–424.
28. Lin, Y. S., Nguyen, C., Mendoza, J. L., Escandon, E., Fei, D., Meng, Y. G. & Modi, N. B. (1999) *J. Pharmacol. Exp. Ther.* **288,** 371–378.
29. Kim, E. S., Serur, A., Huang, J., Manley, C. A., McCrudden, K. W., Frischer, J. S., Soffer, S. Z., Ring, L., New, T., Zabski, S., *et al.* (2002) *Proc. Natl. Acad. Sci. USA* **99,** 11399–11404.
30. Mordenti, J., Thomsen, K., Licko, V., Chen, H., Meng, Y. G. & Ferrara, N. (1999) *Toxicol. Pathol.* **27,** 14–21.
31. Adamis, A. P., Miller, J. W., Bernal, M. T., D'Amico, D. J., Folkman, J., Yeo, T. K. & Yeo, K. T. (1994) *Am. J. Ophthalmol.* **118,** 445–450.
32. Detmar, M., Brown, L. F., Claffey, K. P., Yeo, K. T., Kocher, O., Jackman, R. W., Berse, B. & Dvorak, H. F. (1994) *J. Exp. Med.* **180,** 1141–1146.
33. Wong, A. K., Alfert, M., Castrillon, D. H., Shen, Q., Holash, J., Yancopoulos, G. D. & Chin, L. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 7481–7486.
34. Joussen, A. M., Poulaki, V., Qin, W., Kirchhof, B., Mitsiades, N., Wiegand, S. J., Rudge, J., Yancopoulos, G. D. & Adamis, A. P. (2002) *Am. J. Pathol.* **160,** 501–509.
35. Poulaki, V., Qin, W., Joussen, A. M., Hurlbut, P., Wiegand, S. J., Rudge, J., Yancopoulos, G. D. & Adamis, A. P. (2002) *J. Clin. Invest.* **109,** 805–815.
36. Qaum, T., Xu, Q., Joussen, A. M., Clemens, M. W., Qin, W., Miyamoto, K., Hassessian, H., Wiegand, S. J., Rudge, J., Yancopoulos, G. D. & Adamis, A. P. (2001) *Invest. Ophthalmol. Visual Sci.* **42,** 2408–2413.

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Inhibitors of growth factor receptors, signaling pathways and angiogenesis as therapeutic molecular agents*

Jocelyn Holash · Gavin Thurston · John S. Rudge · George D. Yancopoulos · Alex A. Adjei · Gabriele Bergers · Bronislaw Pytowski · Mark Pegram · Michael S. Gordon

Introduction

There are currently more than 100 agents officially approved for the treatment of cancer world-wide. However, the most common epithelial cancers, which cause greater than 75% of cancer deaths, remain incurable. Most therapeutic agents have been developed empirically by testing large numbers of chemicals on rapidly growing transplantable rodent tumors, and more recently, human tumor xenografts. This approach has predominantly identified DNA-active drugs, which have limited efficacy and considerable toxicity. Novel agents, which selectively target aberrant elements in neoplastic cells and their microenvironment, are needed to improve the cure rates of epithelial malignancies. Due to advances in molecular biology multiple targets and multiple agents inhibiting these targets have been discovered (Fig. 1) [1]. These targets can be conceptualized as supportive vessels, connective tissues, and signaling elements. Agents directed against these targets are those that interfere with signal transduction pathways, cell cycle regulation, and apoptosis (signals), malignant angiogenesis (vessels) and the tumor stroma (connective tissue). As anti-cancer therapeutics with distinct targeting capabilities against malignant cells become available for clinical evaluations, prioritization of these therapies for efficient allotment of clinical trial resources, identification of patients whose malignancies most likely express the molecular constituents resembling the true target, and derivation of relevant endpoints for both screening and assessment of clinical relevance will be critical to their ultimate development and success. These targets include signal transduction pathways such as growth factor receptors and their receptor tyrosine kinases, cytoplasmic second messengers such as ras, raf and MEK, inhibitors of protein trafficking, and inhibitors of protein degradation. As well, angiogenesis, apoptosis, the cell cycle machinery and their regulatory proteins are possible targets. Regardless of which pathway is targeted, Target selection at present appears to be based on the following [2]:

1. Genes encoding mutated targets (*c-kit, B-Raf*)

Raf Kinase Inhibitors. Raf is a family of serine-threonin kinases comprising 3 isoforms, A-Raf, B-Raf and C-Raf (or Raf-1), that is involved in cell signaling, downstream of Ras. Mutated Raf1 has been shown to be constitutively active, with transforming ability. In addition, raf mutations have been

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J. Holash (✉) · G. Thurston · J. S. Rudge · G. D. Yancopoulos
Regeneron Pharmaceuticals, Tarrytown, NY, USA

A. A. Adjei
Mayo Clinic Foundation, Rochester, MN, USA

G. Bergers
Department of Neurological Surgery,
Brain Tumor Research Center and UCSF
Comprehensive Cancer Center,
University of California, San Francisco, CA, USA

B. Pytowski
ImClone Systems, New York, NY, USA

M. Pegram
Department of Medical Oncology, UCLA, Los Angeles, CA, USA

M. S. Gordon
Premiere Oncology of Arizona, Scottsdale, AZ, USA

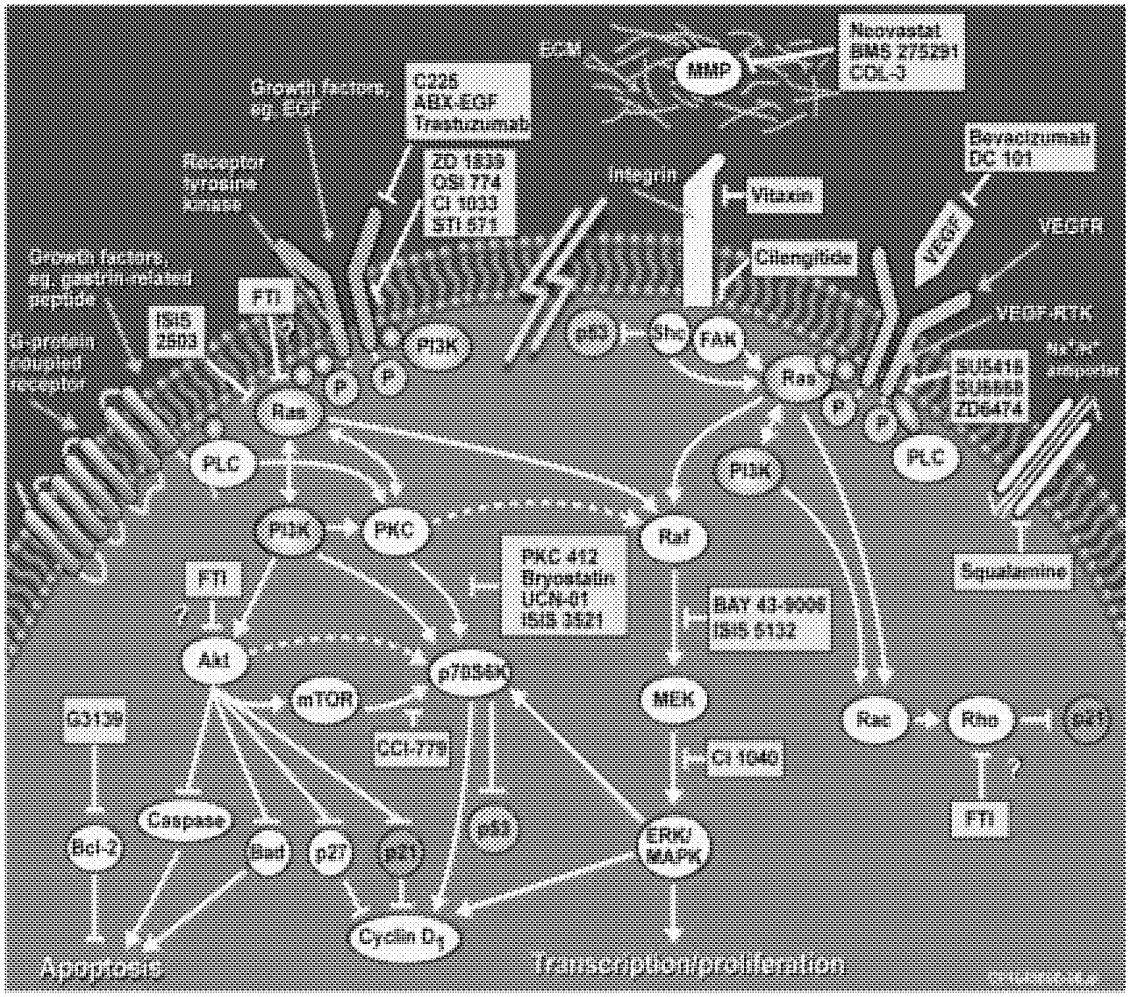

Fig. 1

identified in a range of human cancers [3]. Wild type raf can also be activated in tumor cells with enhanced growth factor signaling pathways, such as those induced by mutant Ras or activation of EGF receptor family members.

A potent oral selective small molecule inhibitor of Raf-1 (BAY 43-9006) is the first in its class to enter clinical trials. In multiple xenograft tumor models, dose-dependent tumor growth inhibition from 44–81% was observed, suggesting cytostatic properties. Toxicities observed to date in phase I trials have generally been mild to moderate, and include skin rash, fatigue, diarrhea and palmar-plantar erthyrodysesthesia, which is dose-limiting. Partial responses have been documented in renal cell and hepatocellular carcinoma. Disease stabilization for 6+ months has been reported in colorectal, ovarian, and head and neck cancers, respectively. Patient accrual continues [4]. Recently, B-Raf mutations have been identified in a number of human tumors, including 63% of melanomas [5]. The role of B-raf/Raf-1 in human cancers therefore remains to be defined.

2. Amplified targets (HER-2/neu)

Therapy directed against HER2/neu will be further discussed.

3. Proteins with aberrant upstream regulators (mTOR and PTEN)

Activation of the phosphoinositide-3' kinase (PI3K)/Akt pathway by RTK results in the production of a number of second messengers that affect downstream targets, among which p70 S6k is as a potential target for inhibition. p70 S6k mediates activation of the 40S ribosomal protein S6, which is necessary for cell cycle progression from G, into S phase. Recent evidence suggests that the mammalian target of rapamycin (mTOR) kinase is structurally related to the PI3K. CC-779, an ester of rapamycin, binds to the cytosolic receptor FKBP (FK506 binding protein) and this complex inhibits the serine/threonine kinase mTOR. Inhibition

of mTOR kinase leads to rapid inactivation of ribosomal p70 S6 kinase and abrogation of uncontrolled proliferation of malignant cells that exhibit increased constitutive PI3K activity resulting in high basal Akt and P70s6k levels independent of activating ras mutations. Tumors with *PTEN* mutations that activate the PI3K pathway are particularly sensitive to rapamycin and its analogs with resultant growth arrest or apoptosis. CCI-779 has shown activity in a wide variety of tumors in several phase I trials [6, 7]. Common toxicities are thrombocytopenia, hypersensitivity reactions, low-grade fever and mild fatigue. RAD001, a hydroxyethyl ether of rapamycin, is also an mTOR inhibitor being developed by Novartis, which has completed phase I testing, and is undergoing phase II trials.

4. Regulators of key cellular proteins (ubiquitin-proteasome system)

The ubiquitin-proteasome system is a highly conserved, intracellular pathway for the degradation of proteins. Many of the short-lived key regulatory proteins such as cyclins, cdk inhibitors (p21, p27), and anaphase-inhibitory proteins, are substances that are degraded by the proteasome upon conjugation of proteins to ubiquitin. In addition, various cell adhesion molecules involved in tumor metastasis and angiogenesis in vivo, such as E-selectin, ICAM-1, VCAM-1, are under the regulation by NF-κB [8], whose activation is regulated by proteasome-mediated degradation of the inhibitor protein I-κB [9]. Thus, reduced NF-κB expression by proteasome inhibition regulates neoplastic growth and metastasis. PS-341 is a modified depeptidyl boronic acid derivative of leucine and pheylalanine. It is a cell-permeable molecule that exerts reversible inhibition of the proteasome. It blocks cell division in the G_2-M phase, leading to cytotoxicity via apoptosis. It inhibits the degradation of wild-type p53, stabilizes the cdk inhibitor p21 which induces G_1 cell cycle arrest by inhibiting the cyclin D, E, and A-dependent kinases, and inhibits the activation of NF-κB as described previously. In ongoing phase I studies, clinical responses have been documented in patients with multiple myeloma, prostate and renal cell carcinomas (RCC) [10]. Phase II studies in multiple myeloma have demonstrated promising activity and phase III studies are ongoing. In solid tumors, the promise of PS-341 lies in combination therapy with chemotherapy agents [10].

5. Over-expressed protein (EGFR, MEK)

The Epidermal Growth Factor (EGF) is the prototype of a large family of closely related growth factors, which includes transforming growth factor-α (TGF-α), amphiregulin, heparin binding-EGF, and betacellullin. TGF has been well-characterized as a key modulator of both normal and malignant cell proliferation. TGF binds to its specific cell membrane receptor, the Epidermal Growth Factor Receptor (EGFR), with subsequent activation of the EGFR tyrosine kinase catalytic activity that activates cytoplasmic and nuclear signaling leading to cell proliferation and survival. The biochemical pathways involved in EGFR signaling have been elucidated. Catalytic activity is initiated after ligand binds to the receptor. As previously mentioned, erbB2 has no known ligand, but participates in receptor signaling by heterodimerization with other ligand-bound family members. Dimerization results in a conformational change, activation of the kinase domain autophosphorylation and initiation of cytoplasmic signaling [11]. Downstream effectors include the proliferative Ras/Raf/MEK/ERK pathway [12], and the anti-apoptic phosphoinositol 3-kinase pathway/Akt pathway [13]. Thus, EGFR signaling appears to be important for the maintenance of the neoplastic phenotype, and is a rational target for anti-cancer therapy.

VEGF trap: A potent anti-angiogenic agent in clinical trials

Following the pioneering studies of Dvorak [14, 15] and Ferrara [16–18] that identified vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) and defined key roles for it during vascular development, we initiated studies that led to the discovery and characterization of additional requisite angiogenic growth factors, including the Angiopoietins and Ephrins. Using both genetic knockout approaches as well as animal models of diseases such as cancer, we identified roles that these factors appear to play during vascular development and disease progression. While investigating the actions of these factors in a variety of settings, we also recognized the pivotal role that VEGF plays in promoting the progression and pathology of many diseases and the tremendous therapeutic potential of a drug that could block the action of VEGF. This approach was recently validated when it was demonstrated in clinical trials that a humanized monoclonal antibody that blocks VEGF efficaciously treats cancer [19].

In order to create a very potent, high affinity VEGF blocker, we employed our Cytokine and Growth Factor Trap Technology platform [20]. Blocking growth factors with soluble "decoy" receptors that are comprised of the extracellular domain of a receptor fused to the Fc portion of human immunoglobulin has proven to be an effective means to block the action of some cytokines and growth factors; however, this approach does not always yield a drug with desirable affinity or pharmacokinetic properties. The Trap Technology platform utilizes portions of multiple receptors for a ligand to engineer a drug with more desirable characteristics [21]. In the case of VEGF, historical versions of soluble "decoy" receptors that were based on the extracellular

domain of VEGF Receptor (VEGFR1) had proven to have high affinity for VEGF but relatively short *in vivo* half lives. Analysis of these constructs revealed that they had a high basic charge and we reasoned that this could make them "sticky" when in the milieu of extracellular matrix. To increase the *in vivo* half life of our construct, a soluble receptor was engineered with decreased basic charge by creating a fusion of the second immunoglobulin domain of VEGFR1 and the third immunoglobulin domain of VEGFR2 which is in turn fused to the Fc portion of human immunoglobulin 1. The hybrid receptor—Fc fusion protein is termed the "VEGF Trap". Using a range of *in vitro* and *in vivo* assays, we have demonstrated that the VEGF Trap has a prolonged *in vivo* half life as well as excellent VEGF blocking properties [21].

To investigate the potential of the VEGF Trap as a cancer therapeutic, initiallystudies were performed to verify that VEGF Trap could access and block VEGF in tumors, and as a consequence could inhibit tumor angiogenesis and growth. This was accomplished by employing subcutaneous tumor models in mice. Using cells lines derived from a variety of types of cancer such as mouse B16F10.1 melanoma, human A673 rhabdomyosarcoma and rat C6 glioma, mice bearing these tumors were treated shortly after tumor inoculation in each of the models, and it was found that VEGF Trap very effectively blocked tumor angiogenesis, resulting in largely avascular tumors, the growth of which was dramatically inhibited [21]. Studies were expanded to evaluate drug efficacy in small, established tumors. By testing a wide range of doses in these studies, information about the relationship between circulating levels of free drug and efficacywas acquired, which was proven to be valuable in ongoing clinical trials. Additionally tumor samples from these studies were obtained for microarray analysis that revealed gene changes that provide a fingerprint of drug efficacy. Genes whose expression is regulated in a dose dependent fashion by VEGF blockade are now being evaluated as potential surrogates of drug efficacy. Temporal studies have also been performed in the C6 glioma model and have found that VEGF Trap activity is apparent within 4 hr after drug treatment at which time vascular remodeling is already apparent. Substantial vascular pruning and regression continues to occur over a period over 72 hr. A profile of genetic changes that occurs concurrently with the vascular remodeling has been identified. The genes identified in this screen, combined with the results of our previous microarray analyses, are allowing us to identify new anti-angiogenic targets.

While subcutaneous models have provided a great deal of information about the action of VEGF Trap in cancer settings, we also wanted to determine if it would be efficacious in models that may share additional characteristics of human cancers. In a number of different laboratories, VEGF Trap has also been tested in a range of orthotopic models of cancer. It has been shown that it can inhibit both primary tumor growth and metastasis in orthotopic mouse models of pancreatic cancer [22], renal cell carcinoma and Wilms tumor [23]. In the setting of Wilms tumor, VEGF Trap can not only inhibit, but can actually shrink both primary tumors and lung metastasis. While the decrease in size of metastases in mice treated with VEGF Trap as compared to that of mice at the initiation of the study was dramatic, it should be noted that the average number of metastases was the same. Thus it appears that even in very responsive cancers, combination therapy with traditional cytotoxic agents or new biological agents will be appropriate. Recent studies in a mouse model of advanced human ovarian cancer demonstrate that combining VEGF Trap with paclitaxel can produce very dramatic and sustained tumor control. VEGF Trap as either a single agent or in combination with other agents is able to control tumor growth in a variety of settings [24].

The range of preclinical models in which VEGF Trap has been efficacious provides us with optimism for success in clinical trials, which are currently ongoing. It does seem likely, however, that some types of tumors will be able to attain a vascular supply independently of VEGF. Consequently it is important to investigate the mechanisms by which this can occur, as well as to search for novel anti-angiogenic targets. Regeneron has developed a multi-faceted approach to identify candidates and to assess their function in tumor and general angiogenesis. As a first step, microarray analysis is used to identify gene changes in tumors harvested from mice treated with various VEGF Trap regimens as compared to tumors from control mice. This approach has already yielded a number of potential angiogenesis gene targets. The role of each newly identified target in developmental and tumor angiogenesis is being evaluated by creating genetically engineered mice in which the gene of interest is replaced with a reporter gene. The ability to screen a large number of gene candidates using genetically modified mice is possible because Regeneron has developed a new technology termed VelociGene which allows for the expeditious generation of knockouts, knockins and transgenics [25]. Additionally, retroviral technology is being used to engineer populations of tumor cells to produce high levels of each gene of interest and the resultant cells are used in *in vivo* tumor assays where the effect of each gene of interest can be evaluated in the presence or absence of VEGF blockade.

As an example of the effectiveness of our approach to identify new angiogenesis targets, a genome-wide screen revealed that Delta-like ligand 4 (Dll4), a ligand for the Notch family of receptors, is strikingly down-regulated in tumors by VEGF blockade. Using VelociGene technology, mice in which Dll4 was replaced with the lacZ reporter gene were created and it was revealed that Dll4 is essential for normal vascular development. This was not unexpected as members of the Notch family and their ligands have previously been shown to be involved in this process [26]; however, more unexpectedly,

it was also observed that loss of only a single copy of Dll4 resulted in embryonic lethality [27]. Gene expression analysis revealed that Dll4 is expressed in major arteries in early development. By adulthood, expression becomes restricted to smaller arteries and microvessels, with expression ending abruptly where capillaries merge into venules. Dll4 is highly expressed in tumors, where it continues to be excluded from the venous side of the circulation. To further explore the role of Dll4 in tumor angiogenesis, C6 glioma cells were engineered to express high levels of full length or a soluble form of Dll4. When implanted into mice, the resultant tumors had aberrant vasculatures, the nature of which is currently being explored.

In summary, using our Trap Technology platform, VEGF Trap can be created, a high affinity VEGF blocking agent with an extended *in vivo* half life. This drug has proven to be highly efficacious in a number of diverse preclinical models: It dramatically inhibits the growth of a variety of types of subcutaneous and orthotopic tumors, and can even cause frank tumor regression in some settings. In other preclinical settings, it has been found that combination of VEGF Trap with a cytotoxic agent can result in potency far greater than that of either single agent. In addition to its efficacy as an anti-cancer agent, the VEGF Trap is also an invaluable research tool. Microarray analysis of tumors from mice treated with VEGF Trap, as compared to those from control mice, has provided a number of gene candidates that are being evaluated using gene knockout and retroviral technology. This strategy is allowing us to identify and validate new targets for anti-angiogenic therapy.

Anti-angiogenic agents in development

Anti-angiogenic therapy has come of age in the treatment and management of patients with cancer. Advances in the use of these agents has improved the overall survival of patients with colorectal cancer and represents the first true advance in the treatment of first-line non-small cell lung cancer in the past decade. Exactly how these agents are causing such an improvement remains to be defined. Original hypotheses focused on the ability of inhibitors of VEGF to inhibit angiogenesis and thereby decrease blood flow to tumors [28]. In this regard, it was thought that anti-angiogenic therapy may be synergistic to other more standard anti-cancer agents such as chemotherapy or even radiation therapy. Despite this simplistic concept the early course of drug development for anti-angiogenic agents was littered with failed phase III trials. In this regard, the inhibitors of vascular endothelial growth factor (VEGF) appear to have alternate possible mechanisms of action that represent a novel concept in development of combinations of cancer therapy [19]. Given the activities of VEGF as a factor that induces permeability within blood vessels it has been demonstrated that tumors with leaky blood vessels have an associated elevation of intra-tumoral pressure related to high interstitial fluid pressure (IFP) [29]. This high IFP results in possible disruption of the delivery of drug therapy to the tumor by "closing off" thin-walled tortuous vessels. Reversal of this effect by inhibitors of permeability such as VEGF inhibitors holds the potential to improve drug delivery by actually improving blood flow to the tumors. Such biology may explain the rapid improvement in response rates and outcome where active chemotherapy is using in combination with VEGF inhibitors.

In addition to the activities of VEGF inhibitors as permeability-affecting agents, newer anti-angiogenic drugs affect angiogenesis by blocking not a single signal but multiple signals critical for the angiogenic pathway. In this regard, agents that disrupt angiogenesis by affecting endothelial cells as well as supporting cells such as pericytes have demonstrated activity in as single agents in diseases such as renal cell carcinoma [30, 31].

The ability to combine anti-angiogenic agents both with chemotherapy as well as with other similarly acting drugs holds great potential in cancer treatment and represents a new forum for combinatorial drug development. Exploration of this potential will take careful development of clinical trials with and without surrogates that need to be validated.

Tumor pericytes as targets for antiangiogenic therapies

Blood vessels are composed of two interacting cell types: endothelial cells, which form the inner lining of the vessel wall and perivascular cells, referred to as pericytes, vascular smooth muscle cells (vSMCs) or mural cells which wrap around the vascular tube. Due to their contractile capabilities and their multiple cytoplasmic processes, pericytes have mainly been associated with stabilization and hemodynamic properties of blood vessels [32, 33] but pericytes are also actively involved in angiogenesis. Angiogenesis starts from a preexisting vasculature, being either the primitive vascular plexus formed by vasculogenesis in the embryo, or the postcapillary venuous compartment of the mature vascular systems during the menstrual cycle, in pregnancy and in wound healing or under pathological conditions like tumor growth [32, 34]. The neovascularization process itself is complex and multi-step involving the concerted action of several molecules [35, 36]. Angiogenic factors (e.g. vascular endothelial growth factor (VEGF)) stimulate the normally quiescent endothelial cells. Pericytes detach from the vessel wall and endothelial cells and pericytes secrete several proteases in order to degrade the vessel basement membrane, which in turn allows endothelial cells to invade into the surrounding matrix. Furthermore endothelial cells

begin to proliferate and migrate, and eventually differentiate in order to form a new, lumen-containing vessel. Finally, endothelial cells secrete growth factors that recruit pericytes to the newly formed blood vessel to stabilize and mature the vascular network. Specifically, PDGF-B has been shown to be a crucial player in pericyte recruitment. During angiogenesis PDGF-B is expressed by sprouting capillary endothelial cells whereas its receptor PDGFRβ is localized on pericytes suggesting a paracrine signaling circuit between the two cell types [37, 38]. Mice that lack either PDGF-B or PDGF R -show a severe deficit in pericyte coverage of blood vessels leading to widespread microvascular leakage, hemorrhage and edema formation which in turn causes embryonic lethality, underscoring the essential role of pericytes in vessel formation during development [39–41].

Much less is known about the functional significance of pericytes for the tumor vasculature, which is quite different from a normal vasculature as it has structural and functional abnormalities. Angiogenesis in tumors leads to a chaotic, poorly organized vasculature with tortuous, irregularly shaped and leaky vessels that are often unable to support efficient blood flow [42, 43]. Due to the imbalanced expression pattern of angiogenic factors, tumor vessels appear to be in a constant state of remodeling, involving simultaneous formation and regression of vascular tubes [36, 44]. As tumor endothelial cells differ from the normal quiescent endothelium, so do tumor pericytes differ from normal pericytes. Pericytes in tumors are more loosely attached, irregularly distributed and often less abundant [45–47] which may result from an inherent inability to properly organize pericytes, but probably also from a limitation to the pool of recruitable perivascular cells [48]. Thus, pericytes in tumors had been suggested as rather "dysfunctional" cells. Consequently, full attention has been given to the tumor endothelial cell as the only critical cell component of tumor vessels.

Yet, there is emerging evidence that have turned back the attention to the hardly appreciated vascular cell type by providing evidence that tumor pericytes are potentially important and functional vascular cell constituents by eliciting survival mechanisms to establish and maintain tumor vessels. In pancreatic islet tumors of Rip1Tag2 mice, PDGF-B is expressed by endothelial cells and its receptor PDGFR is localized on pericytes to form a paracrine signaling circuit similar to the situation in the embryo. Tumor cells express neither the PDGF receptors nor the ligands [36]. When Rip1Tag2 mice were treated with the broad spectrum receptor tyrosine kinase inhibitor SU6668, which preferably targets PDGFR, but also to a lesser extent FGFR-and VEGFR signaling, pericytes in tumors, but not in normal tissue, detached from the vasculature causing blood vessel regression and stabilization of the cancer [49]. Congruently, SU6668 diminished pericytes in xenograft tumors and restricted tumour growth [50, 51]. Recent data also suggest that targeting pericytes in prostate cancer reduces tumor—and lymph angiogenesis [52]. When Rip1Tag2 mice were treated with a specific blocking antibody against PDGFRβ pericytes were reduced by ~85% in comparison to pericytes in untreated tumors [53]. In contrast, neutralizing PDGFRβ-antibodies did not affect pericyte numbers or attachment in normal organs such as pancreas and liver suggesting a new therapeutic window for tumor pericytes. Consistent with the absence of pericytes, tumor vessels became enlarged and hyperdilated indicating that tumor pericytes still have the capability to stabilize blood vessels. There was also an increase in apoptotic cells in PDGFRβ-antibody treated tumors which were predominantly located within the vascular lining. Quantitative analysis revealed that about 80% of all apoptotic cells were endothelial cells in treated [53]. These results further support the notion that tumor pericytes have protective functions for endothelial cells, likely by expressing important survival factors. Indeed, pericytes in the pancreatic islet tumors expressed very high levels of VEGF [53]. The protective capabilities of pericytes for endothelial cells is further supported by the finding that immature blood vessels without pericytes were more vulnerable to anti-VEGF therapy [45].

In summary all these data suggest that targeting two interdependent cellular constituents of the tumor vasculature, pericytes and endothelial cells, should exhibit synergistic effects and regress both immature and large, mature tumor vessels. Indeed, combinatorial treatment with SU5416 (blocking VEGFR-signaling) and SU6668 (blocking PDGFRβ-signaling) in Rip1Tag2 mice was better than either of the single drugs and produced substantial regression of bulky tumors concomitant with severe reduction in vessel density and increased apoptosis and necrosis [49]. These data reveal a new strategy for treating human cancer by targeting both endothelial cells and pericytes in tumors to render anti-angiogenic therapies more efficacious.

In vivo effects of a monoclonal antibody to the murine VEGFR-3 that antagonizes the binding of VEGF-C and receptor signaling

The third member of the VEGF receptor tyrosine kinase family, VEGFR-3 (Flt-4) mediates growth, survival and migration of lymphatic EC [54]. The ligands that activate VEGFR-3, VEGFs-C and D bind only to VEGFR-3 as nascent peptides but bind to and activate both VEGFR-2 and VEGFR-3 after proteolytic processing [55, 56]. The observation that a significant percentage of human tumors express VEGF-C or D has led to speculation that the elevated expression of these factors may contribute to tumor lymphangiogenesis and increased rates of metastasis [54]. Lymphatic vessels are a conduit for the spread of tumor cells to draining lymph nodes and experimental over-expression

of VEGF-C or D in tumor xenografts promotes tumor lymphangiogenesis and metastasis [56, 57]. This increase in metastatic rates can be blocked by systemic administration of soluble VEGFR-3 [56, 58].

The importance of VEGFR-3 in tumor biology led us to develop antagonist monoclonal antibodies to the mouse and human forms of this receptor. We generated a fully human antibody called hF4-3C5 to the human VEGFR-3 that completely inhibits the binding of soluble VEGFR-3 to immobilized VEGF-C and antagonizes in a dose-dependent manner the mitogenic response to VEGF-C of cells that express human VEGFR-3 [59]. However, hF4-3C5 does not cross-react with the murine VEGFR-3. In order to be able to study the effect of inhibiting VEGFR-3 in mouse models, we produced an antagonist rat monoclonal antibody to the mouse VEGFR-3 called mF4-31C1. The current presentation deals primarily with the characterization of this antibody and its effects *in vivo*. mF4-31C1 stains lymphatic endothelial cells lining splenic sinusoids in immunochemical analysis of frozen sections. The antibody inhibits the binding of soluble mouse VEGFR-3 to immobilized VEGF-C and antagonizes VEGF-C-stimulated phosphorylation of VEGFR-3 [60]. Thus, mF4-31C1 is an appropriate proof-of-principle antibody for use in *in vivo* studies of VEGFR-3 function in normal physiology and pathological processes.

It has been reported that VEGFR-3 is re-expressed in adult vascular endothelium under pathological conditions. We investigated the role of VEGFR-3 in tumor angiogenesis in a series of tumor xenograft studies in immunodeficient mice. Systemic treatment with mF4-31C1 inhibited tumor growth of subcutaneously engrafted human pancreatic, renal and colon cell lines. Work of Nicole Roberts in the laboratory of Mihaela Skobe at the Mt. Sinai School of Medicine showed that mF4-31C1 also reduced the growth of breast carcinoma xenografts engineered to over-express VEGF-C. This inhibition was accompanied by a reduction in the frequency of CD31-positive blood vessels [61]. Although the inhibition of xenograft growth was statistically-significant compared to control rat IgG in several models, the magnitude of the effect was modest (60–75% of control). In similar studies, a monoclonal antibody DC101 that antagonizes VEGFR-2 typically gives greater than 80% inhibition of tumor growth at similar doses and with comparable treatment schedules.

In a series of collaborative studies we demonstrated that specific blockade of VEGFR-3 *in vivo* profoundly disrupts normal and pathologic lymphangiogenesis. Work of Jeremy Goldman in the laboratory of Melody Swartz in Switzerland utilized a novel mouse tail model of lymphatic regeneration. In normal mice or in nude mice implanted with VEGF-C overexpressing cells, treatment with mF4-31C1 completely blocked lymphatic regeneration without affecting pre-existing lymphatics vessels [60]. Two additional lines of investigation showed the potent *in vivo* effect of treatment with mF4-31C1 antibody. Our colleagues in the laboratory of Raza Dana in the Scheppens Eye Institute studied corneal pathology of mice null for the gene encoding the cytoskeletal protein destrin. In these mice, lymphangiogenesis and angiogenesis occur postnatally in the normally avascular cornea and both could be blocked with the administration of mF4-31C1 [62]. In the second model, Peter Baluk in the laboratory of Donald McDonald at UCSF used the pathogen Mycoplasma pulmonis to induce airway inflammation in mice. In these mice, pathological lymphangiogenesis in the tracheal lining is driven by VEGF-C and D produced by infiltrating inflammatory cells. Treatment of the mice with mF4-31C1 concurrently with the initiation of infection prevented the occurrence of lymphangiogenesis [63]. Together, these studies show that mF4-31C1 is a potent proof-of-principle tool for investigating the biological role of VEGFR-3 in mouse models. Finally, Nicole Roberts (Skobe Laboratory) demonstrated that selective inhibition of VEGFR-3 signaling with mF4-31C1 suppressed lymph node and lung metastases in a mouse breast cancer xenograft model. This inhibition was statistically significant even if treatment was started as late as 4 weeks after tumor injection. Neutralization of VEGFR-2 decreased primary tumor growth to a much greater degree, inhibited both angiogenesis and lymphangiogenesis but, surprisingly, was less effective than VEGFR-3 blockade in reducing tumor metastases to both lymph nodes and lungs [61].

In conclusion, the fully-human antibody hF4-3C5 represents an excellent candidate for clinical development as an antagonist of VEGFR-3 and its possible clinical activity may be predicted from the results obtained with the anti-murine VEGFR-3 antibody mF4-31C1. However, significant issues remain to be resolved. The direct anti-tumor effect appears modest compared to the results seen with VEGFR-2 antagonists. In addition, while antagonizing VEGFR-3 clearly inhibits active lymphangiogenesis, it is unclear if such inhibition has utility in reducing the dissemination of human cancers. Attempts to define such utility have been hampered by the limitations of the current mouse models of tumor metastasis. Nevertheless, potential therapeutic value of inhibiting VEGFR-3 signaling in tumor therapy appears to warrant further investigation.

HER2/neu (c-erbB2) as a target for monoclonal antibody therapy

Overexpression of the $p185^{erbB2}$ protein results from amplification of the HER2 gene in ~20–25% of primary breast cancers and is associated with poor clinical prognosis [64]. In HER2-amplified breast tumors, there can be as many as

2×10^6 $p185^{erbB2}$ molecules per cell, in contrast to normal breast epithelial cells, which have only 20–50,000 molecules per cell. These elevated levels of HER2 receptors have been shown to play a pathogenic role in the cancers where they occur. Thus HER2 is an attractive target for tumor-selective anti-cancer drug development. A murine monoclonal antibody, 4D5, was found to have dose-dependent antiproliferative effects specific for HER2-overexpressing cell lines and xenografts. In phase I clinical trials at UCLA, this antibody was shown to localize to tumors *in vivo* and had anecdotal efficacy, but treated patients developed human anti-murine antibodies against 4D5 which limited its usefulness as a therapeutic [65]. Antibody humanization technology was applied to 4D5 resulting in an antibody molecule (trastuzumab, Herceptin®) which maintained specificity and binding affinity for HER2, and yet was only ~5% murine. Consequently trastuzumab is non-immunogenic [66].

In preclinical studies, we demonstrated that trastuzumab retained anti-tumor activity relative to 4D5 *in vitro* and *in vivo* [67]. In addition, it was able to elicit antibody-dependent cellular immune responses *ex vivo* against HER2-overexpressing tumor target cells using immune effector cells isolated from breast cancer patients [68]. Trastuzumab also sensitizes tumor cells to the cytotoxic effects of certain chemotherapeutic drugs, as well as tamoxifen and ionizing radiation, making it an attractive agent for integration into existing breast cancer treatment paradigms [69]. Results from phase II and randomized phase III clinical trials demonstrated that trastuzumab has anti-tumor efficacy both as a single agent and in combination with chemotherapy. We reported that trastuzumab in combination with chemotherapy as first line treatment for HER2-overexpressing metastatic breast cancer is associated with a 24% decrease in relative risk of death, and an increased median survival duration from 20.3 to 25.4 months [70]. Subsequently this observation has been confirmed in more recent studies in which a prolongation in median survival from 18.3 to 27.7 months ($P = 0.0002$) was demonstrated through utilization of the synergistic combination of trastuzmab plus docetaxel, versus docetaxel alone [71].

Despite these otherwise encouraging results, the absence of clinical response to trastuzumab in some women with HER2-amplified breast cancers suggests that there must be mechanism(s) by which breast cancers are, or become resistant to, trastuzumab. However, to date there are only a few reports addressing this important clinical observation. Postulated mechanisms for trastuzumab resistance include: (1) insulin-like growth factor receptor co-activation [72], (2) AKT activation [73], (3) co-expression of MUC4 which may sterically inhibit erbB2 antibody binding [74], and (4) reduced expression of PTEN [75]. It is hoped that identification of resistance mechanisms may provide new insights into potential targets for future therapeutic strategies to be used in combination with trastuzumab to optimize the therapeutic efficacy of the drug.

References

1. Adjei AA, Rowinsky EK: Novel anticancer agents in clinical development. Cancer Biol Ther 2(4 Suppl 1): S5–15, 2003
2. Chen EX, Siu LL: Development of molecular targeted anticancer agents: Successes, failures and future directions. Curr Pharm Des 11(2): 265–272, 2005
3. Lyons JF, Wilhelm S, Hibner B, Bollag G: Discovery of a novel Raf kinase inhibitor. Endocr Relat Cancer 8(3): 219–225, 2001
4. Strumberg D, Schuehly U, Moeller J, Hedley D, Hilger R, Stellberg W, Richly H, Heinig R, Ahr G, Wensing G, Kuhlmann J, Schuelen ME, Seeber S: West German Cancer Center/University of Essen, Essen, Germany; Bayer AG, Wuppertal, Germany; Princess Margaret Hospital, University of Toronto, Toronto, Canada.: Phase I clinical, pharmacokinetic, and pharmacodynamic study of the Raf kinase inhibitor BAY 43-9006 in patients with locally advanced or metastatic cancer. Proc Am Soc Clin Oncol 20(abstr 330.), 2001
5. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA: Mutations of the BRAF gene in human cancer. Nature 417(6892): 949–954, 2002
6. Hidalgo M, Rowinsky E, Erlichman C, et al.: Phase I and pharmocological study of CCI-779, a cell cycle inhibitor. Clin Cancer Res 6(suppl): 413, 2000
7. Raymond E, Alexandre J, Depenbrock H, Mekhaldi S, Angevin E, Hanauske A, Baudin E, Escudier B, Frisch J, Boni J, Armand JP: CCI-779, a rapamycin analog with antitumor activity: A Phase I study utilizing a weekly schedule. Proc Am Soc Clin Oncol 19 (abstr): 728, 2000
8. Read MA, Neish AS, Luscinskas FW, Palombella VJ, Maniatis T, Collins T: The proteasome pathway is required for cytokine-induced endothelial-leukocyte adhesion molecule expression. Immunity 2(5): 493–506, 1995
9. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T: The ubiquitin-proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell 78(5): 773–785, 1994
10. Adams J: Proteasome inhibitors as new anticancer drugs. Curr Opin Oncol 14(6): 628–634, 2002
11. Okano J, Gaslightwall I, Birnbaum M, Rustgi A, Nakagawa H: Akt/Protein Kinase B isoforms are differentially regulated by epidermal growth factor stimulation. J Biol Chem 275: 30934–30942, 2000
12. Haluska P, Adjei AA: Receptor tyrosine kinase inhibitors. Curr Opin Investig Drugs 2(2): 280–286, 2001
13. Dy GK, Haluska P, Adjei AA: Novel pharmacological agents in clinical development for solid tumours. Expert Opin Investig Drugs 10(12): 2059–2088, 2001
14. Senger DR, Perruzzi CA, Feder J, Dvorak HF: A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. Cancer Res 46(11): 5629–5632, 1986

15. Senger DR, Connolly DT, Van de Water L, Feder J, Dvorak HF: Purification and NH2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor. Cancer Res 50(6): 1774–1778, 1990
16. Ferrara N, Henzel WJ: Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161(2): 851–858, 1989
17. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N: Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246(4935): 1306–1309, 1989
18. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW: Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380(6573): 439–442, 1996
19. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F: Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350(23): 2335–2342, 2004
20. Economides AN, Carpenter LR, Rudge JS, Wong V, Koehler-Stec EM, Hartnett C, Pyles EA, Xu X, Daly TJ, Young MR, Fandl JP, Lee F, Carver S, McNay J, Bailey K, Ramakanth S, Hutabarat R, Huang TT, Radziejewski C, Yancopoulos GD, Stahl N: Cytokine traps: Multi-component, high-affinity blockers of cytokine action. Nat Med 9(1): 47–52, 2003
21. Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS: VEGF-Trap: A VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA 99(17): 11393–11398, 2002
22. Fukasawa M, Korc M: Vascular endothelial growth factor-trap suppresses tumorigenicity of multiple pancreatic cancer cell lines. Clin Cancer Res 10(10): 3327–3332, 2004
23. Huang J, Frischer JS, Serur A, Kadenhe A, Yokoi A, McCrudden KW, New T, O'Toole K, Zabski S, Rudge JS, Holash J, Yancopoulos GD, Yamashiro DJ, Kandel JJ: Regression of established tumors and metastases by potent vascular endothelial growth factor blockade. Proc Natl Acad Sci USA 100(13): 7785–7790, 2003
24. Byrne AT, Ross L, Holash J, Nakanishi M, Hu L, Hofmann JI, Yancopoulos GD, Jaffe RB: Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. Clin Cancer Res 9(15): 5721–5728, 2003
25. Valenzuela DM, Murphy AJ, Frendewey D, Gale NW, Economides AN, Auerbach W, Poueymirou WT, Adams NC, Rojas J, Yasenchak J, Chernomorsky R, Boucher M, Elsasser AL, Esau L, Zheng J, Griffiths JA, Wang X, Su H, Xue Y, Dominguez MG, Noguera I, Torres R, Macdonald LE, Stewart AF, DeChiara TM, Yancopoulos GD: High-throughput engineering of the mouse genome coupled with high-resolution expression analysis. Nat Biotechnol 21(6): 652–659, 2003
26. Shawber CJ, Kitajewski J: Notch function in the vasculature: Insights from zebrafish, mouse and man. Bioessays 26(3): 225–234, 2004
27. Gale NW, Dominguez MG, Noguera I, Pan L, Hughes V, Valenzuela DM, Murphy AJ, Adams NC, Lin HC, Holash J, Thurston G, Yancopoulos GD: Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc Natl Acad Sci USA 101(45): 15949–15954, 2004
28. Dvorak HF: Vascular permeability factor/vascular endothelial growth factor: A critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. J Clin Oncol 20(21): 4368–4380, 2002
29. Heldin CH, Rubin K, Pietras K, Ostman A: High interstitial fluid pressure—an obstacle in cancer therapy. Nat Rev Cancer 4(10): 806–813, 2004
30. Motzer R, Rini B, Michaelson M, et al.: SU011248, a novel tyrosine kinase inhibitor, shows antihumor activity in second-line therapy for patients with metastatic renal cell carcinoma: Results of phase 2 trial. Proc ASCO 22(14S): 4500, 2004
31. Ratain M, Flaherty K, Stadler W, et al.: Preliminary antitumor activity of BAY 43–9006 in metastatic renal cell carcinoma and other advanced refractory solid tumors in a phase II randomized discontinuation trial (RDT). Proc ASCO 22(14S): 4501, 2004
32. Cleaver O, Melton DA: Endothelial signaling during development. Nat Med 9(6): 661–668, 2003
33. Sims DE: Diversity within pericytes. Clin Exp Pharmacol Physiol 27(10): 842–846, 2000
34. Gerhardt H, Betsholtz C: Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res, 2003
35. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J: Vascular-specific growth factors and blood vessel formation. Nature 407(6801): 242–248, 2000
36. Bergers G, Benjamin LE: Tumorigenesis and the angiogenic switch. Nat Rev Cancer 3(6): 401–410, 2003
37. Hellstrom M, Kalen M, Lindahl P, Abramsson A, Betsholtz C: Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development 126(14): 3047–3055, 1999
38. Lindahl P, Bostrom H, Karlsson L, Hellstrom M, Kalen M, Betsholtz C: Role of platelet-derived growth factors in angiogenesis and alveogenesis. Curr Top Pathol 93: 27–33, 1999
39. Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C: Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Genes Dev 8(16): 1875–1887, 1994
40. Lindahl P, Johansson B, Leveen P, Betsholtz C: Pericyte loss and microaneurysm formation inPDGF-B-deficient mice. Science 126: 3047–3055, 1997
41. Soriano P: Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. Genes Dev 8(16): 1888–1896, 1994
42. Carmeliet P: Angiogenesis in health and disease. Nat Med 9(6): 653–660, 2003
43. Baluk P, Hashizume H, McDonald DM: Cellular abnormalities of blood vessels as targets in cancer. Curr Opin Genet Dev 15(1): 102–11, 2005
44. Folkman J: Tumor angiogenesis. In: Al He (ed) Cancer Medicine, 5th ed, vol In press. B C Decker, Hamilton, Ontario, 2000
45. Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E: Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal [see comments]. J Clin Invest 103(2): 159–165, 1999
46. Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM: Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. Am J Pathol 160(3): 985–1000, 2002
47. Bergers G, Song S: The role of pericytes in blood-vessel formation and maintenance. Neuro-oncol 7(4): 452–464, 2005
48. Abramsson A, Berlin O, Papayan H, Paulin D, Shani M, Betsholtz C: Analysis of mural cell recruitment to tumor vessels. Circulation 105(1): 112–117, 2002
49. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D: Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest 111(9): 1287–1295, 2003

50. Reinmuth N, Liu W, Jung YD, Ahmad SA, Shaheen RM, Fan F, Bucana CD, McMahon G, Gallick GE, Ellis LM: Induction of VEGF in perivascular cells defines a potential paracrine mechanism for endothelial cell survival. FASEB J 15(7): 1239–1241, 2001
51. Shaheen RM, Tseng WW, Davis DW, Liu W, Reinmuth N, Vellagas R, Wieczorek AA, Ogura Y, McConkey DJ, Drazan KE, Bucana CD, McMahon G, Ellis LM.: Tyrosine kinase inhibition of multiple angiogenic growth factor receptors improves survival in mice bearing colon cancer liver metastases by inhibition of endothelial cell survival mechanisms. Cancer Res 61(4): 1464–1468, 2001
52. Ozerdem U: Targeting of pericytes diminishes neovascularization and lymphangiogenesis in prostate cancer. Prostate 66(3): 294–304, 2006
53. Song S, Ewald AJ, Stallcup W, Werb Z, Bergers G: PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. Nat Cell Biol 7(9): 870–879, 2005
54. Alitalo K, Carmeliet P: Molecular mechanisms of lymphangiogenesis in health and disease. Cancer Cell 1(3): 219–227, 2002
55. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, Saksela O, Kalkkinen N, Alitalo K: Proteolytic processing regulates receptor specificity and activity of VEGF-C. EMBO J 16(13): 3898–3911, 1997
56. Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG: VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med 7(2): 186–191, 2001
57. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M: Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med 7(2): 192–198, 2001
58. Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Yla-Herttuala S, Jaattela M, Alitalo K: Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. Cancer Res 61(5): 1786–1790, 2001
59. Persaud K, Tille JC, Liu M, Zhu Z, Jimenez X, Pereira DS, Miao HQ, Brennan LA, Witte L, Pepper MS, Pytowski B: Involvement of the VEGF receptor 3 in tubular morphogenesis demonstrated with a human anti-human VEGFR-3 monoclonal antibody that antagonizes receptor activation by VEGF-C. J Cell Sci 117(Pt 13): 2745–2756, 2004
60. Pytowski B, Goldman J, Persaud K, Wu Y, Witte L, Hicklin DJ, Skobe M, Boardman KC, Swartz MA: Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. J Natl Cancer Inst 97(1): 14–21, 2005
61. Roberts N, Cassella M, Kloos B, et al.: Distinct roles for VEGFR-2 and VEGFR-3 activation in tumor lymphangiogenesis and metastasis to lymph nodes and distant organs. Cancer Research 66: 2650–2657 2006
62. Cursiefen C, Ikeda S, Nishina PM, Smith RS, Ikeda A, Jackson D, Mo JS, Chen L, Dana MR, Pytowski B, Kruse FE, Streilein JW: Spontaneous corneal hem- and lymphangiogenesis in mice with destrin-mutation depend on VEGFR3 signaling. Am J Pathol 166(5): 1367–1377, 2005
63. Baluk P, Tammela T, Ator E, Lyubynska N, Achen MG, Hicklin DJ, Jeltsch M, Petrova TV, Pytowski B, Stacker SA, Yla-Herttuala S, Jackson DG, Alitalo K, McDonald DM: Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. J Clin Invest 115(2): 247–257, 2005
64. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244(4905): 707–712, 1989
65. Pegram M, Slamon D: Biological rationale for HER2/neu (c-erbB2) as a target for monoclonal antibody therapy. Semin Oncol 27(5 Suppl 9): 13–19, 2000
66. Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM: Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci USA 89(10): 4285–4289, 1992
67. Pegram M, Hsu S, Lewis G, Pietras R, Beryt M, Sliwkowski M, Coombs D, Baly D, Kabbinavar F, Slamon D: Inhibitory effects of combinations of HER-2/neu antibody and chemotherapeutic agents used for treatment of human breast cancers. Oncogene 18(13): 2241–2251, 1999
68. Pegram M, Baly D, Wirth C, et al.: Antibody dependant cell-mediated cytotoxicity in breast cancer patients in phase III clinical trials of a humanized anti-HER-2 antibody. Proc Am Assoc Canc Res 38: 602, 1997
69. Pegram MD, Konecny GE, O'Callaghan C, Beryt M, Pietras R, Slamon DJ: Rational combinations of trastuzumab with chemotherapeutic drugs used in the treatment of breast cancer. J Natl Cancer Inst 96(10): 739–749, 2004
70. Slamon D: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. New England Journal of Medicine 344: 783–792, 2001
71. Marty M, Cognetti F, Maraninchi D, Snyder R, Mauriac L, Tubiana-Hulin M, Chan S, Grimes D, Anton A, Lluch A, Kennedy J, O'Byrne K, Conte P, Green M, Ward C, Mayne K, Extra JM: Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first-line treatment: The M77001 study group. J Clin Oncol 23(19): 4265–4274, 2005
72. Lu Y, Zi X, Pollak M: Molecular mechanisms underlying IGF-I-induced attenuation of the growth-inhibitory activity of trastuzumab (Herceptin) on SKBR3 breast cancer cells. Int J Cancer 108(3): 334–341, 2004
73. Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, Arteaga CL: Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt Is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. Cancer Res 62(14): 4132–4141, 2002
74. Price-Schiavi SA, Jepson S, Li P, Arango M, Rudland PS, Yee L, Carraway KL: Rat Muc4 (sialomucin complex) reduces binding of anti-ErbB2 antibodies to tumor cell surfaces, a potential mechanism for herceptin resistance. Int J Cancer 99(6): 783–791, 2002
75. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, Klos KS, Li P, Monia BP, Nguyen NT, Hortobagyi GN, Hung MC, Yu D: PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 6(2): 117–127, 2004

Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer

Calvin J. Kuo*†‡, Filip Farnebo*†, Evan Y. Yu*†, Rolf Christofferson*†, Rebecca A. Swearingen*†, Robert Carter*†, Horst A. von Recum*†, Jenny Yuan§, Junne Kamihara*†, Evelyn Flynn§, Robert D'Amato§, Judah Folkman§, and Richard C. Mulligan*†¶

*Department of Genetics, Harvard Medical School, †Division of Molecular Medicine, and §Department of Surgery, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115

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Although the systemic administration of a number of different gene products has been shown to result in the inhibition of angiogenesis and tumor growth in different animal tumor models, the relative potency of those gene products has not been studied rigorously. To address this issue, recombinant adenoviruses encoding angiostatin, endostatin, and the ligand-binding ectodomains of the vascular endothelial growth factor receptors Flk1, Flt1, and neuropilin were generated and used to systemically deliver the different gene products in several different preexisting murine tumor models. Single i.v. injections of viruses encoding soluble forms of Flk1 or Flt1 resulted in ≈80% inhibition of preexisting tumor growth in murine models involving both murine (Lewis lung carcinoma, T241 fibrosarcoma) and human (BxPC3 pancreatic carcinoma) tumors. In contrast, adenoviruses encoding angiostatin, endostatin, or neuropilin were significantly less effective. A strong correlation was observed between the effects of the different viruses on tumor growth and the activity of the viruses in the inhibition of corneal micropocket angiogenesis. These data underscore the need for comparative analyses of different therapeutic approaches that target tumor angiogenesis and provide a rationale for the selection of specific antiangiogenic gene products as lead candidates for use in gene therapy approaches aimed at the treatment of malignant and ocular disorders.

The central role of angiogenesis in the development of numerous pathologic conditions including cancer, diabetic retinopathy, and vascular malformations is now well appreciated (1). In the case of cancer, the concept of an "angiogenic switch" has been proposed by Hanahan and Folkman (2), wherein angiogenesis both precedes and is necessary for the development of frank tumorigenicity. Recent findings nevertheless have underscored the mechanistic complexity underlying the development of tumor blood supply including delayed angiogenesis into initially avascular tumor masses (3), the early cooption of vasculature from neighboring tissue (4), and the contribution of circulating endothelial stem cells (5).

Extensive data have implicated the vascular endothelial growth factor (VEGF) family and their receptors as critical mediators of physiologic and tumor blood vessel formation, and consequently these molecules have attracted particular attention as targets for antiangiogenic therapy by a variety of strategies (6–13). Recently, the administration of several tumor-derived circulating proteins have been proposed also as an alternative strategy for achieving the systemic inhibition of angiogenesis. In particular, both human and murine forms of angiostatin (AS), a proteolytic fragment of plasminogen, have been described to exert potent antiangiogenic and antitumor activities in a variety of murine tumor models, extending to frank regression of tumors (14, 15). Similarly, a C-terminal fragment of collagen XVIII, termed endostatin (ES), has been reported to exhibit antiangiogenic and tumor-regressing activities accompanied by a lack of acquired tumor resistance (16, 17).

Interestingly, despite the large number of previous studies that have demonstrated the antitumor activity of different gene products that inhibit angiogenesis via either VEGF-dependent or -independent pathways, a systematic comparison of the relative efficacy of the different gene products in the same tumor models has not been described. To begin to address this important issue, we have generated a series of recombinant adenoviral vectors encoding different antiangiogenic gene products and have used the viruses to deliver the different gene products in several different preexisting murine tumor models. Here, we present a comparative evaluation of the antitumor and antiangiogenic activity of those gene products.

Methods

Construction and Purification of Recombinant Adenoviruses. The Flk1-Fc cDNA was a gift from T. Niederman (Children's Hospital, Boston) and contained the murine Flk1 cDNA sequence encoding the signal peptide and the ectodomain (to TIRRVRKEDGG, amino acid 731) fused to a murine IgG2α Fc fragment. The Flk1-Fc fusion gene was released with *Xba*I and *Bam*HI and inserted in the polylinker of the adenovirus shuttle vector HIHG Add2 (J. Gray and R.C.M., unpublished data). In the resulting construct, Flk1-Fc expression is controlled by the human cytomegalovirus promoter and the rabbit β-globin intron and polyadenylation signal. The expression cassette is flanked by the adenovirus type 5 sequences encompassing nucleotides 1–459 and 3328–4619. The murine Flt1(1–3) cDNA was amplified by PCR from Flt-1 cDNA (S. Soker, Children's Hospital, Boston) resulting in amplification of the Flt-1 signal sequence, coding sequence with the first three Ig repeats to FNTSVHV, with an added C-terminal 6× His tag. The tagged cDNA then was ligated into HIHG Add2 as an *Eco*RI–*Sal*I fragment.

For the control Fc fragment, a cDNA encoding the murine IgG2α Fc cDNA (Lexigen, Lexington, MA) was released with *Xho*I and *Xba*I and ligated into HIHG Add2. The human soluble neuropilin (sNRP) cDNA with signal peptide, ABC domains, and a C-terminal 6× His tag (S. Soker) was excised with *Bam*HI and *Xba*I and cloned into HIHG Add2. A fragment comprising the human growth hormone leader peptide-encoding sequence fused to the human AS cDNA (Lys-97–Glu-458, kringle domains 1–4) was synthesized by PCR of human plasminogen cDNA. The PCR product was digested with *Bam*HI and *Xho*I and cloned into the shuttle vector pAd-MDM, which differs from HIHG Add2 only by the plasmid backbone. A cDNA encoding the murine ES coding sequence (HTHQD... TSFSK) fused to the collagen XVIII signal peptide (B. Olsen, Harvard Medical School, Bos-

Abbreviations: VEGF, vascular endothelial growth factor; sNRP, soluble neuropilin; ES, endostatin; AS, angiostatin; pfu, plaque-forming unit; LLC, Lewis lung carcinoma; SCID, severe combined immunodeficient.

‡Present address: Stanford University School of Medicine, Hematology Division, CCSR North 3100, 269 Campus Drive, Stanford, CA 94305.

¶To whom reprint requests should be addressed at: Children's Hospital, Enders 861, 320 Longwood Avenue, Boston, MA 02115. E-mail: mulligan@rascal.med.harvard.edu.

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ton) was cloned into the HIHG Add2 shuttle vector to generate pAdd2 mu endo II. An alternative cDNA containing the same ES sequence fused to the human growth hormone signal sequence (MATGSRTSLLLAFGLLCLPWLQEGSA) was produced by PCR from murine collagen XVIII cDNA (B. Olsen), and the *Bam*HI and *Xho*I-restricted product cloned into pAd-MDM to generate pAdd2 mu endo I. All the PCR-generated DNA fragments were sequenced on both strands to exclude PCR errors.

The HIHG pAdd2-derived recombinant shuttle vectors then were digested with *Pac*I and *Mfe*I to release a fragment where the transgene is flanked by 2.0 and 1.4 kb of homology with the adenovirus plasmid pAd-GM-CSF. The GM-CSF insert of the adenovirus plasmid is replaced with our transgene by homologous recombination in *Escherichia coli* (18). The Flk1-Fc, Flt1(1–3), Fc, sNRP, and mu endo II adeno vectors were rescued by transfection of the *Pac*I-restricted adenovirus plasmids in 293 cells. Homologous recombination between the pAd-MDM-derived shuttle vectors and viral DNA in 293 cells (19) allowed the rescue of the AS and mu endo I recombinant Ad vectors. The viral vectors are propagated on 293 cells and purified by CsCl banding as described (18).

Protein Analysis of Virally Produced ES and Flt1(1–3). C57BL/6 mice were injected with Ad mu endo II or Ad Flt1(1–3) [10^9 plaque-forming units (pfu) by tail vein]. After 3 days mice were bled, and the respective proteins were purified from plasma by using either heparin-Sepharose chromatography with NaCl elution (ES) or Ni-agarose chromatography with imidazole elution [Flt1(1–3)]. These purified proteins were transferred to poly(vinylidene difluoride) membrane and digested *in situ* with trypsin followed by N-terminal sequencing and mass spectroscopy.

ELISA Determination of Transgene Expression. Plasma samples were obtained by retroorbital puncture with heparinized capillary tubes after anesthesia. Murine Flk1-Fc concentrations were determined by sandwich ELISA with anti-murine Flk1 primary (PharMingen) and anti-murine IgG2α Fc-horseradish peroxidase secondary (Jackson ImmunoResearch). Murine ES plasma levels were quantitated by competition ELISA (CytImmune Sciences, College Park, MD) and human AS plasma levels by sandwich ELISA (Entremed, Rockville, MD).

Western Blot Determination of Transgene Expression. Plasma was analyzed by Western blot for Flk1-Fc (rat anti-murine Flk1, PharMingen, or goat anti-murine Fc, Jackson ImmunoResearch), Flt1(1–3) (rabbit anti-His, Santa Cruz Biotechnology), ES (rabbit anti-mouse ES, gift of K. Javaherian, Children's Hospital, Boston), AS (rabbit anti-human plasminogen, Accurate, Westbury, NY) or sNRP (rabbit anti-His, Santa Cruz Biotechnology). Flt(1–3) and sNRP levels were estimated by Western blot against purified standards. Development was performed with species-specific secondary Ab-horseradish peroxidase conjugates and chemiluminescence.

Tumor Cell Lines, Mice, and Adenoviral Injections. Murine Lewis lung carcinoma (LLC) cells were passaged on the dorsal midline of C57BL/6 mice or in DMEM/10% FCS/penicillin/streptomycin (PNS)/L-glutamine. T241 murine fibrosarcoma was grown in DMEM/10% FCS/PNS/L-glutamine and human pancreatic BxPc3 adenocarcinoma in RPMI medium 1640/10% FCS/PNS. Tumor cells (10^6) were injected s.c. into the dorsal midline of C57BL/6 mice (8–10 weeks old) for murine tumors and severe combined immunodeficient (SCID) mice for human tumors, grown to 100–200 mm^3 (typically 10–14 days) to demonstrate tumor take, and 10^9 pfu of antiangiogenic adenoviruses or the control adenovirus Ad Fc given by i.v. tail-vein injection. In Fig. 2*B*, seven Flt1 control animals received Ad GFP instead of Ad Fc, although we have not observed any differences in tumor inhibition with either control construct. Ad mu endo II was used in all ES experiments, except in Fig. 2*B* in which Ad mu endo I was used. Tumor size in mm^3 was calculated by caliper measurements over a 10- to 14-day period by using the formula $0.52 \times$ length (mm) $\times$ width (2) (mm), using width as the smaller dimension. *P* values were determined by using a two-tailed *t* test assuming unequal variances (Microsoft EXCEL).

Corneal Micropocket Assay. C57BL/6 mice received 10^9 pfu i.v. of antiangiogenic adenoviruses or the control adenovirus Ad Fc 2 days before assay. Mice were anesthetized with avertin i.p. and the eye was treated with topical proparacaine·HCl (Allergan, Irvine, CA). Hydron/sucralfate pellets containing VEGF-A_{165} (R & D Systems) were implanted into a corneal micropocket at 1 mm from the limbus of both eyes under an operating microscope (Zeiss) followed by intrastomal linear keratotomy by using a microknife (Medtroni Xomed, Jacksonville, FL). A corneal micropocket was dissected toward the limbus with a von Graefe knife #3 (2×30 mm), followed by pellet implantation and application of topical erythromycin. After 5 days, neovascularization was quantitated by using a slit lamp biomicroscope and the formula $2\pi \times$ (vessel length/10) $\times$ (clock hours). *P* values were determined by using a two-tailed *t* test assuming unequal variances (Microsoft EXCEL).

Immunohistochemistry. C57BL/6 mice bearing LLC tumors on the dorsal midline at 50 mm^3 received 10^9 pfu i.v. of Ad Fc, Ad Flk1-Fc, or Ad Flt1(1–3). After tumor growth to $\approx$200 mm^3, tumors were harvested, fixed in formalin, and paraffin-embedded sections were stained for CD31 by using a biotin-streptavidin horseradish peroxidase system (Vectastain). Microvessel areas were quantitated by manual counting of hotspots in sections.

Results

Construction and Characterization of Adenoviruses Encoding Antiangiogenic Gene Products. By using homologous recombination techniques in bacteria (18), DNA sequences encoding human AS, murine ES, and the ligand-binding ectodomains of the VEGF receptors Flk1, Flt1, and neuropilin were introduced into the E1 region of a standard E1-deleted adenoviral vector (Fig. 1*A*; see *Methods* for details of construction). Viruses encoding each of the gene products were generated after transfection of the different vector DNAs into 293 cells as described (18). In the case of each vector, particle titers of $\approx 10^{13}$/ml and infectious titers of $\approx 10^{11}$ pfu/ml were obtained routinely, with a particle/infectivity ratio of 40:60.

To evaluate the *in vivo* expression potential of the different viruses, 10^9 pfu of each virus was administered by i.v. or i.m. routes into immunocompetent C57BL/6 mice. Transgene expression was easily detectable in the plasma of infected mice by Western blotting (Fig. 1*B*). In the case of Flk-Fc, AS, and ES, plasma expression levels at different times after injection of virus were quantitated by sandwich ELISA (Fig. 1*C*). Ad Flk1-Fc virus provided very high levels of protein expression (2–8 mg/ml) compared with Ad AS (100–250 μg/ml) or Ad ES (>10 μg/ml), and the expression of all gene products declined progressively with time, consistent with the known transient nature of transgene expression afforded by first-generation adenoviral vectors (20). In the case of animals injected with viruses encoding Flt(1–3) or sNRP, Western blot analysis, in conjunction with purified protein standards, was used to estimate the serum concentration of each gene product. By this method, peak Flt(1–3) ectodomain plasma levels were 3–10 μg/ml, whereas peak sNRP levels were estimated to be >50 μg/ml (data not shown).

In vitro assays were used to confirm the functional activity of

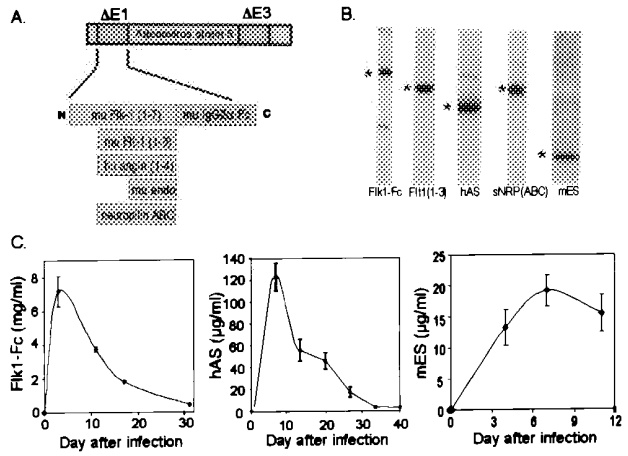

Fig. 1. Construction and characterization of antiangiogenic adenoviruses. (*A*) Schematic of insertion of various antiangiogenic cDNAs into the E1 region of E3-deleted adenovirus type 5. (*B*) Western blot analysis of adenovirus-expressed antiangiogenic proteins in mouse plasma. C57BL/6 mice received i.v. injection of 10^9 particles of the appropriate adenovirus, followed after 2–3 days by Western blot of 1 μl of plasma, except for Flk1-Fc which was taken at day 17 and was a 1:10 dilution. *, position of transgene products: Flk1-Fc (180 kDa), Flt1(1–3) (53 kDa), ES (20 kDa), AS (55 kDa), and sNRP-ABC (120 kDa). Levels in adjacent blots are not comparable because of different enhanced chemiluminescence exposure times. (*C*) Pharmacokinetics of expression from antiangiogenic adenoviruses. Plasma from mice infected i.v. with 10^9 pfu of the appropriate adenovirus was analyzed after the indicated times for expression by ELISA (Flk1-Fc, $n = 4$; ES, $n = 4$; AS, $n = 3$). See the text for further details. Error bars, ± 1 SD.

several of the adenovirus-expressed gene products. Vector encoded Flt1(1–3) and Flk1-Fc proteins both were shown to inhibit VEGF-induced human umbilical vein endothelial cells (HUVEC) proliferation *in vitro*, with IC_{50}s of ≈5 and 100 ng/ml, respectively (data not shown), paralleling reports of the relative affinities of the two receptors for VEGF (21). Because endothelial proliferation assays take at least 3 days and migration assays through a Boyden chamber are demanding technically, we used a bioassay for virally encoded ES on the basis of the dispersion of endothelial cells from endothelial tubes in Matrigel *in vitro*. In this assay, we were able to show that the virus-encoded protein consistently inhibited endothelial migration in Matrigel cultures in a manner similar to that observed with recombinant ES produced in yeast, baculovirus, or myeloma cells (C.J.K., unpublished observations). In addition, as an additional biochemical measure of the structural integrity of the virally encoded ES, we were able to demonstrate by both mass spectroscopy and N-terminal sequencing analysis that the virally encoded ES purified from the serum of mice injected with ES-encoding virus possessed the expected protein sequence (K. Javaherian and C.J.K., unpublished data).

Systemic Inhibition of Tumor Growth by Antiangiogenic Adenoviruses. The ability of each recombinant adenovirus vector to provide systemic inhibition of preestablished tumors was evaluated first in the aggressive LLC model in which recombinant AS and ES had been evaluated previously (14–17). LLC cells were implanted s.c. on the dorsum of C57BL/6 mice for 10–14 days to a size of 100–150 mm^3, consistent with definitive tumor engraftment, followed by i.v. injection of 10^9 pfu of the various adenoviruses. Under these conditions, adenoviral infection occurs primarily in liver without significant intratumoral infection (data not shown); consequently, any inhibition of tumor growth on the dorsum from protein produced in a remote site (i.e., liver) would presumably occur by a systemic mechanism.

In mice bearing preexisting LLC tumors, i.v. injection of Ad Fc did not inhibit tumor growth, with animals often requiring sacrifice by 14 days after virus injection, and no significant difference was observed between tumor growth in Ad Fc- and PBS-treated animals (F.F., C.J.K., and R.C.M., unpublished observations). In contrast, after 10–14 days of treatment, tumors in either Ad Flk1-Fc- or Ad Flt1-injected mice exhibited ≈80% growth inhibition relative to controls, which was statistically significant compared with the Ad Fc control virus ($P < 0.000001$; Fig. 2 *A* and *E*). On the other hand, LLC growth was inhibited less strongly by Ad ES (27%, $P = 0.004$), Ad AS (24%, $P = 0.001$), or Ad neuropilin (14%, $P = 0.15$; Fig. 2*A*). The antitumor effects of both Ad Flk1-Fc and Ad Flt1 were dose-dependent, with the minimal day-3 plasma concentrations for effective systemic tumor suppression being approximately >1 mg/ml for Flk1-Fc and >2 μg/ml for Flt1(1–3) (F.F., E.Y., B.S., and C.K., unpublished data). In most cases, tumor growth eventually supervened after 3–4 weeks (data not shown). Although the studies do not rule out acquired endothelial and/or tumor resistance as the mechanism underlying the observed escape from inhibition, the rapid decline of vector-mediated gene expression over time most likely accounts for the observed results.

A similar relative efficacy of the different viruses was observed in a syngeneic murine T241 fibrosarcoma-C57BL/6 tumor model (Fig. 2 *B–D*) and in a xenogeneic BxPc3-SCID tumor model (Fig. 3 *A* and *B*). In the case of the T241 model, strong tumor suppression was exhibited again by Ad Flk1-Fc (83%, $P < 0.000001$) and Ad Flt1 (87%, $P < 0.000001$); yet in this model, little or no inhibition of tumor growth was achieved by Ad ES (6%, $P = 0.71$), Ad AS (6%, $P = 0.86$), or Ad neuropilin (6%, $P = 0.77$) (Fig. 2 *B–D*). In the case of the BxPc3 model, Ad Flk1-Fc produced a strong suppression of tumor growth (83%, $P = 0.025$), whereas Ad ES, Ad sNRP, or Ad AS did not inhibit growth of preestablished BxPC3 tumors significantly with <12% inhibition ($P = 0.60–0.98$) (Fig. 3 *A* and *B*). For these latter studies, the data for Ad Flt1-injected animals was not included because of the death of the animals before completion of the experiments (see *Discussion*). In a last series of experiments, Ad Flk-Fc was shown also to strongly inhibit tumor growth in another xenogenic tumor model involving LS174T human colon carcinoma and SCID mice (79%, $P = 0.0003$; Fig. 3*C*).

Systemic Inhibition of Tumor Angiogenesis by Ad Flk1-Fc and Ad Flt1. Microvessel density has been used extensively as a marker for tumor angiogenesis, tumor grade, and inhibition of microvessel density as a measure of antiangiogenic activity (22). To evaluate the mechanism for Ad Flk1-Fc and Ad Flt1 suppression of tumor growth, the microvessel density of treated vs. nontreated tumors was measured. LLC cells (10^6) were implanted s.c. in the dorsal midline of C57BL/6 mice, and tumors were allowed to grow to ≈50 mm^3. The tumor-bearing mice then received i.v. injections of Ad Flk1-Fc, Ad Flt1(1–3), or Ad Fc followed by confirmation of expression levels by ELISA and were killed for histologic analysis after reaching a size of 200 mm^3. Immunohistochemistry for the endothelial antigen CD31 demonstrated an ≈50% reduction of microvessel density in Flt1(1–3) and Flk1-Fc mice relative to Fc mice (Fig. 4). Parallel administration of Ad *lacZ* virus produced strong staining in liver and minor staining in lung but did not produce significant intratumoral β-galactosidase staining (data not shown).

Systemic Inhibition of VEGF-Stimulated Corneal Angiogenesis by Antiangiogenic Adenoviruses. The ability of the different adenovirus-produced proteins to provide systemic inhibition of angiogenesis *in vivo* was evaluated also in a VEGF-dependent corneal neovascularization model. C57BL/6 mice received i.v. injections of the various adenoviruses followed after 2 days by implantation

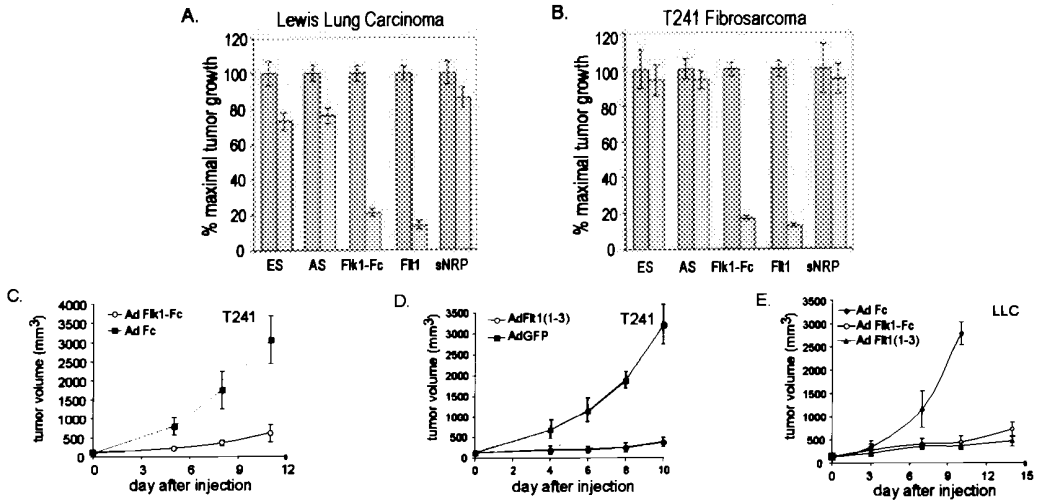

Fig. 2. Inhibition of preexisting tumor growth by antiangiogenic adenoviruses. C57BL/6 mice were implanted s.c. with 10^6 cells of murine LLC (*A*) or murine T241 fibrosarcoma (*B*). At a tumor volume of 100–150 mm^3, tumor-bearing mice received i.v. injection of 10^9 pfu of the control virus Ad Fc (green bars) or the appropriate antiangiogenic adenovirus (yellow bars), and tumor volume was calculated after 10–14 days. Tumor size is expressed as percentage of maximal tumor volume standardized to 100% for Ad Fc, which did not produce significant inhibition relative to PBS controls. Percentage of inhibition of animals receiving antiangiogenic adenoviruses relative to animals injected with the control virus Ad Fc is calculated. Error bars, ± 1 SEM; N, number of individual mice assayed with each adenovirus. For LLC, the number of animals was as follows for Fc and the treatment group: ES, $n = 24,22$; AS, $n = 11,9$; Flk1-Fc, $n = 18,17$; Flt1, $n = 8,10$; sNRP, $n = 8,8$. For T241, the number of animals was as follows for Fc and the treatment group: ES, $n = 6,10$; AS, $n = 6,7$; Flk1-Fc, $n = 24,25$; Flt1, $n = 19,20$; sNRP, $n = 7,5$. (*C* and *D*) Representative growth curves of T241 fibrosarcoma in C57BL/6 mice treated with Ad Flk1-Fc ($n = 6$) (*C*) or Ad Flt1(1–3; $n = 7$) (*D*). C57BL/6 mice bearing preexisting T241 tumors of 100–150 mm^3 received 10^9 pfu i.v. of the appropriate adenoviruses, and tumor size was measured over time. Error bars, ± 1 SD. (*E*) Suppression of LLC growth by Ad Flk1-Fc. Mice with preexisting tumors of 150 mm^3 received i.v. injections of 10^9 particles of Ad Fc ($n = 4$), Ad Flk1-Fc ($n = 5$), or Ad Flt1(1–3) ($n = 5$), and tumor growth was measured over time. Error bars, ± 1 SD.

of hydron pellets containing human VEGF-A_{165} into the mouse cornea. Plasma expression of the appropriate transgene was confirmed by ELISA or Western blotting followed by quantitation of corneal neovascularization 5 days after pellet implantation. In mice receiving VEGF pellets, corneal neovascularization was inhibited strongly by Ad Flk1-Fc (74%, $P < 0.0000001$) or Ad Flt1 (80%, $P < 0.0000001$), which was statistically significant relative to the Ad Fc control virus (Fig. 5 *A* and *B*). VEGF-stimulated corneal angiogenesis was inhibited to a lesser degree by Ad ES (33%, $P = 0.0001$), Ad AS (23%, $P = 0.002$), or Ad neuropilin (35%, $P = 0.027$) Fig. 5 *A* and *B*).

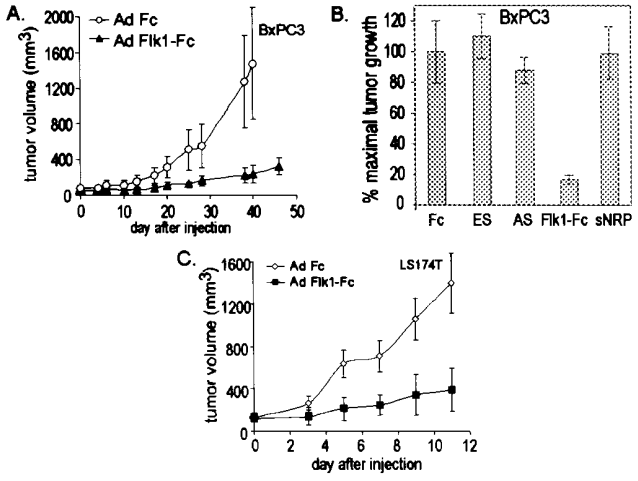

Fig. 3. Suppression of human tumor xenografts in SCID mice by Ad Flk1-Fc. (*A*) Treatment of BxPC3 human pancreatic carcinoma with Ad Flk1-Fc. CB17 SCID mice bearing preexisting tumors BxPC3 tumors of 60 mm^3 received 10^9 pfu i.v. of the appropriate adenoviruses, and tumor size was measured over time. Error bars, ± 1 SD; Fc, $n = 6$; Flk1-Fc, $n = 7$. (*B*) Comparative inhibition of preexisting BxPC3 tumor growth by antiangiogenic adenoviruses. Ad Fc and Ad Flk1-Fc mice in *A* were compared with tumor-bearing mice in the same experiment that received Ad ES ($n = 7$), Ad AS ($n = 7$), or Ad sNRP ($n = 6$). Tumor size is expressed as percentage of maximal tumor volume standardized to 100% for Ad Fc, which did not produce significant inhibition relative to PBS controls. Error bars, ± 1 SEM; N, number of individual mice assayed with each adenovirus. (*C*) Treatment of human LS174T colon adenocarcinoma in SCID mice with Ad Flk1-Fc. $n = 5$ per group. Error bars, ± 1 SD.

Discussion

The studies presented above provide important information regarding the relative potency of a number of antiangiogenic gene products previously shown to possess antitumor activity and specifically identify soluble forms of Flk1 and Flt1 as candidates for future gene therapy strategies. Our finding that soluble forms of Flk1 and Flt1 possessed significantly more potent antitumor

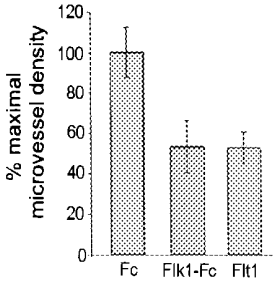

Fig. 4. Decreased microvessel density in tumors treated with Ad Flk1-Fc or Ad Flt1(1–3). C57BL/6 mice bearing LLC tumors of ≈50 mm^3 received i.v. injection of 10^9 pfu of Ad Fc, Ad Flk1-Fc, or Ad Flt1(1–3). Tumors were harvested at a size of 200 mm^3 for CD31 immunohistochemistry, magnification, and manual quantitation of microvessel density. Error bars, ± 1 SD with four representative fields counted per condition.

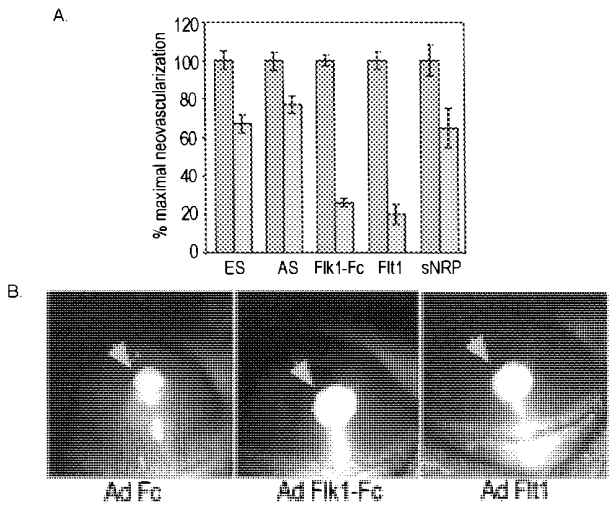

Fig. 5. Systemic inhibition of corneal angiogenesis by antiangiogenic adenoviruses. (*A*) Comparative activity in VEGF corneal micropocket assays. C57BL/6 mice received i.v. injection of 10^9 pfu of the appropriate adenovirus, followed after 2 days by implantation of VEGF-A_{165}-containing hydron pellets into the mouse cornea. Five days after pellet implantation, corneal neovascularization was quantitated by slit lamp examination. Results are presented as percentage of maximal neovascularization relative to the control virus Ad Fc, which was standardized at 100% and produced <5% inhibition relative to PBS. Error bars, ± 1 SEM. The number of eyes examined was as follows for Fc and the treatment group: ES, $n = 13,18$; AS, $n = 13,14$; Flk1-Fc, $n = 16,15$; Flt1, $n = 21,25$; sNRP, $n = 10,8$. (*B*) Systemic inhibition of corneal neovascularization by Ad Flk1-Fc or Ad Flt1(1–3). Representative corneas from experiments in *A* with preinjection of Ad Fc, Ad Flk1-Fc, or Ad Flt1(1–3) were photographed 5 days after pellet implantation. The position of the VEGF pellet is indicated by the arrow. Robust blood vessel ingrowth toward the pellet is noted in Ad Fc but not Ad Flk1-Fc or Ad Flt1(1–3) mice.

activity than AS or ES when delivered via gene transfer was quite unexpected and is of particular interest in light of previous reports of the extremely potent antitumor effects of ES and AS delivered via conventional protein administration (14–16). The reasons for this important discrepancy are unclear currently. Although the serum levels of AS and ES achieved in the previous studies that reported frank tumor regression were not measured (14–17), it is highly likely that the levels of the proteins obtained after adenoviral-mediated gene transfer are far greater. In addition, although differences in protein structure, folding, or posttranslational processing between the conventionally produced molecules and those produced via gene transfer could account for differences in their bioactivity, at least in the case of vector-encoded ES, mass spectroscopy and N-terminal sequencing demonstrated that the expected protein structure was present in mouse serum after gene transfer. Moreover, in this regard, the adenovirus-produced ES exhibits motility-inhibiting properties comparable to that of recombinant ES produced in yeast, baculovirus, or myeloma cells in matrigel assays. Taken together, the data suggests that, at a minimum, ES or AS will not be as easily utilizable as soluble VEGF receptors in conventional single-injection adenoviral strategies aimed at the systemic delivery of protein and may require more innovative approaches with different vector systems, modified transgenes, or alternative routes of administration. Clearly, further studies aimed at understanding the discrepancy between our results and those involving the administration of conventionally produced ES and AS are warranted.

Although several previous reports also had documented the antitumor effects of vector-mediated delivery of AS, ES, soluble Flt1 ectodomains, and sNRP domains (13, 23–27), the ability of the gene products to provide for the potent inhibition of large (>100 mm^3) aggressive preexisting tumors such as LLC had not been demonstrated previously. For example, although it has been shown that tumor lines stably transfected with AS cDNA exhibit impaired tumor growth, systemic gene therapy with AS has not been well documented to strongly suppress preexisting tumor growth (23, 25). Additionally, although several studies report the inhibition of tumor growth and metastases in mice after vector-mediated delivery of ES, no strong activity against preexisting tumors has been reported (24–27). In the case of soluble Flt-1 ectodomains, Kong *et al.* (28) have documented the efficacy of adenovirus vector-encoded Flt when delivered locally but not systemically, whereas Takayama *et al.* (13) have reported systemic antitumor efficacy of adenovirus Flt, but only against coinjected and not preexisting tumor burdens. In this latter case, the inability to observe significant activity against preexisting tumors may have resulted from insufficient production of Flt ectodomains, as our preliminary dosing studies suggest that high levels of gene product (>2 μg/ml) may be necessary for activity against preexisting tumors of >100 mm^3. In the case of soluble forms of neuropilin (sNRP), previous studies have shown that a soluble form of neuropilin representing a naturally occurring spliced form of the gene product was able to inhibit the ability of rat prostatic carcinoma cell lines engineered to express the gene product to grow as tumors (29). The inability of our Ad sNRP to inhibit tumor growth could reflect either the stringency of the tumor models used in our study or the use of a suboptimal soluble form of NRP (the sNRP gene used in the current studies differs from that used in previous studies in that the "C" domain is included). It is noteworthy that sNRP binds to regions of VEGF encoded by exon 7 (30, 31), whereas Flk1 and Flt1 bind to more N-terminal domains of VEGF (32).

In addition to identifying candidate gene products of potential use in cancer therapy, our studies also represent the first comparative study of systemically administered antiangiogenic agents against ocular angiogenesis. Small molecule inhibitors of the Flk1/KDR kinase domain, direct intraocular injection of soluble VEGF receptors, or adenoviral production of soluble Flt-1 have been shown previously to inhibit experimental retinal vascularization (33–35). Potentially, a variety of conditions accompanied by pathologic eye angiogenesis, such as diabetic retinopathy, macular degeneration, retinal ischemia, and ocular melanomas (36, 37) could benefit from the sustained delivery afforded by single injection dosing of gene transfer vectors.

Lastly, although the comparative analysis we have presented is obviously imperfect in that we were not able to provide for the same level of each gene product in the circulation, the expression levels we have achieved likely represent a theoretical "maximum" that reflects the inherent pharmacokinetic properties governing the circulating levels of each protein that can be achieved via gene transfer. As such, the results provide important practical information regarding which antiangiogenic gene products are most likely to be therapeutically effective when delivered via gene therapy. In addition to the need to evaluate the use of vector systems that can provide for the sustained high level expression of genes *in vivo* such as the recently developed "gutless" adenoviral vectors (38), considerably more effort will need to be paid to the issue of the safety and long-term sequelae of systemic, soluble receptor-mediated VEGF inhibition in adult organisms. In this regard, we have observed that although non-tumor-bearing animals injected with Ad Flk1-Fc and viruses encoding ES, AS, and sNRP remained grossly asymptomatic for >1 year, ≈30% of animals injected with Ad Flt1(1–3) develop ascites after 22–28 days followed by frequent mortality despite a several log lower serum concentration of Flt than Flk1-Fc (unpublished results). Determination of whether the toxicity we have observed after injection of Ad Flt1 results from either excessive VEGF chelation by higher affinity Flt1 (21) or the

distinct VEGF binding spectra of these receptors should aid the safety assessment of chronic VEGF-based antiangiogenic therapies.

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1. Folkman, J. (1995) *N. Engl. J. Med.* **333,** 1757–1763.
2. Hanahan, D. & Folkman, J. (1996) *Cell* **86,** 353–364.
3. Folkman, J. & D'Amore, P. A. (1996) *Cell* **87,** 1153–1155.
4. Holash, J., Maisonpierre, P. C., Compton, D., Boland, P., Alexander, C. R., Zagzag, D., Yancopoulos, G. D. & Wiegand, S. J. (1999) *Science* **284,** 1994–1998.
5. Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., Kearne, M., Magner, M. & Isner, J. M. (1999) *Circ. Res.* **85,** 221–228.
6. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S. & Ferrara, N. (1993) *Nature (London)* **362,** 841–844.
7. Presta, L. G., Chen, H., O'Connor, S. J., Chisholm, V., Meng, Y. G., Krummen, L., Winkler, M. & Ferrara, N. (1997) *Cancer Res.* **57,** 4593–4599.
8. Lin, P., Sankar, S., Shan, S., Dewhirst, M. W., Polverini, P. J., Quinn, T. Q. & Peters, K. G. (1998) *Cell Growth Differ.* **9,** 49–58.
9. Ferrara, N. & Alitalo, K. (1999) *Nat. Med.* **5,** 1359–1364.
10. Asano, M., Yukita, A. & Suzuki, H. (1999) *Jpn. J. Cancer Res.* **90,** 93–100.
11. Fong, T. A., Shawver, L. K., Sun, L., Tang, C., App, H., Powell, T. J., Kim, Y. H., Schreck, R., Wang, X., Risau, W., *et al.* (1999) *Cancer Res.* **59,** 99–106.
12. Wedge, S. R., Ogilvie, D. J., Dukes, M., Kendrew, J., Curwen, J. O., Hennequin, L. F., Thomas, A. P., Stokes, E. S., Curry, B., Richmond, G. H. & Wadsworth, P. F. (2000) *Cancer Res.* **60,** 970–975.
13. Takayama, K., Ueno, H., Nakanishi, Y., Sakamoto, T., Inoue, K., Shimizu, K., Oohashi, H. & Hara, N. (2000) *Cancer Res.* **60,** 2169–2177.
14. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H. & Folkman, J. (1994) *Cell* **79,** 315–328.
15. O'Reilly, M. S., Holmgren, L., Chen, C. & Folkman, J. (1996) *Nat. Med.* **2,** 689–692.
16. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R. & Folkman, J. (1997) *Cell* **88,** 277–285.
17. Boehm, T., Folkman, J., Browder, T. & O'Reilly, M. S. (1997) *Nature (London)* **390,** 404–407.
18. Chartier, C., Degryse, E., Gantzer, M., Dieterle, A., Pavirani, A. & Mehtali, M. (1996) *J. Virol.* **70,** 4805–4810.
19. Berkner, K. L. (1992) *Curr. Top. Microbiol. Immunol.* **158,** 39–66.
20. Yang, Y., Nunes, F. A., Berencsi, K., Furth, E. E., Gonczol, E. & Wilson, J. M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4407–4411.
21. Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. & Heldin, C. H. (1994) *J. Biol. Chem.* **269,** 26988–26995.
22. Weidner, N. (1995) *Am. J. Pathol.* **147,** 9–19.
23. Griscelli, F., Li, H., Bennaceur-Griscelli, A., Soria, J., Opolon, P., Soria, C., Perricaudet, M., Yeh, P. & Lu, H. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 6367–6372.
24. Blezinger, P., Wang, J., Gondo, M., Quezada, A., Mehrens, D., French, M., Singhal, A., Sullivan, S., Rolland, A., Ralston, R. & Min, W. (1999) *Nat. Biotechnol.* **17,** 343–348.
25. Chen, Q. R., Kumar, D., Stass, S. A. & Mixson, A. J. (1999) *Cancer Res.* **59,** 3308–3312.
26. Sauter, B. V., Martinet, O., Zhang, W. J., Mandeli, J. & Woo, S. L. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4802–4807. (First Published April 11, 2000; 10.1073/pnas.090065597)
27. Feldman, A. L., Restifo, N. P., Alexander, H. R., Bartlett, D. L., Hwu, P., Seth, P. & Libutti, S. K. (2000) *Cancer Res.* **60,** 1503–1506.
28. Kong, H. L., Hecht, D., Song, W., Kovesdi, I., Hackett, N. R., Yayon, A. & Crystal, R. G. (1998) *Hum. Gene Ther.* **9,** 823–833.
29. Gagnon, M. L., Bielenberg, D. R., Gechtman, Z., Miao, H. Q., Takashima, S., Soker, S. & Klagsbrun, M. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 2573–2578. (First Published February 25, 2000; 10.1073/pnas.040337597)
30. Soker, S., Takashima, S., Miao, H. Q., Neufeld, G. & Klagsbrun, M. (1998) *Cell* **92,** 735–745.
31. Soker, S., Fidder, H., Neufeld, G. & Klagsbrun, M. (1996) *J. Biol. Chem.* **271,** 5761–5767.
32. Keyt, B. A., Nguyen, H. V., Berleau, L. T., Duarte, C. M., Park, J., Chen, H. & Ferrara, N. (1996) *J. Biol. Chem.* **271,** 5638–5646.
33. Aiello, L. P., Pierce, E. A., Foley, E. D., Takagi, H., Chen, H., Riddle, L., Ferrara, N., King, G. L. & Smith, L. E. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 10457–10461.
34. Honda, M., Sakamoto, T., Ishibashi, T., Inomata, H. & Ueno, H. (2000) *Gene Ther.* **7,** 978–985.
35. Ozaki, H., Seo, M. S., Ozaki, K., Yamada, H., Yamada, E., Okamoto, N., Hofmann, F., Wood, J. M. & Campochiaro, P. A. (2000) *Am. J. Pathol.* **156,** 697–707.
36. Aiello, L. P. (1997) *Ophthalmic Res.* **29,** 354–362.
37. Aiello, L. P. (1997) *Curr. Opin. Ophthalmol.* **8,** 19–31.
38. Mountain, A. (2000) *Trends Biotechnol.* **18,** 119–128.

Aflibercept in wet age-related macular degeneration: a perspective review

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Matthew Ohr and Peter K. Kaiser

Abstract: In the treatment of neovascular age-related macular degeneration (AMD), vascular endothelial growth factor (VEGF) has emerged as a key target of therapy. Currently, patients with neovascular AMD are treated with monthly intravitreal injections of anti-VEGF medications. Aflibercept is a novel recombinant fusion protein engineered to bind all isoforms of VEGF-A, VEGF-B, and placental growth factor. It is the latest medication to receive US Federal Drug Administration (FDA) approval for the treatment of neovascular AMD. Theoretical models suggest this molecule may have a longer duration of action compared with current treatments. The results of the VEGF Trap-Eye: Investigation of Efficacy and Safety in wet Age-related Macular Degeneration studies (VIEW 1 and VIEW 2) support this by demonstrating that aflibercept, dosed every 2 months after a monthly loading dose for 3 months, was noninferior in the proportion of patients who maintained or improved vision at 52 weeks compared with monthly injections of ranibizumab. These results were maintained over the 2 years of the studies. Aflibercept (Eylea; Regeneron Pharmaceuticals, Inc., Tarrytown, NY, USA and Bayer, Basel, Switzerland) was approved by the FDA for the treatment of neovascular AMD on 18 November 2011.

Keywords: aflibercept, neovascular age-related macular degeneration, ranibizumab, vascular endothelial growth factor, wet age-related macular degeneration

Introduction

In the USA, age-related macular degeneration (AMD) is the leading cause of vision loss in older patients. It is estimated that the prevalence of AMD is 6.5% among people aged 40 years and older [Klein *et al.* 2011]. AMD also remains a leading cause of vision loss among older adults in other Western countries. Most of this vision loss stems from advanced AMD. Advanced AMD can be classified into two major forms: the non-neovascular, atrophic (dry) form or the neovascular (wet) form. The majority of people with severe vision loss (20/200 or worse) from AMD have the neovascular form, which is estimated to occur in 10–20% of patients [Ferris *et al.* 1984]. Currently, there is no effective treatment for advanced, dry AMD [Meleth *et al.* 2011]. However, neovascular AMD has been successfully targeted by a number of treatment strategies.

Overview of current therapy

The hallmark of wet AMD is the formation of new, anomalous blood vessels that typically arise from the choroidal vasculature and can grow into the subretinal pigment epithelial or subretinal space. Rarely, this process may originate from the retina and extend posteriorly into the subretinal space, a form of neovascular AMD termed retinal angiomatous proliferation. These neovascular vessels commonly hemorrhage and leak and can compromise vision by distorting the retinal and subretinal architecture with fluid, blood, or fibrovascular tissue [Spilsbury *et al.* 2000]. Untreated, choroidal neovascularization (CNV) usually leads to permanents loss of central vision.

The pathogenesis of CNV is not completely understood. However, the overexpression of vascular endothelial grown factor (VEGF), a pro-angiogenic cytokine, has been shown to play a crucial role [Spilsbury *et al.* 2000]. Previous studies have demonstrated increased levels of VEGF in the presence of inflammatory cytokines, suggesting that inflammation is a key component of AMD [Nagineni *et al.* 2012]. Others have suggested that ischemia, also associated with increased VEGF [Witmer *et al.* 2003], may play a role in AMD

Correspondence to:
Peter K. Kaiser, MD
Cleveland Clinic – Cole Eye Institute, 9500 Euclid Avenue, Desk i3, Cleveland, OH 44122, USA
pkkaiser@mac.com

Matthew Ohr, MD
Cleveland Clinic – Cole Eye Institute, Cleveland, OH, USA

[Feigl, 2009]. All of these reports clearly indicate that VEGF is vital to the pathogenesis of CNV in AMD.

Early treatment strategies focused on destruction of choroidal neovascular membranes using laser photocoagulation. The Macular Photocoagulation Study (MPS) established guidelines for treatment of these lesions [Macular Photocoagulation Study Group, 1982, 1986, 1991]. Although this treatment reduced the likelihood of severe vision loss compared with the natural course of the disease, there were many limitations, especially when treating lesions in the fovea. The primary downsides were related to the fact that the laser induced a permanent scotoma, and recurrence of the CNV occurred in over 50% of treated eyes [Macular Photocoagulation Study Group, 1991].

Until 1999, laser photocoagulation was the only treatment for neovascular AMD that had been shown to reduce the risk of vision loss. At that time, the Treatment of Age-Related Macular Degeneration with Photodynamic Therapy (TAP) Study reported that photodynamic therapy (PDT) with verteporfin (Visudyne; Novartis Pharma AG, Basel, Switzerland) reduced the risk of moderate to severe vision loss for at least 5 years in patients who presented with subfoveal lesions classified as predominantly classic [TAP Study Group, 1999; Azab *et al.* 2004; Blumenkranz *et al.* 2002; Bressler and TAP Study Group, 2001; Bressler *et al.* 2002; Kaiser *et al.* 2006]. PDT is a two-step process that involves the intravenous injection of verteporfin, a photosensitizing molecule, which is taken up by dividing cells within CNV. The drug is activated by local application of energy from a diode laser source at a wavelength that corresponds to an absorption peak of the molecule. A photochemical reaction occurs and activated free radicals are generated that can lead to capillary endothelial cell damage and vessel thrombosis. At 2 years, 59% of verteporfin treated eyes *versus* 31% of placebo eyes avoided at least moderate vision loss [Bressler and TAP Study Group, 2001]. While PDT improved the results seen with laser photocoagulation, there remained a pressing need for better treatment modalities.

The first VEGF inhibitor to obtain US Federal Drug Administration (FDA) approval for CNV in AMD was pegaptanib (Macugen; OSI/Eyetech Pharmaceuticals, New York, NY, USA) in 2004. Pegaptanib is an RNA aptamer that binds human $VEGF_{165}$ with high affinity and specificity [Gragoudas *et al.* 2004]. The drug, however, did not bind other active VEGF isoforms such as $VEGF_{121}$. Pegaptanib is administered as an intravitreal injection every 6 weeks. The VEGF Inhibition Study in Ocular Neovascularization (VISION) trial was a prospective, randomized, double-masked, controlled, dose-ranging phase III clinical trial in which 1186 patients with AMD and subfoveal CNV received one of three doses of pegaptanib or sham injections every 6 weeks for 48 weeks [Gragoudas *et al.* 2004]. The results of this study were promising, with 70% of patients losing less than three lines of vision compared with 55% of controls ($p < 0.001$). Unfortunately, similar to the results with PDT, a minority of patients gained vision with this therapy.

One of the most exciting advances in the treatment of CNV in AMD came with the introduction of ranibizumab (Lucentis; Genentech, South San Francisco, CA, USA) in 2006. Ranibizumab is a recombinantly produced, humanized, antibody (Fab) fragment that binds VEGF [Rosenfeld *et al.* 2006]. Unlike pegaptanib, ranibizumab binds to and inhibits the biological activity of all active forms of VEGF-A. The Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular AMD (MARINA) study was a randomized, double-masked, sham-controlled clinical trial of 716 patients with minimally classic or occult CNV secondary to AMD treated with one of two different doses of intravitreal ranibizumab or sham injections given every 4 weeks for 2 years [Rosenfeld *et al.* 2006]. The results of this study were revolutionary with 94.5% of patients treated with ranibizumab 0.3 mg and 94.6% of patients treated with ranibizumab 0.5 mg experiencing vision stabilization or improvement compared with 62.2% of patients receiving sham injections ($p < 0.001$). In fact, visual acuity improved by 15 letters or more in 24.8% of patients receiving 0.3 mg and 33.8% of patients receiving 0.5 mg ranibizumab compared with 5.0% of the sham injection group ($p < 0.001$). These results were further supported by the Anti-VEGF Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization in AMD (ANCHOR) study, which was a randomized, controlled, double-masked phase III clinical trial of 423 patients that compared patients treated with ranibizumab with patients treated with PDT with verteporfin treatment [Brown *et al.* 2006, 2009]. These results showed that 94.3% of patients treated with

0.3 mg ranibizumab and 96.4% of patients treated with 0.5 mg ranibizumab lost less than 15 letters of vision compared with 64.3% of patients treated with PDT at 1 year ($p < 0.001$). Patients receiving ranibizumab again showed increased vision in 35.7% of patients treated with 0.3 mg ranibizumab and 40.3% of patients treated with 0.5 mg ranibizumab compared with only 5.6% of patients treated with PDT ($p < 0.001$). The results of these trials resulted in anti-VEGF therapies largely replacing previous treatment modalities.

Bevacizumab (Avastin; Genentech) is a full-length monoclonal antibody that binds all isoforms of VEGF-A. The FDA originally approved it in February 2004 for the treatment of metastatic colorectal cancer. Soon thereafter, physicians started to use it off label as an intravenous or intravitreal treatment for neovascular AMD. Despite the lack of clinical research to support its safety or efficacy, anecdotal evidence led to its widespread popularity prior to the approval of ranibizumab in 2006. To deliver an intravitreal injection, a physician or pharmacy takes a vial of bevacizumab and makes numerous unit doses. This dramatically lowers the cost of the drug. The approximate cost differential between ranibizumab (US$2000) and bevacizumab (US$S50) was prohibitive for some patients, and bevacizumab continued to be utilized, despite not being FDA approved for intravitreal use. In fact, in a review of Medicare claims for neovascular AMD in 2008, it was noted that 58% of all intravitreal injections given were bevacizumab and 41% were ranibizumab [Brechner *et al.* 2011].

To address the safety and therapeutic concerns of the widespread, off-label use of bevacizumab in the treatment of wet AMD, the National Eye Institute commissioned the Comparison of Age-Related Macular Degeneration Treatment Trial (CATT) [CATT Research Group *et al.* 2011]. In this multicenter, single-blind, noninferiority trial, 1208 patients with neovascular AMD were randomized into four groups. After the first mandatory intravitreal injection, patients received ranibizumab every 28 days (ranibizumab monthly), bevacizumab every 28 days (bevacizumab monthly), ranibizumab only when signs of active neovascularization were present (ranibizumab as needed), and bevacizumab only when signs of active neovascularization were present (bevacizumab as needed). The 1-year results of this study demonstrated that monthly bevacizumab was equivalent to monthly ranibizumab with 8.0 and 8.5 letters gained, respectively. Bevacizumab as needed was found to be equivalent to ranibizumab as needed with 5.9 and 6.8 letters gained, respectively. While ranibizumab as needed was found to be equivalent to ranibizumab monthly, the equivalence of bevacizumab as needed compared with bevacizumab monthly was found to be inconclusive.

Aflibercept

Aflibercept (Eylea; Regeneron, Tarrytown, NY, USA and Bayer, Basel, Switzerland) is a fully human, recombinant fusion protein composed of the second immunoglobulin (Ig) binding domain of VEGF receptor 1 and the third Ig binding domain of VEGF receptor 2, fused to the Fc region of human IgG1. It binds to all VEGF-A isoforms, VEGF-B, and placental growth factor (PlGF) [Papadopoulos *et al.* 2012]. Aflibercept is a member of Regeneron's proprietary family of 'Trap' products that catch, hold, and block (i.e. trap) certain cytokines [Adis R&D Profile, 2008]. Aflibercept is being developed for the treatment of cancer (Zaltrap; Regeneron and Sanofi, Bridgewater, NJ, USA) and eye disorders. The eye formulation, also referred to in the literature as VEGF Trap-Eye, is identical in structure to the intravenous cancer treatment, with further purification steps and buffer modification to allow for comfortable, nonirritating intravitreal injection [Dixon *et al.* 2009].

Unlike currently available anti-VEGF therapies, aflibercept binds PlGF in addition to all isoforms of VEGF-A and VEGF-B. Like VEGF, PlGF is present in human CNV membranes, and animal studies have shown that PlGF contributes to the development of experimental CNV [Rakic *et al.* 2003]. Another differentiating feature of aflibercept is that the binding affinity for VEGF is 0.5 pM Kd, which is considerably stronger than ranibizumab, bevacizumab, or native VEGF receptors. This allows for effective blocking of VEGF, even at low concentrations, which may translate into a longer duration of action and extended dosing intervals [Stewart and Rosenfeld, 2008].

The results of preclinical studies were promising. In Matrigel-induced models of CNV in rats, aflibercept was shown to arrest the growth of CNV and led to the regression of recently established lesions [Cao *et al.* 2010]. Primate studies of laser-induced CNV also showed promise for the drug. When aflibercept was given prior to and following

attempted laser induction of CNV, minimal neovascularization was noted compared with placebo. In drug-naïve eyes with previously established CNV, aflibercept was successful in causing regression of the CNV and resolving vascular leakage [Nork *et al.* 2011]. These encouraging results coupled with the apparent safety of the drug, fueled the demand for human clinical trials.

Phase I

A phase I, randomized, multicenter, masked, placebo-controlled clinical trial of *intravenous* aflibercept in patients with subfoveal CNV from AMD showed a dose-dependent decrease in retinal thickness [Nguyen *et al.* 2006]. However, at systemic doses of 3 mg/kg, hypertension and proteinuria were observed, and the study was halted for safety concerns. This led to investigation of alternative delivery methods.

The safety, tolerability, maximum tolerated dose, and bioactivity of intravitreal injection of aflibercept were evaluated in a phase I, multicenter, dose-escalation study [Nguyen *et al.* 2009]. In the study, 21 patients received a single dose of aflibercept. Patients were monitored for 12 weeks after injection. There were no serious ocular or systemic events noted. With any dose of aflibercept, stable or improved vision was seen in 95% of patients at 6 weeks. The mean decrease in foveal thickness was −105.5 μm, and the mean increase in visual acuity was +4.43 letters. Half of the patients receiving 2 or 4 mg doses showed no retinal leakage and maintained vision gains at 12 weeks after a single injection. These positive results paved the way for further development of an intravitreal formulation of aflibercept.

Phase II

The clinical evaluation of anti-angiogenesis in the retina study (CLEAR-IT) 2 trial was a phase II multicenter, prospective, randomized, double-masked clinical trial designed to study the effect of intravitreal aflibercept in patients with neovascular AMD [Brown *et al.* 2011; Heier *et al.* 2011]. This trial was divided into two parts. In the first part, patients were treated with a fixed dosing interval up to 12 weeks. The second part of the study was designed to be as needed (PRN) dosing and took place from week 16 to 52. The primary endpoint of the study was the change in central retinal thickness. The mean change in best corrected visual acuity (BCVA) was evaluated as a secondary outcome. The study included 159 patients who were randomized into five treatment groups. The first two groups received treatment every 4 weeks and were dosed at 0.5 mg (group 1) or 2 mg (group 2). The last three groups were treated every 12 weeks and were dosed at 0.5 mg (group 3), 2 mg (group 4), or 4 mg (group 5). The primary outcome was at 12 weeks, following the fixed dosing period. The mean decrease in central retinal thickness from baseline to 12 weeks in all groups was −119 μm. Monthly dosing with either 0.5 or 2 mg (groups 1 and 2) provided a more profound and consistent effect than any of the groups treated every 12 weeks. Overall, there was a mean increase in BCVA of +5.7 Early Treatment Diabetic Retinopathy Study (ETDRS) letters in all groups. The greatest mean increase in BCVA, more than +8 letters, was seen in the monthly dosing groups compared with the patients receiving only one injection [Brown *et al.* 2011].

For the PRN dosing arm of the study, patients were evaluated every 4 weeks to determine the need for continued treatment. Patients received an injection of the baseline dose at week 12. At week 16 and thereafter, eyes were reinjected with aflibercept if any of the following conditions were noted: increase in central retinal thickness of at least 100 μm by optical coherence tomography (OCT); loss of at least 5 ETDRS letters with recurrent fluid on OCT; persistent fluid on OCT; new-onset of classic CNV; new or persistent leak on fluorescein angiography; or new macular hemorrhage on clinical examination. Using these criteria, the mean decrease in central retinal thickness in all groups from baseline to 52 weeks was −130 μm. The mean increase in BCVA was +5.3 ETDRS letters in all groups. The greatest increase in BCVA occurred in the group initially treated with 2 mg every 4 weeks for 12 weeks before PRN dosing with a mean increase of +9.0 letters at 1 year. To achieve these excellent visual gains, an average of two additional injections was administered after the 12-week fixed-dosing phase across all groups. The mean time to the first reinjection was 129 days, with 19% of patients receiving no injections and 45% receiving one or two additional injections [Heier *et al.* 2011].

Phase III

Two parallel, phase III, double-masked, randomized studies were initiated in August 2007. The VEGF Trap-Eye: Investigation of Efficacy and Safety in wet Age-Related Macular Degeneration

Table 1. Summary of the 1-year results of VIEW 1 and VIEW 2 studies compared with MARINA and CATT trials.

	MARINA		CATT		VIEW 1 (12 months)				VIEW 2 (12 months)			
	Ran 0.5 mg	Sham	Ran 0.5 mg	Bev 0.5 mg	Aft 0.5 mg	Aft 2 mg	Aft 2 mg Q*	Ran 0.5 mg	Aft 0.5 mg	Aft 2 mg	Aft 2 mg Q*	Ran 0.5 mg
≥15 letter gain [%]	33	4	34	31								
Stable vision [%]	90	53	94	94	96	95	95	94	96	96	96	94
Mean gain in VA from baseline at 12 months	+7.2	−10.4	+8.5	+8.0	+8.1	+10.9	+7.9	+8.7	+9.7	+7.6	+8.9	+9.4
Number of injections	12		12	12								

*Dosed every 8 weeks after treatment initiation with 3 monthly doses.
Aft, aflibercept; Bev, bevacizumab; CATT, Comparison of Age-Related Macular Degeneration Treatment Trial; MARINA, Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular AMD; Ran, ranibizumab; VA, visual acuity; VIEW, VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet Age-related Macular Degeneration.

(VIEW 1) study was performed in North America. The VIEW 2 study was an international study including Europe, Asia Pacific, Japan, and Latin America. The studies were designed as noninferiority studies comparing intravitreal aflibercept with ranibizumab. Patients with subfoveal CNV due to AMD were randomized into four groups. The first two groups received intravitreal injections of aflibercept at doses of 0.5 and 2 mg administered at 4-week intervals. The third group received 2 mg of aflibercept at 8-week intervals following three loading doses given every 4 weeks. These were compared with the fourth group, the control, receiving 0.5 mg of ranibizumab administered every 4 weeks. The primary endpoint was statistical noninferiority in the proportion of patients who maintained or improved vision over 52 weeks compared with ranibizumab.

The 1-year results of the VIEW 1 study showed that vision was maintained, defined as losing fewer than 15 ETDRS letters, in 96% of patients receiving aflibercept 0.5 mg monthly, 95% of patients receiving 2 mg monthly, and 95% of patients receiving 2 mg every 2 months. These results compared favorably with the 94% of patients maintaining vision in the group receiving ranibizumab 0.5 mg monthly [Regeneron, 2010; Heier, 2011]. The patients receiving aflibercept 2 mg monthly on average gained 10.9 letters compared with a mean 8.1 letter gain with ranibizumab 0.5 mg dosed every month ($p < 0.01$). The VIEW 2 study showed similar results, with maintenance of vision in 96% of patients receiving 0.5 mg monthly, 96% of patients receiving 2 mg monthly, and 96% of patients receiving 2 mg every 2 months. These results also compared favorably with the 94% of patients maintaining vision in the group treated with ranibizumab 0.5 mg monthly [Schmidt-Erfurth, 2011]. They are similar to results found in the MARINA and CATT trial (Table 1). The safety of both VIEW 1 and VIEW 2 studies was excellent with no difference seen between any aflibercept group and the ranibizumab group. The fact that 2 mg aflibercept dosed every 8 weeks after three loading doses was noninferior to ranibizumab dosed every 4 weeks in terms of safety and efficacy is exciting, as it offers the hope of similar visual gains with less treatment burden.

The 2-year results of the VIEW 1 and VIEW 2 studies were recently released [Regeneron, 2011]. The integrated analysis of these two studies (Table 2) shows that patients receiving aflibercept 2 mg every 8 weeks gained +7.6 letters from baseline at week 96 compared with +8.4 letters at week 52. The visual acuity gain in from baseline in patients receiving monthly ranibizumab was +7.9 letters at week 96 compared with +8.7 letters at week 52. Patients receiving aflibercept 2 mg every 8 weeks received an average of 11.2 injections over 2 years while patients treated with ranibizumab had an average of 16.5 injections over 2 years. Aflibercept (Eylea) was approved by the FDA for the treatment of wet AMD on 18 November 2011.

Conclusions

The evolution of treatment strategies for neovascular AMD has resulted in a paradigm shift in terms of expectations among patients and physicians. Prior to these recent advances, patients

Table 2. Summary of combined 2-year results of VIEW 1 and VIEW 2 studies.

	Aflibercept	Ranibizumab
Study arm	2 mg Q*	0.5 mg
Stabilization of VA (%)	95	94
Mean gain in VA (ETDRS letters)		
Year 1	+8.4	+8.7
Year 2	+7.6	+7.9
Average number of injections over 24 months	11.2	16.5

*Dosed every 8 weeks after treatment initiation with 3 monthly doses.
ETDRS, Early Treatment Diabetic Retinopathy Study; VA, visual acuity; VIEW, VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet Age-related Macular Degeneration.

who developed neovascular changes could anticipate a progressive and permanent decrease in vision. While destruction of the CNV lesion with laser photocoagulation was possible, in particular when the lesion was located outside of the visual axis, it offered only modest hope of maintaining vision compared with the natural history of the disease, and results were less than satisfactory. The introduction of PDT with verteporfin offered some improvement, especially to patients with subfoveal disease. However, many patients continued to lose vision, and only minimal visual gains were achieved.

The advent of anti-VEGF therapy marked a turning point in the treatment of neovascular AMD. The first FDA-approved anti-VEGF therapy for neovascular AMD was pegaptanib. Unfortunately, the specific targeting of $VEGF_{165}$ seemed to limit its effectiveness, and as with PDT, patients generally continued to slowly lose vision. It was the phase III results of ranibizumab and the off-label intravitreal use of the cancer drug, bevacizumab, that began to change expectations. Anecdotal evidence of improvements in vision and retinal thickness after a single treatment were reported in patients whose condition had failed to respond to pegaptanib therapy [Rosenfeld *et al.* 2005]. With the publication of the ANCHOR and MARINA trials, and the subsequent FDA approval of ranibizumab, vision could be expected to stabilize in close to 95% of cases, with improvement of BCVA by three or more lines in approximately 40% of patients.

While anti-VEGF therapy has changed the efficacy of treatment, it is not without drawbacks. Patients are subjected to intravitreal injections as often as every 4 weeks to maintain vision. Frequent office visits, testing, and medication costs represent a burden to patients, physicians, and society. Although there have been no proven, adverse systemic effects from intravitreal injections, every injection puts patients at risk for endophthalmitis, intraocular inflammation, vitreous hemorrhage, retinal tear, retinal detachment, and iatrogenic cataract. Recent studies have also suggested a sustained rise in intraocular pressure may occur with serial injections of anti-VEGF agents [Tseng *et al.* 2011]. Aside from serious complications, patients are commonly subject to anxiety, discomfort, and the undesirable aesthetics of conjunctival hyperemia or hemorrhage.

Current efforts have focused on extending the benefits of anti-VEGF treatment with less frequent dosing. In the phase IIIb, multi-center, randomized, double-masked, sham injection-controlled study of efficacy and safety of ranibizumab in subjects with subfoveal CNV with or without Classic CNV secondary to AMD (PIER) study, patients received monthly injections of ranibizumab for 3 months followed by quarterly dosing. Gains in visual acuity were noted at 3 months, only to be lost during the quarterly dosing phase of the trial [Regillo *et al.* 2008]. In the prospective optical coherence tomography OCT imaging of patients with neovascular age-related macular degeneration treated with intra-ocular lucentis (PrONTO) study patients also received monthly injections of ranibizumab for 3 months. Following the initial dosing, additional treatments were given on a PRN basis. After 2 years, 78% of patients maintained vision and 43% of patients showed improvement in more than three lines of vision. An average of 9.9 injections was given over the 2-year period [Lalwani *et al.* 2009].

One of the criticisms of PRN dosing is the fact that monthly visits are still required. One approach to treatment that aims to reduce the number of injections and visits is the 'inject and extend' method [Spaide, 2007]. This strategy involves treatment with 3-monthly injections followed by a follow up appointment extended to 6 weeks. At the follow-up visit OCT and biomicroscopy are performed. If edema or any other sign of exudation is present, the patient is given an injection and told to follow up in 4 weeks. Patients without any evidence of exudation are injected and have

their follow-up visit extended to 8 weeks. The same evaluation occurs at the 8-week visit. However, patients with signs of exudation are injected and scheduled to follow up at 6 weeks. Patients without evidence of exudation are further extended to follow up at 10 weeks. In this way, an optimal, individualized treatment strategy can be obtained. While a few small studies have reported positive results with this method, there have been no large, prospective studies to support its effectiveness [Engelbert *et al.* 2009; Oubraham *et al.* 2011].

Twelve-month results from the VIEW 1 and VIEW 2 studies appear promising for aflibercept. Although the data will have to be analyzed further, the apparent noninferiority of the various aflibercept dosing regimens compared with ranibizumab represents a new milestone in the treatment of CNV due to AMD. Of particular interest is the 2 mg every 8 weeks dosing interval studied in the trials. The recent results of the 2-year data for the VIEW 1 and VIEW 2 studies have further demonstrated the ability of aflibercept to maintain the visual gains attained in the first year of the study with a significantly smaller number of injections compared with ranibizumab. Aflibercept was recently approved by the FDA for the treatment of CNV in AMD. The wholesale price of aflibercept (US$1850) is slightly below that of ranibizumab. However, the reduced treatment requirements of every 8-week dosing *versus* monthly dosing of ranibizumab may represent a substantial savings in cost and treatment burden to patients.

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Dr Kaiser is a consultant for Regeneron and Bayer that has been disclosed and approved by the Cleveland Clinic Conflict of Interest Committee. In addition, the Cole Eye Institute, the employer of Drs Ohr and Kaiser has received research grant support from Regeneron.

References

Adis R&D Profile (2008) Aflibercept: AVE 0005, AVE 005, AVE0005, VEGF Trap – Regeneron, VEGF Trap (R1R2), VEGF Trap-Eye. *Drugs R D* 9: 261–269.

Azab, M., Benchaboune, M., Blinder, K.J., Bressler, N.M., Bressler, S.B., Gragoudas, E.S. *et al.* (2004) Verteporfin therapy of subfoveal choroidal neovascularization in age-related macular degeneration: meta-analysis of 2-year safety results in three randomized clinical trials: Treatment of Age-Related Macular Degeneration With Photodynamic Therapy and Verteporfin in Photodynamic Therapy Study report no. 4. *Retina* 24: 1–12.

Blumenkranz, M.S., Bressler, N.M., Bressler, S.B., Donati, G., Fish, G.E., Haynes, L.A. *et al.* (2002) Verteporfin therapy for subfoveal choroidal neovascularization in age-related macular degeneration: three-year results of an open-label extension of 2 randomized clinical trials – TAP report no. 5. *Arch Ophthalmol* 120: 1307–1314.

Brechner, R.J., Rosenfeld, P.J., Babish, J.D. and Caplan, S. (2011) Pharmacotherapy for neovascular age-related macular degeneration: an analysis of the 100% 2008 Medicare fee-for-service part B claims file. *Am J Ophthalmol* 151: 887–895.e1.

Bressler, N.M., Arnold, J., Benchaboune, M., Blumenkranz, M.S., Fish, G.E., Gragoudas, E.S. *et al.* (2002) Verteporfin therapy of subfoveal choroidal neovascularization in patients with age-related macular degeneration: additional information regarding baseline lesion composition's impact on vision outcomes – TAP report no. 3. *Arch Ophthalmol* 120: 1443–1454.

Bressler, N.M. and Treatment of Age-Related Macular Degeneration with Photodynamic Therapy (TAP) Study Group (2001) Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: two-year results of 2 randomized clinical trials – TAP report 2. *Arch Ophthalmol* 119: 198–207.

Brown, D.M., Heier, J.S., Ciulla, T., Benz, M., Abraham, P., Yancopoulos, G. *et al.* (2011) Primary endpoint results of a phase II study of vascular endothelial growth factor trap-eye in wet age-related macular degeneration. *Ophthalmology* 118: 1089–1097.

Brown, D.M., Kaiser, P.K., Michels, M., Soubrane, G., Heier, J.S., Kim, R.Y. *et al.* (2006) Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *N Engl J Med* 355: 1432–1444.

Brown, D.M., Michels, M., Kaiser, P.K., Heier, J.S., Sy, J.P., Ianchulev, T. *et al.* (2009) Ranibizumab versus verteporfin photodynamic therapy for neovascular age-related macular degeneration: two-year results of the ANCHOR study. *Ophthalmology* 116: 57–65.e5.

Cao, J., Zhao, L., Li, Y., Liu, Y., Xiao, W., Song, Y. *et al.* (2010) A subretinal matrigel rat choroidal neovascularization (CNV) model and inhibition of CNV and associated inflammation

and fibrosis by VEGF trap. *Investigative Ophthalmol Vis Sci* 51: 6009–6017.

CATT Research Group, Martin, D.F., Maguire, M.G., Ying, G.S., Grunwald, J.E., Fine, S.L., Jaffe, G.J. *et al.* (2011) Ranibizumab and bevacizumab for neovascular age-related macular degeneration. *N Engl J Med* 364: 1897–1908.

Dixon, J.A., Oliver, S.C., Olson, J.L. and Mandava, N. (2009) VEGF Trap-Eye for the treatment of neovascular age-related macular degeneration. *Exp Opin Invest Drugs* 18: 1573–1580.

Engelbert, M., Zweifel, S.A. and Freund, K.B. (2009) 'Treat and extend' dosing of intravitreal antivascular endothelial growth factor therapy for type 3 neovascularization/retinal angiomatous proliferation. *Retina* 29: 1424–1431.

Feigl, B. (2009) Age-related maculopathy – linking aetiology and pathophysiological changes to the ischaemia hypothesis. *Prog Retin Eye Res* 28: 63–86.

Ferris, F.L., 3rd, Fine, S.L. and Hyman, L. (1984) Age-related macular degeneration and blindness due to neovascular maculopathy. *Arch Ophthalmol* 102: 1640–1642.

Gragoudas, E.S., Adamis, A.P., Cunningham, E.T., Jr, Feinsod, M., Guyer, D.R. and VEGF Inhibition Study in Ocular Neovascularization Clinical Trial Group (2004) Pegaptanib for neovascular age-related macular degeneration. *N Engl J Med* 351: 2805–2816.

Heier, J.S.(2011). *VEGF Trap-Eye Phase III Trial Results. VIEW 1 results*. Presented at: Angiogenesis, Exudation, and Degeneration, February 12, 2011; Miami FL.

Heier, J.S., Boyer, D., Nguyen, Q.D., Marcus, D., Roth, D.B., Yancopoulos, G. *et al.* (2011) The 1-year results of CLEAR-IT 2, a phase 2 study of vascular endothelial growth factor trap-eye dosed as-needed after 12-week fixed dosing. *Ophthalmology* 118: 1098–1106.

Kaiser, P.K. and Treatment of Age-Related Macular Degeneration with Photodynamic Therapy (TAP) Study Group (2006) Verteporfin therapy of subfoveal choroidal neovascularization in age-related macular degeneration: 5-year results of two randomized clinical trials with an open-label extension: TAP report no. 8. *Graefes Arch Clin Exp Ophthalmol* 244: 1132–1142.

Klein, R., Chou, C.F., Klein, B.E., Zhang, X., Meuer, S.M. and Saaddine, J.B. (2011) Prevalence of age-related macular degeneration in the US population. *Arch Ophthalmol* 129: 75–80.

Lalwani, G.A., Rosenfeld, P.J., Fung, A.E., Dubovy, S.R., Michels, S., Feuer, W. *et al.* (2009) A variable-dosing regimen with intravitreal ranibizumab for neovascular age-related macular degeneration: year 2 of the PrONTO Study. *Am J Ophthalmol* 148: 43–58.e1.

Macular Photocoagulation Study Group (1982) Argon laser photocoagulation for senile macular degeneration. Results of a randomized clinical trial. *Arch Ophthalmol* 100: 912–918.

Macular Photocoagulation Study Group (1986) Argon laser photocoagulation for neovascular maculopathy. Three-year results from randomized clinical trials. *Arch Ophthalmol* 104: 694–701.

Macular Photocoagulation Study Group (1991) Argon laser photocoagulation for neovascular maculopathy. Five-year results from randomized clinical trials. *Arch Ophthalmol* 109: 1109–1114.

Meleth, A.D., Wong, W.T. and Chew, E.Y. (2011) Treatment for atrophic macular degeneration. *Curr Opin Ophthalmol* 22(3): 190–193.

Nagineni, C.N., Kommineni, V.K., William, A., Detrick, B. and Hooks, J.J. (2012) Regulation of VEGF expression in human retinal cells by cytokines: implications for the role of inflammation in age-related macular degeneration. *J Cell Physiol* 227: 116–126.

Nguyen, Q.D., Shah, S.M., Browning, D.J., Hudson, H., Sonkin, P., Hariprasad, S.M. *et al.* (2009) A phase I study of intravitreal vascular endothelial growth factor trap-eye in patients with neovascular age-related macular degeneration. *Ophthalmology* 116: 2141–2148.e1.

Nguyen, Q.D., Shah, S.M., Hafiz, G., Quinlan, E., Sung, J., Chu, K. *et al.* (2006) A phase I trial of an IV-administered vascular endothelial growth factor trap for treatment in patients with choroidal neovascularization due to age-related macular degeneration. *Ophthalmology* 113: 1522.e1–1522.e14.

Nork, T.M., Dubielzig, R.R., Christian, B.J., Miller, P.E., Miller, J.M., Cao, J. *et al.* (2011) Prevention of experimental choroidal neovascularization and resolution of active lesions by VEGF trap in nonhuman primates. *Arch Ophthalmol* 129: 1042–1052.

Oubraham, H., Cohen, S.Y., Samimi, S., Marotte, D., Bouzaher, I., Bonicel, P. *et al.* (2011) Inject and extend dosing versus dosing as needed: a comparative retrospective study of ranibizumab in exudative age-related macular degeneration. *Retina* 31: 26–30.

Papadopoulos, N., Martin, J., Ruan, Q., Rafique, A., Rosconi, M.P., Shi, E. *et al.* (2012) Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab. *Angiogenesis* 3 February [Epub ahead of print].

Rakic, J.M., Lambert, V., Devy, L., Luttun, A., Carmeliet, P., Claes, C. *et al.* (2003) Placental growth factor, a member of the VEGF family, contributes

to the development of choroidal neovascularization. *Invest Ophthalmol Vis Sci* 44: 3186–3193.

Regeneron (2010) Bayer and Regeneron report positive top-line results of two phase 3 studies with VEGF Trap-Eye in wet age-related macular degeneration (press release). Available at: http://investor.regeneron.com/releasedetail.cfm?ReleaseID=532099 (accessed February 2012).

Regeneron (2011) Two year results of phase 3 studies with EYLEA™ (aflibercept) injection in wet AMD show sustained improvement in visual acuity (press release). Available at: http://investor.regeneron.com/releasedetail.cfm?ReleaseID=629800 (accessed February 2012).

Regillo, C.D., Brown, D.M., Abraham, P., Yue, H., Ianchulev, T., Schneider, S. *et al.* (2008) Randomized, double-masked, sham-controlled trial of ranibizumab for neovascular age-related macular degeneration: PIER Study year 1. *Am J Ophthalmol* 145: 239–248.

Rosenfeld, P.J., Brown, D.M., Heier, J.S., Boyer, D.S., Kaiser, P.K., Chung, C.Y. *et al.* (2006) Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med* 355: 1419–1431.

Rosenfeld, P.J., Moshfeghi, A.A. and Puliafito, C.A. (2005) Optical coherence tomography findings after an intravitreal injection of bevacizumab (avastin) for neovascular age-related macular degeneration. *Ophthal Surg Lasers Imaging* 36: 331–335.

Schmidt-Erfurth, U. (2011). *VEGF Trap-Eye Phase III Trial Results. VIEW 2 results*. Presented at: Angiogenesis, Exudation, and Degeneration, February 12, 2011; Miami FL.

Spaide, R. (2007) Ranibizumab according to need: a treatment for age-related macular degeneration. *Am J Ophthalmol* 143: 679–680.

Spilsbury, K., Garrett, K.L., Shen, W.Y., Constable, I.J. and Rakoczy, P.E. (2000) Overexpression of vascular endothelial growth factor (VEGF) in the retinal pigment epithelium leads to the development of choroidal neovascularization. *Am J Pathol* 157: 135–144.

Stewart, M.W. and Rosenfeld, P.J. (2008) Predicted biological activity of intravitreal VEGF Trap. *Br J Ophthalmol* 92: 667–668.

Treatment of Age-related Macular Degeneration with Photodynamic Therapy (TAP) Study Group (1999) Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one-year results of 2 randomized clinical trials–TAP report. *Arch Ophthalmol* 117: 1329–1345.

Tseng, J.J., Vance, S.K., Della Torre, K.E., Mendonca, L.S., Cooney, M.J., Klancnik, J.M. *et al.* (2011) Sustained increased intraocular pressure related to intravitreal antivascular endothelial growth factor therapy for neovascular age-related macular degeneration. *J Glaucoma* 21: 241–247.

Witmer, A.N., Vrensen, G.F., Van Noorden, C.J. and Schlingemann, R.O. (2003) Vascular endothelial growth factors and angiogenesis in eye disease. *Prog Retin Eye Res* 22: 1–29.

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ORIGINAL PAPER

Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab

Nicholas Papadopoulos · Joel Martin · Qin Ruan · Ashique Rafique · Michael P. Rosconi · Ergang Shi · Erica A. Pyles · George D. Yancopoulos · Neil Stahl · Stanley J. Wiegand

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Abstract Pharmacological inhibition of VEGF-A has proven to be effective in inhibiting angiogenesis and vascular leak associated with cancers and various eye diseases. However, little information is currently available on the binding kinetics and relative biological activity of various VEGF inhibitors. Therefore, we have evaluated the binding kinetics of two anti-VEGF antibodies, ranibizumab and bevacizumab, and VEGF Trap (also known as aflibercept), a novel type of soluble decoy receptor, with substantially higher affinity than conventional soluble VEGF receptors. VEGF Trap bound to all isoforms of human VEGF-A tested with subpicomolar affinity. Ranibizumab and bevacizumab also bound human VEGF-A, but with markedly lower affinity. The association rate for VEGF Trap binding to VEGF-A was orders of magnitude faster than that measured for bevacizumab and ranibizumab. Similarly, in cell-based bioassays, VEGF Trap inhibited the activation of VEGFR1 and VEGFR2, as well as VEGF-A induced calcium mobilization and migration in human endothelial cells more potently than ranibizumab or bevacizumab. Only VEGF Trap bound human PlGF and VEGF-B, and inhibited VEGFR1 activation and HUVEC migration induced by PlGF. These data differentiate VEGF Trap from ranibizumab and bevacizumab in terms of its markedly higher affinity for VEGF-A, as well as its ability to bind VEGF-B and PlGF.

Keywords VEGF receptor · Aflibercept · Affinity · Age-related macular degeneration · Placental growth factor

Abbreviations

AMD	Age-related macular degeneration
HUVEC	Human umbilical vein endothelial cells
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
PlGF	Placental growth factor

Introduction

Angiogenesis is the process by which new vessels are created from pre-existing vasculature. Abnormal angiogenesis is a hallmark of diseases such as cancer [1] and the neovascular or 'wet' form of age-related macular degeneration (AMD) [2], the leading cause of blindness in the elderly population [3]. The process is characterized by an increase in the number of proliferating endothelial and stromal cells, and altered morphology of the vasculature [4, 5]. Several proangiogenic factors are consistently upregulated during diverse forms of pathological angiogenesis, including two members of the vascular endothelial growth factor (VEGF) family, VEGF-A and placental growth factor (PlGF) [6–8]. These factors activate quiescent endothelial cells and promote cell proliferation, migration and vascular permeability [5–9]. As in cancer, VEGF-A is the major driver of pathological angiogenesis and vascular leak in wet AMD, as well as in other ocular vascular diseases, such as diabetic and ischemic

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N. Papadopoulos · J. Martin · Q. Ruan · A. Rafique · M. P. Rosconi · E. Shi · E. A. Pyles · G. D. Yancopoulos · N. Stahl · S. J. Wiegand (✉)
Regeneron Pharmaceuticals Inc., 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA
e-mail: stanley.wiegand@regeneron.com

retinopathies. Moreover, growing evidence suggests that PlGF synergizes with VEGF-A in promoting vascular pathology in these diverse conditions [10–16].

In humans and other mammals, the VEGF family of factors consists of five related glycoproteins, VEGF-A, -B, -C, -D and PlGF [17, 18]. VEGF-A is the first, and most well studied member of the VEGF family and is currently a key target for antiangiogenic therapy [17]. Although encoded by a single gene, several distinct isoforms of VEGF-A exist as a result of alternative splicing and/or proteolytic cleavage. The various VEGF-A isoforms are all active as dimers, differing principally in their size and their ability to bind heparin or accessory, non-signaling binding proteins called neuropilins. For example, VEGF-A_{165} binds heparin and neuropilins with low affinity, and is the predominant isoform expressed in humans. VEGF-A_{121} is also expressed at high levels in many tissues and in pathological conditions, but it lacks the domains that mediate binding to heparin and neuropilins [17, 18] and is thus freely diffusible. Other isoforms such as VEGF-A_{189} and VEGF-A_{206} bind heparin with high affinity and thus accumulate in the extracellular matrix. Isoforms of VEGF-B and PlGF, which differ in their capacity to bind heparin and/or neuropilins are also produced by alternative splicing.

VEGF family ligands bind with high affinity to and signal through three receptor tyrosine kinases, VEGFR1, VEGFR2 and VEGFR3 [8, 17–19]. VEGFR2 is expressed predominantly on vascular endothelial cells. In addition to being expressed on the vascular endothelium, VEGFR1 is also expressed by several other cell types including neutrophils, monocytes, macrophages, mural cells, and endothelial progenitor cells. Although VEGFR1 has a higher affinity for VEGF-A than does VEGFR2, in endothelial cells VEGFR1 exhibits only weak tyrosine phosphorylation when activated by VEGF-A induced dimerization. Thus, the effects of all isoforms of VEGF-A on the vascular endothelium are thought to be mediated primarily through activation of VEGFR2. PlGF and VEGF-B bind only to VEGFR1, and in further contrast to VEGF-A, neither PlGF nor VEGF-B are essential for normal vascular development or physiological angiogenesis in the adult. However, like VEGF-A, both PlGF and VEGF-B have been implicated in pathological vascular remodeling [8, 11, 18]. The remaining VEGF family members, VEGF-C and VEGF-D, bind with high affinity to VEGFR3. VEGFR3 is found primarily on lymphatic endothelial cells in the adult. Consequently, VEGF-C and VEGF-D are involved primarily in the regulation of lymphangiogenesis [19], although VEGFR3 signaling is also thought to be important for both developmental and tumor angiogenesis [20–22].

The arsenal of VEGF blockers has evolved over time, with newer generations offering potentially improved anti-angiogenic activity by increasing their affinity for VEGF-A, and/or the number of VEGF-isoforms and family members that they inhibit. Pegaptanib (Macugen™, Eyetech, Inc.) is an aptamer that selectively binds to and neutralizes VEGF-A_{165}, but not VEGF-A_{121}, and was the first anti-VEGF therapy approved for the treatment of wet AMD [23, 24]. Bevacizumab (Avastin®, Genentech, Inc.) is a recombinant, humanized monoclonal antibody that binds all isoforms of VEGF-A, and has been approved for the treatment of metastatic colorectal cancer, non-small-cell lung cancer, and glioblastoma multiforme [1, 25]. Ranibizumab (Lucentis®, Genentech, Inc.) was developed specifically for intravitreal administration to treat vascular eye diseases, notably the wet or neovascular form of AMD [26, 27]. Ranibizumab is an affinity-matured antigen-binding fragment (Fab) derived from bevacizumab, and thus has a higher affinity for VEGF-A relative to that of the parental bevacizumab Fab molecule (Fab-12) [28]. Ranibizumab was developed as a Fab because the smaller size was thought to enhance its diffusion from the vitreous into the retina and choroid, relative to full-length antibodies [26]. Being an antibody Fab fragment, each ranibizumab molecule has one binding site for VEGF (compared to bevacizumab's two), such that two molecules of ranibizumab are bound by each VEGF dimer. In clinical trials, pegaptanib was shown to have a modest effect in slowing the rate of vision loss in patients with wet AMD, while ranibizumab has proven to be highly effective not only in reducing macular edema and preventing further vision loss, but also in producing clinically meaningful improvements in vision in significant numbers of patients [26, 29, 30]. Ranibizumab has been approved by the FDA for the treatment of wet AMD, while bevacizumab is also currently used off-label to treat AMD by intravitreal administration. While the comparative safety and efficacy of bevacizumab for the treatment of wet AMD have not yet been definitively established, several large, controlled clinical trials comparing the relative efficacy of ranibizumab and bevacizumab in the wet AMD are in progress [31, 32].

VEGF Trap (aflibercept, Regeneron Pharmaceuticals, Inc.) is a novel type of soluble decoy receptor generated with Trap technology [33], which employs the fusion of components from multiple endogenous receptors. VEGF Trap consists of an all human amino-acid sequence and comprises the second Ig domain of human VEGFR1 and the third Ig domain of human VEGFR2 expressed as an inline fusion with the constant region (Fc) of human IgG1 [34]. Like bevacizumab and ranibizumab, VEGF Trap binds multiple isoforms of VEGF-A [35] but in contrast to these antibodies the VEGF Trap was designed to also bind the related VEGFR1 ligands, VEGF-B and PlGF. An intravenous formulation of VEGF Trap, generically known as aflibercept, is being developed for use in oncology

[ZALTRAP™ (aflibercept)]; this formulation is hyperosmotic and diluted prior to infusion. An alternate formulation of aflibercept, known as VEGF Trap-Eye [EYLEA™ (aflibercept) Injection)], is an ultra-purified and iso-osmotic drug product that has been developed specifically for intravitreal injection for use in the treatment of various ophthalmological conditions.

Although some data on the binding affinities and in vitro activities of bevacizumab, ranibizumab and VEGF Trap have been published [28, 34, 36–40], the available data are incomplete. Moreover, comparison of the currently available data for these agents across publications is problematic as the experimental methods, cell lines, and particular conditions employed differ significantly from study to study. For example, the equilibrium dissociation constant (K_D) of the Fab fragment of bevacizumab (Fab-12) for VEGF-A has been variously reported as 1.8 and 20 nM, as determined by surface plasmon resonance (SPR) technology (Biacore) [28, 36], while the binding characteristics of the full bivalent bevacizumab molecule have not been reported. Thus, the goal of the present work was to assess the binding properties and in vitro activity of VEGF Trap, ranibizumab and bevacizumab under identical experimental conditions.

The results of these experiments show that VEGF Trap binds to VEGF-A with higher affinity and a faster association rate than ranibizumab or bevacizumab, and that VEGF Trap has the unique ability to additionally bind VEGF-B and PlGF. Consistent with its higher affinity for VEGF-A and faster association rate, VEGF Trap demonstrates increased potency relative to ranibizumab and bevacizumab in blocking VEGF-A induced activation of VEGFR1 and VEGFR2 in cell-based assays, and also in blocking VEGF-mediated calcium mobilization and migration in human endothelial cells. Finally, the high affinity binding of VEGF Trap to PlGF is borne out by the finding that only VEGF Trap can markedly inhibit VEGFR1 activation and endothelial cell migration induced by PlGF.

Materials and methods

VEGF reagents

Human VEGF-A_{121}, human PlGF-1, human VEGF-C, human VEGF-D, murine VEGF-A_{164}, murine VEGF-A_{120}, murine PlGF-2, rat VEGF-A_{164}, human VEGFR1-hFc, human VEGFR2-hFc and hVEGFR3-hFc were purchased from R&D Systems (Minneapolis, MN). VEGF Trap, rabbit VEGF-A_{165}, human PlGF-2, human VEGF-$B_{(10\text{-}108)}$ and human VEGF-A_{165} were made at Regeneron Pharmaceuticals, Inc. (Tarrytown, NY). Bevacizumab and ranibizumab (Genentech, Inc., South San Francisco, CA) were purchased.

Surface plasmon resonance (SPR)

SPR experiments were performed on a Biacore 3000 instrument using a dextran-coated (CM5) chip at 25°C. The running buffer was filtered HBS-T (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.05% polysorbate 20, pH 7.4). A capture sensor surface was prepared by covalently immobilizing recombinant Protein A (Pierce, Rockford, IL) or an anti-human Fab polyclonal antibody (human Fab capture kit, GE Healthcare, Piscataway, NJ) to the chip surface using (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride)/N-hydroxysuccinimide (EDC/NHS) coupling chemistry. Following surface activation, Protein A or anti-human Fab polyclonal antibody in coupling buffer (0.1 M acetate buffer, pH 4.5) was injected over the activated chip surface until a resonance unit (RU) signal of about 2,000 RU (Protein A) or 1,000 RU (anti-human Fab polyclonal antibody) was reached. The activated coupled chip surfaces were then washed and treated with 10 mM glycine–HCl, pH 1.5, to remove uncoupled residual proteins.

VEGF Trap, bevacizumab or ranibizumab were diluted into the running buffer and captured on the coupled Protein A (VEGF Trap and bevacizumab) or anti-human Fab polyclonal antibody (ranibizumab) chip surface. Following the capture step, a range of concentrations of test ligands (1.0–0.062 nM for VEGF-A ligands, 2.5–0.156 nM for VEGF-$B_{(10\text{-}108)}$ and 5.0–0.078 nM for PlGF ligands) were individually injected over VEGF inhibitor captured surfaces. For all ligands, the association rate constant (k_a) was determined from data obtained at multiple test ligand concentrations. The dissociation rate constant (k_d), which is independent of test ligand concentration, was determined from the change in VEGF inhibitor-bound test ligand RU over time ($\sim$10–70 min) for PlGF and VEGF-B ligands. Since the dissociation rate (k_d) of VEGF-A family ligands is too slow to allow for sufficient RU change within ligand dissociation time periods typically employed, the dissociation rates for these ligands were measured on a Biacore 2000 instrument using the "fixed k_d" procedure as described by Drake et al. [41]. This format uses a saturating concentration of ligand for binding, followed by monitoring the dissociation rate for an extended period of time ($\sim$2–3 h). Specific Biacore kinetic sensorgrams (Online Resource 1, Figures 1–5) were obtained by a double referencing procedure as described by Myszka et al. [42]. The data were then processed using Scrubber software (version 2.0, BioLogic Software) and kinetic analyses performed using BiaEvaluation (version 4.1, Biacore). The equilibrium dissociation constant (K_D) was calculated from the ratio of the dissociation rate constant divided by the association rate constant ($K_D = k_d/k_a$). Similar studies were conducted to evaluate the binding kinetics of VEGF-A_{165}

to the extracellular domains of native VEGFR1 and VEGFR2 fused to human Fc (Online Resource, Fig. 5) and several other VEGF family related ligands from multiple species (Online Resource 1, Table 1). Additional studies demonstrated no detectable binding of VEGF Trap to human VEGF-C and human VEGF-D, however a positive control binding experiment confirmed the ability of VEGF-C and VEGF-D to associate with VEGFR3 (Online Resource 1, Fig. 5).

KinExA equilibrium assays

In addition to surface capture kinetic experiments, solution binding studies were also conducted at room temperature (25°C) using a KinExA 3000 instrument (Sapidyne Instruments, Boise, ID) to quantify the equilibrium binding constants of VEGF inhibitors in solution, using varying concentrations of VEGF-A_{165},VEGF-A_{121}, hPlGF-2 or VEGF-$B_{(10\text{-}108)}$. Inhibitor-ligand mixtures were equilibrated at room temperature for 10–96 h. Fifty microgram of human VEGF-A_{165} was immobilized onto 75 mg Azlactone beads, suspended in 1.5 ml PBS and rotated at 4°C overnight. The supernatant was removed and the beads were incubated for another hour at room temperature in 1.0 ml PBS with 10 mg/ml BSA to block nonspecific binding sites. The blocked beads were washed three times with PBS, resuspended in 30 ml of PBS, and used immediately. Co-complex mixtures contained: VEGF Trap (concentration range 1–50 pM) with VEGF-A_{165} or VEGF-A_{121} (concentration range 19.5 fM–100 pM) or hPLGF-2 (concentration range 0.5pM–5 nM) or VEGF–$B_{(10\text{–}108)}$ (concentration range 0.61 pM–1.25 nM): Ranibizumab (concentration range 50–400 pM) with VEGF-A_{165} (concentration range 0.73 pM–15 nM): Bevacizumab (concentration range 25–50 pM) with VEGF-A_{165} (concentration range 0.49 pM–5 nM). Human VEGF-A_{165} was coupled to Azlactone beads and was used to capture unbound inhibitor. Equilibrated mixtures were injected through a column of VEGF-A_{165}-coupled micro-beads in the KinExA system at a flow rate of 0.25 ml/min. Bead contact time was <0.5 s, permitting unbound VEGF inhibitors to be captured by the beads without perturbing the equilibrium state of the solution. Captured VEGF inhibitors were quantified with Cy5-conjugated goat polyclonal anti-human IgG or anti-human $F(ab')_2$ fragment specific for light-chain antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The K_D was obtained from non-linear regression analysis of the data using a one-site homogeneous binding model contained within the KinExA software (Version 1.0.3; Sapidyne Instruments) using the 'standard analysis' method. The software calculates the K_D and determines the 95% confidence interval by fitting the data points to a theoretical K_D curve (Online Resource 1, Figures 6 and 7). The 95% confidence interval is given as K_D low and K_D high as described by Darling et al. [43].

Cell-based bioassays

VEGFR1/VEGFR2 cell lines and VEGF assay

In order evaluate the ability of VEGF Trap, ranibizumab and bevacizumab to specifically block ligand-mediated dimerization and activation of VEGFR1 or VEGFR2, two separate cell lines expressing these receptors were created. Two chimeric VEGFR1 receptors were constructed that incorporated the VEGFR1 extracellular domain (1–756, Genbank # NP_002010) fused to the transmembrane and cytoplasmic domain of either IL18Rα (328–541, Genbank # NP_003846.1) or IL18Rβ. 355–549, Genbank # NP_003844.1). The VEGFR1/IL18Rα chimeric receptor was cloned into a plasmid with a G418 resistance marker, while the VEGFR1/IL18Rβ chimeric receptor was cloned into a plasmid with a hygromycin resistance marker. The chimeric receptors were transfected into an HEK293 cell line with an integrated NFκB-luciferase-IRES-eGFP reporter gene using Lipofectamine plus (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Likewise, similar chimeric receptors incorporating the VEGFR2 extracellular domain (1–764, Genbank # NP_002244.1) fused to the transmembrane and cytoplasmic domain of either IL18Rα or IL18Rβ were constructed and transfected into the same HEK293 reporter cell line. In order to isolate cells for use in a bioassay, the cells were grown in G418 (Invitrogen, Inc.) and hygromycin (Calbiochem) to ensure the presence of both chimeric receptors. Cells underwent further selection by stimulating the cells with VEGF and then sorting cells expressing GFP by fluorescence activated cell sorting (FACS). When the extracellular VEGFR1 or VEGFR2 is dimerized by binding VEGF, the IL18Rα and β intracellular domains interact and are able to signal through the NFκB driven luciferase reporter gene.

VEGF and PlGF activation of the VEGFR1 and VEGFR2 cell lines

Cells expressing either VEGFR1 or VEGFR2 were resuspended at 1.25×10^5 cells/ml in Optimem (Invitrogen, Inc.) plus 0.1% fetal calf serum (FCS) and 80 µl was placed in each well of a 96 well plate (10,000 cells/well). The cells were incubated overnight at 37°C, 5% CO_2. The dose response curve for VEGFR1 activation was determined by adding 20 µl of VEGF-A_{165}, VEGF-A_{121} or PlGF-2 (human or mouse) to the cells at concentrations ranging from 0.022 pM to 4.0 nM. One well served as the negative control with no test ligand added. The dose

response curve for VEGFR2 activation was determined by adding 20 µl of VEGF-A_{165}, VEGF-A_{121}, or hPlGF-2 to the cells at the same concentrations used above. Each dose response curve was done in quadruplicate. After addition of the VEGF or PlGF, the plates were incubated at 37°C and 5% CO_2 for 6 h, and then equilibrated to room temperature for 30 min. An equal volume of One-glo luciferase substrate (Promega, Madison, WI) was added to each well and the plate was incubated at room temperature for a further 15 min. Plates were read on Victor X instrument and the values were analyzed by a four-parameter logistic equation over a 12–point dose response curve (Prism, GraphPad Software, version 5.03, La Jolla, CA).

VEGF Trap, bevacizumab and ranibizumab were tested with both the VEGFR1 and VEGFR2 cell lines. VEGF Trap was added to the cells at concentrations ranging from 0.8 pM to 50 nM and included a control well with buffer. Bevacizumab and ranibizumab were added to the cells at concentrations ranging from 8.5 pM to 500 nM and included a control well. Immediately after addition of VEGF Trap or the antibodies to the VEGFR1 cell line, VEGF-A_{165}, VEGF-A_{121}, or hPlGF-2 was added to the cells at a constant concentration of 20 pM (VEGF) or 40 pM (hPlGF-2). The VEGFR2 cell line was stimulated with 20 pM VEGF-A_{165} or 20 pM VEGF-A_{121}. The plates were incubated at 37°C and 5% CO_2 for 6 h and then equilibrated to room temperature for 30 min. An equal volume of One-glo luciferase substrate (Promega) was added to each well and the plate was incubated at room temperature for a further 15 min. Plates were read on Victor X instrument and the values were analyzed by a four-parameter logistic equation over a 12-point response curve (GraphPad Prism). Each inhibition curve was done in triplicate.

VEGF dependent calcium mobilization in human umbilical vein endothelial cells (HUVEC)

HUVEC (Vec Technologies, Inc., Rensselaer, NY) were diluted to 3×10^5 cells/ml in MCDB-131 complete medium (Vec Technologies, Inc.), and 100 µl was added to each well of a 96 well plate. The plates were incubated overnight at 37°C and 5% CO_2. The media was then removed and the HUVEC loaded with a calcium sensitive dye, Fluo4 NW (Invitrogen, Inc), in ECB media (BD Biosciences) with 0.25 mM of probenicid and 0.3% BSA (80 µl per well). The solution was incubated with the cells for 30 min at 37°C and 5% CO_2 followed by another 30 min at room temperature.

To measure the dose response, HUVEC were simulated with buffer or VEGF-A_{165} at concentrations ranging from 0.023 pM to 4.0 nM. The cellular response was recorded at a fluorescence emission wavelength of 575 nm with an excitation of 515 nm for 6 min, using the FLIPR^{TETRA} (Molecular Devices, Sunnyvale, CA). Each dose response curve was done in duplicate.

Inhibition of VEGF-A_{165} was determined by adding VEGF Trap at concentrations ranging from 0.17 pM to 10.0 nM and for bevacizumab and ranibizumab at concentrations ranging from 8.4 pM to 500 nM. VEGF Trap, bevacizumab, or ranibizumab were incubated with 20 pM VEGF-A_{165} for 10 min and then added to the cells, and the calcium response recorded as above. The data were analyzed using the average peak fluorescence at each inhibitor concentration tested in triplicate.

Cell migration assays

Cell culture

HUVEC, at first passage, were purchased from VEC Technologies and grown at 37°C in a 5% CO_2 humidified incubator, in MCDB-131 complete media. Cells grown to confluency in 10 cm^2 culture dishes, were washed twice with Hank's Buffered Saline Solution (HBSS; Mediatech, Manassas, VA.) without calcium, magnesium or phenol red, and dissociated with Trypsin/EDTA (Lonza, Walkersville, MD). Cells were then seeded at approximately 2×10^5 cells/dish and typically reached confluency in 3–4 days. Prior to use in cell migration assays, cells were serum-starved for 5 h in MCDB-131 basal media (MBM; VEC Technologies) supplemented with 2 mM L-glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 10 µg/ml heparin, and 0.1% fetal bovine serum.

HUVEC migration

HUVEC migration was assessed using a modified Boyden chamber [BD FluoroBlok^{TM} 24-well Biocoat angiogenesis system: Endothelial cell migration (ECM); 3 µm pore size] according to the manufacturer's suggested protocol. Briefly, serum-starved HUVECs were dissociated using enzyme-free cell dissociation media (Millipore, Billerica, MA) and resuspended in MBM to a final concentration of $2–3 \times 10^5$ cells/ml. An aliquot of resuspended cells (250 µl; ~50,000 cells/well) was placed in the upper well of the ECM plate, and MBM (750 µl) with or without ligand (130 pM human VEGF-A_{165}, 7.1 nM human PLGF-2, or 3.5 nM mouse PLGF-2), was mixed with VEGF Trap, bevacizumab, or ranibizumab (inhibitor concentration range 0.013–13 nM) and placed in the lower well following a 1 h incubation of the mixture at room temperature. The ECM plate was incubated for 18–20 h in a 37°C/5% CO_2 incubator to allow cells from the upper well to migrate through the FluoroBlok^{TM} membrane towards the lower well. Following migration, cells attached to the underside

of the FluoroBlok™ membrane were stained with 500 μL of a 2 μg/mL solution of the fluorescent dye Calcein AM (Anaspec, Freemont, CA) for 1.5 h in a 37°C/5% CO_2 incubator. Fluorescence emission was measured at 580 nm with excitation at 485 nm in a Flexstation 3 (Molecular Devices, Sunnyvale CA) bottom-reading fluorescent plate reader. Statistical analyses were carried out using a 1-way ANOVA followed by a Dunnett's multiple comparison post hoc test (Prism, GraphPad Software, version 5.03, La Jolla, CA).

Results

VEGF Trap binds VEGF-A, VEGF-B and PlGF from multiple species with high affinity

The interaction between VEGF Trap and VEGF family ligands was measured using SPR-Biacore technology. Kinetic binding data was generated using an amine-coupled Protein A surface and subsequent VEGF Trap capture at low density. VEGF Trap bound heparin binding and non-heparin binding isoforms of human VEGF-A, and PlGF, as well as VEGF-$B_{(10\text{-}108)}$ with high affinity (Table 1 and Online Resource 1, Table 1). Notably, the equilibrium dissociation constant (K_D) of VEGF Trap for VEGF-A_{165} (0.490 pM) was significantly lower (tighter binding) than that of the extracellular domains of dimerized human VEGFR1 (9.33 pM) or VEGFR2 (88.8 pM) fused inline to hFc (Table 1 and Online Resource 1, Fig. 5). The above absolute and relative K_D values for VEGFR1-Fc and VEGFR2-Fc are comparable to those previously reported for native VEGFR1 and VEGFR2 using cell-based bindings assays [44, 45]. VEGF Trap did not bind human VEGF-C or human VEGF-D (Online Resource 1, Fig. 5). The K_D values for the interaction between VEGF Trap and VEGF-A from mouse, rat and rabbit were similar to those of human and ranged from 0.471 to 0.776 pM. VEGF Trap also bound human and murine PlGF-2 with a K_D of 38.9 and 3.32 pM, respectively (Table 1 and Online Resource 1, Table 1). In contrast, bevacizumab and ranibizumab are specific for human and non-human primate VEGF-A, and do not effectively bind or neutralize rodent VEGF [46–48].

Binding parameters for VEGF Trap, ranibizumab and bevacizumab interactions with human VEGF-A_{165} and PlGF-2

While all three VEGF inhibitors bound human VEGF-A_{165} with high affinity, the K_D for VEGF Trap binding of VEGF-A_{165} was approximately 100-fold lower (i.e. the binding affinity was ~100-fold tighter) than that for ranibizumab or bevacizumab (Table 1). Specifically, the K_D value for VEGF Trap was 0.490 pM, while those for ranibizumab and bevacizumab were 46 and 58 pM, respectively. The lower K_D value for VEGF Trap binding VEGF-A_{165} was primarily attributable to a significantly faster association rate (k_a) that was 77- and 256-fold faster than that for bevacizumab and ranibizumab, respectively (Table 1). VEGF Trap also bound human PlGF-2 with high affinity (K_D = 38.9 pM), whereas no binding was detected between ranibizumab or bevacizumab and human PlGF-2 (Table 1). Biacore kinetic sensorgrams analyzed for association and dissociation rate constants are provided in Online Resource 1, Figures 1–4.

To confirm the surface kinetic data determined using SPR-Biacore, the binding interactions between soluble VEGF Trap, bevacizumab or ranibizumab and human VEGF-A_{165} were also compared in solution equilibrium assays using KinExA methodology. As shown in Table 2 and Online Resource 1, Figures 6 and 7, the absolute K_D values and 95% confidence interval obtained for the VEGF inhibitors binding to VEGF-A_{165}, were comparable to those obtained with SPR-based measurements. Similarly, VEGF Trap binding affinities for VEGF-A_{121}, VEGF-$B_{(10\text{-}108)}$, and PlGF were also comparable between SPR and solution based equilibrium assays.

Effects of VEGF Trap, ranibizumab and bevacizumab on VEGF-A or PlGF-2 induced activation of VEGFR1

To determine the ability of VEGF Trap, ranibizumab and bevacizumab to block human VEGF-A or PlGF-2 induced VEGFR1 activation in vitro, a VEGFR1 specific luciferase assay was developed, which used the human cell line HEK293 transfected with an NFκB-luciferase reporter plasmid and human VEGFR1 (Fig. 1). Notably in this assay, the potency of ranibizumab for blocking 20 pM VEGF-A_{121} or VEGF-A_{165} induced luciferase activity through VEGFR1 was only slightly greater than that of bevacizumab. Ranibizumab exhibited IC_{50} values (50% inhibitory concentration) of 675 and 1,140 pM, while IC_{50} values for bevacizumab were 845 and 1,476 pM for VEGF-A_{121} or VEGF-A_{165}, respectively. In contrast, VEGF Trap exhibited a 45–92-fold greater blocking potency compared to either ranibizumab or bevacizumab, with IC_{50} values of 15 and 16 pM for blocking VEGFR1 activation by 20 pM VEGF-A_{121} or VEGF-A_{165}, respectively (Table 3; Fig. 1). VEGF Trap also blocked luciferase activity induced by human PLGF-2 (40 pM) or mouse PlGF-2 (20 pM) with IC_{50} values of 2.9 nM and 104 pM, respectively. In contrast, neither bevacizumab nor ranibizumab showed ability to block human or mouse PlGF-2 under these experimental conditions.

Table 1 Kinetic binding parameters for VEGF Trap, ranibizumab and bevacizumab binding to human VEGF family ligands determined by SPR-Biacore

VEGF inhibitor	Ligand	Kinetic binding parameters		
		$k_a/10^5$ (M^{-1} s^{-1})	$k_d/10^{-5}$ (s^{-1})	K_D (pM)
VEGF Trap[a]	VEGF-A_{121}	375.0 (5.0)	1.35 (.02)	0.360
VEGF Trap[a]	VEGF-A_{165}	410.0 (10.0)	2.01 (.01)	0.490
Ranibizumab[b]	VEGF-A_{165}	1.6 (0.003)	0.73 (.005)	46
Bevacizumab[a]	VEGF-A_{165}	5.3 (0.01)	3.10 (.02)	58
hVEGFR1-Fc[a]	VEGF-A_{165}	300.0 (20.0)	28.0 (1.0)	9.33
hVEGFR2-Fc[a]	VEGF-A_{165}	152.0 (5.0)	135 (6.0)	88.8
VEGF Trap[a]	PlGF-2	17.5 (0.06)	6.81 (.03)	38.9
Ranibizumab[b]	PlGF-2	NB	NB	NB
Bevacizumab[a]	PlGF-2	NB	NB	NB
VEGF Trap[a]	VEGF-$B_{(10\text{-}108)}$	352.0 (3.0)	6.74 (.09)	1.92

Numbers in parentheses represent the standard error of the kinetic fit

NB No binding under assay conditions used

[a] VEGF inhibitor captured on a Protein A-coupled sensor chip

[b] VEGF inhibitor captured on an anti-human Fab polyclonal antibody-captured sensor chip

Table 2 Solution binding parameters for VEGF Trap, ranibizumab and bevacizumab binding to human VEGF family ligands determined by KinExA equilibrium assays

VEGF inhibitor	Ligand	Kinexa equilibrium binding parameters	
		K_D (pM)	K_D range (pM)[a]
VEGF Trap	VEGF-A_{165}	0.66	0.36–1.06
Ranibizumab	VEGF-A_{165}	20.6	10.9–36.3
Bevacizumab	VEGF-A_{165}	35.1	12.2–82.9
VEGF Trap	VEGF-A_{121}	0.18	0.08–0.32
VEGF Trap	PlGF-2	20.7	13.7–29.3
VEGF Trap	VEGF-$B_{(10\text{-}108)}$	17.5	12.9–22.9

[a] 95% confidence interval

Effects of VEGF Trap, ranibizumab and bevacizumab on VEGF-A induced activation of VEGFR2

To determine the ability of VEGF Trap, ranibizumab and bevacizumab to block VEGFR2 activation in vitro, a VEGFR2 specific luciferase assay was developed, which used the human cell line HEK293 transfected with an NFκB-luciferase reporter plasmid and human VEGFR2 (Fig. 2). As for VEGFR1, VEGF Trap efficiently blocked VEGFR2 signaling induced by 20 pM of human VEGF-A_{121} or VEGF-A_{165} (IC_{50} of 16 and 26 pM, respectively). VEGF Trap was again markedly more potent in blocking VEGF-mediated VEGFR2 activation than either ranibizumab or bevacizumab (33–51-fold more potent, see Fig. 2; Table 3). As expected, hPlGF-2 was not able to activate VEGFR2 in this assay.

The effect of VEGF Trap, ranibizumab and bevacizumab on VEGF-A_{165} induced calcium mobilization in human endothelial cells

The ability of the three VEGF inhibitors to block human VEGF-A_{165} induced activation of VEGF receptors was also tested in human endothelial cells. A VEGF-A_{165} induced calcium mobilization assay was developed using HUVEC [49, 50], which express native VEGFR1 and VEGFR2 (Fig. 3). Interestingly in this assay, bevacizumab was ~5-fold more potent than ranibizumab at blocking VEGF-A_{165} induced calcium mobilization. Nevertheless, the IC_{50} for VEGF Trap was ~27-fold lower than that of bevacizumab and ~129-fold lower than ranibizumab, confirming the greater potency of VEGF Trap for blocking VEGFR1 and VEGFR2 activation in vitro (Table 3; Fig. 3). The relative potency of VEGF blockers in this acute assay may reflect differences in their association rate constants.

The effect of VEGF Trap, bevacizumab and ranibizumab on HUVEC migration induced by $VEGF_{165}$ or PlGF-2

Endothelial cell migration plays a central part in the process of angiogenesis and, consistent with its pro-angiogenic profile, VEGF acts as a chemoattractant for endothelial cells [51]. To determine the ability of VEGF Trap, ranibizumab and bevacizumab to block human VEGF-A_{165} induced cell migration, HUVEC mobility was assessed in a modified Boyden chamber assay. None of the VEGF inhibitors affected basal endothelial cell migration in the absence of test ligands (data not shown). In the presence of VEGF-A_{165} (130 pM), VEGF Trap blocked VEGF-A_{165} induced cell migration in a dose-dependent manner (Fig. 4). At a 1:1 molar ratio of VEGF Trap and VEGF-A_{165}, cell migration was reduced by approximately 90%. Ranibizumab and bevacizumab also inhibited cell migration in a dose–dependent manner (Fig. 4) but were less potent than VEGF Trap, requiring a 10- to 100-fold greater molar concentration of inhibitor to produce an equivalent level of inhibition of cell migration due to VEGF-A_{165} activation.

PlGF also acts as a chemoattractant for endothelial cells through VEGFR1 [52]. Again, the modified Boyden chamber assay was used to test the ability of the VEGF inhibitors to block HUVEC migration stimulated by human PlGF-2. As shown in Fig. 4 (inset), a 100-fold excess of VEGF Trap blocked cell migration induced by human

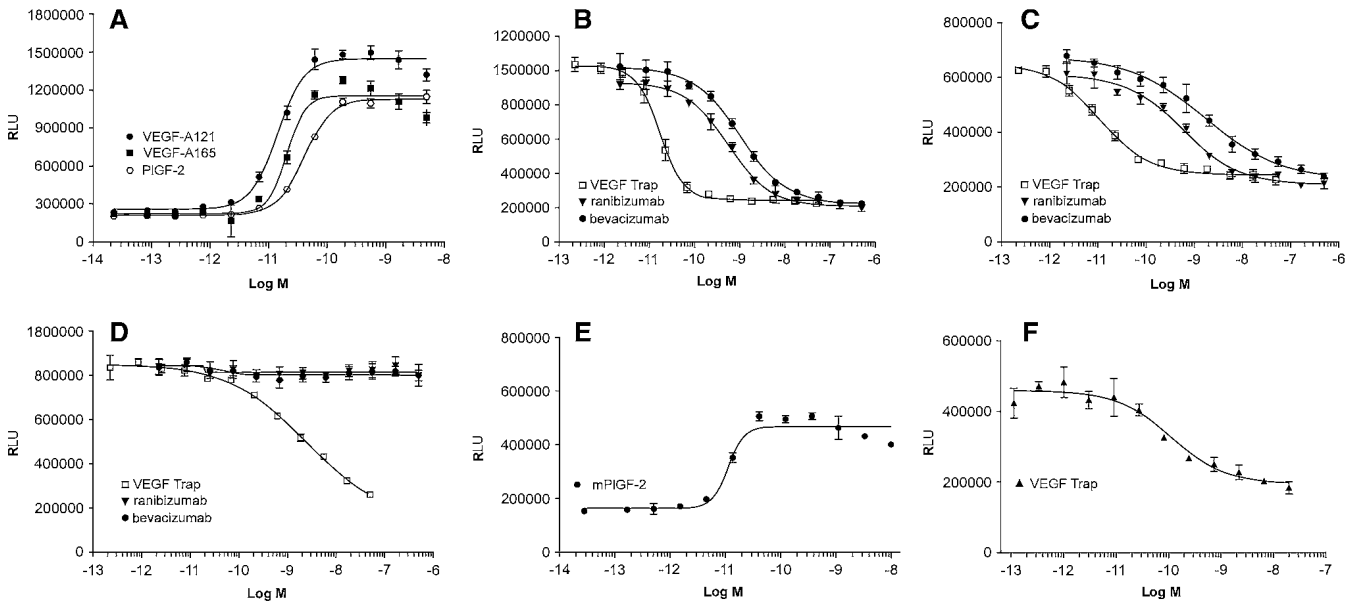

Fig. 1 The effects of VEGF Trap, ranibizumab and bevacizumab on luciferase activation induced by VEGF-A_{121}, VEGF-A_{165}, human PlGF-2 (hPlGF-2) or mouse PlGF-2 (mPLGF-2) in HEK293/VEGFR1 cells. **a** Dose response curves for VEGF-A_{121}, VEGF-A_{165} and hPlGF-2 yielded EC_{50} values of 13, 17, and 29 pM, respectively. **b** Serial dilutions of VEGF Trap (*open box*), ranibizumab (*triangle*), or bevacizumab (*closed circle*) were added to HEK293/VEGFR1 cells along with 20 pM of VEGF-A_{121}. **c** Serial dilutions of VEGF Trap (*open box*), ranibizumab (*triangle*), or bevacizumab (*closed circle*) were added to HEK293/VEGFR1 cells along with 20 pM of VEGF-A_{165}. **d** Serial dilutions of VEGF Trap (*open box*), ranibizumab (*triangle*), or bevacizumab (*closed circle*) were added to HEK293/VEGFR1 cells along with 40 pM of human PlGF-2. **e** Dose response curve for mPlGF-2 yielded an EC_{50} value of 10 pM (**f**). Serial dilutions of VEGF Trap were added to HEK293/VEGFR1 cells along with 20 pM of mPlGF-2. The cells were incubated for 6 h and OneGlo luciferase substrate was then added to each well. The plates were read on a luminometer and the data were plotted using a four parameter curve fit with GraphPad Prism. Each point represents a replica of 3 wells at each concentration

Table 3 Summary of IC_{50} values for VEGF Trap, ranibizumab and bevacizumab blocking VEGF-A or PlGF-2 induced activation of VEGFR1 and VEGFR2

VEGF inhibitor	VEGFR1 cell line				VEGFR2 cell line		Ca^{2+} mobilization in HUVE cells
	IC_{50} at 20 pM hVEGF-A_{121}	IC_{50} at 20 pM hVEGF-A_{165}	IC_{50} at 40 pM hPlGF-2	IC_{50} at 20 pM mPlGF-2	IC_{50} at 20 pM hVEGF-A_{121}	IC_{50} at 20 pM hVEGF-A_{165}	IC_{50} at 20 pM hVEGF-A_{165}
VEGF Trap	15 pM (2.4)	16 pM (2.2)	2,890 pM (227)	104 pM (23)	16 pM (2.5)	26 pM (11)	2.6 pM (1.2)
Ranibizumab	675 pM (165)	1,140 pM (226)	NB	NB	576 pM (84)	845 pM (185)	334.9 pM (61.1)
Bevacizumab	854 pM (214)	1,476 pM (288)	NB	NB	630 pM (66)	1,323 pM (491)	70.8 pM (20.1)

Numbers in parentheses represent standard error of the mean

The IC_{50} numbers were obtained from at least 3 separate experiments

hVEGF: human VEGF; hPlGF-2: human PlGF-2; mPLGF-2: mouse PlGF-2

NB No blocking activity observed under the assay conditions used

PlGF-2 (7.1 nM) or mouse PlGF-2 (3.5 nM) by approximately 80%. In contrast, ranibizumab and bevacizumab did not inhibit cell migration induced by either human or mouse PlGF–2.

Discussion

The experiments described herein provide a comprehensive assessment of the ability of VEGF Trap, ranibizumab and bevacizumab to bind and block the activity of VEGF family ligands in vitro, under identical experimental conditions. The data demonstrate that VEGF Trap binds human VEGF-A with higher affinity and a significantly faster association rate, thus neutralizing VEGF-A with greater potency than ranibizumab or bevacizumab. In addition, the studies show that VEGF Trap has the unique ability to bind the additional VEGF family ligands, VEGF-B and PlGF. Moreover, VEGF Trap also bound VEGF-A and PlGF isoforms from all mammalian species tested with similar high affinity, while neither ranibizumab nor bevacizumab efficiently bind and neutralize mouse or rat VEGF-A [46–48].

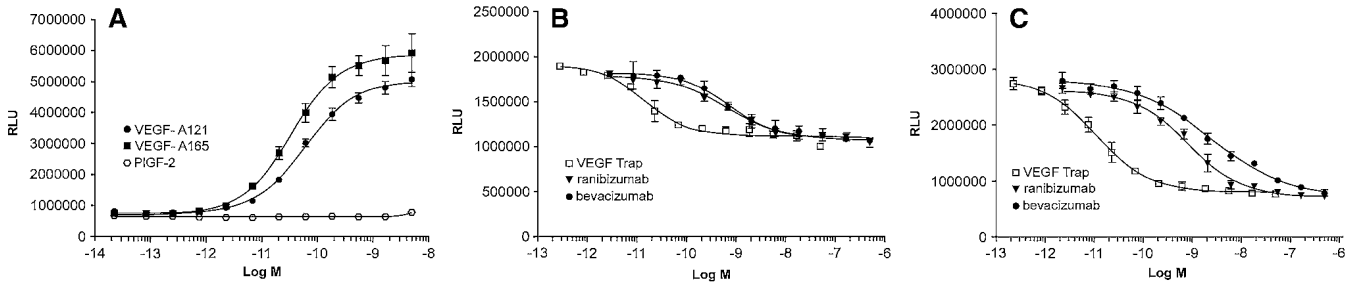

Fig. 2 The effects of VEGF Trap, ranibizumab and bevacizumab on luciferase activation induced by VEGF-A_{121} and VEGF-A_{165} in HEK293/VEGFR2 cells. **a** Dose response curves for VEGF-A_{121} and VEGF-A_{165} with EC_{50} values of 70 and 30 pM, respectively. PlGF-2 was not active in this assay. **b** Serial dilutions of VEGF Trap (*open box*), ranibizumab (*triangle*) or bevacizumab (*closed circle*) were added to HEK293/VEGFR2 cells along with 20 pM of VEGF-A_{121}. **c** Serial dilutions of VEGF Trap (*open box*), ranibizumab (*triangle*) or bevacizumab (*closed circle*) were added to HEK293/VEGFR2 cells along with 20 pM of VEGF-A_{165}. The cells were incubated for 6 h and OneGlo luciferase substrate was then added to each well. The plates were read on a luminometer and the data were plotted using a four parameter curve fit with GraphPad Prism. Each point represents a replica of 3 wells at each concentration

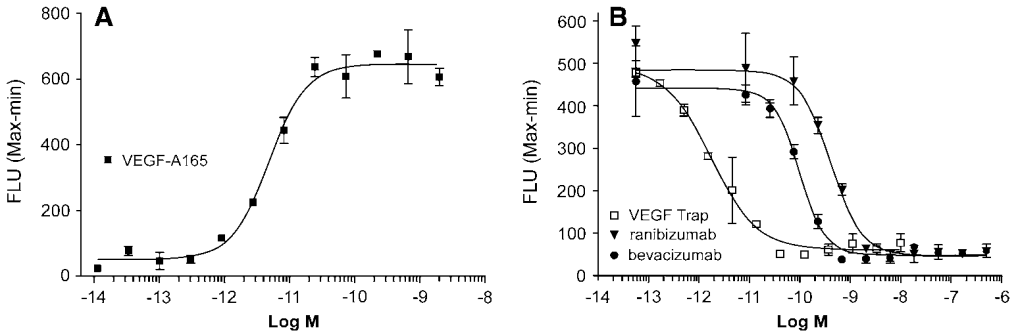

Fig. 3 The effects of VEGF Trap, ranibizumab and bevacizumab on calcium mobilization induced byVEGF-A_{165} in HUVEC. **a** A dose–response curve generated using serial dilutions of VEGF-A_{165} (4.0 nM–0.023 pM) resulted in an EC_{50} value of 5 pM. **b** Serial dilutions of VEGF Trap (*open box*), ranibizumab (*triangle*) or bevacizumab (*closed circle*) were added to HUVEC along with 20 pM of VEGF-A_{165}. The VEGF-A_{165} was preincubated with the inhibitors for 10 min at 25°C. The solution was added to HUVEC preloaded with fluo-4 and the fluorescence of the well was determined on a FLIPR instrument. The data were plotted using a four parameter curve fit with GraphPad Prism. Each point represents duplicate wells at each concentration

Several published papers have provided binding affinity data for ranibizumab's interactions with human VEGF-A [28, 36, 37]. However, to date, binding affinity and specificity data have been provided only for the monovalent Fab fragment of bevacizumab (Fab-12), and not the full bivalent bevacizumab molecule itself. The equilibrium dissociation constant (K_D) for Fab-12 has been variously reported as 1.8 nM [36] or 20 nM [28], indicating an affinity improvement of ranibizumab over Fab-12 of 10–100-fold. Likewise, ranibizumab has been reported to be 30–100-fold more potent than Fab-12 in bioassays measuring VEGF-induced endothelial cell mitogenesis [26]. However, measuring the kinetic binding parameters or in vitro activity of the Fab-12 fragment does not take into account potential avidity interactions of bivalent antibodies, especially when the binding partner is a dimeric ligand such as VEGF-A. These types of avidity driven interactions can significantly increase binding affinity, and potentially the potency of the bivalent antibody relative to that of the monovalent antigen binding fragment in cell-based assays and in vivo.

In the present study, Biacore and KinExA analyses have demonstrated that the equilibrium dissociation constants for VEGF Trap binding VEGF-A_{121} and VEGF-A_{165}, were less than 1 pM, in close agreement with earlier reports [34]. In contrast, ranibizumab exhibited a K_D of 46 pM for VEGF-A_{165}. While this represents an approximately 3–4-fold greater affinity for VEGF-A relative to SPR Biacore values previously reported for ranibizumab ($K_D \leq 140$ pM, [28]; $\leq$179 pM, [37]), it is nevertheless an ~94-fold weaker binding for VEGF-A_{165} relative to VEGF Trap (0.490 pM) (Table 4). Similarly, the K_D of soluble VEGF Trap for VEGF-A_{165}, as determined by KinExA was 0.66 pM, while that of ranibizumab was 20.6 pM, approximately 30-fold lower than that of VEGF Trap.

Interestingly, the K_D of bevacizumab for VEGF-A_{165} as determined by Biacore was 58 pM, markedly lower than that reported previously for Fab-12 [28, 36] and within

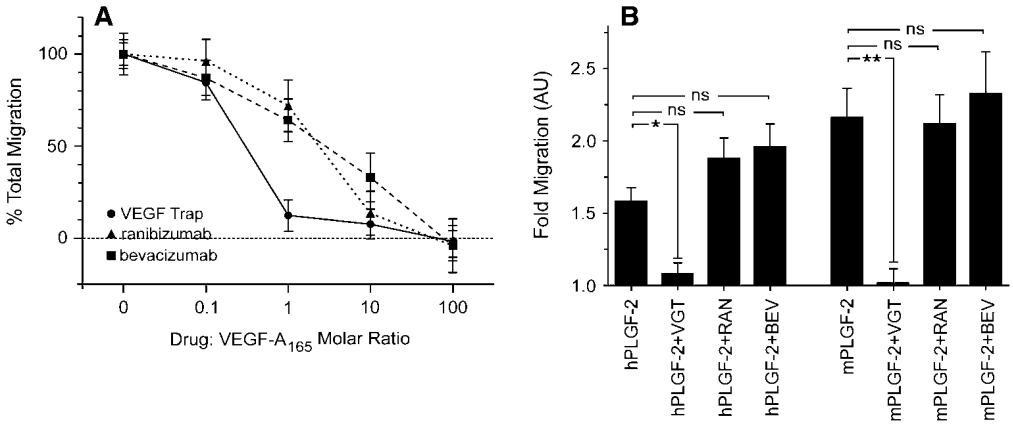

Fig. 4 The effects of VEGF Trap, ranibizumab and bevacizumab on HUVEC migration. **a** HUVEC were placed in the upper compartment of the Boyden chamber and allowed to migrate towards basal media containing 0.1% fetal bovine serum with or without VEGF-A_{165} or VEGF-A_{165} mixed with four concentrations each of VEGF Trap (*circles*, *solid line*), ranibizumab (*triangles*, *dotted line*) or bevacizumab (*squares*, *dashed line*) ranging from 0.013 to 13 nM. The percentage of total migration (*y*-axis) was calculated as $(F_{Drug} - F_{Basal})/(F_{Total} - F_{Basal}) \times 100$; where F_{Total} is fluorescence in the presence of VEGF-A_{165}, F_{Basal} is fluorescence in the absence of VEGF-A_{165}, and F_{Drug} is fluorescence in the presence of VEGF-A_{165} mixed with drug at a specific molar ratio (*x*-axis). **b** HUVEC migration was assessed in the absence and presence of human PLGF-2 (hPLGF-2) or mouse PLGF-2 (mPLGF-2) with and without a 100-fold molar excess of VEGF Trap (VGT), ranibizumab (RAN) or bevacizumab (BEV). Fold migration (*y*-axis) was calculated as the ratio F/F_{Basal}; where F is the total fluorescence measured for the indicated condition (*x*-axis) and F_{Basal} is the fluorescence in the absence of either hPLGF-2 or mPLGF-2. Statistical significance: $^*P < 0.05$; $^{**}P < 0.01$; ns, no significance. Values and *error bars* represent the average value and standard error of the mean from at least three independent experiments with each experiment containing four biological replicates per condition (total $n = 12$–16 per condition) for all conditions tested. *AU* arbitrary units

Table 4 Relative VEGF binding affinities and potency of VEGFR signaling blockade

Parameter	Ranibizumab	Bevacizumab	VEGF Trap
Affinity for VEGF-A_{165} (Biacore)	1.0	0.79	94.0
Potency of blocking VEGF (20 pM) mediated signaling			
VEGFR1			
VEGF-A_{121}	1.0	0.79	45.0
VEGF-A_{165}	1.0	0.77	71.3
VEGFR2			
VEGF-A_{121}	1.0	0.91	36.0
VEGF-A_{165}	1.0	0.64	32.5
HUVEC			
VEGF-A_{165}	1.0	4.73	128.8

The relative fold differences for the K_D and IC_{50} values for bevacizumab and VEGF Trap are expressed relative to values for ranibizumab (set at 1). Higher numbers reflect tighter binding or increased potency in the indicated assays. Raw values used to calculate relative fold differences were taken from Table 1 and Table 3

twofold of the binding affinity of ranibizumab. This was also the case for soluble equilibrium binding of bevacizumab in the Kinexa assay (K_D of 35.1 pM for bevacizumab and 20.6 pM for ranibizumab), and most likely reflects avidity interactions of the bivalent, full antibody molecule. However, like other conventional antibodies that bind dimeric targets, bevacizumab has the potential to form higher order complexes with VEGF, which under some conditions may act as immune complexes [53]. In contrast, each molecule of VEGF Trap forms an inert 1 to 1 complex with VEGF, and cannot form higher order complexes [35].

The K_D for VEGF Trap binding of VEGF-A documented in the SPR Biacore and KinExA assays translated into increased potency relative to ranibizumab and bevacizumab in all of the bioassays employed. Specifically, VEGF Trap was ~33–71-fold more potent than ranibizumab at inhibiting VEGF-A induced receptor activation in cell lines expressing either VEGFR1 or VEGR2 (Table 4). Moreover, VEGF Trap was highly effective at reducing VEGF-A-induced calcium signaling in HUVEC, where it was ~130-fold more potent than ranibizumab (Table 3). In addition to promoting endothelial cell proliferation and vascular permeability, VEGF-A is powerful mediator of endothelial cell migration [25]. Consistent with the high potency of VEGF Trap to neutralize VEGF receptor activation, VEGF Trap was highly effective at blocking HUVEC migration induced by VEGF-A_{165}. In agreement with previous reports [38, 54], ranibizumab and bevacizumab were also effective at decreasing HUVEC migration, though they were less potent than VEGF Trap, such that a 10- to 100-fold molar excess of ranibizumab or bevacizumab was required to completely block VEGF-induced HUVEC migration, while VEGF Trap was effective at equimolar concentrations.

In the present studies, the ability of ranibizumab to neutralize VEGF-A activity in cell-based assays was only moderately better than that of bevacizumab. For example, the IC_{50} values for inhibition of activation of VEGFR1 and VEGFR2 by 20 pM VEGF-A were less than twofold lower for ranibizumab than bevacizumab (Table 3). This corresponded closely to the observed differences in the binding kinetics of ranibizumab and the full length bivalent bevacizumab antibody, where the K_D of bevacizumab for VEGF-A was within twofold of that of ranibizumab, as determined by both Biacore and KinExA assays (Tables 1, 2, 4). Interestingly, bevacizumab was ~fivefold more potent than ranibizumab at neutralizing VEGF-A induced calcium influx in HUVEC. This finding may reflect the ~threefold faster association rate of bevacizumab (Table 1), as k_a is a critical determinant of potency in relatively acute cell-based assays.

The above findings stand in contrast to those recently described by Yu et al. [40]. Specifically, ranibizumab and VEGF Trap were reported to be equally effective in blocking endothelial cell proliferation and migration in HUVEC, while bevacizumab was approximately tenfold less potent. Evaluation of MAPK phosphorylation, which reflects activation of intracellular signaling pathways downstream of the VEGF receptors, showed that all three agents completely blocked MAPK phosphorylation when the VEGF inhibitors were pre-incubated with VEGF-A overnight, before addition to the cells, while VEGF Trap was more potent than either ranibizumab or bevacizumab when preincubated with VEGF-A for shorter time periods (5 and 30 min). The apparent discrepancies with findings of the present study are likely attributable to the fact that Yu et al. [40] utilized higher concentrations of exogenous VEGF-A in all of their cell-based assays, in the range of 0.15–1.25 nM. In other words, the concentration of ligand was above the K_D values for ranibizumab and bevacizumab, as well as VEGF Trap (Table 1); under these assay conditions the IC_{50} is determined primarily by the concentration of ligand relative to that of the blocker, rather than by the binding affinity. Therefore, precise evaluation of the relative activity of different inhibitors in bioassays requires utilization of the lowest amount of VEGF-A practicable, so that the IC_{50} can reflect differences in binding affinity and not simply inhibition of activity at stoichiometric concentrations of inhibitor, which predominates under conditions where both antibody and ligand concentrations are well above the K_D.

For example, several studies published to date have reported that ranibizumab and bevacizumab are equally effective in neutralizing VEGF-induced endothelial cell proliferation at 'clinically relevant' concentrations, i.e., those that obtain in the eye shortly following intravitreal injection [38, 55], which are well above the equilibrium dissociation constants for both antibodies. Differences in activity emerge only when lower concentrations of drug are evaluated, or where acute bioassay readouts reflect differences in association rate constants. For example, Klettner et al. [39], reported that at lower concentrations ranibizumab more efficiently neutralized VEGF secreted from retinal-choroidal cultures than did bevacizumab. Costa et al. [54] also reported that ranibizumab was moderately more effective at inhibiting endothelial cell proliferation than bevacizumab, while in an acute assay bevacizumab more effectively inhibited VEGF-stimulated VEGFR2 and MAPK phosphorylation in human microvascular endothelial cells.

Binding kinetics and affinity are key determinants of the biological activity of antibody-like drugs. In addition to binding affinity, the activity of a drug is also influenced by the concentration present at the site of target activity, which is in turn dependent on tissue distribution and clearance, with larger molecules typically having longer half-lives. With respect to ocular delivery, it was estimated that biologically active concentrations of ranibizumab would be maintained in the vitreous for approximately 4 weeks following intravitreal injections of 0.5 mg [26, 56]. Indeed, monthly injection of 0.5 mg ranibizumab has proven to be the most effective regimen for the treatment of neovascular AMD, based on the outcomes of several phase III clinical trials [29, 57–60], and is the currently approved regimen for treating this disease. Using mathematical modeling, and the then available information on intravitreal clearance and binding affinities, Stewart [61] predicted that the anti-VEGF bioactivity present in the vitreous 30 days following intravitreal (IVT) injection of 0.5 mg ranibizumab would be equivalent to that present at 27–38 days following an injection of 1.25 mg bevacizumab. More recently, using the same modeling approach, Stewart and Rosenfeld [62] predicted the intraocular biological activity comparable to that of 0.5 mg ranibizumab at 30 days post-injection would be maintained for approximately twice that time following injection of 0.5 mg VEGF Trap, and potentially as long as 12 weeks following IVT injection of 2 mg VEGF Trap. This substantial theoretical increase in the relative duration of VEGF neutralizing activity was driven primarily by the higher binding affinity of VEGF Trap for VEGF-A compared to ranibizumab, with a lesser contribution of the predicted longer intravitreal half-life of VEGF Trap (e.g. 4.7 days in rabbits, compared to ~2.9 days for ranibizumab, [63, 64]. Thus, modeling studies suggested that intravitreal administration of the current clinical doses of ranibizumab and bevacizumab would result in effective VEGF-A inhibition of relatively similar duration, while VEGF Trap might be as efficacious as ranibizumab, but with less frequent dosing.

While it remains to be unequivocally determined whether the durations of bioactivity of these VEGF blockers predicted by the above modeling studies will be confirmed by clinical experience, data available to date suggest that the results of these modeling studies may prove reasonably accurate. For example, several clinical studies have investigated alternative strategies to monthly ranibizumab injection, including quarterly (every 3 months) or pro renata (PRN) injections following a treatment initiation phase comprising 3 monthly loading doses. Most large, well-controlled studies conducted to date have found that improvements in visual acuity attained during the initiation phase are lost during the quarterly or PRN maintenance phases [58–60, 65]. The recent CATT Trial produced the best results obtained to date using PRN dosing of ranibizumab, which was statistically non-inferior to that of monthly ranibizumab. This may reflect the fact that in the CATT study patients were followed monthly and rigorous criteria were established for retreatment [32]. Nevertheless, the mean improvement in visual acuity attained in CATT using PRN ranibizumab was 1.6 letters below that of monthly ranibizumab, at the end of 1 year. Importantly, the effect of bevacizumab given monthly on visual outcomes was within 0.4 letters of that obtained with ranibizumab given monthly. However, bevacizumab administered PRN failed non-inferiority comparisons to monthly regimens for both antibodies, despite the fact that it was administered more frequently than ranibizumab PRN. These findings are in line with the predictions of modeling studies, as well as the results of the present report, which indicate that the binding affinity and in vitro activity of bevacizumab are moderately less than those of ranibizumab. Several additional large scale controlled trials are currently in progress to evaluate the effects of these two antibodies in patients with neovascular AMD, using both fixed and PRN dosing schedules [31]. These studies, together with outcomes from the CATT trial following longer-term treatment, should provide a clearer picture of the relative clinical activity, and safety, of ranibizumab and bevacizumab.

Although fewer clinical trials have been conducted to date with VEGF Trap-Eye, the available data suggest that, as predicted in modeling studies, the increased affinity of VEGF Trap for VEGF-A may be reflected in clinical activity. For example, in a recent double masked phase 2 trial (CLEAR-IT 2) patients with exudative AMD were randomized to an initiation phase of either a single, or monthly IVT injections of VEGF Trap for 12 weeks at doses of either 0.5 or 2 mg. Patients were then switched to a PRN regimen at their originally assigned doses. Reports of the 1 year results described maintenance of statistically significant improvements in vision, retinal thickness and size of the CNV lesions [66, 67]. Here, patients initially dosed on a 2.0 mg monthly schedule received, on average, only 1.6 additional injections during the 40 week PRN period, and those initially dosed on a 0.5 mg monthly schedule received, on average, 2.5 injections. More recently, 1 year results have been reported from two phase 3 clinical trials (VIEW 1 and VIEW 2) in which VEGF Trap-Eye was dosed monthly at 0.5 or 2.0 mg in patients with wet AMD, or at 2.0 mg every other month following an initiation phase of 3 monthly doses. All VEGF Trap-Eye treatment arms, including the 2.0 mg every other month treatment regimen, produced improvements in visual acuity that were equivalent to that obtained in patients dosed with 0.5 mg ranibizumab monthly [68, 69].

The development of ranibizumab has demonstrated that binding multiple VEGF-A isoforms is of substantial benefit in the treatment of neovascular AMD, compared to treatment with pegaptanib, which binds only the 165 isoform of VEGF-A [23, 29, 57, 70–72]. Recent studies have implicated additional VEGF family members, notably PlGF and VEGF-B, in the pathology of ocular vascular diseases as well as some cancers [8, 16, 73]. Therefore, a unique potential advantage of VEGF Trap relative to ranibizumab and bevacizumab is that it also binds VEGF-B and PlGF with high affinity. PlGF in particular has been shown to act in concert with VEGF-A to promote pathological angiogenesis, vascular leak and inflammation [8, 11, 18, 74], and like VEGF-A, levels of PlGF are elevated in the eyes of patients with diverse ocular vascular diseases, including wet AMD [15, 75]. Furthermore, genetic deletion or pharmacological inhibition of PlGF has been shown to inhibit choroidal neovascularization and inflammation, and to enhance the activity of VEGF-A targeted molecules in animal models of choroidal neovascularization [13, 16]. More recently, it has been reported that overexpression of VEGF-B in the murine retina, via adeno-associated virus gene transfer, also promotes retinal and choroidal neovascularization and blood-retinal barrier breakdown [76]. These studies suggest that targeting PlGF and VEGF-B, in addition to VEGF-A, could be of added benefit in treating angiogenic ocular disorders.

Similarly, targeting these additional factors may be important in the oncology setting. First, these VEGF family ligands, most notably PlGF, have been implicated in promoting tumor growth [8, 16, 73], therefore inhibiting these factors, in addition to VEGF-A, may prove therapeutically beneficial in treating cancer. Bevacizumab, which inhibits only VEGF-A, is approved for use in various cancer treatment settings. VEGF Trap, while not currently approved for use, has also exhibited efficacy in the oncology setting. Most recently it was reported to have an overall survival benefit in metastatic colorectal cancer [77]. Changes in the levels of PlGF and other factors have been observed in patients with metastatic colorectal cancer treated with bevacizumab, during and following cessation

of treatment [78, 79], and the authors of both studies suggested that increases in other pro-angiogenic factors may be one mechanism underlying the development of resistance to anti-VEGF therapy. However, further prospective evaluations are needed to confirm these hypotheses.

In summary, VEGF Trap demonstrated higher binding affinity for VEGF-A isoforms and greater potency in vitro than ranibizumab or bevacizumab. These attributes, in addition to its ability to bind VEGF-B and PlGF, could be of added benefit in treating various ocular disorders and cancers.

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Conflict of interest All of the authors are employed by Regeneron Pharmaceuticals, Inc. and hold equity positions in the company.

References

1. Grothey A, Galanis E (2009) Targeting angiogenesis: progress with anti-VEGF treatment with large molecules. Nat Rev Clin Oncol 6:507–518
2. Bressler SB (2009) Introduction: understanding the role of angiogenesis and antiangiogenic agents in age-related macular degeneration. Ophthalmology 116:S1–S7
3. Bressler NM (2004) Age-related macular degeneration is the leading cause of blindness. JAMA 291:1900–1901
4. Folkman J (2007) Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug 6:273–286
5. Baluk P, Hashizume H, McDonald DM (2005) Cellular abnormalities of blood vessels as targets in cancer. Curr Opin Genet Dev 15:102–111
6. Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257
7. Crawford Y, Ferrara N (2009) VEGF inhibition: insights from preclinical and clinical studies. Cell Tissue Res 335:261–269
8. Cao Y (2009) Positive and negative modulation of angiogenesis by VEGFR1 ligands. Sci Signal 2:rel
9. Dvorak HF, Nagy JA, Feng D, Brown LF, Dvorak AM (1999) Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. Curr Top Microbiol Immunol 237:97–132
10. Miller JW, Adamis AP, Aiello LP (1997) Vascular endothelial growth factor in ocular neovascularization and proliferative diabetic retinopathy. Diabetes Metab Rev 13:37–50
11. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG (2001) Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 7:575–583
12. Mitamura Y, Tashimo A, Nakamura Y, Tagawa H, Ohtsuka K, Mizue Y, Nishihira J (2002) Vitreous levels of placenta growth factor and vascular endothelial growth factor in patients with proliferative diabetic retinopathy. Diabetes Care 25:2352
13. Rakic JM, Lambert V, Devy L, Luttun A, Carmeliet P, Claes C, Nguyen L, Foidart JM, Noel A, Munaut C (2003) Placental growth factor, a member of the VEGF family, contributes to the development of choroidal neovascularization. Invest Ophthalmol Vis Sci 44:3186–3193
14. Schlingemann RO, Witmer AN (2009) Treatment of retinal diseases with VEGF antagonists. Prog Brain Res 175:253–267
15. Witmer AN, Vrensen GF, Van Noorden CJ, Schlingemann RO (2003) Vascular endothelial growth factors and angiogenesis in eye disease. Prog Retin Eye Res. 22:1–29
16. Van de Veire S, Stalmans I, Heindryckx F, Oura H, Tijeras-Raballand A, Schmidt T, Loges S, Albrecht I, Jonckx B, Vinckier S, Van Steenkiste C, Tugues S, Rolny C, De Mol M, Dettori D, Hainaud P, Coenegrachts L, Contreres JO, Van Bergen T, Cuervo H, Xiao WH, Le Henaff C, Buysschaert I, Kharabi Masouleh B, Geerts A, Schomber T, Bonnin P, Lambert V, Haustraete J, Zacchigna S, Rakic JM, Jimenez W, Noel A, Giacca M, Colle I, Foidart JM, Tobelem G, Morales-Ruiz M, Vilar J, Maxwell P, Vinores SA, Carmeliet G, Dewerchin M, Claesson-Welsh L, Dupuy E, Van Vlierberghe H, Christofori G, Mazzone M, Detmar M, Collen D, Carmeliet P (2010) Further pharmacological and genetic evidence for the efficacy of PlGF inhibition in cancer and eye disease. Cell 141:178–190
17. Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9:669–676
18. Takahashi H, Shibuya M (2005) The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. Clin Sci (Lond) 109:227–241
19. Alitalo K, Carmeliet P (2002) Molecular mechanisms of lymphangiogenesis in health and disease. Cancer Cell 1:219–227
20. Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K, Breitman M, Alitalo K (1998) Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. Science 282:946–949
21. Tammela T, Zarkada G, Wallgard E, Murtomäki A, Suchting S, Wirzenius M, Waltari M, Hellström M, Schomber T, Peltonen R, Freitas C, Duarte A, Isoniemi H, Laakkonen P, Christofori G, Ylä-Herttuala S, Shibuya M, Pytowski B, Eichmann A, Betsholtz C, Alitalo K (2008) Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature 454:656–660
22. Tammela T, Zarkada G, Nurmi H, Jakobsson L, Heinolainen K, Tvorogov D, Zheng W, Franco CA, Murtomäki A, Aranda E, Miura N, Ylä-Herttuala S, Fruttiger M, Mäkinen T, Eichmann A, Pollard JW, Gerhardt H, Alitalo K (2011) VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling. Nat Cell Biol 13:1202–1213
23. Gragoudas ES, Adamis AP, Cunningham ET Jr, Feinsod M, Guyer DR (2004) Pegaptanib for neovascular age-related macular degeneration. N Engl J Med 351:2805–2816
24. Ng EW, Shima DT, Calias P, Cunningham ET Jr, Guyer DR, Adamis AP (2006) Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. Nat Rev Drug Discov 5:123–132

25. Ferrara N, Hillan KJ, Gerber HP, Novotny W (2004) Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 3:391–400
26. Ferrara N, Damico L, Shams N, Lowman H, Kim R (2006) Development of ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration. Retina 26:859–870
27. Campochiaro PA (2007) Targeted pharmacotherapy of retinal diseases with ranibizumab. Drugs Today (Barc) 43:529–537
28. Chen Y, Wiesmann C, Fuh G, Li B, Christinger HW, McKay P, de Vos AM, Lowman HB (1999) Selection and analysis of an optimized anti-VEGF antibody: crystal structure of an affinity-matured Fab in complex with antigen. J Mol Biol 293:865–881
29. Rosenfeld PJ, Brown DM, Heier JS, Boyer DS, Kaiser PK, Chung CY, Kim RY (2006) Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 355:1419–1431
30. Monés J (2010) A review of ranibizumab clinical trial data in exudative age-related macular degeneration and how to translate it into daily practice. Ophthalmologica 225:112–119
31. Ziemssen F, Grisanti S, Bartz-Schmidt KU, Spitzer MS (2009) Off-label use of bevacizumab for the treatment of age-related macular degeneration: what is the evidence? Drugs Aging 26:295–320
32. The CATT Research Group, Martin DF, Maguire MG, Ying GS, Grunwald JE, Fine SL, Jaffe GJ (2011) Ranibizumab and bevacizumab for neovascular age-realted macular degeneration. N Engl J Med 364:1897–1908
33. Economides AN, Carpenter LR, Rudge JR, Wong V, Koehler-Stec EM, Hartnett C, Pyles EA, Xu X, Daly TJ, Young MR, Fandl JP, Lee F, Carver S, McNay J, Bailey K, Ramakanth S, Hutabarat R, Huang TT, Radziejewski C, Yancopoulos GD, Stahl N (2003) Cytokine traps: multi-component, high-affinity blockers of cytokine action. Nat Med 9:47–52
34. Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS (2002) VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA 99:11393–11398
35. Rudge JS, Holash J, Hylton D, Russell M, Jiang S, Leidich R, Papadopoulos N, Pyles EA, Torri A, Wiegand SJ, Thurston G, Stahl N, Yancopoulos GD (2007) VEGF Trap complex formation measures production rates of VEGF, providing a biomarker for predicting efficacious angiogenic blockade. Proc Natl Acad Sci USA 104:18363–18370
36. Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, Winkler M, Ferrara N (1997) Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res 57:4593–4599
37. Lowe J, Araujo J, Yang J, Reich M, Oldendorp A, Shiu V, Quarmby V, Lowman H, Lien S, Gaudreault J, Maia M (2007) Ranibizumab inhibits multiple forms of biologically active vascular endothelial growth factor in vitro and in vivo. Exp Eye Res 85:425–430
38. Carneiro A, Falcao M, Pirraco A, Milheiro-Oliveira P, Falcao-Reis F, Soares R (2009) Comparative effects of bevacizumab, ranibizumab and pegaptanib at intravitreal dose range on endothelial cells. Exp Eye Res 88:522–527
39. Klettner A, Roider J (2008) Comparison of bevacizumab, ranibizumab, and pegaptanib in vitro: efficiency and possible additional pathways. Invest Ophthalmol Vis Sci 49:4523–4527
40. Yu L, Liang XH, Ferrara N (2011) Comparing protein VEGF inhibitors: in vitro biological studies. Biochem Biophys Res Commun 408:276–281
41. Drake AW, Myszka DG, Klakamp SL (2004) Characterizing high-affinity antigen/antibody complexes by kinetic- and equilibrium-based methods. Anal Biochem 328:35–43
42. Myszka DG (1999) Improving biosensor analysis. J Mol Recognit 12:279–284
43. Darling RJ, Brault PA (2004) Kinetic exclusion assay technology: characterization of molecular interactions. Assay Drug Dev Technol 2:647–657
44. Quinn TP, Peters KG, De Vries C, Ferrara N, Williams LT (1993) Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proc Natl Acad Sci USA 90:7533–7537
45. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. J Biol Chem 269:26988–26995
46. Fuh G, Wu P, Liang WC, Ultsch M, Lee CV, Moffat B, Wiesmann C (2006) Structure-function studies of two synthetic anti-vascular endothelial growth factor Fabs and comparison with the Avastin Fab. J Biol Chem 10;281:6625–6631
47. Yu L, Wu X, Cheng Z, Lee CV, LeCouter J, Campa C, Fuh G, Lowman H, Ferrara N (2008) Interaction between bevacizumab and murine VEGF-A: a reassessment. Invest Ophthalmol Vis Sci 49:522–527
48. Lu F, Adelman RA (2009) Are intravitreal bevacizumab and ranibizumab effective in a rat model of choroidal neovascularization? Graefes Arch Clin Exp Ophthalmol 247:171–177
49. Meyer RD, Singh A, Majnoun F, Latz C, Lashkari K, Rahimi N (2004) Substitution of C-terminus of VEGFR-2 with VEGFR-1 promotes VEGFR-1 activation and endothelial cell proliferation. Oncogene 23:5523–5531
50. Dawson NS, Zawieja DC, Wu MH, Granger HJ (2006) Signaling pathways mediating VEGF165-induced calcium transients and membrane depolarization in human endothelial cells. FASEB J 20:991–993
51. Yoshida A, Anand-Apte B, Zetter BR (1996) Differential endothelial migration and proliferation to basic fibroblast growth factor and vascular endothelial growth factor. Growth Factors 13:57–64
52. Hauser S, Weich HA (1993) A heparin-binding form of placenta growth factor (PlGF-2) is expressed in human umbilical vein endothelial cells and in placenta. Growth Factors 9:259–268
53. Meyer T, Robles-Carrillo L, Robson T, Langer F, Desai H, Davila M, Amaya M, Francis JL, Amirkhosravi A (2009) Bevacizumab immune complexes activate platelets and induce thrombosis in FCGR2A transgenic mice. J Thromb Haemost 7:171–181
54. Costa R, Carneiro A, Rocha A, Pirraco A, Falcao M, Vasques L, Soares R (2009) Bevacizumab and ranibizumab on microvascular endothelial cells: a comparative study. J Cell Biochem 108:1410–1417
55. Spitzer MS, Yoeruek E, Sierra A, Wallenfels-Thilo B, Schraermeyer U, Spitzer B, Bartz-Schmidt KU, Szurman P (2007) Comparative antiproliferative and cytotoxic profile of bevacizumab (Avastin), pegaptanib (Macugen) and ranibizumab (Lucentis) on different ocular cells. Graefes Arc Clin Exp Ophthalmol 245:1837–1842
56. Gaudreault J, Fei D, Rusit J, Suboc P, Shiu V (2005) Preclinical pharmacokinetics of Ranibizumab (rhuFabV2) after a single intravitreal administration. Invest Ophthalmol Vis Sci 46:726–733
57. Brown DM, Kaiser PK, Michels M, Soubrane G, Heier JS, Kim RY, Sy JP, Schneider S (2006) Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N Engl J Med 355:1432–1444
58. Regillo CD, Brown DM, Abraham P, Yue H, Ianchulev T, Schneider S, Shams N (2008) Randomized, double-masked,

sham-controlled trial of ranibizumab for neovascular age-related macular degeneration: PIER Study year 1. Am J Ophthalmol 145:239–248
59. Schmidt-Erfurth U, Eldem B, Guymer R, Korobelnik JF, Schlingemann RO, Axer-Siegel R, Wiedemann P, Simader C, Gekkieva M, Weichselberger A (2011) Efficacy and safety of monthly versus quarterly ranibizumab treatment in neovascular age-related macular degeneration the EXCITE study. Ophthalmology 118:831–839
60. Holz FG, Amoaku W, Donate J, Guymer RH, Kellner U, Schlingemann RO, Weichselberger A, Staurenghi G (2011) Safety and efficacy of a flexible dosing regimen of ranibizumab in neovascular age-related macular degeneration: the SUSTAIN study. Ophthalmology 118:663–671
61. Stewart MW (2007) Predicted biologic activity of intravitreal bevacizumab. Retina 27:1196–1200
62. Stewart MW, Rosenfeld PJ (2008) Predicted biological activity of intravitreal VEGF Trap. Br J Ophthalmol 92:667–668
63. Furfine E, Coppi A, Koehler-Stec E, Zimmer E, Tu W, Struble C. Pharmacokinetics and ocular tissue penetration of VEGF Trap after intravitreal injections in rabbits. Invest Opthalmol Vis Sci 47:E-abstract 1430
64. Bakri SJ, Snyder MR, Reid JM, Pulido JS, Ezzat MK, Singh RJ (2007) Pharmacokinetics of intravitreal ranibizumab (Lucentis). Ophthalmology 114:2179–2182
65. Boyer DS, Heier JS, Brown DM, Francom SF, Ianchulev T, Rubio RG (2009) A Phase IIIb study to evaluate the safety of ranibizumab in subjects with neovascular age-related macular degeneration. Ophthalmology 116:1731–1739
66. Brown DM, Heier JS, Ciulla T, Benz M, Abraham P, Yancopoulos G, Stahl N, Ingerman A, Vitti R, Berliner AJ, Yang K, Nguyen QD, CLEAR-IT 2 Investigators (2011) Primary endpoint results of a phase II study of vascular endothelial growth factor trap-eye in wet age-related macular degeneration. Ophthalmology 118:1089–1097
67. Heier JS, Boyer D, Nguyen QD, Marcus D, Roth DB, Yancopoulos G, Stahl N, Ingerman A, Vitti R, Berliner AJ, Yang K, Brown DM, CLEAR-IT 2 Investigators (2011) The 1-year results of CLEAR-IT 2, a phase 2 study of vascular endothelial growth factor trap-eye dosed as-needed after 12-week fixed dosing. Ophthalmology 118:1098–1106
68. Nguyen QD, Heier J, Brown D, Ho A, Kaiser P, Vitti R, VIEW 1 Study Group (2011) Randomized, double-masked, active-controlled phase 3 trial of the efficacy and safety of intravitreal VEGF Trap-eye in Wet AMD: one-year results of the view-1 study. Invest Ophthalmol Vis Sci 52:E-abstract 3073
69. Schmidt-Erfurth U, Chong V, Kirchhof B, Korobelnik JF, Papp A, Anderesi M, Groetzbach G, Sommerauer B, Sandbrink R, Ogura Y (2011) Primary results of an international phase III study using intravitreal VEGF trap-eye compared to ranibizumab in patients with Wet AMD (VIEW2). Invest Ophthalmol Vis Sci 52:E-abstract 1650
70. VEGF Inhibition Study in Ocular Neovascularization (V.I.S.I.O.N.) Clinical Trial Group, Chakravarthy U, Adamis AP, Cunningham ET Jr, Goldbaum M, Guyer DR, Katz B, Patel M (2006) Year-2 efficacy results of 2 randomized controlled clinical trials of pegaptanib for neovascular age-related macular degeneration. Ophthalmology 113:1508.e1–1508.e25
71. Ciulla TA, Rosenfeld PJ (2009) Antivascular endothelial growth factor therapy for neovascular age-related macular degeneration. Curr Opin Ophthalmol 20:158–165
72. Moussa S, Ansari-Shahrezaei S, Smretschnig E, Hagen S, Steindl-Kuscher K, Krebs I, Binder S (2010) Contrast sensitivity after intravitreal antivascular endothelial growth factor therapy for myopic choroidal neovascularization. Graefes Arch Clin Exp Ophthalmol 248:1087–1090
73. Yao J, Wu X, Kasman IM, Vogt T, Phan V, Shibuya M, Ferrara N, Bais C (2011) Expression of a functional VEGFR-1 in tumor cells is a major determinant of anti-PlGF antibodies efficacy. Proc Natl Acad Sci USA 108:11590–11595
74. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P (2002) Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. Nat Med 8:831–840
75. Miyamoto N, de Kozak Y, Jeanny JC, Glotin A, Mascarelli F, Massin P, BenEzra D, Behar-Cohen F (2007) Placental growth factor-1 and epithelial haemato-retinal barrier breakdown: potential implication in the pathogenesis of diabetic retinopathy. Diabetologia 50:461–470
76. Zhong X, Huang H, Shen J, Zacchigna S, Zentilin L, Giacca M, Vinores SA (2011) Vascular endothelial growth factor-B gene transfer exacerbates retinal and choroidal neovascularization and vasopermeability without promoting inflammation. Mol Vis 17:492–507
77. Van Cutsem E, Tabernero J, Lakomy R et al. Intravenous (IV) aflibercept versus placebo in combination with irinotecan/5-FU (FOLFIRI) for second-line treatment of metastatic colorectal cancer (MCRC): Results of a multinational phase III trial (EFC10262-VELOUR). Ann Oncol 22(supplement 5): v10–v18. Abstract O-0024
78. Kopetz S, Hoff PM, Morris JS, Wolff RA, Eng C, Glover KY, Adinin R, Overman MJ, Valero V, Wen S, Lieu C, Yan S, Tran HT, Ellis LM, Abbruzzese JL, Heymach JV (2010) Phase II trial of infusional fluorouracil, irinotecan, and bevacizumab for metastatic colorectal cancer: efficacy and circulating angiogenic biomarkers associated with therapeutic resistance. J Clin Oncol 28:453–459
79. Lieu C, Tran H, Jian Z, Mao M, Overman M, Eng C, Morris J, Ellis L, Heymach J, Kopetz S. The association of alternate VEGF ligands with resistance to anti-VEGF therapy in metastatic colorectal cancer (mCRC). J Clin Oncol 29:suppl. abstr 3533

REGENERON

September 14, 2009

Enrollment Completed in Regeneron and Bayer HealthCare Phase 3 Studies of VEGF Trap-Eye in Neovascular Age-Related Macular Degeneration (Wet AMD)

One-year primary endpoint data expected in Q4 2010

TARRYTOWN, N.Y., Sept 14, 2009 /PRNewswire-FirstCall via COMTEX News Network/ -- Regeneron Pharmaceuticals, Inc. (Nasdaq: REGN) today announced the completion of patient enrollment in two randomized, double-masked, Phase 3 clinical trials evaluating VEGF Trap-Eye in the treatment of the neovascular form of age-related macular degeneration (wet AMD). In each study of the VIEW (VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet AMD) program, VEGF Trap-Eye is being evaluated for its effect on maintaining and improving vision when dosed as an intravitreal injection on a schedule of 0.5 milligram (mg) every four weeks, 2.0 mg every four weeks, or 2.0 mg every eight weeks (following three monthly doses), as compared with intravitreal ranibizumab (Lucentis((R)), a registered trademark of Genentech, Inc.) administered 0.5 mg every four weeks during the first year of the studies. As-needed (PRN) dosing with both agents is being evaluated during the second year of each study. These studies are part of the global development program for VEGF Trap-Eye being conducted by Regeneron and Bayer HealthCare AG. Each study has enrolled in excess of the targeted 1,200 patient goal. One-year primary endpoint data from both studies are expected in the fourth quarter of 2010.

VEGF Trap-Eye, an investigational drug, is being developed by Regeneron and Bayer HealthCare AG for the potential treatment of eye diseases, including wet AMD, diabetic macular edema (DME), and Central Retinal Vein Occlusion (CRVO). Regeneron maintains exclusive rights to VEGF Trap-Eye in the United States. Bayer HealthCare has exclusive rights to market VEGF Trap-Eye outside the United States, where the companies will share equally in profits from any future sales of VEGF Trap-Eye.

"Even with recent advances in the treatment of wet AMD, vision is not improved or stabilized in all patients despite monthly office visits and examinations that are inconvenient for these often elderly patients," said George D. Yancopoulos, M.D., Ph.D., President of Regeneron Research Laboratories. "This Phase 3 program is exploring various doses and dosing schedules with our novel anti-VEGF investigational agent to evaluate whether further improvements in vision and/or longer dosing intervals than monthly administration are possible."

About the VIEW Program

The VIEW 1 study is being conducted in the United States and Canada by Regeneron and the VIEW 2 study is being conducted in Europe, Asia Pacific, Japan, and Latin America by Bayer HealthCare. In the first year of the studies, the safety and efficacy of VEGF Trap-Eye at doses of 0.5 mg and 2.0 mg administered at four-week intervals and 2.0 mg at an eight-week dosing interval following one additional 2.0 mg dose at week four are being evaluated. Patients randomized to the ranibizumab arm of the trial will receive a 0.5 mg dose every four weeks. After the first year of treatment, patients will continue to be followed and treated for another year on a flexible, criteria-based extended PRN regimen with a dose administered at least every 12 weeks, but not more often than every four weeks until the end of the study.

The primary endpoint of these non-inferiority studies is the proportion of patients treated with VEGF Trap-Eye who maintain vision at the end of one year, compared to ranibizumab patients. Visual acuity is defined as the total number of letters read correctly on the Early Treatment Diabetic Retinopathy Study (ETDRS) chart, a standard chart used in research to measure visual acuity. Maintenance of vision is defined as losing fewer than three lines (equivalent to 15 letters) on the ETDRS chart. Key secondary endpoints include the mean change from baseline in visual acuity as measured by ETDRS and the proportion of patients who gained at least 15 letters of vision at week 52.

About VEGF Trap-Eye

Vascular Endothelial Growth Factor (VEGF) is a naturally occurring protein in the body whose normal role is to trigger the formation of new blood vessels (angiogenesis) to support the growth of the body's tissues and organs. It has also been associated with the abnormal growth and fragility of new blood vessels in the eye, which lead to the development of wet AMD. VEGF Trap-Eye is a fully human, soluble VEGF receptor fusion protein that binds all forms of VEGF-A along with the related placental growth factor (PlGF). Investigational VEGF Trap-Eye is a specific blocker of VEGF-A and PlGF that has been demonstrated in preclinical models to bind these growth factors with greater affinity than their natural receptors. Blockade of VEGF can prevent abnormal blood vessel formation as well as vascular leak and has proven beneficial in the treatment of wet AMD.

VEGF Trap-Eye is also in Phase 3 development for the treatment of Central Retinal Vein Occlusion (CRVO), another cause of blindness. The COPERNICUS (COntrolled Phase 3 Evaluation of Repeated iNtravitreal administration of VEGF Trap-Eye In Central retinal vein occlusion: Utility and Safety) study is being led by Regeneron and the GALILEO (General Assessment Limiting InfiLtration of Exudates in central retinal vein Occlusion with VEGF Trap-Eye) study is being led by Bayer HealthCare. Patients in both studies will receive six monthly intravitreal injections of either VEGF Trap-Eye at a dose of 2 mg or sham control injections. The primary endpoint of both studies is improvement in visual acuity versus baseline after six months of treatment. At the end of the initial six months, patients will be dosed on a PRN (as needed) basis for another six months. All patients will be eligible for rescue laser treatment. Initial data from the program are anticipated in early 2011.

VEGF Trap-Eye is also in Phase 2 development for the treatment of Diabetic Macular Edema (DME). VEGF Trap-Eye dosed at 0.5 mg or 2 mg monthly, 2 mg every eight weeks after three monthly loading doses, or 2 mg on an as-needed (PRN) basis after three monthly loading doses is being compared to focal laser treatment, the current standard of care in DME. The primary efficacy endpoint evaluation is mean improvement in visual acuity at six months. Patient enrollment has been completed with initial data expected in the first half of 2010.

About Wet AMD

Age-related Macular Degeneration (AMD) is a leading cause of acquired blindness. Macular degeneration is diagnosed as either dry (non-exudative) or wet (exudative). In wet AMD, new blood vessels grow beneath the retina and leak blood and fluid. This leakage causes disruption and dysfunction of the retina creating blind spots in central vision, and it can account for blindness in wet AMD patients. Wet AMD is the leading cause of blindness for people over the age of 65 in the U.S. and Europe.

About Regeneron

Regeneron is a fully integrated biopharmaceutical company that discovers, develops, and commercializes medicines for the treatment of serious medical conditions. In addition to ARCALYST((R))(rilonacept) Injection for Subcutaneous Use, its first commercialized product, Regeneron has therapeutic candidates in clinical trials for the potential treatment of cancer, eye diseases, inflammatory diseases, and pain, and has preclinical programs in other diseases and disorders. Additional information about Regeneron and recent news releases are available on Regeneron's Web site at www.regeneron.com.

Forward Looking Statement - Regeneron Pharmaceuticals, Inc.

This news release discusses historical information and includes forward-looking statements about Regeneron and its products, development programs, finances, and business, all of which involve a number of risks and uncertainties, such as risks associated with preclinical and clinical development of VEGF Trap-Eye, determinations by regulatory and administrative governmental authorities which may delay or restrict Regeneron's ability to continue to develop or commercialize VEGF Trap-Eye, competing drugs that may be superior to VEGF Trap-Eye, uncertainty of market acceptance of VEGF Trap-Eye, the potential for any collaboration agreement, including Regeneron's agreements with the sanofi-aventis Group and Bayer HealthCare, to be canceled or to terminate without any product success, risks associated with third party intellectual property, and other material risks. A more complete description of these and other material risks can be found in Regeneron's filings with the United States Securities and Exchange Commission (SEC), including its Form 10-K for the year ended December 31, 2008 and Form 10-Q for the quarter ending June 30, 2009. Regeneron does not undertake any obligation to update publicly any forward-looking statement, whether as a result of new information, future events, or otherwise unless required by law.

Contact Information:

Peter Dworkin	Laura Lindsay
Investor Relations	Media Relations
914.345.7640	914.345.7800
peter.dworkin@regeneron.com	laura.lindsay@regeneron.com

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http://www.regeneron.com

myopia: a randomized prospective study. Ophthalmology 1998;105:1512–1522; discussion 1522–1523.
6. Magallanes R, Shah S, Zadok D, et al. Stability after laser in situ keratomileusis in moderately and extremely myopic eyes. J Cataract Refract Surg 2001;27:1007–1012.
7. Miyata K, Kamiya K, Takahashi T, et al. Time course of changes in corneal forward shift after excimer laser photorefractive keratectomy. Arch Ophthalmol 2002;120:896–900.
8. Kamiya K, Oshika T. Corneal forward shift after excimer laser keratorefractive surgery. Semin Ophthalmol 2003;18:17–22.
9. Toshino A, Uno T, Ohashi Y, et al. Transient keratectasia caused by intraocular pressure elevation after laser in situ keratomileusis. J Cataract Refract Surg 2005;31:202–204.
10. Hiatt JA, Wachler BS, Grant C. Reversal of laser in situ keratomileusis-induced ectasia with intraocular pressure reduction. J Cataract Refract Surg 2005;31:1652–1655.
11. Qi H, Hao Y, Xia Y, Chen Y. Regression-related factors before and after laser in situ keratomileusis. Ophthalmologica 2006;220:272–276.
12. Wang Z, Chen J, Yang B. Posterior corneal surface topographic changes after laser in situ keratomileusis are related to residual corneal bed thickness. Ophthalmology 1999;106:406–410.
13. Kamiya K, Oshika T, Amano S, et al. Influence of excimer laser photorefractive keratectomy on the posterior corneal surface. J Cataract Refract Surg 2000;26:867–871.
14. Baek T, Lee K, Kagaya F, et al. Factors affecting the forward shift of posterior corneal surface after laser in situ keratomileusis. Ophthalmology 2001;108:317–320.
15. Pan Q, Gu YS, Wang J, et al. Differences between regressive eyes and non-regressive eyes after LASIK for myopia in the time course of corneal changes assessed with the Orbscan. Ophthalmologica 2004;218:96–101.
16. Gauthier CA, Holden BA, Epstein D, et al. Role of epithelial hyperplasia in regression following photorefractive keratectomy. Br J Ophthalmol 1996;80:545–548.
17. Gauthier CA, Holden BA, Epstein D, et al. Factors affecting epithelial hyperplasia after photorefractive keratectomy. J Cataract Refract Surg 1997;23:1042–1050.
18. Moller-Pedersen T, Li HF, Petroll WM, et al. Confocal microscopic characterization of wound repair after photorefractive keratectomy. Invest Ophthalmol Vis Sci 1998;39:487–501.
19. Lohmann CP, Reischl U, Marshall J. Regression and epithelial hyperplasia after myopic photorefractive keratectomy in a human cornea. J Cataract Refract Surg 1999;25:712–715.
20. Park CK, Kim JH. Comparison of wound healing after photorefractive keratectomy and laser in situ keratomileusis in rabbits. J Cataract Refract Surg 1999;25:842–850.
21. Moller-Pedersen T, Cavanagh HD, Petroll WM, Jester JV. Stromal wound healing explains refractive instability and haze development after photorefractive keratectomy: a one-year confocal microscopic study. Ophthalmology 2000;107:1235–1245.
22. Duch S, Serra A, Castanera J, et al. Tonometry after laser in situ keratomileusis treatment. J Glaucoma 2001;10:261–265.
23. Arimoto A, Shimizu K, Shoji N, et al. Underestimation of intraocular pressure in eyes after laser in situ keratomileusis. Jpn J Ophthalmol 2002;46:645–649.
24. Kniestedt C, Kanngiesser H, Stonpet RL. Assessment of Pascal dynamic contour tonometer in monitoring IOP after LASIK. J Cataract Refract Surg 2005;31:458–459.
25. Doyle A, Lachkar Y. Comparison of dynamic contour tonometry with Goldmann applanation tonometry over a wide range of central corneal thickness. J Glaucoma 2005;14:288–292.
26. Martinez-de-la-Casa JM, Garcia-Feijoo J, Vico E, et al. Effect of corneal thickness on dynamic contour, rebound, and Goldmann tonometry. Ophthalmology 2006;113:2156–2162.
27. Hamedo T, Ogawa Y, Akaishi T, et al. Efficacy of long-term treatment with nipradilol, a nitrooester-containing beta-blocker, in patients with mild-to-moderate essential hypertension. Clin Ther 1995;17:667–679.
28. Kanno M, Araie M, Kotbuchi H, Masuda K. Effects of topical nipradilol, a beta blocking agent with alpha blocking and nitroglycerin-like activities, on intraocular pressure and aqueous dynamics in humans. Br J Ophthalmol 2000;84:293–299.
29. Kanno M, Araie M, Masuda K, et al. Phase III long-term study and comparative clinical study of nipradilol ophthalmic solution in patients with primary open-angle glaucoma and ocular hypertension. Part 2. Arzneimittelforschung 2006;56:820–825.
30. Wilkerson M, Cyrlin M, Lippa EA, et al. Four-week safety and efficacy study of dorzolamide, a novel, oral active topical carbonic anhydrase inhibitor. Arch Ophthalmol 1995;113:1343–1350.
31. Herndon LW, Choudhri SA, Cox T, Damji KF, Shields MB, Allingham RR. Central corneal thickness in normal, glaucomatous, and ocular hypertensive eyes. Arch Ophthalmol 1997;115:1137–1141.
32. Kaminski S, Hommer A, Koyuncu D, Biowski R, Barisani T, Baumgartner I. Influence of dorzolamide on corneal thickness, endothelial cell count and corneal sensibility. Acta Ophthalmol Scand 1998;76:78–79.
33. Inoue K, Okugawa K, Oshika T, Amano S. Influence of dorzolamide on corneal endothelium. Jpn J Ophthalmol 2003;47:129–133.
34. Groh M, Leitritz M, Mielke J, Reinthal E, Bartz-Schmidt KU, Rohrbach JM. Effect of timolol on central corneal thickness and endothelial cell density. Klin Monatsbl Augenheilkd 2006;223:894–898.
35. Herreras JM, Pastor JC, Calonge M, Asensio VM. Ocular surface alteration after long-term treatment with an antiglaucomatous drug. Ophthalmology 1992;99:1082–1088.
36. Yalvac IS, Gedikoglu G, Kanguz Y, et al. Effects of antiglaucoma drugs on ocular surface. Acta Ophthalmol Scand 1995;73:246–248.
37. Arici MK, Arici DS, Topalkara A, Guler C. Adverse effects of topical antiglaucoma drugs on the ocular surface. Clin Experiment Ophthalmol 2000;28:113–117.
38. Aoyama Y, Motoki M, Hashimoto M. Effect of various anti-glaucoma eye drops on human corneal epithelial cells. Nippon Ganka Gakkai Zasshi 2004;108:75–83.
39. Hirano S, Sagara T, Suzuki K, Seki K, Nishida T. Inhibitory effects of anti-glaucoma drugs on corneal epithelial migration in a rabbit organ culture system. J Glaucoma 2004;13:196–199.
40. Nawa Y, Masuda K, Ueda T, et al. Evaluation of apparent ectasia of the posterior surface of the cornea after keratorefractive surgery. J Cataract Refract Surg 2005;31:571–573.
41. Ueda T, Nawa Y, Masuda K, et al. Posterior corneal surface changes after hyperopic laser in situ keratomileusis. J Cataract Refract Surg 2005;31:2084–2087.

Randomized, Double-Masked, Sham-Controlled Trial of Ranibizumab for Neovascular Age-related Macular Degeneration: PIER Study Year 1

CARL D. REGILLO, DAVID M. BROWN, PREMA ABRAHAM, HUIBIN YUE, TSONTCHO IANCHULEV, SUSAN SCHNEIDER, AND NAVEED SHAMS, ON BEHALF OF THE PIER STUDY GROUP

• PURPOSE: To evaluate the efficacy and safety of ranibizumab administered monthly for three months and then quarterly in patients with subfoveal choroidal neovascularization (CNV) secondary to age-related macular degeneration (AMD).

• DESIGN: Phase IIIb, multicenter, randomized, double-masked, sham injection-controlled trial in patients with predominantly or minimally classic or occult with no classic CNV lesions.

• METHODS: Patients were randomized 1:1:1 to 0.3 mg ranibizumab (n = 60), 0.5 mg ranibizumab (n = 61), or sham (n = 63) treatment groups. The primary efficacy endpoint was mean change from baseline visual acuity (VA) at month 12.

• RESULTS: Mean changes from baseline VA at 12 months were −16.3, −1.6, and −0.2 letters for the sham, 0.3 mg, and 0.5 mg groups, respectively ($P \leq .0001$, each ranibizumab dose vs sham). Ranibizumab arrested CNV growth and reduced leakage from CNV. However, the treatment effect declined in the ranibizumab groups during quarterly dosing (e.g., at three months the mean changes from baseline VA had been gains of 2.9 and 4.3 letters for the 0.3 mg and 0.5 mg doses, respectively). Results of subgroups analyses of mean change from baseline VA at 12 months by baseline age, VA, and lesion characteristics were consistent with the overall results. Few serious ocular or nonocular adverse events occurred in any group.

• CONCLUSIONS: Ranibizumab administered monthly for three months and then quarterly provided significant VA benefit to patients with AMD-related subfoveal CNV and was well tolerated. The incidence of serious ocular or nonocular adverse events was low. (Am J Ophthalmol 2008;145:239–248. © 2006 by Elsevier Inc. All rights reserved.)

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From the Retina Service, Wills Eye Institute, Philadelphia, Pennsylvania (CDR); Vitreoretinal Consultants, The Methodist Hospital, Houston, Texas (DMB); Mid Kansas Eye Institute, Retina Care, South Dakota (PA); and Genentech, Inc, South San Francisco, California (HY, TI, SS, NS).

Inquiries to Carl D. Regillo, Wills Eye Institute, 840 Walnut Street, Suite 1020, Philadelphia, PA 19107; e-mail: cregillo@aol.com

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RANIBIZUMAB (LUCENTIS; GENENTECH, INC, SOUTH San Francisco, California, USA) is an intravitreally administered recombinant, humanized, monoclonal antibody antigen-binding fragment (Fab) that neutralizes all known active forms of vascular endothelial growth factor-A (VEGF-A). It is the first treatment shown to not only prevent loss of visual acuity (VA) but also improve VA on average in patients with subfoveal choroidal neovascularization (CNV) secondary to age-related macular degeneration (AMD). In the two pivotal phase III trials—the MARINA Study in patients with minimally classic or occult with no classic CNV[1] and the ANCHOR Study in patients with predominantly classic CNV[2]—ranibizumab was injected monthly.

The phase IIIb PIER Study was designed to determine whether a less frequent ranibizumab dosing schedule (monthly for three months and then once every three months) would also prevent loss of VA in patients with AMD-related subfoveal CNV with or without a classic component, and to provide additional safety information. This alternative dosing regimen was selected for testing based on evidence from phase I and II studies indicating that the pharmacodynamic activity of ranibizumab (0.3 and 0.5 mg) administered intravitreally monthly for three doses may last 90 days.[3,4]

METHODS

PIER IS A TWO-YEAR, PHASE IIIB, MULTICENTER, RANDOMized, double-masked, sham injection–controlled study of the efficacy and safety of ranibizumab in patients with AMD-related subfoveal CNV, with or without classic CNV. After providing written informed consent, patients entered a screening period (≤28 days), with eligibility determined by the investigator. A central reading center (University of Wisconsin Fundus Photograph Reading Center, Madison, Wisconsin) later re-assessed the CNV types based on fluorescein angiograms, but this did not affect patients' eligibility. See Supplemental Table A (available at AJO.com) for full eligibility criteria.

Only patients ≥50 years old were eligible. One eye per subject (the "study eye") received study treatment. If both eyes were eligible, the one with better VA was selected

unless, for medical reasons, the other was more appropriate. Key inclusion criteria for the study eye were primary or recurrent subfoveal CNV secondary to AMD, with the total CNV area (classic plus occult CNV) composing ≥50% of the total AMD lesion area; total AMD lesion size ≤12 disk areas (DA); and best-corrected VA of 20/40 to 20/320 (Snellen equivalent) measured per a standard testing protocol using Early Treatment Diabetic Retinopathy Study (ETDRS) charts at a distance of 4 meters. Eyes with minimally classic or occult with no classic CNV were eligible only if they met any of three criteria for presumed disease progression: ≥10% increase in lesion size based on a fluorescein angiogram obtained ≤one month before day zero, inclusive, vs one obtained ≤six months before day zero, inclusive; or >one Snellen line (or equivalent) VA loss within the prior six months; or CNV-associated subretinal hemorrhage ≤one month before day zero. Eyes with predominantly (>50% of the lesion) classic CNV were not required to meet the criteria for presumed disease progression. Key exclusion criteria were any prior treatment with verteporfin photodynamic therapy (PDT), external-beam radiation therapy, transpupillary thermotherapy, or subfoveal laser photocoagulation (or juxtafoveal or extrafoveal laser photocoagulation ≤one month before day zero); permanent structural damage to the central fovea, or subretinal hemorrhage involving the fovea if ≥1 DA or ≥50% of the total lesion area. Patients were excluded if either eye had been treated in a prior antiangiogenic drug trial, or if the nonstudy eye received PDT ≤seven days before day zero.

Using a dynamic randomization algorithm, subjects were randomly assigned 1:1:1 to receive 0.3 mg ranibizumab, 0.5 mg ranibizumab, or sham injections. Randomization was stratified by VA score at day zero (≤54 letters [approximately worse than 20/80] vs ≥55 letters [approximately 20/80 or better]), CNV type (minimally classic vs occult with no classic vs predominantly classic CNV), and study center.

To achieve double-masking of treatment assignment, at least two investigators participated at each study site: an "injecting" ophthalmologist unmasked to treatment assignment (ranibizumab vs sham) but masked to ranibizumab dose, and a masked "evaluating" ophthalmologist for efficacy and safety assessments. All other study site personnel (other than those assisting with study treatment administration), central reading center personnel, and the subjects were masked to treatment assignment.

The ranibizumab groups received their assigned dose by intravitreal injection every month for three doses (day zero, months one and two), followed by doses every three months (months five, eight, 11, 14, 17, 20, and 23). Ranibizumab injection procedures have been described previously.[12] For the sham-injected control group, an empty syringe without a needle was used, with pressure applied to the anesthetized and antiseptically prepared eye at the site of a typical intravitreal injection. Pre- and postinjection procedures (described previously[12]) were identical for all groups.

The original study protocol specified that each treatment group would follow the same injection schedule. Thus, during the 24-month study, a total of 10 ranibizumab or sham injections were to be given, with six of the 10 during the first 12 months. After careful review of recent clinical data, including 12-month data from the two pivotal phase III studies,[1,2] the study protocol was amended on February 17, 2006 to allow control subjects who had completed the month-12 visit (the assessment timepoint for the primary efficacy analysis) to cross over to 0.5 mg ranibizumab for the remainder of the treatment period (subjects in the ranibizumab groups continued their originally assigned dose of 0.3 or 0.5 mg). On August 21, 2006, the protocol was again amended to increase assessments from quarterly to monthly after month 12, and to switch subjects randomized to the 0.3 mg dose to the 0.5 mg dose for the remainder of their study treatment. Also, because ranibizumab was by this time approved by the U.S. Food and Drug Administration (FDA), subjects were allowed to receive ranibizumab in the fellow eye as well as the study eye. No subjects were unmasked to their original treatment assignment as a result of these protocol amendments.

Assessments were performed at scheduled clinic visits. The first ranibizumab (0.3 or 0.5 mg) or sham treatment was administered on day zero. At subsequent injection visits, subjects underwent a preinjection safety evaluation. In addition to injection visits (day zero and months one, two, five, eight, 11, 14, 17, 20, and 23), clinic visits were scheduled at months three, 12, and 24. At each scheduled visit, subjects received a full ophthalmologic assessment, including VA testing using ETDRS charts at a test distance of 4 meters, slit-lamp biomicroscopy, fundoscopy, and intraocular pressure (IOP) measurement. Fundus photography and fluorescein angiography (FA) were done at day zero and months three, five, eight, 12, and 24. Optical coherence tomography (OCT) was done at selected study sites at day zero and months one, two, three, five, eight, 12, and 24. The primary efficacy endpoint was mean change from baseline to 12 months in VA score. The following key secondary VA endpoints were also assessed at 12 months: proportion of subjects losing ≤15 letters (≈3 lines) from baseline; proportion gaining ≥15 letters from baseline; proportion with a Snellen equivalent of 20/200 or worse (legal blindness = 20/200 or worse in both eyes); mean change from baseline in the near activities, distance activities, and vision-specific dependency NEI VFQ-25 subscales; and mean change from baseline in total area of CNV and total area of leakage from CNV (based on central reading center assessment). Prespecified exploratory endpoints included the proportion of subjects who had lost ≤30 letters (≈6 lines) from baseline VA at 12 months, the mean change from baseline at three months, and mean change from three months to 12 months.

Key safety assessments were the incidence and severity of ocular and nonocular adverse events, changes in vital

TABLE 1. Ranibizumab for Neovascular Age-Related Macular Degeneration: Subject Demographics and Baseline Study Eye Characteristics

Characteristic	Sham (N = 66)	Ranibizumab 0.3 mg (n = 66)	Ranibizumab 0.5 mg (N = 67)
Gender—no. (%)			
Male	20 (31.7)	26 (43.3)	26 (45.9)
Female	43 (68.3)	34 (56.7)	33 (54.1)
Race—no. (%)			
White	59 (93.7)	57 (95.0)	56 (91.8)
Other	4 (6.3)	3 (5.0)	5 (8.2)
Age—years			
Mean (SD)	77.8 (7.3)	78.7 (6.3)	78.5 (7.9)
Range	59–92	60–93	54–94
Age group—no. (%)			
50–64 years	4 (6.3)	1 (1.7)	4 (6.6)
65–74 years	12 (19.0)	12 (20.0)	10 (16.7)
75–84 years	36 (57.1)	37 (61.7)	31 (50.8)
≥85 years	11 (17.5)	10 (16.7)	14 (23.0)
Prior therapy for AMD—no. (%)			
Any	35 (53.8)	35 (58.3)	33 (54.1)
Laser photocoagulation	3 (4.6)	5 (8.3)	7 (11.5)
Medication[*]	1 (1.5)	1 (1.7)	3 (4.9)
Supplements	34 (54.0)	33 (55.0)	28 (45.9)
Years since first diagnosis of neovascular AMD[†]			
Mean (SD)	0.3 (0.5)	0.7 (1.6)	0.7 (1.2)
Range	0.0–3.0	0.0–9.1	0.0–5.0
Visual acuity (letters with approximate Snellen equivalent)[‡]			
Mean (SD)	56.1 (13.5)	55.8 (12.2)	53.7 (15.5)
≤54, 20/80—no. (%)	25 (38.7)	28 (46.3)	27 (44.3)
≥55, 20/80—no. (%)	56 (60.9)	31 (51.7)	34 (55.7)
Visual acuity (approximate Snellen equivalent)[‡]—no. (%)			
20/200 or worse	10 (15.9)	3 (5.0)	10 (16.4)
Better than 20/200 but worse than 20/40	42 (66.7)	49 (81.7)	36 (59.0)
20/40 or better	11 (17.5)	8 (13.3)	15 (24.6)
CNV lesion subtype—no. (%)			
Occult with no classic	20 (31.7)	29 (48.3)	30 (49.2)
Minimally classic	29 (46.0)	22 (36.7)	18 (29.5)
Predominantly classic	14 (22.2)	8 (13.3)	13 (21.3)
Cannot classify	0	1 (1.7)	0
Total area of lesion[§]			
Mean (SD) DA	4.24 (3.25)	4.38 (3.30)	4.01 (2.64)
Range DA	0.10–17.00	0.09–20.30	0.09–10.00
≤4 DA—no. (%)	33 (52.4)	32 (54.2)	31 (50.8)
>4 DA—no. (%)	30 (47.6)	27 (45.8)	30 (49.2)
Total area of CNV (DA)[§]			
Mean (SD)	3.58 (3.25)	3.80 (3.43)	3.29 (2.27)
Range	0.02–17.00	0.00–20.30	0.02–9.65
Leakage from CNV, plus RPE staining (DA)[§]			
Mean (SD)	4.25 (5.55)	4.48 (5.56)	3.99 (2.61)
Range	0.10–48.00	0.00–42.00	0.50–9.70

AMD = age-related macular degeneration; CNV = choroidal neovascularization; DA = disk areas; RPE = retinal pigment epithelium; SD = standard deviation.

*Triamcinolone acetonide in the sham and 0.3 mg ranibizumab groups; alteplase and a multiple vitamin / mineral formulation in the 0.5 mg ranibizumab group.

†For this parameter, the numbers of subjects are as follows: sham, n = 62; 0.3 mg ranibizumab, n = 59; 0.5 mg ranibizumab, n = 61.

‡Measured using Early Treatment Diabetic Retinopathy Study (ETDRS) charts at a starting distance of 4 meters.

§For this parameter, the numbers of subjects are as follows: sham, n = 63; 0.3 mg ranibizumab, n = 59; 0.5 mg ranibizumab, n = 61.

signs, and the incidence of positive serum antibodies to ranibizumab. Slit-lamp examination and indirect ophthalmoscopy were performed before each study injection. Grading scales for flare/cells and vitreous hemorrhage density (see Supplemental Tables B1 to B3 for grading criteria) were used to grade intraocular inflammation or vitreous hemorrhage, assessed by slit-lamp examination. IOP was measured using applanation tonometry before and 60 ± 10 minutes after each study treatment.

Safety analyses, performed using descriptive statistics and including all treated subjects, were based on the treatment actually received. Efficacy analyses used the intent-to-treat approach and included all subjects as randomized. Missing values were imputed using the last-observation-carried-forward method. All pairwise comparisons between the ranibizumab groups and the sham group used a statistical model including only two treatment groups (active vs control) at a time. For the primary efficacy endpoint, a Hochberg-Bonferroni adjustment[7] was made for multiple treatment comparisons of each ranibizumab dose group with the sham group. For secondary efficacy endpoints, a Type I error management plan was used to adjust for multiplicity of treatment comparisons and secondary endpoints. Unless otherwise noted, efficacy analyses were stratified by CNV classification at baseline (minimally classic vs occult with no classic vs predominantly classic CNV), as determined by the central reading center, and by baseline VA (≤54 vs ≥55 letters). For binary endpoints, stratified Cochran χ^2 tests were used for between-groups comparisons of proportions of subjects meeting the endpoint. Analysis of variance or analysis of covariance models were used to analyze continuous endpoints.

The study sample size was based on the primary efficacy endpoint. Calculations were based on a 1:1:1 randomization ratio (0.3 mg vs 0.5 mg ranibizumab vs sham), the Student *t* test for comparing mean changes from baseline to 12 months in VA (for each ranibizumab group vs sham), and the Hochberg-Bonferroni multiple comparison procedure at an overall α level of .05. The power of the Hochberg-Bonferroni multiple comparison procedure was evaluated using Monte Carlo simulations. The target sample size of 180 subjects provided 90% power in the intent-to-treat analysis to detect a nine-letter difference between one or both ranibizumab dose groups and the sham group in mean change in VA at month 12, according to the Hochberg-Bonferroni criterion (assumptions based on results of the TAP[6] and VIP[5] trials and anticipated proportions of each CNV type).

Prior PDT in the study eye was an exclusion criterion, but subjects with predominantly classic CNV at study entry or whose CNV was confirmed by the central reading center to have converted during the study from minimally classic or occult with no classic to predominantly classic CNV could receive verteporfin PDT treatment in the study eye given according to the Visudyne prescribing information[8] (i.e., the physician should reevaluate the patient every three months and if CNV leakage is detected on FA, therapy should be repeated) and at the discretion of the investigator per standard of care. Treatment of minimally classic or occult with no classic CNV with PDT is not approved by the U.S. FDA, but was permitted in this study if the investigator deemed PDT to be indicated and the lesion met all of the following criteria: ≥ 30-letter loss from baseline VA recorded at all study visits over a three-month period that included at least two study visits, total CNV lesion area ≤4 DA, and active CNV as defined in the inclusion criteria (Supplemental Table A1). Subjects receiving PDT in the study eye could continue study treatment, but PDT could not be given less than 28 days before or less than 21 days after a study injection. Also, PDT in the nonstudy eye could not be given less than five days before or less than 21 days after a study injection. No independent check was done to determine if investigators followed the instructions regarding PDT administration that were provided in the study protocol, nor was the clinical judgment of the investigator regarding suitability of the subject for PDT questioned or independently verified.

Treatment of either eye with other anti-VEGF drugs was prohibited. When pegaptanib sodium (Macugen) was approved by the U.S. FDA in January 2006, subjects were allowed to opt for treatment with this agent but were to be discontinued from their randomized study treatment and followed for the remainder of the study period.

RESULTS

BETWEEN SEPTEMBER 7, 2006 AND MARCH 16, 2007, 184 SUBJECTS were enrolled at 43 investigative sites in the U.S. and were randomly assigned to study treatment: 60 to 0.3 mg ranibizumab, 61 to 0.5 mg ranibizumab, and 63 to sham injection. Subject disposition is summarized in Supplemental Table C (available at AJO.com). Treatment compliance was good in the ranibizumab groups, with 85% or more of subjects receiving each scheduled injection. In the sham group, 27% of subjects permanently discontinued treatment before month 12, most often because the subject's condition mandated another therapeutic intervention. A month-12 VA score was obtained from 97% of each ranibizumab group and 86% of the sham group.

The treatment groups were well balanced overall for demographic and baseline ocular characteristics (Table 1). Each group was predominantly White and nearly two-thirds female, with a mean age of ≈78 years. The baseline mean VA score was 53 to 56 letters (approximate Snellen equivalent, 20/63 to 20/80) across groups. The time since diagnosis of neovascular AMD was within the prior year in 87% of subjects. Overall, 80% of subjects had either occult with no classic or minimally classic CNV lesions, but occult with no classic CNV was more common in the ranibizumab groups than in the sham group (nearly half vs less than one-third of study eye lesions, respectively). Nearly half of each group had lesion sizes ≥4 DA. The mean total areas of the AMD lesion, the CNV component, and leakage from CNV plus retinal pigment epithelium (RPE) staining were similar among the groups.

Of the 184 randomized subjects, 19 (10.3%) received one or more treatments with PDT in the study eye during the first treatment year: 17 subjects in the sham-injection group (27.8%), one subject in the 0.3 mg group (1.7%), and one subject in the 0.5 mg group (1.6%). Of the 14 subjects (22.2%) in the sham group who had predominantly classic CNV at study entry, four received at least one PDT treatment in the first year (total = five PDT administrations). None of the 21 subjects (17.4%) in the ranibizumab groups with predominantly classic CNV at study entry received PDT.

Figure 1 shows the mean change from baseline VA by study month for the first treatment year. At 12 months (primary endpoint), sham-treated subjects had lost a mean of 16.5 letters, whereas ranibizumab-treated subjects had lost a mean of 1.6 letters (0.3 mg dose, $P = .0001$ vs sham) or 0.2 letters (0.5 mg dose; $P < .0001$ vs sham). Thus, the difference from the sham group after one year of treatment was 14.7 letters in the 0.3 mg ranibizumab group and 16.1 letters in the 0.5 mg ranibizumab group. Moreover, each of the ranibizumab groups was statistically significantly different from the sham group at month one, following a single injection of ranibizumab ($P = .02$ for 0.3 mg dose, $P < .0001$ for 0.5 mg dose), and at each monthly assessment (all $P < .02$). After the initial three monthly doses, both ranibizumab groups showed a more than 10-letter benefit in mean VA compared with the sham group.

Results for key vision-related secondary endpoints at 12 months are summarized in Figure 2. Significantly greater proportions of the ranibizumab groups than the sham group

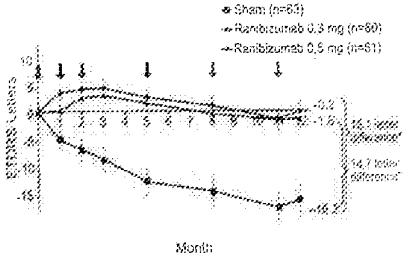


FIGURE 1. Ranibizumab for neovascular age-related macular degeneration (AMD). Mean change from baseline visual acuity, measured as letters read on the Early Treatment of Diabetic Retinopathy Study (ETDRS) chart, at monthly intervals. At month 12, the 0.3 mg ranibizumab group and the 0.5 mg ranibizumab group differed from the sham group by 14.7 and 16.1 letters, respectively ($P < .0001$). The arrows indicate that ranibizumab or sham injections occurred at day zero, month one, month two, month five, month eight, and month 11.

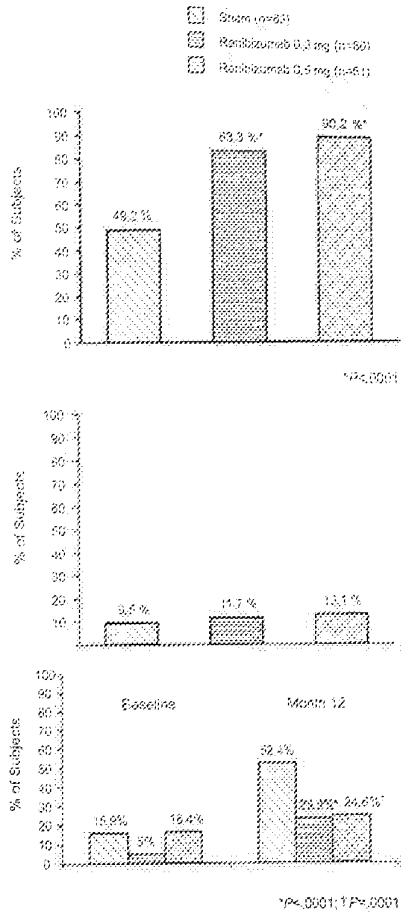


FIGURE 2. Ranibizumab for neovascular AMD. Percentages of the three treatment groups who (Top) at 12 months had lost fewer than 15 letters from baseline visual acuity score, (Middle) at 12 months had gained 15 or more letters from baseline visual acuity score, and (Bottom) had a Snellen equivalent visual acuity of 20/200 or worse at baseline (left) and at month 12 (right). *P* values are vs the sham treatment group.

TABLE 2. Ranibizumab for Neovascular Age-Related Macular Degeneration: Mean Change from Baseline in the Total Area of Choroidal Neovascularization and the Total Area of Leakage from Choroidal Neovascularization at Month 12

Change from Baseline at Month 12	Sham Injection (n = 58)	Ranibizumab 0.3 mg (n = 59)	Ranibizumab 0.5 mg (n = 61)
Change in total area of CNV (DA)			
Mean (SD)	2.08 (2.66)	0.18 (2.13)	0.43 (1.86)
95% CI for mean	(1.41 to 2.75)	(−0.37 to 0.74)	(−0.04 to 0.91)
P value (vs Sham)[a]		<.0001	<.0002
Change in total area of leakage from CNV + intense RPE staining (DA)			
Mean (SD)	1.40 (3.77)	−1.41 (2.69)	−1.29 (2.48)
95% CI for mean	(0.45 to 2.36)	(−2.10 to −0.71)	(−1.93 to −0.66)
P value (vs Sham)		<.0001	<.0001

CI = confidence interval; CNV = choroidal neovascularization; DA = disk areas; RPE = retinal pigment epithelium; SD = standard deviation.

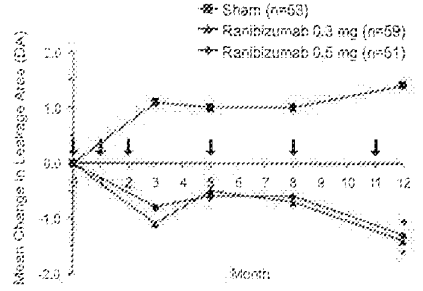
Note: The last-observation-carried-forward method was used to impute missing data. Strata were defined using two factors: baseline CNV classification (minimally classic vs occult with no classic vs predominantly classic) and baseline visual acuity score (≤54 vs ≥55 letters).

[a]One subject randomized to the 0.3 mg ranibizumab group withdrew before receiving any study treatment.

[b]*P* values are based on pairwise analysis of covariance (ANCOVA) models adjusted for the two stratification factors and baseline value of the endpoint.

had lost fewer than 15 letters from baseline VA: 83.3% and 90.2% of the 0.3 mg and 0.5 mg groups, respectively, compared with 49.2% of the sham group ($P < .0001$ for each dose level vs sham). However, the three treatment groups did not differ significantly in the proportions gaining at least 15 letters: 9.5% in the sham group, 11.7% in the 0.3 mg ranibizumab group, and 13.1% in the 0.5 ranibizumab group. Significantly smaller proportions of the ranibizumab groups than the sham group had VA of 20/200 or worse Snellen equivalent at month 12: 23.3% and 24.6% of the 0.3 mg and 0.5 mg groups, respectively, compared with 52.4% of the sham group ($P = .0002$ and $P < .001$, respectively vs sham). However, the proportion with VA this poor at baseline was smaller in the 0.3 mg dose group (5%) than in the 0.5 mg ranibizumab group (16.4%) and sham group (16.4%), which may partially account for the smaller proportion in this dose group at month 12. There was no statistically significant difference between either ranibizumab dose group and the sham control for any of the three NEI VFQ-25 subscales that were prespecified as secondary endpoints. However, post hoc analysis indicated that significantly more subjects in the ranibizumab groups reported clinically meaningful (≥10-point increases) in the near activities subscale scores compared with sham (32% for 0.3 mg ranibizumab group, 31% for 0.5 mg ranibizumab group and 14% for sham group, $P < .05$ vs sham for both ranibizumab groups).

Prespecified subgroup analyses of the month 12 VA results were performed for several baseline characteristics: age (less than 75 and 75 or over), gender, and, for the study eye, VA score (≤54 letters vs ≥55 letters), total lesion size (≤4 vs >4), occult CNV present at baseline (yes vs no), and CNV angiographic type at baseline. In these subgroup analyses the treatment effect of ranibizumab at both doses compared with sham injection was consistent with the overall results. For the subgroups of age, gender, baseline VA greater than or equal to 55 letters, baseline lesion size less than or equal to 4 DA, occult CNV present at baseline, and occult with no classic CNV, both ranibizumab dose groups were significantly different from the sham injection group ($P < .05$). For the subgroups of occult CNV absent at baseline and predominantly classic CNV, the 0.3 mg dose group was significantly different from the sham injection group ($P < .05$). For the baseline lesion size less than 4 DA subgroup, the 0.5 mg ranibizumab group was significantly different from the sham injection group ($P < .05$). The other subgroup comparisons did not achieve statistical significance.



*P<.0001

FIGURE 3. Ranibizumab for neovascular AMD. Mean change over time in the area of leakage from choroidal neovascularization (CNV) plus intense progressive retinal pigment epithelium staining. At month 12, each of the ranibizumab-treated groups showed significantly less leakage than the sham-treated group ($P < .0001$). The arrows indicate that ranibizumab or sham injections occurred at day zero, month one, month two, month five, month eight, and month 11.

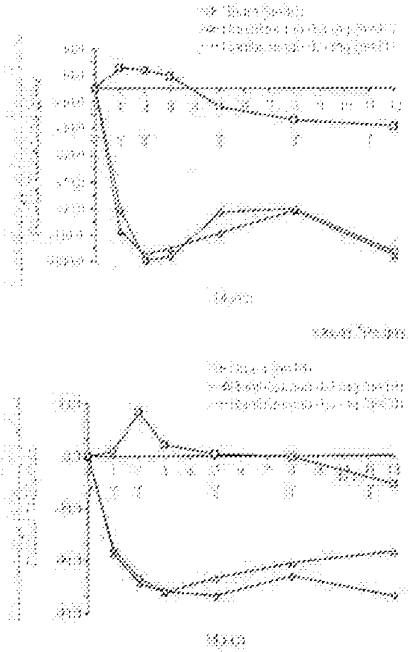


FIGURE 4. Ranibizumab for neovascular AMD. Mean change over time in (Top) foveal center point retinal thickness ($P = .01$ for 0.3 mg ranibizumab and $P < .001$ for 0.5 ranibizumab, vs sham at 12 months) and (Bottom) central subfield retinal thickness ($P < .05$ for 0.5 ranibizumab, vs sham at 12 months). The number of subjects contributing data for central subfield retinal thickness is less than that for foveal center point retinal thickness because calculation of the former requires nine data points on the optical coherence tomography (OCT) scan whereas calculation of the latter requires only six. Therefore, there was a greater likelihood of missing data points (and consequently an inability to calculate thickness) for central subfield retinal thickness than for foveal center point retinal thickness. The arrows indicate that ranibizumab or sham injections occurred at day zero, month one, month two, month five, month eight, and month 11.

The results for key secondary endpoints related to lesion morphologic characteristics at 12 months are summarized in Table 2. Ranibizumab arrested CNV growth, on average, as indicated by the difference between the ranibizumab and sham groups for change from baseline in total area of CNV (both ranibizumab doses $P < .001$ vs sham). Ranibizumab also reduced the total area of leakage from CNV plus intense progressive RPE staining on average, whereas the sham group exhibited an increase trend (both ranibizumab doses $P < .0001$ vs sham); this pattern was evident at each of the four postbaseline assessments, and was most pronounced at month 12 (Figure 3).

Comparisons between month three and month 12 for the VA endpoints were considered indicative of the efficacy of the quarterly dosing schedule as a maintenance therapy, and therefore several prespecified exploratory analyses were conducted. On average, there was 4.5-letter decline in VA between month three and month 12 for both ranibizumab dose groups. Because neither of the 95% confidence intervals for these values contained zero ([−8.6 to −0.3] and [−7.2 to −1.7] for the 0.3 mg and 0.5 mg groups, respectively), these declines were considered statistically significant. Over the same timeline, VA in the sham group declined by a mean of 1.6 letters. The differences between the ranibizumab dose groups and the sham group in mean change in VA from month three to month 12 were not statistically significant.

Data for comparisons of OCT-assessed anatomical outcomes over time were available from 40, 37, and 41 subjects from the sham, 0.3, and 0.5 mg groups, respectively (Figure 4). On average, both ranibizumab groups showed decreases in retinal thickness over the 12-month period. In ranibizumab-treated subjects, a statistically significant within-group reduction from baseline was seen as early as month one for both foveal center point retinal thickness ($P < .001$, each dose group) and central subfield retinal thickness ($P < .007$, each dose group). At month 12, compared with the sham group, ranibizumab-treated subjects showed significantly greater mean decreases from baseline in foveal center point thickness ($P = 0.01$ for 0.3 mg and $P = .0006$ for 0.5 mg) and, in the 0.5 mg group, central subfield retinal thickness ($P = .04$ for 0.5 mg). There was a maximal decrease in foveal center point thickness at months two and three for both ranibizumab groups, compared with a continued increase in thickness in the sham group during the same period. During the ensuing quarterly dosing interval, at assessments made three months after the previous dose (months five and eight), foveal center point thickness was on average greater than that at months two and three, and also greater than that at month 12, which followed a ranibizumab dose at month 11.

Fluorescein angiographic data for comparisons of change over time were available from 63, 59, and 61 subjects from the sham, 0.3, and 0.5 mg groups, respectively. The leakage from CNV plus RPE staining increased by a mean of approximately 1.4 DA at 12 months in sham-treated subjects while decreasing by a mean of 1.4 DA and 1.3 DA for the 0.3 mg group and the 0.5 mg group at 12 months. During the quarterly dosing interval (months three to 12),

TABLE 3. Ranibizumab for Neovascular Age-Related Macular Degeneration: Summary of Safety During First 12 Months

	Sham Injection (n = 63)	Ranibizumab 0.3 mg (n = 66)	Ranibizumab 0.5 mg (n = 64)
Key serious ocular adverse events[1]—no. (%)			
Endophthalmitis, uveitis, retinal detachment, or lens damage	0	0	0
Ocular hemorrhage[2]	2 (3.2)	2 (3.4)	0
Macular edema	2 (3.2)	1 (1.7)	0
Most severe ocular inflammation, regardless of cause (slit-lamp examination)[3]—no. (%)			
None	58 (95.2)	65 (93.2)	59 (96.7)
Trace	2 (3.2)	1 (1.7)	1 (1.6)
1+	0	2 (3.4)	0
2+	1 (1.6)	1 (1.7)	1 (1.6)
3+ or 4+	0	0	0
Key nonocular adverse events—no. (%)			
Nonocular hemorrhage	3 (4.8)	3 (5.4)	4 (6.6)[4]
Hypertension	5 (8.1)	4 (6.8)	6 (9.8)
Arterial thromboembolic events			
Myocardial infarction	0	0	0
Cerebrovascular accident	0	0	0
Ischemic cardiomyopathy	1 (1.6)	0	0
Death	0	0	0

Note. Multiple occurrences of the same event for a subject were counted once in the overall incidence.

[1]One subject randomized to the 0.3 mg ranibizumab group withdrew before receiving any study treatment.

[2]Ocular adverse events in study eye.

[3]The two events in the Sham group and 1 of the events in the 0.3 mg ranibizumab group were reported as "retinal hemorrhage." One event in the 0.3 mg ranibizumab group was reported as "eye hemorrhage."

[4]Includes one event (catheter-site hemorrhage) categorized as serious.

[5]For slit-lamp examination, the n = 62 for the Sham group.

leakage from CNV plus RPE staining, on average, was greater at months five and eight than that at month three and also greater than that at month 12, which followed the dose at month 11.

Key safety results through month 12 are summarized in Table 3. There was no overall imbalance among the three treatment groups in the proportions of subjects experiencing these key ocular or nonocular adverse events. No cases of endophthalmitis, serious uveitis, rhegmatogenous, or other retinal detachment, retinal tear, vitreous hemorrhage, or lens damage (traumatic cataract) were reported. Serious ocular hemorrhage of any type occurred in two subjects in the sham group (1.6% both retinal hemorrhages) and two subjects in the 0.3 ranibizumab group (3.4%: one retinal hemorrhage and one reported as "eye hemorrhage"). Serious macular edema was reported in two subjects in the sham group (1.6%) and one subject in the 0.3 mg ranibizumab group (1.7%). Few subjects in any group exhibited intraocular inflammation upon slit-lamp examination, and no cases of 3+ or 4+ inflammation were reported. Cataract event rates were similar across treatment groups, reported in 6.5% of the sham, 5.1% of the 0.3 mg ranibizumab, and 6.6% of the 0.5 mg ranibizumab groups.

As in previous ranibizumab trials, and consistent with intravitreal injection of a small volume of fluid, changes in IOP occurred transiently after injection and were generally mild. Within one hour after injection, IOP was elevated from the preinjection value. On average, postdose IOP was elevated from the predose value by approximately 1.9 mm Hg to 2.3 mm Hg in the ranibizumab groups, compared with approximately 1.4 mm Hg in the sham-injection group. Most of the IOP elevations following the injection were less than 10 mm Hg.

Any postbaseline occurrence of IOP ≥30 mm Hg (pre- or postinjection) was reported in one subject (1.7%) in the 0.3 mg group, five subjects (8.2%) in the 0.5 mg group, and in one subject (1.7%) in the sham groups in only one subject (in the 0.5 mg group; 1.6%) was this elevation reported as a preinjection occurrence.

Assessment of serious or nonserious nonocular adverse events revealed no overall imbalance among groups. No deaths occurred during the first year of treatment. Nonocular adverse events known to be associated with systemic VEGF inhibition were of particular interest, and key events are summarized in Table 3. Hypertension in subjects who were not hypertensive at baseline was reported as an adverse event in 8.1% of sham-treated subjects, 6.8% of subjects at the 0.3 mg ranibizumab dose, and 9.8% of subjects at the 0.5 mg dose. Routinely measured systolic and diastolic blood pressures were very similar among groups at baseline and at 12 months. No adverse event of proteinuria was reported during the first year in any treatment group. The rates of nonocular hemorrhage were 4.8% and 6.6% for the 0.3 mg and 0.5 mg ranibizumab groups, respectively, compared with 4.8% in the sham group. Only one serious nonocular hemorrhage (hemorrhage at the site of an intravenous catheter in a subject in the 0.5 mg ranibizumab group) was reported, and there were no GI perforations. No arterial thromboembolic events classified using Antiplatelet Trialists' Collaboration (APTC) criteria (i.e., nonfatal myocardial infarction, nonfatal ischemic stroke, nonfatal hemorrhagic stroke, or death due to vascular or unknown cause)[9] were reported in any treatment group. The only serious or nonserious arterial thromboembolic event during the first year was ischemic cardiomyopathy in a sham-treated subject.

Immunoreactivity to ranibizumab was assessed at screening and at month 12 or early termination. During screening, one subject randomized to sham treatment tested positive, possibly due attributable to preexisting anti-Fab antibodies. No ranibizumab subjects tested positive before study treatment, but two subjects in the 0.5 mg ranibizumab group tested positive at month 12. Neither of these two subjects who received ranibizumab experienced any severe or serious adverse event during the study.

DISCUSSION

RESULTS OF THE PIER STUDY INDICATE THAT RANIBIZUMAB administered on a schedule of monthly dosing for three months followed by quarterly dosing provides significant visual acuity benefit to patients with all angiographic subtypes of CNV (i.e., minimally or predominantly classic or occult with no classic component), with a low incidence of serious ocular and nonocular adverse events and an acceptable rate of nonserious adverse events. Both of the ranibizumab dose groups (0.3 mg and 0.5 mg) maintained baseline VA on average at 12 months, and exhibited a clinically meaningful (at least three-line) benefit relative to the sham-injected control group.

Approximately 90% of ranibizumab treated subjects lost fewer than 15 letters in VA, compared with approximately 50% of sham control subjects. In addition, at 12 months maintenance of visual acuity was accompanied, on average, by a significant reduction in vascular leakage (a more than 2.8-DA mean reduction in leakage), reduced foveal retinal thickness, and inhibition of CNV lesion growth in the ranibizumab groups.

Although quarterly ranibizumab therapy during months three to 12 maintained baseline VA, this regimen was associated on average with a 4.5-letter decline in the maximal VA benefit achieved with monthly induction therapy during months one to three. This loss was accompanied, on average, with an increase in vascular leakage on fluorescein angiography and increases in mean retinal thickness once the quarterly dosing regimen began. This overall increase in mean retinal thickness and increase in leakage suggests that at least some of the subjects needed ranibizumab injections more frequently to control the neovascular leakage. Further subgroup analysis, particularly of individual OCT responses, may support this initial impression. Unfortunately, there are no data available at months four, six, seven, nine, and 10 to allow assessment of duration of action of ranibizumab in individual subjects and the temporal association with retinal thickness. It is possible that the return of retinal edema in at least some subjects contributed to the decline in VA, on average, between months three and 12, in contrast to the patients in the two pivotal ranibizumab trials.[1,2] Also, in the present study there was no benefit demonstrated in the percentage improving by at least 15 letters, whereas in the two pivotal trials a significant improvement was observed. Finally, there were no statistically significant differences between the ranibizumab groups and the sham group in the NEI VFQ-25 subscales, although this may have been attributable to the smaller sample size than in the pivotal studies. Considered together, we conclude that although this regimen provided clinically meaningful and statistically significant benefit to patients, the outcomes were not as strong as those observed with monthly dosing of ranibizumab, suggesting that some patients may require dosing on a more frequent schedule such as that used in the MARINA and ANCHOR studies.[1,2] It is possible that OCT-guided administration of ranibizumab retreatment, as was used with favorable results in a recently reported small, uncontrolled study,[10] may allow greater individualization of ranibizumab dosing intervals, and merits further study.

The overall safety profile of ranibizumab in the PIER study was consistent with that seen during the first year of the MARINA and ANCHOR studies, and indicates that repeated intravitreal injection of ranibizumab is generally well tolerated. Rates of specific ocular and nonocular adverse events that have a hypothesized or documented relationship to ranibizumab, based on either the route of administration (e.g., endophthalmitis) or mechanism of action (e.g., ocular and nonocular hemorrhages; arterio-thromboembolic events), were very low or absent in the present study, and intraocular inflammation was infrequent and never above a grade of 2+.

In conclusion, ranibizumab administered monthly for three months and then quarterly provided VA benefit to patients with neovascular AMD and was well tolerated. However, observations from the MARINA and ANCHOR trials suggest that the PIER regimen of dosing every three months after three monthly doses provides less benefit in VA on average than continued monthly dosing. Monthly dosing may be necessary in some patients to achieve maximal treatment benefit from ranibizumab.

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REFERENCES

1. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–1431.
2. Brown DM, Kaiser PK, Michels M, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N Engl J Med 2006;355:1432–1444.
3. Heier JS, Antoszyk AN, Pavan PR, et al. Ranibizumab for treatment of neovascular age-related macular degeneration: a phase I/II multicenter, controlled, multidose study. Ophthalmology 2006;113:633–642.
4. Rosenfeld PJ, Heier JS, Hantsbarger G, Shams N. Tolerability and efficacy of multiple escalating doses of ranibizumab (Lucentis) for neovascular age-related macular degeneration. Ophthalmology 2006;113:623–632.
5. Hochberg Y. A sharper Bonferroni procedure for multiple tests of significance. Biometrika 1988;75:800–802.
6. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one-year results of two randomized clinical trials—TAP report 1. Treatment of age-related macular degeneration with Photodynamic Therapy (TAP) Study Group. Arch Ophthalmol 1999;117:1329–1345.
7. Photodynamic therapy of subfoveal choroidal neovascularization in pathologic myopia with verteporfin. 1-year results of a randomized clinical trial—VIP Report No. 1. Ophthalmology 2001;108:841–52.
8. Visudyne [package insert]. East Hanover, New Jersey: Novartis Pharmaceuticals Corp; 2005.
9. Collaborative overview of randomized trials of antiplatelet therapy—I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. Antiplatelet Trialists' Collaboration. BMJ 1994;308:81–106.
10. Fung AE, Lalwani GA, Rosenfeld PJ, et al. An optical coherence tomography-guided, variable dosing regimen with intravitreal ranibizumab (Lucentis) for neovascular age-related macular degeneration. Am J Ophthalmol 2007;143:566–583.

Intravitreal Bevacizumab for Treatment of Neovascular Age-related Macular Degeneration: A One-year Prospective Study

ZIAD F. BASHSHUR, ZEINA A. HADDAD, ALEXANDRE SCHAKAL, ROLA F. JAAFAR, MARC SAAB, AND BAHA' N. NOUREDDIN

• PURPOSE: To investigate the efficacy of intravitreal bevacizumab for treatment of neovascular age-related macular degeneration (AMD).

• DESIGN: Prospective, open-label, nonrandomized clinical study.

• METHODS: Sixty patients (60 eyes) with subfoveal choroidal neovascular membrane (CNV) attributable to AMD participated in this study at the American University of Beirut and Hotel Dieu de France Retina Clinics. All lesion types were included except for retinal angiomatous proliferation. In the initial treatment phase, intravitreal bevacizumab (2.5 mg/0.1 ml) was given at baseline, and then two additional monthly injections were given if the macula was not dry on optical coherence tomography. The criteria for re-injection after the induction phase were presence of new fluid in the macula, increased central retinal thickness (CRT) at least 100 μm, loss of at least five letters of vision with increased fluid in the macula, new classic CNV or new macular hemorrhages. Main outcome measure was the proportion of eyes losing <15 letters of vision after 12 months.

• RESULTS: Fifty-one patients (51 eyes) completed the 12 months. Mean visual acuity improved from 45.7 letters at baseline to 53.1 letters at 12 months ($P = .004$), and 47 eyes (92.2%) lost <15 letters. Mean CRT decreased from 327.4 μm at baseline to 227.8 μm at 12 months ($P < .001$). A mean of 3.4 injections were given over the course of the study, and no ocular or systemic side-effects were noted.

• CONCLUSION: Eyes with neovascular AMD treated with intravitreal bevacizumab over 12 months had significant anatomical and functional improvement. Further studies need to confirm the long-term efficacy of this treatment. (Am J Ophthalmol 2008;145:249–256. © 2008 by Elsevier Inc. All rights reserved.)

NEOVASCULAR AGE-RELATED MACULAR DEGENERATION (AMD) is responsible for 90% of significant visual disability related to AMD.[1] Visual acuity loss typically results from development of a choroidal neovascular membrane (CNV) beneath the macula. Vascular endothelial growth factor (VEGF), a central mediator of angiogenesis and a potent permeability factor, seems to be a major stimulus for choroidal neovascularization in AMD.[2–4]

Photodynamic therapy with verteporfin and intravitreal injection of pegaptanib sodium are approved by the Food and Drug Administration (FDA) for the management of subfoveal CNV in AMD. Although both treatments are able to reduce the risk of severe visual acuity loss, neither is able to result in significant visual improvement.[5,6]

On June 30, 2006, the FDA approved ranibizumab for the treatment of neovascular AMD. Ranibizumab is the Fab fragment of a recombinant monoclonal humanized antibody that inhibits all isoforms of VEGF.[7] In the MARINA study, monthly injections of intravitreal ranibizumab were shown to decrease the chance for vision loss and improve visual acuity during two years of follow-up in patients with minimally classic or occult CNV in AMD.[8] The ANCHOR study showed similar results with predominantly classic CNV secondary to AMD.[9] Moreover, intravitreal ranibizumab was found to be superior to photodynamic therapy.[9] Recently, the PrONTO study used a variable dosing regimen depending mostly on optical coherence tomography (OCT) changes which was found to promote visual stability and improvement.[10]

Bevacizumab is a full length humanized monoclonal antibody derived from the same precursor as ranibizumab and binds all isoforms of VEGF. It is approved by the FDA for the treatment of metastatic colorectal cancer.[11] In the SANA study, systemic bevacizumab was found to reduce macular thickness and improve visual acuity.[12] However, there was a safety concern with systemic side-effects, namely elevated arterial blood pressure. In 2005, Rosenfeld and associates published the first case report about the intravitreal use of bevacizumab for CNV.[13] Since then, there have been several reports that seem to show that intravitreal bevacizumab is an effective treatment for neovascular AMD.[14–16] Moreover, the relatively low cost of bevacizumab has made it appealing for treatment of

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From the Departments of Ophthalmology, American University of Beirut (Z.F.B., Z.A.H., R.F.J., B.N.N.), and the Department of Ophthalmology, Hotel Dieu de France, St Joseph University (A.S., M.S.), Beirut, Lebanon.

Inquiries to Ziad F. Bashshur, American University of Beirut Medical Center, PO Box 11-0236/B11, Beirut, Lebanon; e-mail: [illegible]

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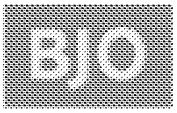

Comparative toxicity and proliferation testing of aflibercept, bevacizumab and ranibizumab on different ocular cells

Sven Schnichels, Ulrike Hagemann, Kai Januschowski, et al.

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Comparative toxicity and proliferation testing of aflibercept, bevacizumab and ranibizumab on different ocular cells

Sven Schnichels, Ulrike Hagemann, Kai Januschowski, Johanna Hofmann, Karl-Ulrich Bartz-Schmidt, Peter Szurman, Martin S Spitzer, Sabine Aisenbrey

Centre of Ophthalmology, University Eye Hospital Tübingen, Tübingen, Germany

Correspondence to
Kai Januschowski, Centre of Ophthalmology, University Eye Hospital Tübingen, Schleichstr. 12/1, Tübingen D-72076, Germany; Kai.januschowski@med.uni-tuebingen.de

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ABSTRACT

Background/aims Vascular endothelial growth factor (VEGF) is a key factor in the pathogenesis of neovascular retinal diseases including age-related macular degeneration. VEGF inhibitors including ranibizumab, pegaptanib or bevacizumab improve retinal morphology and vision in many patients. The recently approved drug aflibercept (VEGF Trap-Eye/Eyelea, Regeneron, Tarrytown, New York, USA) offers a new therapy modality. We therefore tested for toxic and anti-proliferating effects of aflibercept.

Methods The effects of aflibercept (0.125, 0.5, 2 mg), ranibizumab (0.125 mg) and bevacizumab (0.3125 mg) after 1, 24, 48 and 72 h on cell morphology via phase contrast pictures, cell viability via MTS assay, total cell amount via crystal violet staining, apoptosis induction via caspase 3/7 assay and proliferation via BrdU assay were investigated. Three ocular cell lines were chosen for toxicology testing: ARPE19 cells, RGC-5 cells and 661W cells.

Results Aflibercept did not cause changes in cell morphology, induce apoptosis or cause permanent decrease in cell viability, cell density or proliferation in any cell line or concentration investigated. In general, aflibercept had fewer effects (upregulation or downregulation) compared with controls than bevacizumab or ranibizumab.

Conclusions In our experiments, aflibercept did not lead to any negative effects on retinal cell lines and might therefore be used safely in clinical applications.

INTRODUCTION

Clinical trials have shown that inhibition of vascular endothelial growth factor (VEGF) by either intravitreal pegaptanib (Macugen, Eyetech Pharmaceutical, New York, USA), ranibizumab (Lucentis, Genentech, San Francisco, California, USA) or bevacizumab (Avastin, Roche, Basel, Switzerland) can result in stabilisation or improvement of retinal morphology and vision in many patients with neovascular age-related macular degeneration (AMD)[1–4] and other retinal diseases.[5–9]

Aflibercept (VEGF Trap-Eye/Eyelea, Regeneron, Tarrytown, New York, USA) is a new agent available for the treatment of exudative AMD. It is a decoy receptor with a longer half-life in rabbits and a higher affinity to VEGF compared with ranibizumab or bevacizumab.[10 11] In addition to its effect on VEGF, aflibercept inhibits placental growth factors 1 and 2.[11 12] Aflibercept has shown benefits in treating wet AMD in phase III trials comparable with those of ranibizumab. Since it was approved by the FDA, the drug seems to represent a safe treatment option for patients suffering from exudative AMD.[13]

In this study, we compare the proliferative and cytotoxic effects of three different aflibercept concentrations (0.125, 0.5 and 2.0 mg/ml), bevacizumab and ranibizumab on three different ocular cell lines (ARPE19, RGC-5 and 661W) at four different time-points (1, 24, 48, 72 h). We used the aflibercept diluent without aflibercept as an additional control.

MATERIALS AND METHODS

Cell culture

The rat ganglion cell-like/neuronal progenitor cell line —RGC-5—was kindly provided by Professor Neeraj Agarwal (UNT Health Science Center, Fort Worth, Texas, USA). The human retinal pigment epithelium cell line—ARPE19—was purchased from American Type Culture Collection (Manassas, Virginia, USA). The mouse photoreceptor cell line—661W—originated from Professor Dr Muayyad Al-Ubaidi.[14 15] All cell lines were maintained throughout the experiments in Dulbecco's modified Eagle's medium containing 4 mM L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin sulphate at 37°C and 5% CO_2. Depending on the specific experiment and cell line, ARPE19 cells were seeded either at a density of 30 000 cells/well in a 24-well plate or at a density of 10 000 cells/well in a 96-well plate; RGC-5 and 661W cells were seeded at a density of 10 000 cells/well in a 24-well plate or at a density of 5000 cells/well in a 96-well plate. Bevacizumab, ranibizumab and aflibercept were diluted with culture medium to obtain bevacizumab at concentrations of 0.3125 mg/ml, ranibizumab at concentrations of 0.125 mg/ml and aflibercept at concentrations of 0.125, 0.5 and 2.0 mg/ml, representing the injection doses, epiretinal doses and taking into account presumed dilution by the vitreous humor. The formula for the aflibercept diluent was prepared by the university pharmacy, Tübingen. In addition, the apoptosis inducer staurosporine (600 nM) was included in this study. Twenty-four hours after seeding, diluent, bevacizumab, ranibizumab, aflibercept or staurosporine at the desired concentration was added to the medium. Twenty-four, 48 and 72 hours after supplementation photographs were taken (100× magnification) from the 24-well plates using a phase contrast/fluorescence microscope (Axiovert 135, Zeiss, Göttingen, Germany) and AxioVision 4.6 software (Zeiss)

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MTS viability assay

One, 24, 48 and 72 hours after supplementation, 20 µl of the CellTiter 96 AQueous One Solution Reagent (Promega) was directly added to the culture wells and incubated for 90 min. Then the absorbance was recorded at 490 nm with a Microplate Reader (BioTek, Synergy HT, Bad Friedrichshall, Germany) with the correction of interference at 690 nm.[16]

Crystal violet staining

After the MTS assay medium was removed and the cells fixed overnight with 4% paraformaldehyde, the cells were washed three times, stained with crystal violet solution (Sigma Aldrich, Steinheim, Germany), washed again, and incubated with 1% SDS for 1 h. Absorbance was determined at 595 nm (BioTek).[16]

Caspase 3/7 activity assay

One, 24, 48 and 72 hours after supplementation, caspase 3/7 activity was determined using the CaspaseGlo 3/7 activity kit (Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol. Luminescence was measured with a luminometer (BioTek).[16]

BrdU cell proliferation assay

Cellular proliferative activity was directly monitored by quantification of 5′-bromo-2′-deoxyuridine (BrdU) incorporation into the genomic DNA during cell growth. DNA synthesis was assessed by BrdU Cell Proliferation Assay (Calbiochem, La Jolla, California, USA). Briefly, 24, 48 and 72 h after supplementation, cell proliferation was monitored by adding 20 µl BrdU label to the media 24 h prior to the desired time-point. All additional steps were performed according to the manufacturer's protocol. Absorbance was determined at 450 and 595 nM (BioTek).

Statistical analysis

Data are represented as mean±SD. With every assay, four to ten different experiments were conducted per cell line and concentration or control, respectively (n=4–10). Statistical analysis was performed using JMP (V.9.0.0, SAS Institute Inc., Cary, North Carolina, USA). Dunnett's analysis was used for comparison between medium versus diluent or drugs and diluent versus drugs. Differences were considered to be significant at $p<0.05$. Results differing more than 0.2 or 20% were considered to be relevant.

RESULTS

Effect of aflibercept on RGC-5 cells

Untreated RGC-5 cells appeared as a spindle-type shape (figure 1A) as observed by phase contrast pictures. This shape did not change after the treatment of aflibercept, bevacizumab or ranibizumab, independent of the time-point (figure 1A). Only staurosporine changed the morphology at every time-point investigated (figure 1A).

Cell viability of RGC-5 cells treated with varying concentrations of aflibercept (0.125, 0.5 or 2 mg), bevacizumab, ranibizumab and diluent did not show a relevant decrease in cell viability. However, some significant differences were detected (figure 1B). For all substances, cell viability was significantly reduced 1 h after treatment compared with medium only treated cells. The cell viability at all other time-points varied between 98% and 137% of the control. Only 2 mg aflibercept compared with medium and diluent 24 h, bevacizumab compared with medium 48 h and bevacizumab compared with medium and diluent 72 h after supplementation showed a significant difference with only bevacizumab 72 h after supplementation differing relevantly. In contrast, staurosporine significantly reduced cell viability down to 11%. No substance decreased cell viability below the amount of the apoptosis inducer staurosporine at any time-point (figure 1B).

Accordingly, cell density via crystal violet staining did not show any relevant differences. Cell density was significantly lower 1 h after application of aflibercept (2 mg) and bevacizumab compared with diluent. Cell density increased significantly for bevacizumab 24 h after treatment compared with diluent supplemented probes. All other drugs or time-points investigated did not show a significant or even relevant difference (figure 1C).

With regard to cell viability and cell density, a significant effect of the applied drugs could only be observed on caspase 3/7 activity at the 1-hour time-point. All drugs (except for 0.125 mg aflibercept) showed a significant increase of the caspase 3/7 activity. All effects were diminished at the 24-hour and later time-points, except for staurosporine treated probes with a caspase activity up to 13-fold higher compared with the control (figure 1D).

Proliferation was similar in all measurements except for 0.125 mg aflibercept 24 h and bevacizumab 48 h after supplementation. The proliferation of staurosporine treated probes was significantly reduced at all time-points (figure 1E).

Effect of aflibercept on ARPE19 cells

Untreated and treated ARPE19 cells showed no obvious morphological changes as observed by phase contrast pictures (figure 2A). Staurosporine changed the morphology at every time-point investigated (figure 2A).

Cell viability of ARPE19 cells treated with varying concentrations of aflibercept, bevacizumab, ranibizumab and diluent did not show relevant decrease in cell viability. Only ranibizumab at 1 h was significantly different from medium only and diluent supplemented probes. Staurosporine decreased the cell viability in a time-dependent manner (figure 2B).

Accordingly, cell density via crystal violet staining did not show any relevant negative effects: no significant differences were found 1 h after treatment. At all other time-points, an increase in cell density could be observed in treated probes. Only a few were significant to the medium only probes (figure 2C). No significant difference was found for any drug compared with diluent (figure 2C). In contrast, staurosporine caused a time-dependent decrease in cell density (figure 2C).

Few significant differences in caspase 3/7 activity were observable immediately after application of the drugs (1 h) compared with medium or diluent supplemented probes. No differences were observed at 24, 48 and 72 h after treatment. In contrast, staurosporine induced apoptosis in a time-dependent manner (figure 2D).

All drugs had a positive effect on proliferation 24 h after application compared with control. At 48- and 72-hour time-points, the proliferation rate of treated probes ranged below the proliferation rate of control cells. These findings were significant for 2 mg aflibercept, bevacizumab and ranibizumab 48 h after application and all drugs except for ranibizumab at 72 h after application. No change in proliferation rate was defined as relevant. Staurosporine reduced proliferation in a time-dependent manner.

Effect of aflibercept on 661W cells

Untreated 661W cells showed spindle-type shape (figure 3A) as observed by phase contrast pictures. Treatment of the cells with aflibercept, bevacizumab or ranibizumab, independent of the

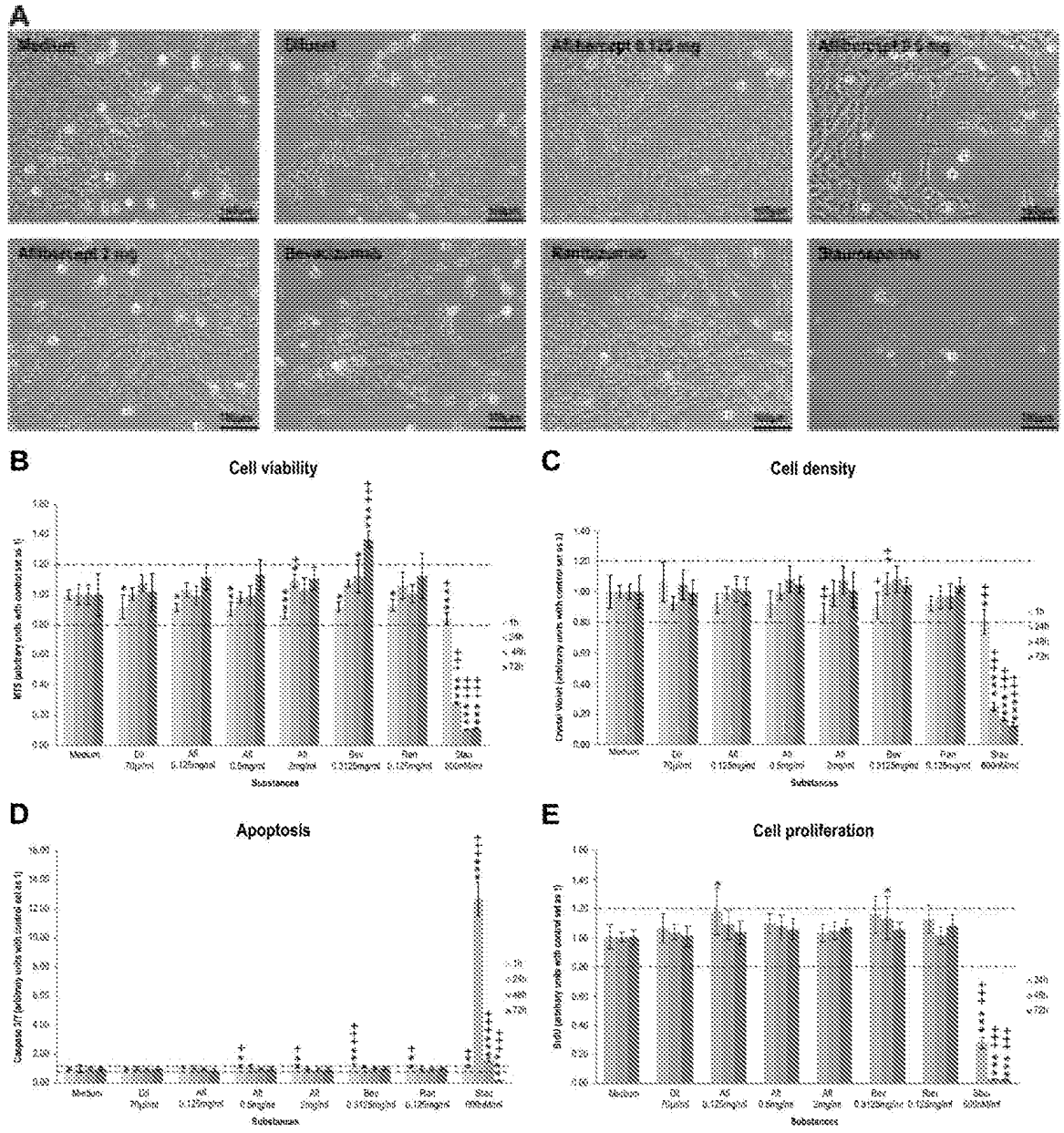

Figure 1 Effects of aflibercept, bevacizumab or ranibizumab on RGC-5 cells. Representative phase contrast pictures of RGC-5 cells 72 h after supplementation with medium, diluent, aflibercept (0.125, 0.5, 2 mg), ranibizumab (0.125 mg) or bevacizumab (0.3125 mg) and staurosporine (600 nM): no morphological changes of the RGC-5 cells were observed by phase contrast pictures at any time-point or concentration or drug, except for staurosporine (A). No drug (except staurosporine) had a relevant negative effect on cell viability, total cell amount, apoptosis and proliferation in RGC-5 cells. Bar graphs represent cell viability (B), total cell amount (C), caspase 3/7 activity (D) and proliferation (E) expressed as arbitrary units with the control set as 1 of RGC-5 cells 1, 24, 48 and 72 h after supplementation with different concentrations of aflibercept (0.125, 0.5, 2 mg), bevacizumab (0.3125 mg) and ranibizumab (0.125 mg) (n=4–6). (B) Although the application of the drugs shows an immediate decrease of cell viability, no drug permanently negatively affects the cell viability of the RGC-5 cells. Even more, the cell viability increased continuously with time. In contrast, staurosporine decreased the cell viability. (C) The application of the drugs does not affect the amount of cells in a relevant way. All cell amounts vary between 86% and 108% of the medium controls. In contrast, staurosporine decreased the cell amount in a time-dependent manner. (D) One hour after treatment, the caspase 3/7 activity was significantly higher in all cells supplemented with a drug. This effect was not observable at any other time-point, at which the caspase 3/7 activity varied between 89% and 115% of the control. However, staurosporine induced apoptosis, with a peak caspase 3/7 activity at the 24-hour time-point. (E) Cell proliferation was significantly changed in 3 of 18 investigated treatments: no relevant increase or decrease was observed. Proliferation varied between 101% and 118% compared with the medium only control. Only staurosporine reduced the proliferation to a minimum (2%).

time-point did not result in obvious changes in morphology (figure 3A). In contrast, staurosporine obviously changed morphology at every time-point investigated (figure 3A).

Application of the drugs or the diluent led to immediate, sometimes significant increase of cell viability 1 h after treatment (103% and 125% of the control) (figure 3B). Cell viability of most treated 661W cells did not show a significant or relevant difference 24 and 48 h after application. Only two samples differed significantly: 0.125 mg aflibercept versus diluent and 2 mg aflibercept versus medium supplemented samples 48 h after stimulation. In contrast, at the 72-hour time-point, all treated probes showed significant and relevant increase in cell

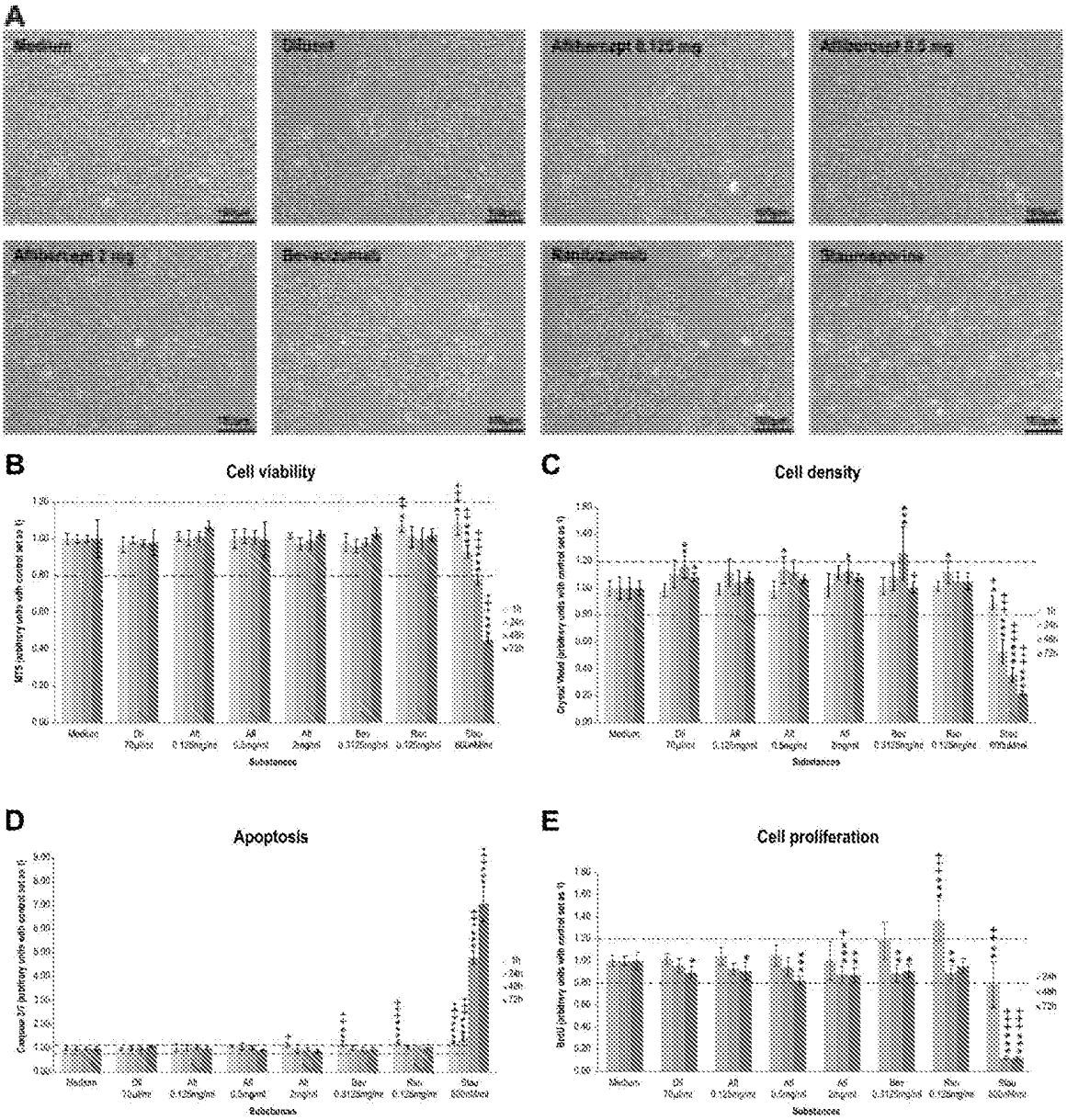

Figure 2 Effects of aflibercept, bevacizumab or ranibizumab on ARPE19 cells. Representative phase contrast pictures of ARPE19 cells 72 h after supplementation with medium, diluent, aflibercept (0.125, 0.5, 2 mg), ranibizumab (0.125 mg) or bevacizumab (0.3125 mg) and staurosporine (600 nM): no morphological changes of the ARPE19 cells were observed by phase contrast pictures at any time-point or concentration or drug, except for staurosporine (A). No drug (except staurosporine) had a permanent and relevant negative effect on cell viability, total cell amount, apoptosis and proliferation in ARPE19 cells. Bar graphs represent cell viability (B), total cell amount (C) caspase 3/7 activity (D) and proliferation (E) expressed as arbitrary units with the control set as 1 of ARPE19 cells 1, 24, 48 and 72 h after supplementation with different concentrations of aflibercept (0.125, 0.5, 2 mg), bevacizumab (0.3125 mg) and ranibizumab (0.125 mg) (n=5–10). (B) No drug showed a relevant reduction in cell viability at any time-point. Although some variations between the medium were significant, none of these are regarded relevant, as the cell viability varied between 96% and 107% of the medium only vials. However, staurosporine, which served as an additional control for a negative induction, decreased the cell viability in a time-dependent manner. (C) No significant or even relevant negative effect on the cell density was observed at any time-point or condition investigated except for staurosporine, which reduced the amount of cells in a time-dependent manner. In contrast, the cell amount was even higher at many time-points compared with the controls. (D) Although the caspase 3/7 activity was significantly and relevantly higher in some drug treated probes 1 h after application, these effects were not permanent and not found at further time-points. Staurosporine induced apoptosis in a time-dependent manner. (E) At the 24-hour time-point, all drugs caused an increased proliferation. Moreover, ranibizumab even caused a relevant increase. At the 48- and 72-hour time-point, the proliferation rate was lower than that of the controls. Staurosporine reduced proliferation in a time-dependent manner.

viability compared with untreated cells. Compared with the cells treated with diluent only, the 2 mg aflibercept treated cells had significant and relevant higher cell viability. Cell viability of staurosporine treated cells was reduced at every time-point from 24 h onwards.

Except for the 0.125 and 0.5 mg aflibercept, bevacizumab and ranibizumab treated probes 24 h, 0.125 and 2 mg aflibercept 48 h, ranibizumab 72 h after treatment, we did not record a difference in cell density. Only the 0.125 and 0.5 mg aflibercept treated probes 24 h after stimulation showed a relevant

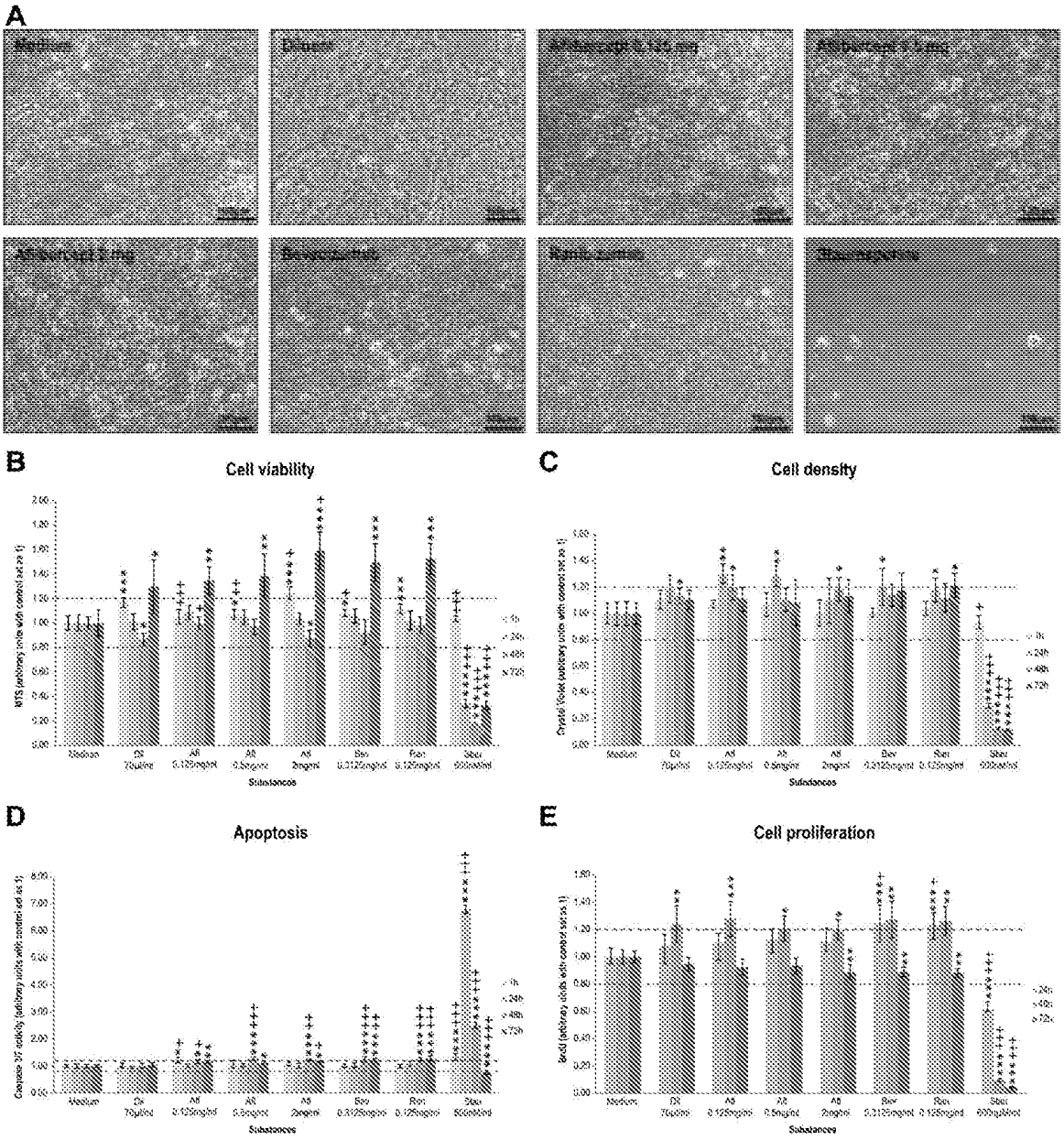

Figure 3 Effects of aflibercept, bevacizumab or ranibizumab on 661W cells. Representative phase contrast pictures of 661W cells 72 h after supplementation with medium, diluent, aflibercept (0.125, 0.5, 2 mg), ranibizumab (0.125 mg) or bevacizumab (0.3125 mg) and staurosporine (600 nM): no morphological changes of the 661W cells were observed by phase contrast pictures at any time-point or concentration or drug, except for staurosporine (A). No drug (except staurosporine) had a distinct negative permanent effect on cell viability, total cell amount and proliferation in 661W cells. However, an increased caspase 3/7 activity was measured in some settings. Bar graphs represent cell viability (B), total cell amount (C), caspase 3/7 activity (D) and proliferation (E) expressed as arbitrary units with the control (medium only) set as 1 of 661W cells 1, 24, 48 and 72 h after supplementation with different concentrations of aflibercept (0.125 mg; 0.5 mg; 2 mg), bevacizumab (0.3125 mg) and ranibizumab (0.125 mg) (n=4–6). (B) One and 24 hours after supplementation no drug showed a negative effect. However, 48 h after treatment, cell viability was reduced (87%–100%) in many probes compared with the medium only treated samples. In contrast, 72 h after treatment cell viability was higher in every drug treated sample than in the controls (1.30–1.59-fold higher). In contrast, staurosporine decreased the cell viability permanently. (C) The amount of cells was always higher than the amount of cells in the medium only treated cells. In contrast, staurosporine reduced the amount of cell in a time-dependent manner. (D) The caspase 3/7 activity was not more than 1.3-fold higher than that of the controls at any time-point investigated after application of the drugs or diluents. However, based on our definition that any increase of more than 20% should be regarded as relevant, the following settings are crucial: aflibercept 0.125 mg 1 h after treatment, aflibercept 0.5 and 2 mg 48 h after treatment and bevacizumab and ranibizumab 48 and 72 h after treatment. Although one should pay attention to these findings, this does not mean that these drugs are not safe because a real induction of apoptosis would be much higher, as observable with staurosporine. Staurosporine induced caspase 3/7 activity with a peak (6.8-fold) at the 24-hour time-point. (E) Cell proliferation was significantly and sometimes relevantly increased (between 1.1- and 1.28-fold) in several drug treated probes 24 and 48 h after application. In contrast, 72 h after treatment proliferation was reduced to 95%–88% relatively compared with the medium only samples (controls). Only staurosporine reduced the proliferation to a minimum (5%) in a time-dependent manner.

difference compared with medium. In staurosporine treated probes, the cell density was significantly and relevantly lower compared with medium only probes from 24 h onwards.

Neither aflibercept (except 0.125 mg 1 h) nor bevacizumab or ranibizumab significantly increased caspase 3/7 activity in the 661W cells 1 or 24 h after treatment compared with the control or the diluent supplemented probes. In contrast, 48 and 72 h after supplementation the caspase 3/7 activity significantly increased in all drug-treated probes compared with the medium only cells. In addition, caspase 3/7 activity increased significantly in 2 mg aflibercept, bevacizumab or ranibizumab treated probes compared with the diluent 72 h after supplementation. Moreover, except for 0.125 mg aflibercept all differences were relevant 48 h after supplementation compared with medium and diluent, whereas only bevacizumab and ranibizumab showed relevant differences 72 h after treatment. However, the known apoptosis inducer staurosporine increased the caspase 3/7 activity at 24 h up to 6.8-fold compared with control (figure 3C).

The cell proliferation rate was higher in several of the treated probes: the proliferation rate was significantly higher in bevacizumab or ranibizumab at 24 h, all drugs or diluent at 48 h, 2 mg aflibercept, bevacizumab and ranibizumab at 72 h after stimulation compared with medium only cells. Compared with diluent, the proliferation rate of bevacizumab and ranibizumab treated cells were significantly higher 24 h after treatment. Staurosporine decreased proliferation rates in a time-dependent manner.

DISCUSSION

The most important finding of this study is the observation that aflibercept, even at the highest concentration tested, did not lead to any obvious change in cell morphology and did not induce apoptosis or a permanent decrease in cell viability, cell density or proliferation in any of the three cell lines investigated. It is important to consider that while testing aflibercept we used the maximum dose for intravitreal injection taking an inhomogeneous distribution of aflibercept into account.

The tests applied in this study (MTS viability assay, caspase 3/7 activity assay, crystal violet staining and BrdU assay) are well evaluated and standardised tests. Although each of these tests examines only a single effect: MTS for cell viability, caspase 3/7 activity for apoptosis, crystal violet staining for the amount of cells, phase contrast pictures for morphology, BrdU assay for proliferation, the combination of these assays would reveal any negative effects that might be hidden if only a single test is performed. Single tests alone could be misleading: apoptosis as an active process is sometimes characterised by a high cell activity leading to a higher reduction of MTS, which can be misinterpreted as a higher or normal cell viability. With the additional assays performed in this set of experiments, such an effect can be excluded since apoptosis would have been detected with higher caspase 3/7 activity, a less amount of cells or a lower proliferation rate.

Other interesting observations concerning the results need to be discussed further. Aflibercept was constructed to bind human VEGF isoforms.[11] The RGC-5 and 661W cell lines are of rat or mouse origin, whereas the ARPE19 cell line is of human origin. However, Holash *et al* stated that despite its affinity for human proteins, aflibercept binds VEGF in all species tested.[11] Therefore, we assume that independent of the cell line origin any possible effect should be present in these cells. Moreover, several authors successfully reported the use of aflibercept in vivo and in tissue of mice and rat origin under different conditions.[17–21]

In a previous study, we investigated proliferation and cytotoxic effects of bevacizumab and ranibizumab on ARPE19 and RGC-5 cells; in these experiments only bevacizumab at 0.3 mg/ml showed a significant but not relevant inhibition of proliferation.[22] The results of the present study are in accordance with our previous results. We also investigated the effects of staurosporine on RGC-5 cell viability, cell density and apoptosis induction using the same assays and achieved similar results.[23]

Since the diluent of the agent might also cause certain effects, we decided to include the diluent in the tests. It contains sucrose, which is known to cause a higher proliferation rate in cells.[24] This might explain the higher proliferation rates and higher cell density, cell viability and sometimes apoptosis ratio in our experiments and is additionally supported by the increasing cell density rate seen in figures 1C, 2C and 3C. Our results were not normalised for cell density. Therefore, with increasing cell numbers the amount of the converted agents in the assays also increases; this effect has to be taken into consideration in the interpretation of the data (figures 1–3). The diluent in bevacizumab and ranibizumab solutions, both including trehalose instead of sucrose, differs from the diluent used in the aflibercept formula. Our data indicate that the proliferation rate decreases for all drugs at the 72-hour time-point. This effect could be explained by the fact that in general the proliferation rate decreases with increasing confluence. The probes maintained in medium only proliferated slower due to the missing sucrose or trehalose. However, after 72 h, these cells proliferated relatively faster due to less confluence (figures 1D, 2D and 3D).

In the RGC-5 and ARPE19 cells, we observed changes in cell viability, cell density, a slight induction of apoptosis or changes in proliferation rate in some drug-treated probes (figures 1B–D and 2B–D). Bevacizumab only shows long term effects in cell viability in RGC-5 cells. Recently, 24-hour tests with an MTT assay on ARPE19 cells were performed by another group.[25] The results of the two identical concentrations tested in that study (0.125 and 0.5 mg) are in accordance with our results.[25]

In the 661W cell line, we observed a slight increase in the caspase 3/7 activity. However, this is accompanied by a higher cell density and cell viability. Since we also observed a higher proliferation rate at 72 h, we assume that the sugar in the drugs increases the proliferation rate until confluence is reached. Therefore, a higher number of cells is present in the drug-treated probes and accordingly a higher cell viability and caspase 3/7 activity are observable. In conclusion, the slight induction of apoptosis cannot be considered as a concerning effect.

Our data show that the recently approved drug aflibercept does not induce toxic effects over a 72-hour time period at any of the clinically used and tested concentrations. Based on our findings, administration of aflibercept can be considered a safe treatment option for neovascular retinal diseases with regard to local effects on the retina.

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Contributors SS planned the study design, performed the statistical analysis, analysed the data, wrote the manuscript and generated the figures. UH performed the experiments, planned the study design, performed the statistical analysis, cleaned and analysed the data and revised the paper. KJ planned the study design and wrote and revised the manuscript. JH performed the experiments. K-UB-S and PS revised the manuscript. MSS planned the study design and revised the manuscript. SA planned the study design and revised the manuscript.

Competing interests None.

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REFERENCES

1 Brown DM, Kaiser PK, Michels M, *et al*. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *N Engl J Med* 2006;355:1432–44.
2 Gragoudas ES, Adamis AP, Cunningham ET, *et al*. Pegaptanib for neovascular age-related macular degeneration. *N Engl J Med* 2004;351:2805–16.
3 Ng EW, Shima DT, Calias P, *et al*. Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nat Rev Drug Discov* 2006;5:123–32.
4 Rosenfeld PJ, Brown DM, Heier JS, *et al*. Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med* 2006;355:1419–31.
5 Adamis AP, Shima DT. The role of vascular endothelial growth factor in ocular health and disease. *Retina* 2005;25:111–18.
6 Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004;25:581–611.
7 Heier JS, Brown DM, Chong V, *et al*. Intravitreal aflibercept (VEGF Trap-Eye) in wet age-related macular degeneration. *Ophthalmology* 2012;119:2537–48.
8 Brown DM, Heier JS, Clark WL, *et al*. Intravitreal aflibercept injection for macular edema secondary to central retinal vein occlusion: 1-year results from the phase 3 COPERNICUS study. *Am J Ophthalmol* 2013;155:429–37.e7.
9 Do DV, Nguyen QD, Boyer D, *et al*. One-year outcomes of the DA VINCI study of VEGF trap-eye in eyes with diabetic macular edema. *Ophthalmology* 2012;119:1658–65.
10 Papadopoulos N, Martin J, Ruan Q, *et al*. Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab. *Angiogenesis* 2012;15:171–85.
11 Holash J, Davis S, Papadopoulos N, *et al*. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci USA* 2002;99:11393–8.
12 Dixon JA, Oliver SC, Olson JL, *et al*. VEGF Trap-Eye for the treatment of neovascular age-related macular degeneration. *Expert Opin Investig Drugs* 2009;18:1573–80.
13 Browning DJ, Kaiser PK, Rosenfeld PJ, *et al*. Aflibercept for age-related macular degeneration: a game-changer or quiet addition? *Am J Ophthalmol* 154:222–6.
14 al-Ubaidi MR, Font RL, Quiambao AB, *et al*. Bilateral retinal and brain tumors in transgenic mice expressing simian virus 40 large T antigen under control of the human interphotoreceptor retinoid-binding protein promoter. *J Cell Biol* 1992;119:1681–7.
15 Tan E, Ding XQ, Saadi A, *et al*. Expression of cone-photoreceptor-specific antigens in a cell line derived from retinal tumors in transgenic mice. *Invest Ophthalmol Vis Sci* 2004;45:764–8.
16 Schnichels S, Schultheiss M, Hofmann J, *et al*. Trichostatin A induces cell death at the concentration recommended to differentiate the RGC-5 cell line. *Neurochem Int* 2012b;60:581–91.
17 Lal D, Park JA, Demock K, *et al*. Aflibercept exerts antivascular effects and enhances levels of anthracycline chemotherapy in vivo in human acute myeloid leukemia models. *Mol Cancer Ther* 2010;9:2737–51.
18 Cao J, Zhao L, Li Y, *et al*. A subretinal matrigel rat choroidal neovascularization (CNV) model and inhibition of CNV and associated inflammation and fibrosis by VEGF trap. *Invest Ophthalmol Vis Sci* 2011;51:6009–17.
19 Garbow JR, Santeford AC, Anderson JR, *et al*. Magnetic resonance imaging defines cervicovaginal anatomy, cancer, and VEGF trap antiangiogenic efficacy in estrogen-treated K14-HPV16 transgenic mice. *Cancer Res* 2009;69:7945–52.
20 Gomez-Manzano C, Holash J, Fueyo J, *et al*. VEGF Trap induces antiglioma effect at different stages of disease. *Neuro Oncol* 2008;10:940–5.
21 Bachmann BO, Luetjen-Drecoll E, Bock F, *et al*. Transient postoperative vascular endothelial growth factor (VEGF)-neutralisation improves graft survival in corneas with partly regressed inflammatory neovascularisation. *Br J Ophthalmol* 2009;93:1075–80.
22 Spitzer MS, Yoeruek E, Sierra A, *et al*. Comparative antiproliferative and cytotoxic profile of bevacizumab (Avastin), pegaptanib (Macugen) and ranibizumab (Lucentis) on different ocular cells. *Graefes Arch Clin Exp Ophthalmol* 2007;245:1837–42.
23 Schultheiss M, Schnichels S, Miteva K, *et al*. Staurosporine-induced differentiation of the RGC-5 cell line leads to apoptosis and cell death at the lowest differentiating concentration. *Graefes Arch Clin Exp Ophthalmol* 2012;250:1221–9.
24 Kato A, Tohoyama H, Joho M, *et al*. Different effects of galactose and mannose on cell proliferation and intracellular soluble sugar levels in Vigna angularis suspension cultures. *J Plant Res* 2007;120:713–19.
25 Ammar DA, Mandava N, Kahook MY. The effects of aflibercept on the viability and metabolism of ocular cells in vitro. *Retina* 2013;33:1056–61.

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Article

Update on VEGF Trap-Eye Clinical Trials

A new way to block VEGF

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Update on VEGF Trap-Eye Clinical Trials

A new way to block VEGF

Sumit Sharma, MD • Peter K. Kaiser, MD

Multiple studies have implicated vascular endothelial growth factor in the pathogenesis of neovascular eye diseases, including neovascular age-related macular degeneration, diabetic retinopathy, diabetic macular edema and central retinal vein occlusion (CRVO).[1-6] Currently, the only FDA-approved treatments for AMD targeting the VEGF pathway are pegaptanib sodium (Macugen, Eyetech Pharmaceuticals, Inc), an aptamer that binds $VEGF_{165}$ and ranibizumab (Lucentis, Genentech), a humanized, affinity-matured Fab fragment that binds all isoforms of VEGF-A; bevacizumab (Avastin, Genentech), a humanized monoclonal antibody with two VEGF-binding domains against all VEGF-A isoforms, is also used off label as an alternative to ranibizumab given its much lower cost.

Intraocular injection of pegaptanib every six weeks reduced the percentage of patients who experience severe vision loss but did not lead to a significant improvement in visual acuity.[7] In contrast, monthly injections of ranibizumab resulted in a significant improvement in visual acuity in about one-third of patients.[8-9]

It is felt that the difference in efficacy between these two drugs is due to the fact that isoforms other than $VEGF_{165}$ are implicated in the pathogenesis of neovascularization in the eye. Although there have been a few small studies evaluating bevacizumab for the treatment of neovascular AMD, there are no completed randomized clinical trials comparing ranibizumab to bevacizumab, but many are currently in progress, including the Comparison of AMD Treatment Trials (CATT), with results expected in 2011.

VEGF TRAP-EYE

VEGF Trap-Eye (Regeneron Pharmaceuticals and Bayer Healthcare AG) is a 110 kDa fusion protein of portions of the extracellular binding domains of VEGF receptors 1 and 2 (VEGFR-1 and VEGFR-2) and the Fc region of human IgG1. Previous studies have found that one of the most potent ways to block VEGF signaling is to prevent VEGF from binding to its receptor by administering decoy VEGF receptors.[10] VEGF Trap-Eye was engineered to have much higher affinity for VEGF-A (~1 pM),[11] compared to bevacizumab (500-2,200 pM)[12-14] and ranibizumab (140 pM).[15] This may allow VEGF Trap-eye to be more potent than either drug currently in use.

It is mathematically estimated that VEGF Trap-Eye will maintain significant intravitreal VEGF-binding activity for 10-12 weeks after a single intravitreal injection.[16] Another possible advantage that VEGF Trap-Eye has over ranibizumab is that it blocks all isoforms of VEGF-A as well as placental growth factor (PlGF)-1 and -2. PlGF is a

part of an independent angiogenesis cascade (**Figures 1 and 2**).

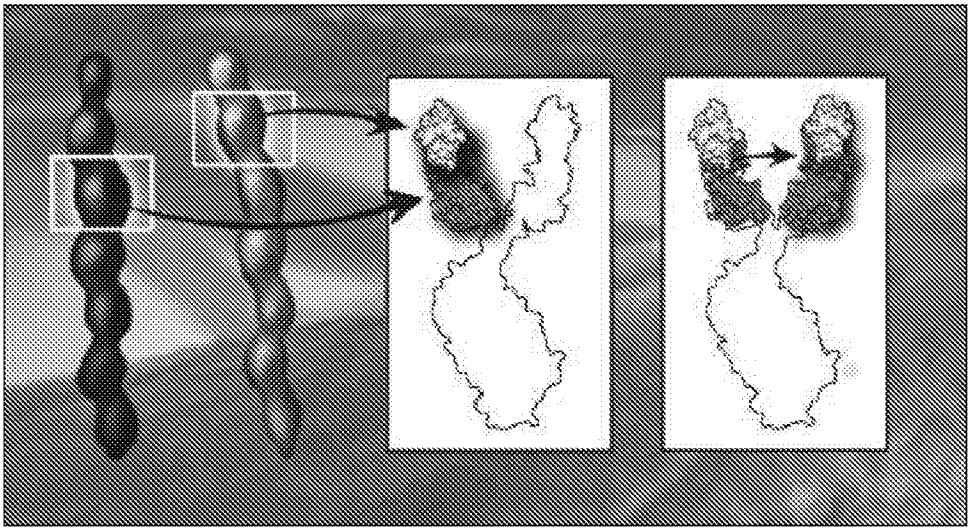

Figure 1. A key binding domain of VEGFR1 and a key binding domain of VEGFR2 (left) are fused for tight binding affinity for both VEGF-A isomers and PlGF (center). Two dual-domain arms are used for one VEGF Trap-Eye molecule to mimic the natural receptor pairing necessary for growth factor signaling (right).

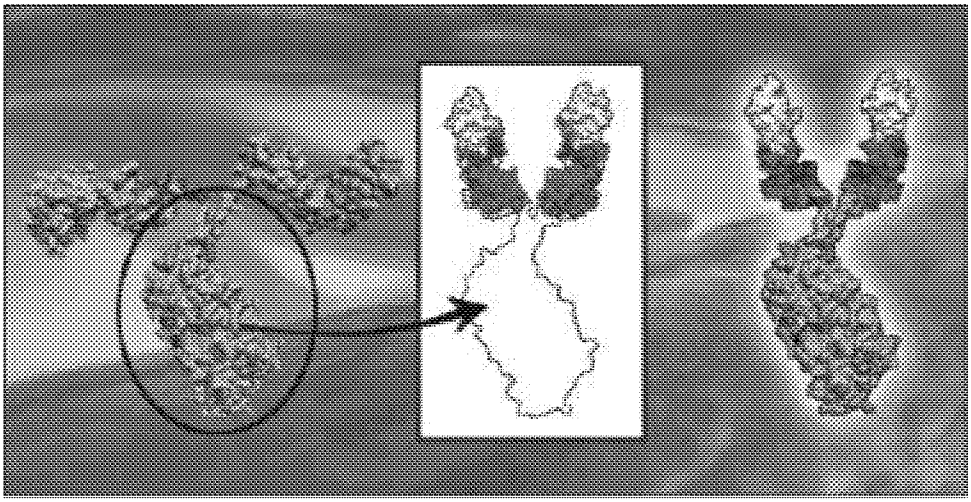

Figure 2. The Fc portion of IgG1 (left) is fused to the two dual-domain arms (center) resulting in the engineered molecule of VEGF Trap-Eye (right). This exemplifies how a molecule can be designed to possess specific properties of different naturally-occurring molecules with a goal of optimizing therapeutic activity.

Herein, we review the results of phase 2 trials evaluating VEGF Trap-Eye for neovascular AMD and diabetic macular edema, as well as describe trials that are currently in progress.

THE TRIALS

The CLEAR-IT-2 trial was a phase 2, randomized, double-masked, multicenter dose-comparison study of the safety and efficacy of VEGF Trap-Eye in patients with neovascular AMD. Subjects were assigned to one of five treatment groups: monthly intravitreal injections of 0.5 or 2.0 mg of VEGF Trap-Eye for the first 12 weeks (for a total of four mandatory injections) or quarterly dosing with one initial intravitreal injection of 0.5, 2.0 or 4.0 mg of VEGF Trap-Eye followed by a second mandatory injection at week 12. After the 12-week primary outcome, all patients were treated on an as-needed basis for another 40 weeks.

At one year of follow-up, there was a mean improvement of +5.3 letters in best corrected visual acuity for all groups combined (P<0.0001). Patients who received four monthly doses of 0.5 or 2.0 mg followed by as-needed dosing achieved a mean improvement of +5.4 letters (P<.085 vs baseline) and +9.0 letters (P<0.0001 vs baseline) from baseline, respectively. Patients receiving quarterly dosing of 0.5 mg gained +2.6 letters (P=0.344 vs baseline), those receiving quarterly dosing of 2.0 mg gained +5.2 letters (P=0.0412 vs baseline), and those receiving quarterly dosing of 4.0 mg gained +4.2 letters (P=0.0154 vs baseline) at one year. Analysis of retinal imaging studies revealed a statistically significant reduction in central retinal thickness and mean CNV lesion size for all groups.

One hundred seventeen patients originally enrolled in CLEAR-IT-2 were followed in an open-label extension study and received injections of 2.0 mg of VEGF Trap-Eye on an as-needed basis with q8-week monitoring. The mean gain in BCVA from baseline in the original trial of the 117 patients who were followed in the extension study was +7.3 letters (P<0.0001 vs baseline) at three months, +8.4 letters (P<0.0001 vs baseline) at one year, +7.1 letters (P<0.0001 vs baseline) at 18 months, and +6.1 letters (P<0.0001 vs baseline) at two years. Of the patients enrolled in the extension study, 92% lost less than 15 letters, 71% gained 0 or more letters, and 30% gained 15 or more letters of visual acuity after treatment with VEGF Trap-Eye. This compares favorably to the pivotal MARINA/ANCHOR studies, but in CLEAR-IT-2 patients did not require monthly injections, as in the Genentech trials. Over the 21 months of the PRN dosage stage of the phase 2 trial and extension study patients received an average of only 4.6 additional injections of VEGF Trap-Eye, with 9% requiring no additional injections.

Serious adverse events in CLEAR-IT-2 were rare: one patient had culture-negative endophthalmitis, five patients died (one from pre-existing pulmonary hypertension, one from pancreatic cancer, one from pulmonary failure, one from squamous cell lung cancer and one from cerebrovascular accident) and four patients had arterial thromboembolic events (two cerebrovascular accidents and two myocardial infarctions). The most commonly reported adverse events were those related to the intravitreal injection: subconjunctival hemorrhage at the injection site and transient increase in intraocular pressure. Subgroup analysis showed that patients less than 75 years old achieved greater BCVA gains compared to patients older than 75. No other subgroup comparisons achieved statistical significance.

The DME And VEGF Trap-Eye: INvestigation of Clinical Impact (DA VINCI) study was a double masked, randomized, active controlled phase 2 study of the safety, tolerability and biological effect of repeated intravitreal administration of VEGF Trap-Eye in patients with clinically significant DME. Data were presented by Diana Do, MD, at the World Ophthalmology Congress in Berlin, Germany. The primary outcome measure was the change in BCVA at 24 weeks. Two hundred nineteen patients were randomized into one of five groups: the control group received macular laser therapy at week one and as-needed repeat laser therapy as often as every 16 weeks; two groups received doses of 0.5 mg or 2.0 mg every four weeks for 24 weeks; two groups received three initial doses of 2.0 mg every four weeks, followed by either every-eight-week dosing or as-needed dosing.

At the six-month primary analysis, patients had a mean change in vision of +2.5 letters with traditional laser photocoagulation. In contrast, all of the VEGF Trap-Eye arms achieved statistically significant improvements in BCVA over the laser control arm, gaining from +8.5 to +11.4 letters. There was no statistically significant difference in outcomes noted between the VEGF Trap-Eye groups compared to the laser group. There were two cases of endophthalmitis, one growing *Staphylococcus epidermidis* and one culture negative. The most common adverse events reported were those related to intravitreal injections and were similar to those described above.

TRIALS STILL UNDER WAY

The Double-Masked Study of Efficacy and Safety of Intravitreal VEGF Trap-Eye in Subjects with Wet AMD (VIEW 1 and VIEW 2) studies are the two pivotal, randomized, active controlled, double-masked, phase 3 studies to compare VEGF Trap-Eye dosed 0.5 mg every four weeks, 2.0 mg every four weeks, or 2.0 mg every eight weeks

(after three monthly 2.0 mg doses) for one year, compared to ranibizumab 0.5 mg delivered every four weeks for one year.

In the second year of the trial, PRN dosing will be evaluated, but patients will receive a treatment at least once every 12 weeks. The primary outcome is the proportion of subjects who maintain or improve vision at week 52, compared to ranibizumab. VIEW 1 is being performed at 188 sites in the United States and Canada, and VIEW 2 is being performed at 190 sites in Europe, Asia, Japan, Australia and South America. Both trials have completed enrollment with one-year results expected in early 2011.

VEGF Trap-Eye: Investigation of Efficacy and Safety in Central Retinal Vein Occlusion (COPERNICUS and GALILEO) studies are randomized, double-masked, sham controlled phase 3 trials of the efficacy, safety and tolerability of repeated intravitreal administration of VEGF Trap-Eye in subjects with macular edema secondary to CRVO. Both trials consist of two arms: injection of 2.0 mg of VEGF Trap-Eye every four weeks for one year vs sham injections every four weeks for one year.

The primary outcome measure is the improvement of BCVA vs baseline at 24 weeks. All patients are eligible for panretinal photocoagulation at any time during the study if they progress to anterior segment neovascularization. Both trials have reached their enrollment goals of 165 patients and will reach completion in early 2011. COPERNICUS is based at 61 locations in the United States, Canada, India, and South America, while GALILEO is based at 73 sites in Europe, Australia and Asia.

CONCLUSION

Results of the phase 2 AMD and DME trials are expected in the coming year. These results, along with data from the phase 3 studies, should position VEGF Trap-Eye for FDA approval by 2012. **RP**

REFERENCES

1. Adamis AP, Miller JW, Bernal MT, et al. Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am J Ophthalmol.* 1994;118:445-450.
2. Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med.* 1994;331:1480-1487.
3. Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. *Arch Ophthalmol.* 2002;120:338-346.
4. Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. *Invest Ophthalmol Vis Sci.* 1996; 37:855-868.
5. Qaum T, Xu Q, Joussen AM, et al. VEGF-initiated blood-retinal barrier breakdown in early diabetes. *Invest Ophthalmol Vis Sci.* 2001;42:2408-2413.
6. Tolentino MJ, Miller JW, Gragoudas ES, et al. Intravitreous injections of vascular endothelial growth factor produce retinal ischemia and micro-angiopathy in an adult primate. *Ophthalmology.* 1996;103:1820-1828.
7. Gragoudas ES, Adamis AP, Cunningham ET, Jr., Feinsod M, Guyer DR. Pegaptanib for neovascular age-related macular degeneration. *N Engl J Med.* 2004;351:2805-2816.
8. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med.* 2006;355:1419-1431.
9. Brown DM, Kaiser PK, Michels M, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *N Engl J Med.* 2006;355:1432-1444.
10. Economides AN, Carpenter LR, Rudge JS, et al. Cytokine traps: multi-component, high-affinity blockers of cytokine action. *Nat Med.* 2003;9:47-52.
11. Holash J, Davis S, Papadopoulos N, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl*

Acad Sci U S A. 2002;99:11393-11398.
12. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov.* 2004;3:391-400.
13. Gerber HP, Ferrara N. Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies. *Cancer Res.* 2005;65:671-680.
14. Liang WC, Wu X, Peale FV, et al. Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. *J Biol Chem.* 2006;281:951-961.
15. Chen Y, Wiesmann C, Fuh G, et al. Selection and analysis of an optimized anti-VEGF antibody: crystal structure of an affinity-matured Fab in complex with antigen. *J Mol Biol.* 1999;293:865-881.
16. Stewart MW, Rosenfeld PJ. Predicted biological activity of intravitreal VEGF Trap. *Br J Ophthalmol.* 2008;92:667-668.

Sumit Sharma, MD, is a resident at the Cole Eye Institute in Cleveland. **Peter K. Kaiser, MD**, is professor of ophthalmology at Cleveland Clinic Lerner College of Medicine, and on staff at Cole. Dr. Kaiser reports minimal financial interest in Regeneron. He can be reached via e-mail at pkkaiser@aol.com.

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Archives

Predicted biological activity of intravitreal VEGF Trap

M W Stewart,[1] P J Rosenfeld[2]

[1] Mayo Clinic College of Medicine, Jacksonville, Florida, USA; [2] Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Miami, Florida, USA

Correspondence to: Dr M W Stewart, Department of Ophthalmology, 4500 San Pablo Rd, Jacksonville, FL 32224, USA; stewart.michael@mayo.edu

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ABSTRACT

Aim: To compare the intravitreal binding activity of VEGF Trap with that of ranibizumab against vascular endothelial growth factor (VEGF) using a time-dependent and dose-dependent mathematical model.

Methods: Intravitreal half-lives and relative equimolar VEGF-binding activities of VEGF Trap and ranibizumab were incorporated into a first-order decay model. Time-dependent VEGF Trap activities (relative to ranibizumab) for different initial doses (0.5, 1.15, 2 and 4 mg) were calculated and plotted.

Results: Seventy-nine days after a single VEGF Trap (1.15 mg) injection, the intravitreal VEGF-binding activity would be comparable to ranibizumab at 30 days. After injection of 0.5, 2 and 4 mg VEGF Trap, the intravitreal VEGF-binding activities (comparable to ranibizumab at 30 days) would occur at 73, 83 and 87 days, respectively

Conclusion: On the basis of this mathematical model, VEGF Trap maintains significant intravitreal VEGF-binding activity for 10–12 weeks after a single injection.

Vascular endothelial growth factor (VEGF), a potent vasoactive cytokine, mediates the pathological angiogenesis and hyperpermeability associated with several chorioretinal vascular disorders including neovascular age-related macular degeneration (AMD). Attempts to stabilise and improve the condition of patients with AMD have led to the development and subsequent Food and Drug Administration (FDA) approval of two drugs with anti-VEGF action: pegaptanib[1] sodium (Macugen, a pegylated aptamer from Eyetech/OSI, New York, USA) and ranibizumab[2 3] (Lucentis), a recombinant, humanised, antibody fragment from Genentech Inc (San Francisco, California, USA). In addition, administration of intravitreal bevacizumab (Avastin), a full-length, recombinant, humanised antibody from Genentech and approved for the systemic treatment of metastatic colon cancer, appears to be useful for the treatment of neovascular AMD.[4] The intravitreal administration of each of these drugs is likely to achieve high intraocular concentrations with low systemic levels and few adverse effects.

VEGF Trap, a 110 kDa soluble protein, contains extracellular VEGF receptor sequences (VEGFR1 and VEGFR2) fused to an IgG backbone.[5] Although its intraocular duration of action is unknown, its high VEGF-binding affinity suggests a longer period of biological activity than ranibizumab.

This report presents a time-dependent mathematical model of intraocular VEGF Trap activity relative to ranibizumab.

METHODS AND RESULTS

VEGF Trap has a very high VEGF-binding affinity (K_d <1 pmol/l),[6] about 140 times that of ranibizumab. On the basis of laboratory and clinical data, significant biological activity of ranibizumab (0.5 mg) persists for 30 days after intravitreal administration.[7]

If we assume that the intravitreal half-lives of antibodies and antibody fragments are proportional to their molecular masses, then we can predict the intravitreal half-life of VEGF Trap in primates even though it is not known. We know that its molecular mass is 110 kDa, approximately half way between that of ranibizumab (48 kDa) and that of bevacizumab (148 kDa). As a monkey model showed that rhuFab VEGF (a 48 kDa antibody fragment) has an intravitreal half-life of 3.2 days and rhuMab HER2 (a 148 kDa antibody similar to bevacizumab) has an intravitreal half-life of 5.6 days,[8] the half-life of VEGF Trap in a primate eye may be reasonably estimated as 4–5 days. In a rabbit model, VEGF Trap concentration decreased according to first-order kinetics, with a half-life of 4.79 days (unpublished data from Regeneron Pharmaceuticals, Tarrytown, New York, USA); this value was used for the calculations that follow.

A pharmacokinetic single-compartment rabbit model showed that intravitreal bevacizumab concentration decreases according to first-order decay.[9] Therefore, after a 1.15 mg injection of VEGF Trap (1.15 mg VEGF Trap is equimolar to 0.5 mg ranibizumab), the intravitreal biological activity of VEGF Trap, relative to ranibizumab, can be calculated according to the following equation:

$$A_t = A_r e^{-kt}$$

where A_t is the time-dependent VEGF activity, A_r is the baseline VEGF activity relative to ranibizumab, and k is a VEGF Trap time-dependent constant. Figure 1 shows this relationship graphically. VEGF Trap activity at 79 days equals that of ranibizumab at 30 days.

One treatment arm in the recently completed phase 2 VEGF Trap trial used a 4 mg dose. After a 4 mg VEGF Trap injection, the time-dependent intravitreal anti-VEGF activity, relative to 0.5 mg ranibizumab, can be calculated according to the following:

$$A_t = A_r C_r e^{-kt}$$

where C_r is the molar concentration of injected VEGF Trap relative to 0.5 mg ranibizumab. Figure 2 shows this relationship graphically. On day 87 after a 4 mg VEGF Trap injection, the relative biological activity would be comparable to ranibizumab at 30 days. Doses of 0.5 mg and 2 mg would provide similar biological activities at 73 days and 83 days, respectively.

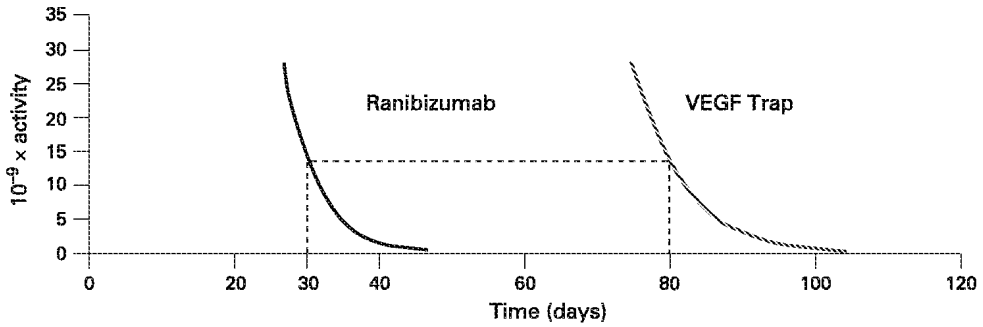

Figure 1 Time-dependent intravitreal activity of 1.15 mg VEGF Trap compared with that of 0.5 mg ranibizumab. The biological activity of VEGF Trap at 79 days is comparable to that of ranibizumab at 30 days.

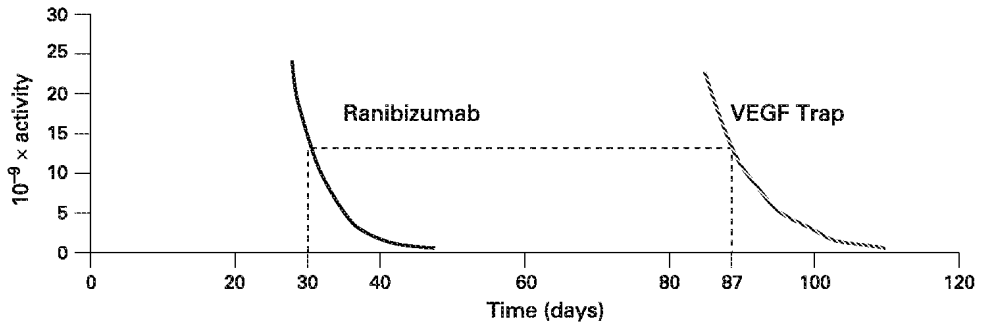

Figure 2 Time-dependent intravitreal activity of 4 mg VEGF Trap compared with that of 0.5 mg ranibizumab. The biological activity of VEGF Trap at 87 days is comparable to that of ranibizumab at 30 days.

DISCUSSION

At 10 weeks after an injection, the intraocular biological activity of VEGF Trap is theoretically comparable to the activity of ranibizumab at 30 days. This prolonged biological activity can be explained by the higher VEGF-binding affinity of VEGF Trap and its presumed longer intravitreal half-life when compared with ranibizumab. If this theoretical model is correct, then the advantages of VEGF Trap will include less frequent drug administration, resulting in fewer physician appointments and ancillary tests, lower overall cost, less cumulative risk from intravitreal injections, and the potential for improved patient compliance. However, it should be appreciated that, by increasing the intravitreal dose of VEGF Trap from 1.15 mg to 4 mg, there is only a marginal increase in the relative biological activity from 79 days to 87 days compared with ranibizumab at 30 days, and this increased dose may not be worth the increase in potential systemic adverse events. Consequently, there seems to be little advantage to increasing the dose above 1 mg unless a much higher initial dose results in greater suppression of VEGF and less frequent dosing overall because of the increased initial potency of the 4 mg dose.

A similar analysis comparing the biological activities of ranibizumab with bevacizumab showed that the two drugs were comparable after 27–38 days.[10] This can be explained by the lower affinity of bevacizumab for VEGF-A combined with longer half-life of bevacizumab compared with ranibizumab. In contrast with bevacizumab, VEGF Trap has both a longer intravitreal half-life because of its larger size and a much higher affinity for VEGF-A than ranibizumab, resulting in the greater theoretical duration of biological activity in the eye.

If our assumptions for the half-life and relative biological activity of VEGF Trap are correct, then the modelling presented in this paper supports less frequent dosing of VEGF Trap compared with ranibizumab for the treatment of neovascular AMD. This approach will be tested in the upcoming phase 3 trial with VEGF Trap.

Competing interests: None.

REFERENCES

1. **Gragoudas ES,** Adamis AP, Cunningham ET Jr, *et al*. Pegaptanib for neovascular age-related macular degeneration. *N Engl J Med* 2004;**351**:2805–16.
2. **Rosenfeld PJ,** Brown DM, Heier JS, *et al*. Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med* 2006;**355**:1419–31.
3. **Brown DM,** Kaiser PK, Michels M, *et al*. Ranibizumab versus verteporfin for age-related macular degeneration. *N Engl J Med* 2006;**355**:1432–44.
4. **Rosenfeld PJ,** Moshfeghi AA, Puliafito CA. Optical coherence tomography findings after an intravitreal injection of bevacizumab (Avastin) for neovascular age-related macular degeneration. *Ophthalmic Surg Lasers Imaging* 2005;**36**:331–5.
5. **Saishin Y,** Saishin Y, Takahasi K, *et al*. VEGF-TRAP$_{R1R2}$ suppresses choroidal neovascularization and VEGF-induced breakdown of the blood-retinal barrier. *J Cell Physiol* 2003;**195**:241–8.
6. **Nguyen QD,** Shah SM, Hafiz G, *et al*. A phase I trial of an IV-administered vascular endothelial growth factor trap for treatment in patients with choroidal neovascularization due to age-related macular degeneration. *Ophthalmology* 2006;**113**:1522–32.
7. **Gaudreault J,** Fei D, Rusit J, *et al*. Preclinical pharmacokinetics of ranibizumab (rhuFabV2) after a single intravitreal administration. *Invest Ophthalmol Vis Sci* 2005;**46**:726–33.
8. **Mordenti J,** Cuthbertson RA, Ferrara N, *et al*. Comparisons of the intraocular tissue distribution, pharmacokinetics, and safety of ^{125}I-labeled full-length and Fab antibodies in rhesus monkeys following intravitreal administration. *Toxicol Pathol* 1999;**27**:536–44.
9. **Bakri SJ,** Snyder MR, Reid JM, *et al*. Pharmacokinetics of intravitreal bevacizumab (Avastin). *Ophthalmology* 2007;**114**:855–9.
10. **Stewart MW.** Predicted biologic activity of intravitreal bevacizumab. *Retina* 2007;**27**:1196–200.

FRESH FROM THE PIPELINE

Aflibercept

Michael W. Stewart, Seden Grippon and Peter Kirkpatrick

In November 2011, aflibercept (Eylea; Regeneron Pharmaceuticals), a recombinant fusion protein that binds to members of the vascular endothelial growth factor family, was approved by the US Food and Drug Administration (FDA) for the treatment of patients with neovascular age-related macular degeneration.

Age-related macular degeneration (AMD) is the leading cause of blindness among the elderly in the developed world[1]. The disorder is classified into two forms: non-neovascular (dry) AMD and neovascular (wet) AMD. The neovascular form involves abnormal neovascularization under the macula (the central part of the retina), which leads to leakage of blood or serum that damages the macula and causes deterioration in sight. Although only ~10% of patients with AMD have the neovascular form, it accounts for ~90% of the severe loss of vision[1].

Treatment strategies for neovascular AMD have progressed substantially in the past decade or so, from thermal laser photocoagulation to specific pharmacotherapies, in particular those that inhibit vascular endothelial growth factor (VEGF), which is important in choroidal neovascularization (CNV)[2]. In 2000, photodynamic therapy with verteporfin (Visudyne; QLT/Novartis), which causes selective destruction of CNV lesions, became the first FDA-approved treatment for neovascular AMD. However, in most patients photocoagulation and photodynamic therapy only slow the deterioration in vision[2].

Basis of discovery

VEGF, which acts via two receptor tyrosine kinases, VEGFR1 and VEGFR2, on the surface of endothelial cells, has a key role in physiological angiogenesis and pathological angiogenesis[1]. Recognition of the importance of VEGF in cancer and pathological ocular neovascularization led to the development of various strategies to inhibit VEGF signalling[1].

Pegaptanib (Macugen; Eyetech), an aptamer that targets the $VEGF_{165}$ isoform, was approved by the FDA for neovascular AMD in 2004. However, its effectiveness in preventing deterioration of vision is not as great as that of ranibizumab (Lucentis; Genentech/Roche), a recombinant VEGF-specific antibody fragment that was approved by the FDA for neovascular AMD in 2006 following trials showing that it not only maintained visual acuity in more than 90% of patients but also improved it in around a third of patients[2]. In addition, bevacizumab (Avastin; Genentech/Roche), a monoclonal VEGF-specific antibody that has been developed and approved for the treatment of various cancers, has been widely used off-label for the treatment of neovascular AMD following studies indicating its effectiveness, largely owing to its lower cost than ranibizumab[1].

Another strategy that has been developed to block the activity of cytokines such as VEGF is to prevent them from binding to their normal receptors by administering soluble decoy receptors that are constructed by fusing binding domains of the normal receptors to an immunoglobulin constant region[3]. Aflibercept (previously known as VEGF-Trap) was developed by optimizing the pharmacokinetic properties of such fusion proteins to improve their *in vivo* anticancer activity[3]. In particular, these efforts focused on selecting portions of the extracellular domains of VEGFR1 and VEGFR2 that were anticipated to lead to fusion proteins with reduced propensity for nonspecific interactions with the extracellular matrix, as well as improved binding potency[3].

Drug properties

Aflibercept is a recombinant fusion protein that consists of portions of human VEGFR1 and VEGFR2 extracellular domains fused to the Fc portion of human immunoglobulin G1 (REFS 3,4). It binds strongly to VEGF and placental growth factor, and thereby inhibits the binding and activation of the cognate VEGFRs[3,4].

Clinical data

The efficacy and safety of aflibercept (administered by intravitreal injection) were assessed in two randomized, double-masked, active-controlled trials in patients with wet AMD[4]. A total of 2,412 patients were treated and evaluable for efficacy in the two trials (known as VIEW1 and VIEW2)[4]. In each of these trials, patients were randomly assigned in a 1:1:1:1 ratio to one of the following four treatment regimens: aflibercept at a dose of 2 mg administered every 8 weeks, following three initial monthly doses; aflibercept at a dose of 2 mg administered every 4 weeks; aflibercept at a dose of 0.5 mg administered every 4 weeks; and ranibizumab at a dose of 0.5 mg administered every 4 weeks[4]. In both trials, the primary efficacy end point was the proportion of patients who maintained vision, defined as losing fewer than 15 letters of best corrected visual acuity (BVCA) at 52 weeks compared with the baseline[4]. Secondary end points included the mean change in BVCA as measured by the ETDRS (Early Treatment Diabetic Retinopathy Study) score from the baseline[4].

After 52 weeks, the efficacy results for the two groups that received aflibercept at the 2 mg dose were clinically equivalent to those from the ranibizumab group[4]. Of the patients who received 2 mg aflibercept every 8 weeks (after the initial three monthly doses), the proportions that maintained visual acuity were 94% in VIEW1 and 95% in VIEW2 (REF. 4). Of the patients who received 2 mg aflibercept every 4 weeks, the proportions that maintained visual acuity were 95% in VIEW1 and 95% in VIEW2 (REF. 4). Of the patients who received ranibizumab, 94% maintained visual acuity in VIEW1, and 95% maintained visual acuity in VIEW2 (REF. 4).

For the secondary end point of mean change in BVCA, of the patients who received 2 mg aflibercept every 8 weeks after three initial monthly doses, the mean changes were 7.9 in VIEW1 and 8.9 in VIEW2 (REF. 4). The mean changes for the groups that received 2 mg aflibercept every 4 weeks were 10.9 in VIEW1 and 7.6 in VIEW2, and for the groups that received ranibizumab the mean changes were 8.1 in VIEW1 and 9.4 in VIEW2 (REF. 4).

Indications

Aflibercept is approved by the FDA for the treatment of patients with neovascular (wet) AMD[4].

ANALYSIS | AGE-RELATED MACULAR DEGENERATION

Analysing issues in the treatment of AMD is Michael W. Stewart, M.D., Chair, Department of Ophthalmology, Mayo Clinic, Mayo School of Medicine, Jacksonville, Florida, USA.

The recent approval of aflibercept provides ophthalmologists and patients with a third excellent anti-VEGF therapy for AMD. Whereas bevacizumab was developed for the treatment of advanced solid tumours and has been used off-label for AMD and other ophthalmic disorders, ranibizumab was developed exclusively for ophthalmic conditions. The pivotal Phase III trials for ranibizumab[2] established monthly injections as the standard against which other drugs and dosing regimens have since been compared. Despite this, many physicians have preferred low-cost bevacizumab (~US$50 per dose) over the higher-cost ranibizumab (~$1,950 per dose) for the initial treatment of AMD, although only recently has bevacizumab (administered monthly) been shown to produce improvements in vision that are comparable to ranibizumab[5].

The year 1 results of the Phase III VIEW trials for aflibercept demonstrated for the first time that injections of an anti-VEGF drug every 8 weeks (aflibercept; 2 mg) improve vision comparably to ranibizumab administered every 4 weeks[4]. For patients treated according to the labelling guidelines (based on the Phase III trial protocols), those receiving aflibercept require fewer office visits and injections than those receiving ranibizumab (7 versus 12) during the first year.

To reduce the burden of clinic visits and intravitreal injections, however, most physicians use 'treat and observe' or 'treat and extend' strategies. Although the VIEW trials did not strictly evaluate either of these strategies, in the second year a 'treat and observe' strategy with a 3-month cap (that required injection) was used. During year 2, the developer Regeneron has reported that both ranibizumab and aflibercept performed well, as patients receiving either drug lost an average of only 0.8 letters of visual acuity. Compared with ranibizumab, aflibercept showed slightly better durability for each group studied, suggesting that the duration between injections for aflibercept could be extended by an additional 2–4 weeks compared with ranibizumab. However, doubling the injection intervals, as suggested by the year 1 result, is probably not achievable for most patients.

For patients treated according to the VIEW protocols, aflibercept (~$1,850 per dose) reduces costs and patient visits by 42% compared with ranibizumab. For those on 'treat and observe' or 'treat and extend' regimens, the savings will be considerably lower. As physicians gain experience with aflibercept, it is possible that many could switch from ranibizumab for cases when a high-affinity anti-VEGF drug is indicated.

Despite the savings resulting from less frequent aflibercept therapy, however, monthly bevacizumab remains the less expensive alternative. When choosing an anti-VEGF drug, physicians and patients will need to consider the trade-offs between lower costs (bevacizumab) versus less frequent visits and injections (aflibercept).

The pivotal AMD trials for ranibizumab and aflibercept all showed that regularly administered anti-VEGF injections improve visual acuity by 8–11 letters over the study period, leading many physicians to believe that anti-VEGF monotherapy has hit an efficacy 'ceiling'. Future anti-VEGF agents, such as the designed ankyrin repeat protein (DARPin) MP0112 (which has completed Phase I/II trials), will need to be differentiated from current drugs based on improved durability.

Improving the efficacy of AMD treatment by reducing the size of the neovascular complex, thereby improving the anatomy and function of the photoreceptors, retinal pigment epithelium and choriocapillaris, will probably require combination drug therapy. Several drugs that inhibit the actions of molecules that are crucial to the growth of the neovascular complex — including integrins, complement component 5 and platelet-derived growth factor — are in various stages of development. Effective combination therapy, however, is still several years away. Given the crucial role of VEGF in wet AMD and the demonstrated efficacy of the currently available drugs, anti-VEGF therapy will remain an important component of AMD therapy for many years.

Box 1 | **Market for age-related macular degeneration**

Analysing the market for therapies for wet age-related macular degeneration (AMD) is Seden Grippon, IMS Health, London, UK.

The global market for wet AMD therapies is currently worth ~US$4 billion annually, according to data from IMS Health. This market is dominated by ranibizumab (Lucentis; Genentech/Roche), an antibody fragment specific for vascular endothelial growth factor (VEGF), which accounts for ~98% of sales.

Aflibercept (Eylea, Regeneron Pharmaceuticals), a fusion protein that also targets VEGF, was launched into this market in the United States in November 2011, following its approval by the US Food and Drug Administration. In Europe, aflibercept is at the preregistration phase. Its less frequent dosing compared with Lucentis (see main text) appears to be perceived by physicians as a moderate advantage, and analysts predict that its uptake will be robust, potentially taking more than half of Lucentis's market share in the next 3 years; in February 2012, the number of patients on Eylea had grown by 50% over the previous 6 weeks, and with rapid uptake it has been predicted that US sales alone may reach $1 billion in 2016 (Nadeau, P. & Bishop, N. Cowen. Company Report on Regeneron Pharmaceuticals. 9 February 2012; Meacham, G. *et al.* JP Morgan Report on Regeneron Pharmaceuticals. 13 February 2012). Aflibercept is also in development for other ophthalmic indications, including diabetic macular oedema, central retinal vein occlusion, myopic choroidal neovascularization and branch retinal vein occlusion. Finally, as only ~30% of patients with wet AMD experience a significant improvement in vision with anti-VEGF therapy, alternative strategies that are currently being investigated in Phase II trials have a high chance of physician uptake if successful. These strategies include: E10030 (developed by Ophthotech), which is an aptamer that targets platelet-derived growth factor B; hI-con1 (developed by Iconic Therapeutics), which is a fusion protein that targets tissue factor; and mesenchymal precursor cells (developed by Mesoblast).

Michael W. Stewart is at the Department of Ophthalmology, Mayo Clinic, Mayo School of Medicine, Jacksonville, Florida 32224, USA.

Seden Grippon is at IMS Health, 7 Harewood Avenue, London NW1 6JB, UK.

Peter Kirkpatrick is at Nature Reviews Drug Discovery.

e-mails: Stewart.Michael@mayo.edu; SGrippon@imscg.com; p.kirkpatrick@nature.com

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1. Stewart, M. W. The expanding role of vascular endothelial growth factor inhibitors in ophthalmology. *Mayo Clin. Proc.* **87**, 77–88 (2012).
2. Narayanan, R. *et al.* Ranibizumab. *Nature Rev. Drug Discov.* **5**, 815–816 (2006).
3. Holash J. *et al.* VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc. Natl Acad. Sci. USA* **99**, 11393–11398 (2002).
4. US Food and Drug Administration. FDA labeling information — Eylea. *FDA website* [online], http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/125387lbl.pdf (2011).
5. Martin, D. F. *et al.* Ranibizumab and bevacizumab for neovascular age-related macular degeneration. *N. Engl. J. Med.* **364**, 1897–1908 (2011).

Competing financial interests
The authors declare competing financial interests: see Web version for details.

Ranibizumab for Macular Edema Due to Retinal Vein Occlusions: Implication of VEGF as a Critical Stimulator

Peter A Campochiaro[1], Gulnar Hafiz[1], Syed Mahmood Shah[1], Quan Dong Nguyen[1], Howard Ying[1], Diana V Do[1], Edward Quinlan[1], Ingrid Zimmer-Galler[1], Julia A Haller[1], Sharon D Solomon[1], Jennifer U Sung[1], Yasmin Hadi[1], Kashif A Janjua[1], Nida Jawed[1], David F Choy[2] and Joseph R Arron[2]

[1]*Department of Ophthalmology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA;* [2]*Immunology, Tissue Growth, and Repair Diagnostics and Biomarkers Genentech, Inc., South San Francisco, California, USA*

Macular edema is a major cause of vision loss in patients with central retinal vein occlusion (CRVO) or branch retinal vein occlusion (BRVO). It is not clear how much of the edema is due to hydrodynamic changes from the obstruction and how much is due to chemical mediators. Patients with macular edema due to CRVO ($n = 20$) or BRVO ($n = 20$) were randomized to receive three monthly injections of 0.3 or 0.5 mg of ranibizumab. At the primary endpoint, month 3, the median improvement in letters read at 4 m was 17 in the 0.3-mg group and 14 in the 0.5-mg group for CRVO, and 10 and 18, respectively for the BRVO group. Optical coherence tomography (OCT) showed that compared to injections of 0.3 mg, injections of 0.5 mg of ranibizumab tended to cause more rapid reductions of central retinal thickening that lasted longer between injections, but in 3 months, excess central retinal thickening which is a quantitative assessment of the macular edema, was reduced by ~90% in all four treatment groups. There was no correlation between the amount of improvement and duration of disease or patient age at baseline, but there was some correlation between the aqueous vascular endothelial growth factor (VEGF) level at baseline and amount of improvement. These data indicate that excess production of VEGF in the retinas of patients with CRVO or BRVO is a major contributor to macular edema and suggest that additional studies investigating the efficacy of intraocular injections of ranibizumab are needed.

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INTRODUCTION

The pathogenesis of central retinal vein occlusions (CRVOs) is poorly understood. In the early stages, there are scattered hemorrhages throughout the entire retina, cotton wool patches, a sign of retinal ischemia, and massive edema of the retina. Fluorescein angiography often shows delayed filling of retinal veins suggesting reduced blood flow, staining of the walls of retinal veins, and leakage of dye into the retina. It has been concluded that this picture most likely occurs from thrombosis within the central retinal vein causing partial obstruction of blood flow from the eye, increased intraluminal pressure in the retinal veins, and increased transudation of blood and plasma into the retina. Histopathology has confirmed the presence of a thrombus in the central retinal vein in several cases.[1] The marked increase in interstitial fluid and protein increases interstitial pressure and is likely to be an impediment to capillary perfusion resulting in ischemia. The subacute stage varies among patients depending primarily upon the amount of retinal ischemia, and patients are classified as ischemic or nonischemic, although it is not an all or none dichotomy. In some patients ischemia increases over time and they are viewed as undergoing a transition from nonischemic to ischemic. Severe retinal ischemia can be complicated by retinal neovascularization, iris neovascularization, neovascular glaucoma, and a very poor visual outcome. Thus the amount of retinal ischemia is one of the major determinants of outcome.

Those patients classified as nonischemic still have retinal ischemia, as demonstrated by cotton wool patches and areas of capillary nonperfusion seen in fluorescein angiograms. These patients often enter a chronic stage in which they have severe macular edema that may last for many months and often years. Eventually the edema may resolve, presumably because there is resolution of the venous obstruction due to recanalization and/or formation of collateral vessels, but generally the visual outcome is poor due to damage to macular photoreceptors from the chronic edema, poor perfusion of perifoveal capillaries, or both.[2]

Hypertension is a major risk factor for branch retinal vein occlusions (BRVOs).[3] Chronic hypertension leads to thickening of the walls of retinal arterioles and since retinal arterioles and veins share a common adventitia at crossings, this may cause constriction of retinal veins that can lead to occlusions.[4,5] The complications of BRVOs are similar to those for CRVOs but more limited in scope, because only part of the retina is drained by the involved branch vein. There is increased luminal pressure distal to the obstruction resulting in increased transudation of blood and plasma, increased interstitial fluid pressure, and reduced

Correspondence: *Peter A. Campochiaro, Maumenee 719, The Wilmer Eye Institute, The Johns Hopkins University School of Medicine, 600 North Wolfe Street, Baltimore, Maryland 21287-9277, USA. E-mail: pcampo@jhmi.edu*

capillary perfusion causing ischemia. Since the area of ischemic retina tends to be much less than that in patients with CRVO, iris neovascularization and neovascular glaucoma are rare, but retinal neovascularization adjacent to the ischemic retina can occur and can cause vitreous hemorrhages necessitating scatter photocoagulation to the ischemic retina. The most common cause of reduced vision is macular edema, but nonperfusion of perifoveal capillaries can also be another contributing factor. Grid laser photocoagulation to the poorly perfused retina adjacent to the fovea can help to resolve macular edema and improve vision.[6]

Thus, in both CRVO and BRVO, retinal ischemia occurs and serves as an exacerbating factor. The ischemic retina releases vascular endothelial growth factor (VEGF) which underlies neovascular complications, but also causes excessive vascular permeability.[7,8] Hence, the release of VEGF is likely to contribute to macular edema. In this study, we investigated the potential contribution of VEGF to macular edema in patients with CRVO and BRVO by testing the effects of intraocular injections of ranibizumab (Lucentis; Genentech, South San Francisco, CA), an Fab fragment that binds and neutralizes all isoforms of VEGF-A.[9]

RESULTS

Baseline characteristics of study patients

The baseline characteristics of patients are shown in Table 1. For CRVO patients, the 0.5-mg group was somewhat older than the 0.3-mg group and had a longer duration of disease.

Table 1 Baseline characteristics

	CRVO		BRVO	
	0.3 mg	0.5 mg	0.3 mg	0.5 mg
Age (years)				
Mean ± SD	63 ± 17	68 ± 13	69 ± 13	65 ± 10
Median	69	70	68	65
Range	34–83	48–83	43–84	50–82
Duration of disease (months)				
Mean ± SD	9 ± 7	16 ± 17	5 ± 3	3 ± 2
Median	7.4	13	5	3
Range	1–26	0.5–53	0.4–9	0.8–6
Systemic disease				
Diabetes	3	3	3	3
Hypertension	5	6	9	8
Hyperlipidema	4	7	7	3
Elevated homocysteine	1	3	3	6
Ocular disease				
Glaucoma	1	3	0	1
Other	2	5	5	3
Prior treatment				
Bevacizumab	0	0	1	2
Steroids	1	2	2	2
Laser	1	3	4	3
Visual acuity (ETDRS letters read at 4 m)				
Mean ± SD	16 ± 13	23 ± 15	26 ± 12	20 ± 14
Median	18	26	29	23
Excess foveal thickness (in μm)				
Mean ± SD	346 ± 88	297 ± 126	252 ± 104	288 ± 101
Median	340	309	270	294

Abbreviations: BRVO, branch retinal vein occlusion; CRVO, central retinal vein occlusion; ETDRS, Early Treatment Diabetic Retinopathy Study.

Effect of ranibizumab on central retinal (foveal) thickening

Figure 1 shows optical coherence tomography (OCT) scans at all time points up to the primary endpoint for five randomly selected patients with CRVO randomized to each dose. Although the response varied somewhat among patients in each group, most patients showed substantial reduction in central retinal thickness over time. Within 7 days of the first injection, 8 out of 10 patients in the 0.3-mg group and 9 out of 10 in the 0.5-mg group had a substantial improvement in edema as assessed by OCT (Figure 2a and b). Four patients in the 0.3-mg group showed a worsening of the thickening between the time points of 1 week and 1 month but improved after the next two injections, while the other six showed persistent improvement 1 month after the first injection that improved further with subsequent injections (Figure 2a). All the patients in the 0.5-mg dose group showed the latter pattern of response (Figure 2b). Figure 3 shows the OCT scans of the five randomly selected patients with BRVO randomized to each group. They tended to show rapid and sustained responses after the first injection of 0.3 or 0.5 mg of ranibizumab with further improvement after subsequent injections. Measurements of central retinal thickening confirmed that this was the case for most patients, but two in the 0.3-mg group and one in the 0.5-mg group showed slight worsening between the time points of 1 week and 1 month (Figure 4).

In patients with CRVO, the median excess foveal thickness was 340 μm at baseline in the 0.3-mg group and 7 days after the first injection it improved to 124 μm, thus eliminating 64% of the edema (Figure 5a). In the 0.5-mg group, the median excess foveal thickness improved from 309 μm at baseline to 53 μm 1 week after the first injection, thus eliminating 83% of the edema (Figure 5b). At the primary endpoint of 3 months, excess foveal thickness was reduced to 25 μm (eliminating 93% of the edema) in the 0.3-mg group, and 35 μm (eliminating 89% of the edema), in the 0.5-mg group. Thus, in patients with CRVO, edema was reduced to a greater extent and the reduction was more sustained after a single injection of 0.5 mg of ranibizumab compared to 0.3 mg, but after three injections of either dose, most of the edema, *i.e.*, ~90%, had been eliminated.

In patients with macular edema due to BRVO, the median excess foveal thickness was 270 μm at baseline in the 0.3-mg group and 7 days after the first injection it improved to 48 μm, thus eliminating 82% of the edema (Figure 5c). In the 0.5-mg group, the median excess foveal thickness was 294 μm at baseline and 7 days after the first injection it improved to 51 μm, eliminating 83% of the edema (Figure 5d). At the primary endpoint, excess foveal thickness was essentially eliminated in each group.

The last injection was at the time point of month 2; by month 4 in some patients and month 6 in most patients there was recurrent edema in patients with CRVO and worsening of the edema

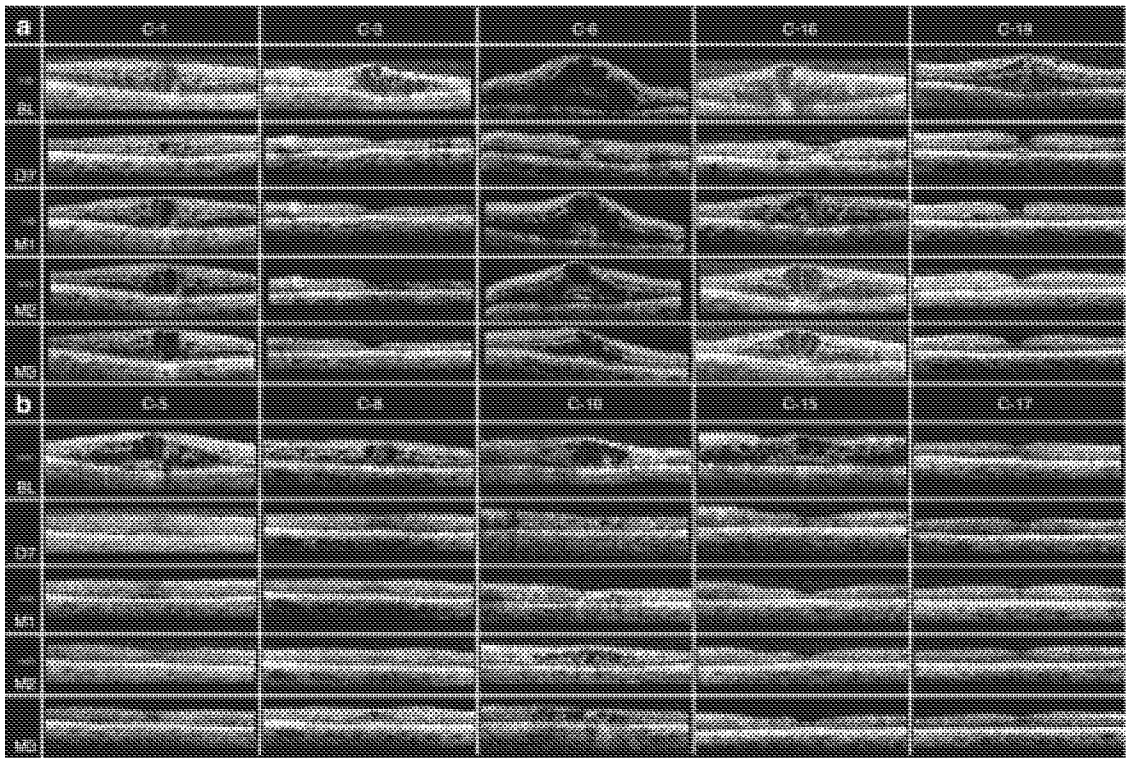

Figure 1 Cross sections through the fovea obtained by optical coherence tomography in patients with central retinal vein occlusion. The horizontal cross sections at baseline (BL), day 7 (D7), month 1 (M1), month 2 (M2), and month 3 (M3, primary endpoint) are shown for five randomly selected patients of the 10 patients treated with (**a**) 0.3 mg or (**b**) 0.5 mg of ranibizumab.

tended to occur sooner after the last injection in the 0.3-mg group compared to the 0.5-mg group. In the 0.3-mg/CRVO group, seven, and then five patients were given another treatment (usually an off-label injection of bevacizumab) at the time points of months 4 and 6, respectively. In the 0.5-mg/CRVO group, three, and then six patients received additional injections at the time points of months 4 and 6, respectively. In patients with BRVO, recurrent edema was somewhat less frequent than in patients with CRVO, but still occurred in several patients. In the 0.3-mg/BRVO group, one, and then two patients received additional injections at the time points of months 4 and 6, respectively, and in the 0.5-mg/BRVO group, four patients, and then one, received additional injections at time points of months 4 and 6, respectively.

Effect of ranibizumab on visual acuity

In the CRVO group at the primary endpoint, the change from baseline (in the median number of letters read on an Early Treatment Diabetic Retinopathy Study chart) at 4 m was 17 and 14 in patients treated with 0.3 or 0.5 mg of ranibizumab, respectively (Figure 5a and b). All the patients in the 0.3-mg group and eight patients in the 0.5-mg group showed improvement in vision at the primary endpoint compared to baseline. One of the patients showed a reduction of 16 letters at the endpoint of 3 months, that was back to baseline at 4 months. The other patient showed a reduction of 11 letters at the endpoint of 3 months that was judged to be due to progression of edema despite administration of ranibizumab; there was no evidence of ischemia or any other problem that could be attributed to the drug. The percentage of patients with clinically significant visual improvement defined as an improvement of ≥15 letters was 70% in the 0.3-mg group and 40% in the 0.5-mg group.

At the primary endpoint in patients with macular edema due to BRVO, the median change in visual acuity (VA) from baseline was 10 letters in the 0.3-mg group, and 18 in the 0.5-mg dose group (Figure 5c and d). One patient in the 0.5-mg dose group showed a reduction in VA of three letters at the primary endpoint, but all other patients showed improved vision. The percentage of patients with clinically significant visual improvement defined as an improvement of ≥15 letters was 40% in the 0.3-mg group and 70% in the 0.5-mg group.

Effect of duration of disease and patient's age on visual outcome

Figure 6 shows scatter plots of change from baseline in VA versus the patient's age or duration of disease for patients with CRVO or BRVO treated with ranibizumab. There was no correlation between the amount of improvement in VA and duration of edema. There were four patients who had edema from CRVO for >2 years prior to starting injections of ranibizumab and three of them improved in VA by >15 letters. Three patients had edema from BRVO for >2 years at baseline and they showed improvements of 41, 21, and 14 letters in 3 months. Therefore, chronic edema from a vein occlusion does not preclude visual improvement as a result of intraocular injections of ranibizumab. There was no correlation between the patient's age and visual improvement; improvement in VA of >15 letters occurred in two out of

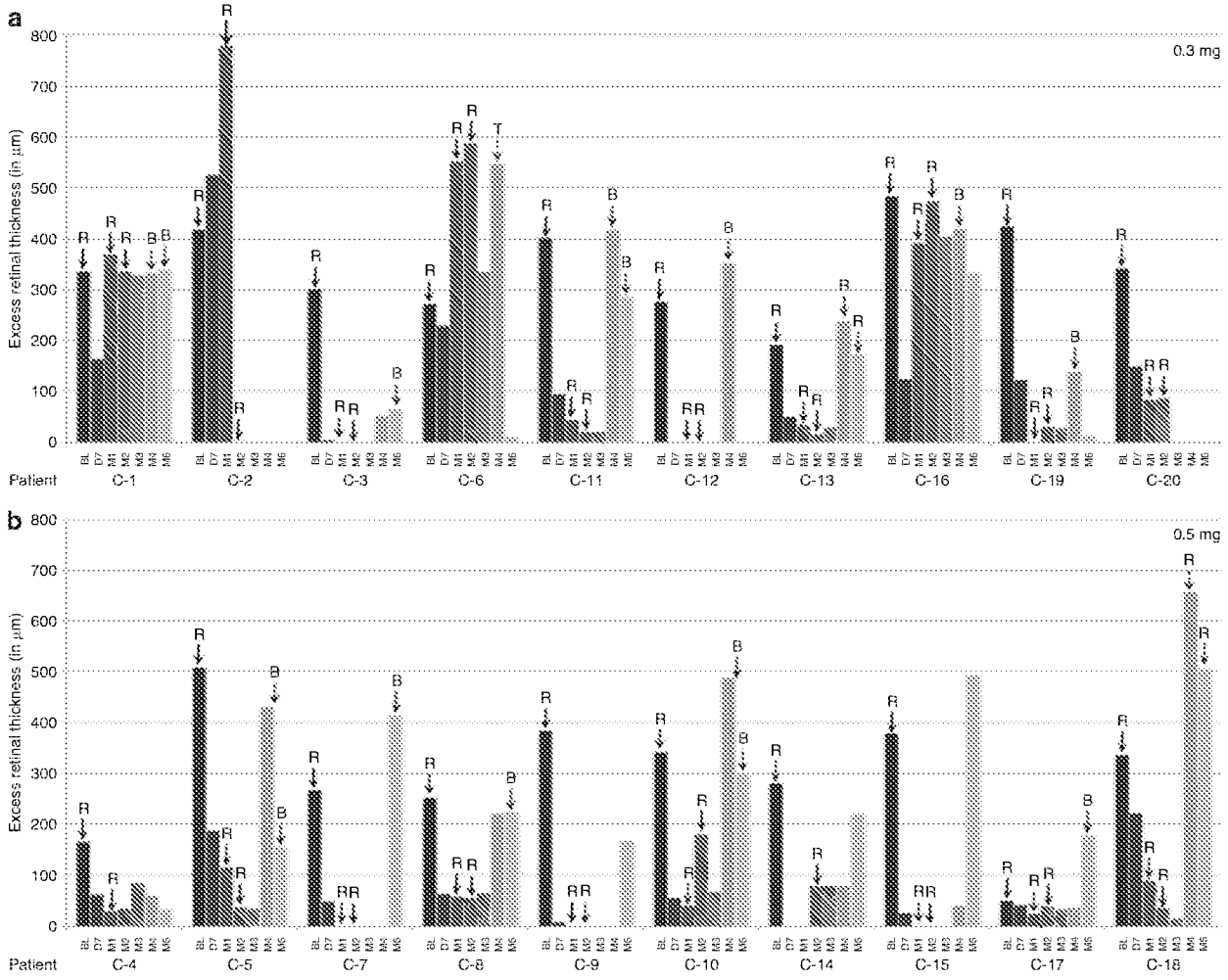

Figure 2 Excess foveal thickness at each visit from baseline (BL) to month 6 (M6) in patients with central retinal vein occlusion. The bars represent excess foveal thickness (central 1 mm retinal thickness—212 µm), which is a measure of the amount of macular edema, at BL just prior to injection of ranibizumab, and at day 7 (D7) and months 1 (M1), 2 (M2), 3 (M3), 4 (M4), and 6 (M6) for patients that received three injections of (**a**) 0.3 mg or (**b**) 0.5 mg of ranibizumab. The arrows show when injections were done (R, ranibizumab; B, bevacizumab; T, triamcinolone). Fairly rapid improvement in excess foveal thickness occurred in essentially all patients in the 0.5-mg dose group and most patients in the 0.3-mg group, but a few patients in the latter group showed small and/or delayed responses. Most patients showed recurrent edema 1–3 months after the last injection.

three patients with CRVO and three out of four patients with BRVO who were >80 years at baseline.

VEGF levels in aqueous

The mean aqueous VEGF level at baseline was 39 pg/ml in 17 patients with BRVO and 380 pg/ml in the 18 patients with CRVO for which measurements could be performed, a difference that was statistically significant ($P = 0.001$). There was an inverse correlation between baseline aqueous VEGF level and visual outcome in CRVO patients considered alone ($P = 0.038$) and for CRVO and BRVO patients considered together ($P = 0.038$); although BRVO patients considered alone did not reach statistical significance (Figure 7). There was no correlation of VEGF levels with excess foveal thickness at baseline or change in excess foveal thickness after treatment with ranibizumab.

Safety

Intraocular injections of ranibizumab were tolerated well with no inflammation or other problems. None of the patients showed elevation of blood pressure, thromboembolic events, or any other systemic problems. One patient, an 83-year-old male with a history of pre-existent heart disease, died from a myocardial infarction 6 months after the last injection of ranibizumab; this was judged to be unrelated to the ranibizumab. As noted above, 38 out of 40 subjects showed improvement in VA at the primary endpoint compared to baseline and in the other two patients the reduction in vision was not felt to be attributable to ranibizumab.

DISCUSSION

Although this is an uncontrolled, open-label trial involving a relatively small number of patients, the results were very consistent among patients and suggest that intraocular injections of ranibizumab have a substantial effect on macular edema due to CRVO or BRVO. In both patient populations, the results were good with either 0.3 or 0.5 mg of ranibizumab and no clear differences could be discerned between the doses except that more patients seemed to have rapid improvements in center subfield thickness and more

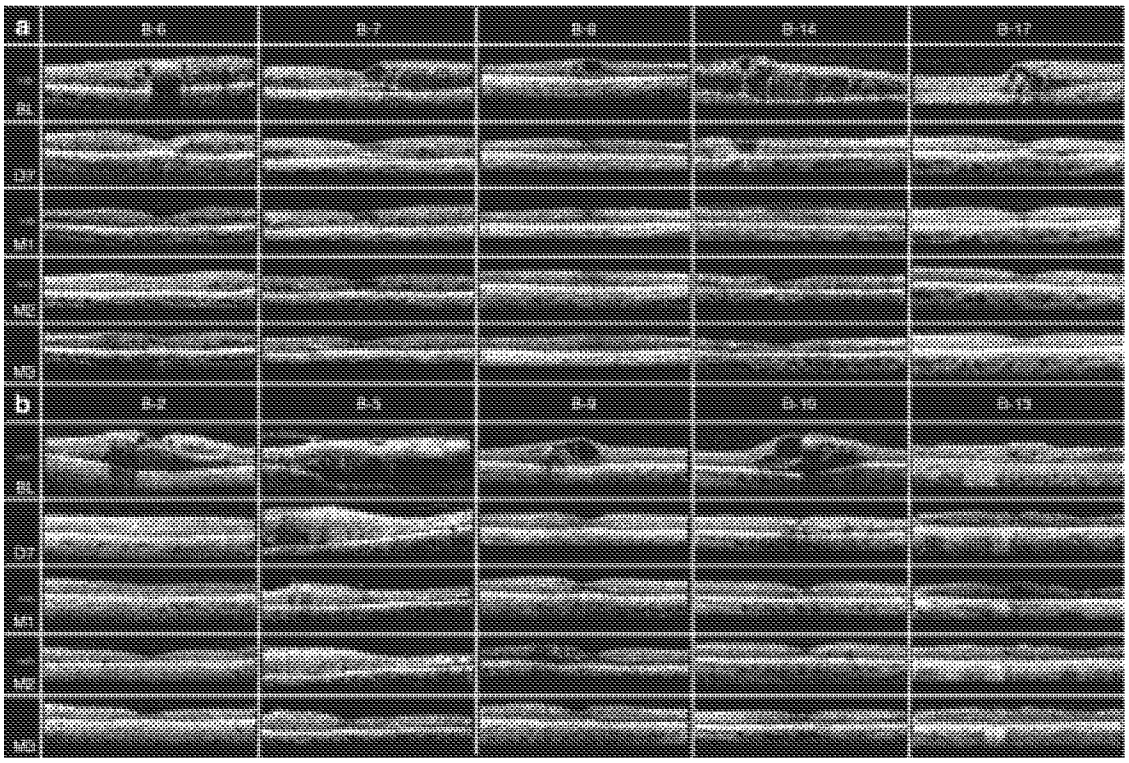

Figure 3 Cross sections through the fovea obtained by optical coherence tomography in patients with branch retinal vein occlusion. The horizontal cross sections at baseline (BL), day 7 (D7), month 1 (M1), month 2 (M2), and month 3 (M3, primary endpoint) are shown for five randomly selected patients of the ten patients treated with (**a**) 0.3 mg or (**b**) 0.5 mg of ranibizumab.

had improvements that lasted for a month after the initial injection in the 0.5-mg groups compared to the 0.3-mg groups. However, at the 3-month primary endpoint, 1 month after the third injection, ~90% of excess foveal thickness was eliminated in both patient populations with either dose. In addition, improvements from baseline in VA were large in both dose groups for patients with macular edema due to CRVO or BRVO. These results are very encouraging and strongly support the performance of larger, more definitive studies.

The Central Retinal Vein Occlusion Study was a large multicenter trial that investigated the effect of grid laser photocoagulation in patients with macular edema due to CRVO.[10] Although 69% of patients in the treated group compared to 0% in the untreated group showed reduction of fluorescein leakage in the macula at the end of 1 year, there was no difference in the final VA (20/200 in treated patients versus 20/160 in untreated patients). It has been felt that a possible explanation is that chronic edema due to CRVO leads to permanent visual loss. Our data demonstrate that visual improvement is possible in some patients who have had edema for >1 year and in some cases several years. In fact, there was no inverse correlation between duration of edema at baseline and improvement in VA after three injections of ranibizumab, suggesting that patients with chronic edema should not be excluded from treatment based solely upon the duration of edema. Our data suggest that chronicity of edema and the relatively slow resolution of edema after grid laser therapy are not likely to explain the poor visual results after grid laser therapy. Our data also suggest that elderly patients with macular edema due to CRVO or BRVO should not be excluded from treatment with intraocular ranibizumab, because the patient's age being advanced at baseline did not negatively impact the outcome.

No drug-related adverse effects such as elevation of blood pressure, thromboembolic events, or any other systemic problems were observed. This provides some preliminary data, suggesting that intraocular injections of ranibizumab are well-tolerated in patients with retinal vein occlusions just as they are in patients with neovascular age-related macular degeneration.[11,12]

Measurement of VEGF levels in aqueous demonstrated a higher mean level in patients with CRVO compared to patients with BRVO. In patients with CRVO there was an inverse correlation with VEGF level at baseline and the visual outcome. Further work is needed to determine the predictive value of baseline aqueous levels of VEGF and to determine the range of VEGF levels that occur in other disease processes such as neovascular age-related macular degeneration and diabetic macular edema.

In addition to grid laser therapy, several treatments have been tried in patients with macular edema due to CRVO including the use of anticoagulants, fibrinolytics, steroids, acetozolamide, isovolemic hemodilution, surgically induced retinochoroidal anastamoses or laser-induced retinochoroidal anastamoses, and radial optic neurotomy. A recent meta-analysis of all published randomized clinical trials concluded that there is no convincing evidence that any of these treatments provide benefit.[13] In contrast, grid laser therapy provides modest benefit in patients with macular edema due to BRVO;[6] after 3 years, patients treated with grid laser photocoagulation improved by 1.33 lines from baseline compared to an improvement of 0.23 lines in the control group. There are several case series suggesting possible benefit

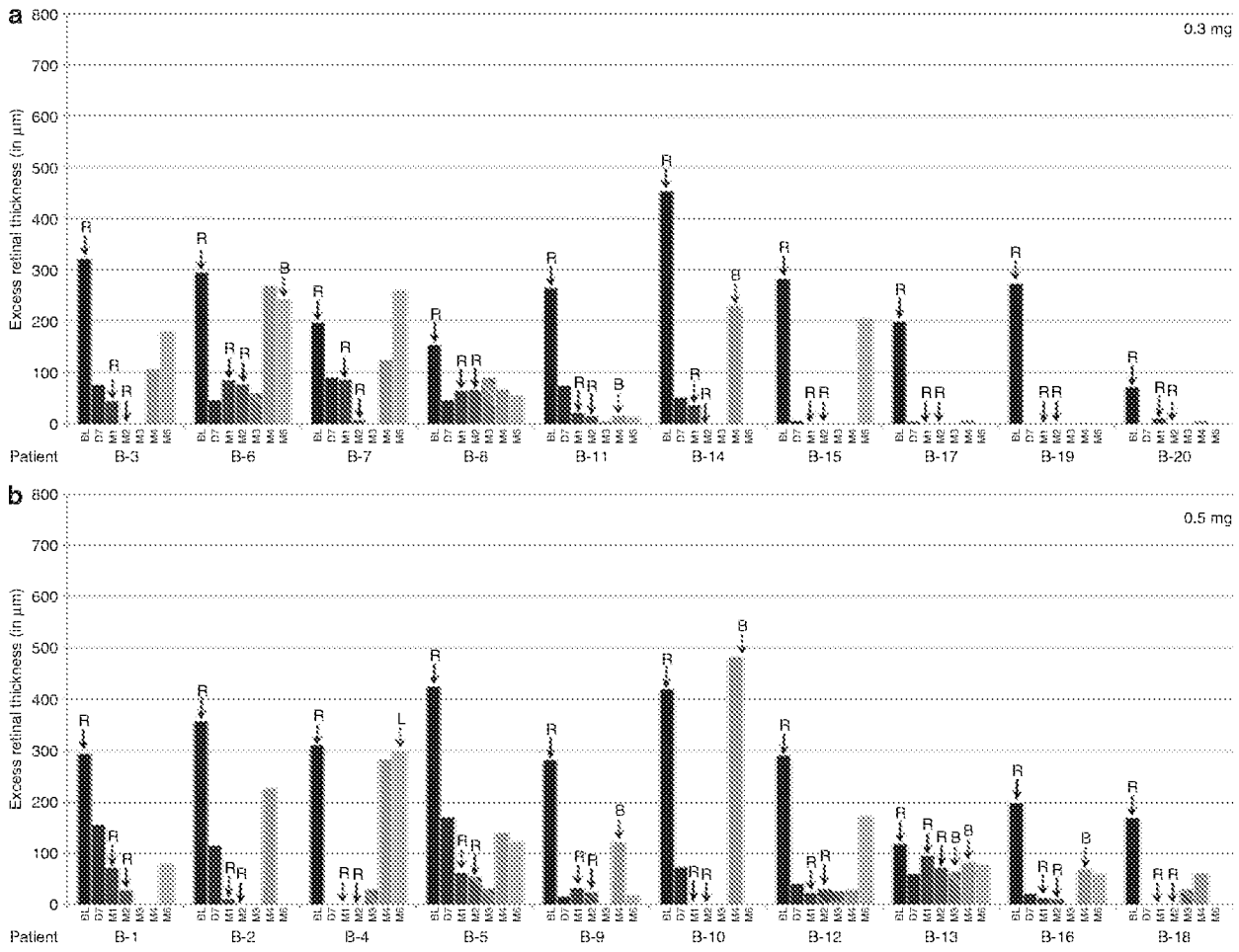

Figure 4 Excess foveal thickness at each visit from baseline (BL) to month 6 (M6) in patients with branch retinal vein occlusion. The bars represent excess foveal thickness (central 1 mm retinal thickness—212 µm), which is a measure of the amount of macular edema, at BL just prior to injection of ranibizumab, and at day 7 (D7) and months 1 (M1), 2 (M2), 3 (M3), 4 (M4), and 6 (M6) for patients who received three injections of (**a**) 0.3 mg or (**b**) 0.5 mg of ranibizumab. The arrows show when injections of ranibizumab were done. The response was uniformly good in all patients of both groups. Several patients in each group showed recurrent edema 1–3 months after the last injection.

from intraocular steroids and the ongoing Standard Care vs. Corticosteroid for Retinal Vein Occlusion Study should determine if there truly is benefit. There are also case series suggesting possible benefit from intraocular injections of bevacizumab.[14–18] When examined together, these reports and our study suggest that VEGF plays an important role in the development of macular edema in retinal vein occlusions. Hemodynamic changes from the vascular occlusion lead to reduced retinal perfusion and ischemia causing increased production of VEGF. The increased production of VEGF is the major cause of macular edema, because blockade of the VEGF results in substantial improvement in the edema.

The results of our study are very encouraging because of the magnitude and consistency of response among patients and the rarity of spontaneous improvements in patients with macular edema due to CRVO;[10] however, they are not definitive because of the relatively small number of patients studied, the lack of a control group, and the short follow-up. A major unanswered question is the duration of treatment that will be needed. Three injections was not sufficient in achieving long-term benefit in most of our patients and it is important to determine if and when injections can be terminated without recurrent edema. The CRUISE and BRAVO phase III trials investigating the effect of ranibizumab in patients with macular edema due to CRVO or BRVO are underway and should provide a definitive answer as to the usefulness of intraocular ranibizumab in these conditions.

MATERIALS AND METHODS

The protocol for this study was designed to test the effect of two doses of ranibizumab, 0.3 and 0.5 mg, in patients with macular edema due to CRVO or BRVO and was approved by the Federal Drug Administration under a physician-initiated investigational new drug application and by the Institutional Review Board of the Johns Hopkins Medical Institutions. This study was conducted in compliance with the Declaration of Helsinki, US Code 21 of Federal Regulations, and the Harmonized Tripartite Guidelines for Good Clinical Practice (1996). Twenty patients with CRVO and 20 patients with BRVO were randomized 1:1 to receive three monthly intraocular injections of 0.3 or 0.5 mg of ranibizumab, with both patients and investigators masked with respect to treatment group. The primary endpoint was 3 months after the first injection and 1 month after the third injection. The primary outcome measure was the percentage of patients who achieved an improvement in VA from baseline for ≥15 letters read on an Early Treatment Diabetic Retinopathy Study VA chart at 4 m. Secondary outcome

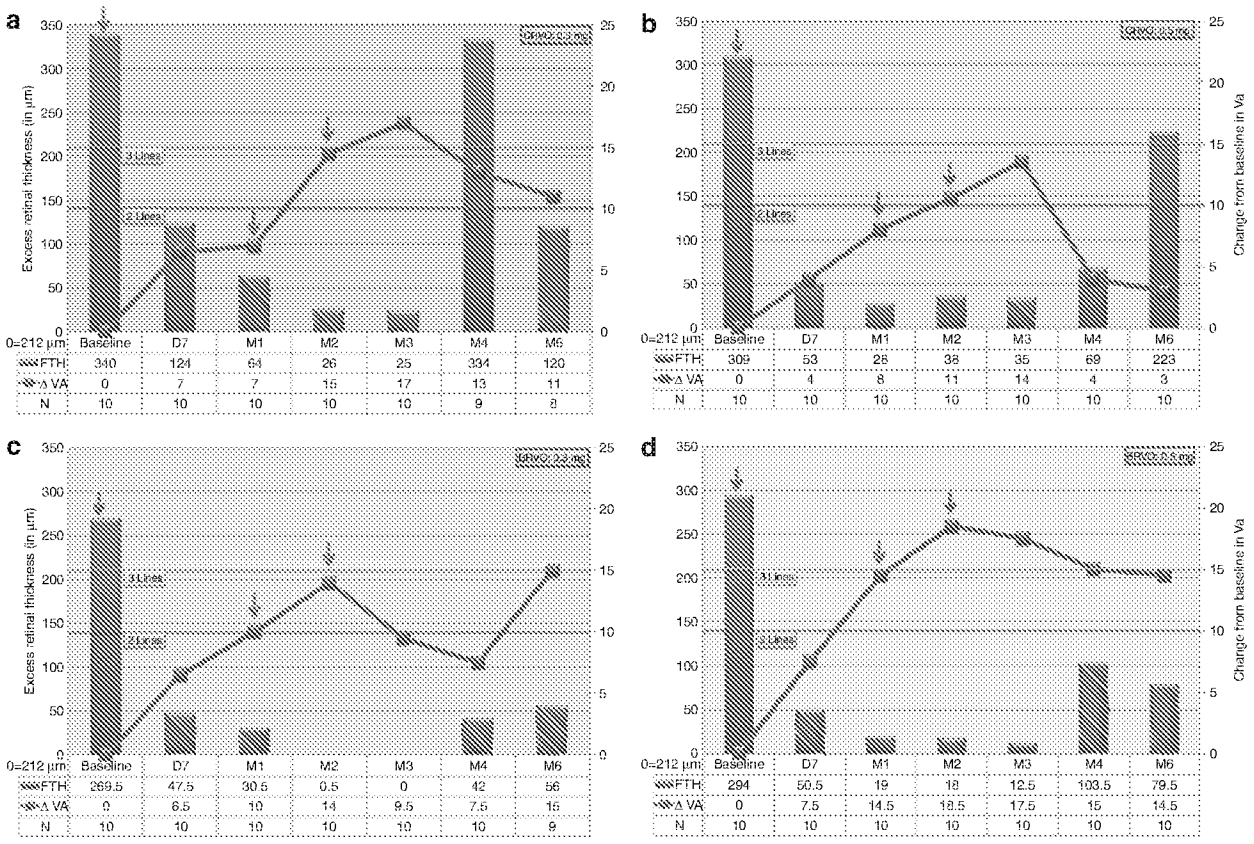

Figure 5 Median excess foveal thickness and median change from baseline in visual acuity (VA) for patients in each of the four treatment groups. The bars show the median excess foveal thickness at baseline, day 7 (D7), and months 1 (M1), 2 (M2), 3 (M3), 4 (M4), and 6 (M6) for patients with central retinal vein occlusion (CRVO) who received three injections of (**a**) 0.3 or (**b**) 0.5 mg of ranibizumab or patients with branch retinal vein occlusion (BRVO) who received (**c**) 0.3 or (**d**) 0.5 mg of ranibizumab. The scale, in microns, for excess foveal thickness is shown along the left side of each graph. The median number of letters for VA that has improved from baseline is shown by the points connected by lines and the scale is located along the right side of each graph. The arrows show when injections of ranibizumab were done. Substantial improvements in edema and VA occurred in each group. After injections were stopped, recurrent edema was more substantial in the CRVO patients, particularly those treated with 0.3 mg of ranibizumab. FTH, foveal thickness.

measures were mean and median change in VA at several time points after study entry and change in excess foveal thickness measured by OCT.[19,20]

Inclusion and exclusion criteria. Patients >18 years with VA between 20/30 and 20/400 from macular edema due to CRVO or BRVO were eligible if foveal thickness (central subfield) was >250 µm and none of the following exclusion criteria were present: (i) VA <20/400 in the fellow eye, (ii) a sign of possible permanent vision loss in the study eye such as atrophy or prominent pigmentary change in the macula, (iii) laser photocoagulation or intraocular surgery within the previous 3 months, (iv) intraocular injection of a VEGF antagonist within the previous 3 months, (v) intraocular steroids within the previous 4 months, or (vi) vitreomacular traction or an epiretinal membrane.

Study protocol. Consenting patients were screened for the study by taking down their medical history, conducting a physical examination, measuring best-corrected VA by an experienced examiner using the Early Treatment Diabetic Retinopathy Study protocol,[21] conducting a complete eye examination, an OCT, doing a fluorescein angiogram, and laboratory tests on blood and urine. Patients who entered the study underwent an anterior chamber tap and were randomized to receive an intraocular injection of 0.3 or 0.5 mg of ranibizumab. Patients returned 1 week later for a repeat examination and OCT. Intraocular injections of ranibizumab were given at time points of 1 and 2 months, and the primary endpoint was at 3 months. Safety evaluations, measurement of best-corrected VA, eye examinations, and OCTs were done at all study visits, and fluorescein angiograms were done at baseline and 3 months.

Administration of study drug. Povidone iodine was used to clean the lids and a lid speculum was inserted. Topical anesthesia was applied and the conjunctiva was irrigated with 5% povidone iodine. A 30-gauge needle was inserted through the pars plana and 0.05 ml of ranibizumab was injected into the vitreous cavity. Funduscopic examination was done to confirm retinal perfusion.

OCTs. OCT scans were performed by an experienced investigator with a StratusOCT3 (Carl Zeiss Meditec, Dublin, CA) using the fast macular scan protocol. This protocol consists of six line scans that are 6.0-mm long centered on fixation and spaced 30° apart around the circumference of a circle. Each line consists of 128 A-scan measurements. With each A-scan, the OCT software measures the distance between the inner surface of the retina and the anterior border of retinal pigmented epithelium-choriocapillaris complex based on changes in reflectivity. In some cases the retinal pigmented epithelium/choriocapillaris layer is obscured due to excessive edema and StratusOCT software misinterprets the outer boundary of the retina. We used the RetinaTomographer (version 1.1; RIRRC, Baltimore, MD) to correct the outer boundary of the retina for cases in which StratusOCT software identified it erroneously.

The center point thickness, also known as the foveolar thickness, is a mean value generated by the StratusOCT software from the six central

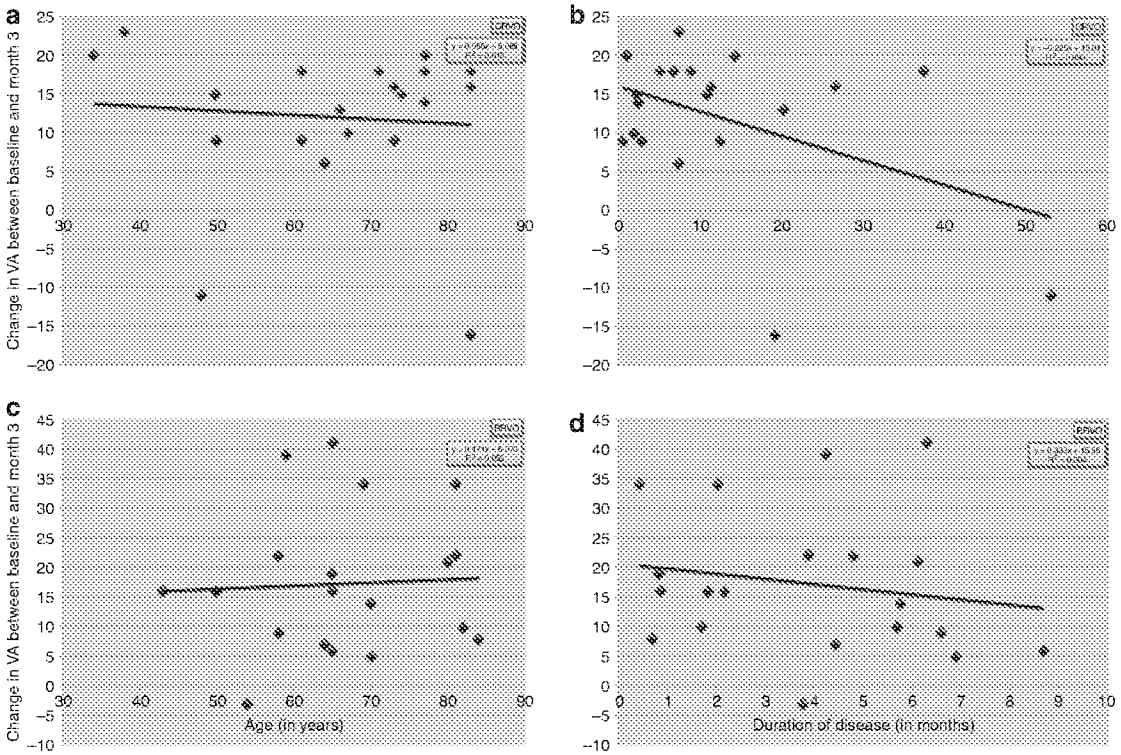

Figure 6 Impact of patient age and duration of disease on visual outcome. (**a**) Visual outcome (expressed as change in visual acuity between baseline and month 3 in number of letters read at 4 m by standardized protocol) was plotted versus patient age for central retinal vein occlusion (CRVO) patients. (**b**) Visual outcome (expressed as change in visual acuity between baseline and month 3 in number of letters read at 4 m by standardized protocol) was plotted versus duration of disease for CRVO. (**c**) Visual outcome (expressed as change in visual acuity between baseline and month 3 in number of letters read at 4 m by standardized protocol) was plotted versus patient age for branch retinal vein occlusion (BRVO) patients. (**d**) Visual outcome (expressed as change in visual acuity between baseline and month 3 in number of letters read at 4 m by standardized protocol) was plotted versus duration of disease for BRVO patients. There was no inverse correlation for either indicating that neither age nor duration of disease had a negative impact on visual outcome.

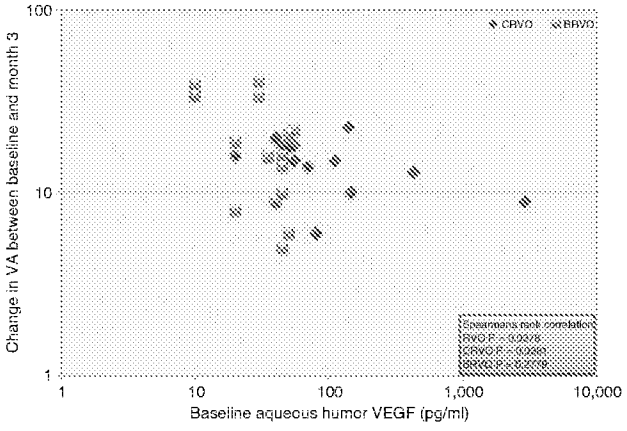

Figure 7 Negative correlation between baseline aqueous vascular endothelial growth factor (VEGF) levels and change in visual acuity (VA) between baseline and month 3. The aqueous humor levels of VEGF at baseline are plotted against the change in VA between baseline and month 3 for all patients for whom measurements could be made [n = 18 for central retinal vein occlusion (CRVO); n = 17 for branch retinal vein occlusion (BRVO)]. Two CRVO patients and one BRVO patient experienced a net loss of VA at 3 months and were included in the analysis, but are not depicted in the graph, as negative values cannot be plotted on a logarithmic scale. Spearman rank order correlation analysis was conducted and a significant correlation was observed for CRVO alone (P = 0.038) and for CRVO and BRVO considered together (P = 0.038); although BRVO alone did not reach statistical significance (P = 0.278). CRVO patients are depicted as closed squares, BRVO patients are depicted as open squares.

A-scan thickness values of each of the radial lines comprising the fast macular thickness map. We did not use this value generated from only six data points for our primary measure of central retinal thickness, but instead used the foveal or central 1-mm thickness, which is an average interpolated value based on central 21 scans of each of the six lines passing through the patient's fixation (126 data points). Macular volume throughout the entire 6-mm zone was calculated using extrapolated values between the line scans. Excess foveal thickness was calculated by subtracting the measured foveal thickness value from the normal mean value of 212 μm calculated from measurements on a large population of subjects.[22]

Measurement of VEGF in aqueous samples. A 30-gauge needle was inserted into the anterior chamber and 0.1 ml of aqueous was removed just prior to each injection of ranibizumab and 1 hour after each injection. This report focuses on the level of VEGF in the aqueous at baseline prior to the first injection of ranibizumab. It was measured by a highly sensitive sandwich enzyme-linked immunosorbent assay designed to detect all isoforms of VEGF-A.[23] Briefly, we used two monoclonal mouse capture antibodies and a biotinylated detection antibody against human VEGF-A. Aqueous humor samples were diluted tenfold in enzyme-linked immunosorbent assay diluent for accurate quantification. VEGF levels were measured by fluorometry for the detection of β-galactosidase conversion of 4-methylumbelliferyl-β-D-galactopyranoside. A four-parameter cure fit program was used to generate a standard curve from which sample and control concentrations were interpolated.

Statistical analysis. Statistical analyses were performed using statistical package for the social sciences (SPSS, Chicago, IL). The likelihood that the change

in foveal thickness or VA from baseline to month 3 was due to ranibizumab rather than chance was determined by the Wilcoxon signed-rank test. To assess the relationship between baseline aqueous VEGF level and visual outcome, Spearman's rank order correlation coefficient was calculated.

ACKNOWLEDGMENTS

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REFERENCES

1. Green, WR, Chan, CC, Hutchins, GM and Terry, JM (1981). Central retinal vein occlusion: a prospective histopathologic study of 29 eyes in 28 cases. *Trans Am Ophthalmol Soc* **79**: 371–422.
2. The Central Vein Occlusion Study Group (1997). Natural history and clinical management of central retinal vein occlusion. *Arch Ophthalmol* **115**: 486–491.
3. The Eye Disease Case-control Study Group (1993). Risk factors for branch retinal vein occlusion. *Am J Ophthalmol* **116**: 286–296.
4. Weinberg, D, Dodweil, DG and Fern, SA (1990). Anatomy of arteriovenous crossings in branch retinal vein occlusion. *Am J Ophthalmol* **109**: 298–302.
5. Zhao, J, Sastry, SM Sperduto, RD, Chew, EY and Remaley, NA (1993). Arteriovenous crossing patterns in branch retinal vein occlusion. The Eye Disease Case-control Study Group. *Ophthalmology* **100**: 423–428.
6. The Branch Vein Occlusion Study Group (1984). Argon laser photocoagulation for macular edema in branch vein occlusion. *Am J Ophthalmol* **98**: 271–282.
7. Ozaki, H, Hayashi, H, Vinores, SA, Moromizato, Y, Campochiaro, PA and Oshima, K (1997). Intravitreal sustained release of VEGF causes retinal neovascularization in rabbits and breakdown of the blood-retinal barrier in rabbits and primates. *Exp Eye Res* **64**: 505–517.
8. Derevjanik, NL, Vinores, SA, Xiao, WH, Mori, K, Turon, T, Hudish, T *et al.* (2002). Quantitative assessment of the integrity of the blood-retinal barrier in mice. *Invest Ophthalmol Vis Sci* **43**: 2462–2467.
9. Ferrara, N, Damico, L, Shams, N, Lowman, H and Kim, R (2006). Development of ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration. *Retina* **26**: 859–870.
10. The Central Vein Occlusion Study Group (1995). Evaluation of grid pattern photocoagulation for macular edema in central vein occlusion. The Central Vein Occlusion Study Group M report. *Ophthalmology* **102**: 1425–1433.
11. Rosenfeld, PJ, Brown, DM, Heier, JS, Boyer, DS, Kaiser, PK, Chung, CY *et al.* (2006). Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med* **355**: 1419–1431.
12. Brown, DM, Kaiser, PK, Michels, M, Soubrane, G, Heier, JS, Kim, RY *et al.* (2006). Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *N Engl J Med* **355**: 1432–1444.
13. Mohamed, Q, McIntosh, RL, Saw, SM and Wong, TY (2007). Interventions for central retinal vein occlusion: an evidence-based systematic review. *Ophthalmology* **114**: 507–519, 524.
14. Iturralde, D, Spaide, RF, Meyerle, CB, Klancnik, JM, Yannuzzi, LA, Fisher, YL *et al.* (2006). Intravitreal bevacizumab (Avastin) treatment of macular edema in central retinal vein occlusion: a short-term study. *Retina* **26**: 279–284.
15. Costa, RA, Jorge, R, Calucci, D, Melo, LA Jr, Cardillo, JA and Scott, IU (2007). Intravitreal bevacizumab (Avastin) for central and hemicentral retinal vein occlusions: the IBeVO study. *Retina* **27**: 141–149.
16. Rabena, MD, Pieramici, DJ, Castellarin, AA, Nasir, MA and Avery, RL (2007). Intravitreal bevacizumab (Avastin) in the treatment of macular edema secondary to branch retinal vein occlusion. *Retina* **27**: 419–425.
17. Pai, SA, Shetty, R, Vijayan, PB, Venkatasubramaniam, G, Yadav, NK, Shetty, BK *et al.* (2007). Clinical, anatomic, and electrophysiologic evaluation following intravitreal bevacizumab for macular edema in retinal vein occlusion. *Am J Ophthalmol* **143**: 601–606.
18. Stahl, A, Agostini, H, Hansen, LL and Feltgen, N (2007). Bevacizumab in retinal vein occlusion-results of a prospective case series. *Graefes Arch Clin Exp Ophthalmol* **245**: 1429–1436.
19. Huang, D, Swanson, EA, Lin, CP, Schuman, JS, Stinson, WG, Chang, W *et al.* (1991). Optical coherence tomography. *Science* **254**: 1178–1181.
20. Hee, MR, Izatt, JA, Swanson, EA, Huang, D, Schuman, JS, Lin, CP *et al.* (1995). Optical coherence tomography of the human retina. *Arch Ophthalmol* **113**: 325–332.
21. The Diabetic Retinopathy Study Research Group (1985). Early vitrectomy for severe vitreous hemorrhage in diabetic retinopathy. Two-year results of a randomized trial. Diabetic Retinopathy Vitrectomy Study report 2. *Arch Ophthalmol* **103**: 1644–1652.
22. Chan, A, Duker, JS, Ko, TH, Fujimoto, JG and Schuman, JS (2006). Normal macular thickness measurements in healthy eyes using Stratus optical coherence tomography. *Arch Ophthalmol* **124**: 193–198.
23. Rodriguez, CR, Fei, DT, Keyt, B and Baly, DL (1998). A sensitive fluorometric enzyme-linked immunosorbent assay that measures vascular endothelial growth facto165 in human plasma. *J Immunol Methods* **219**: 45–55.

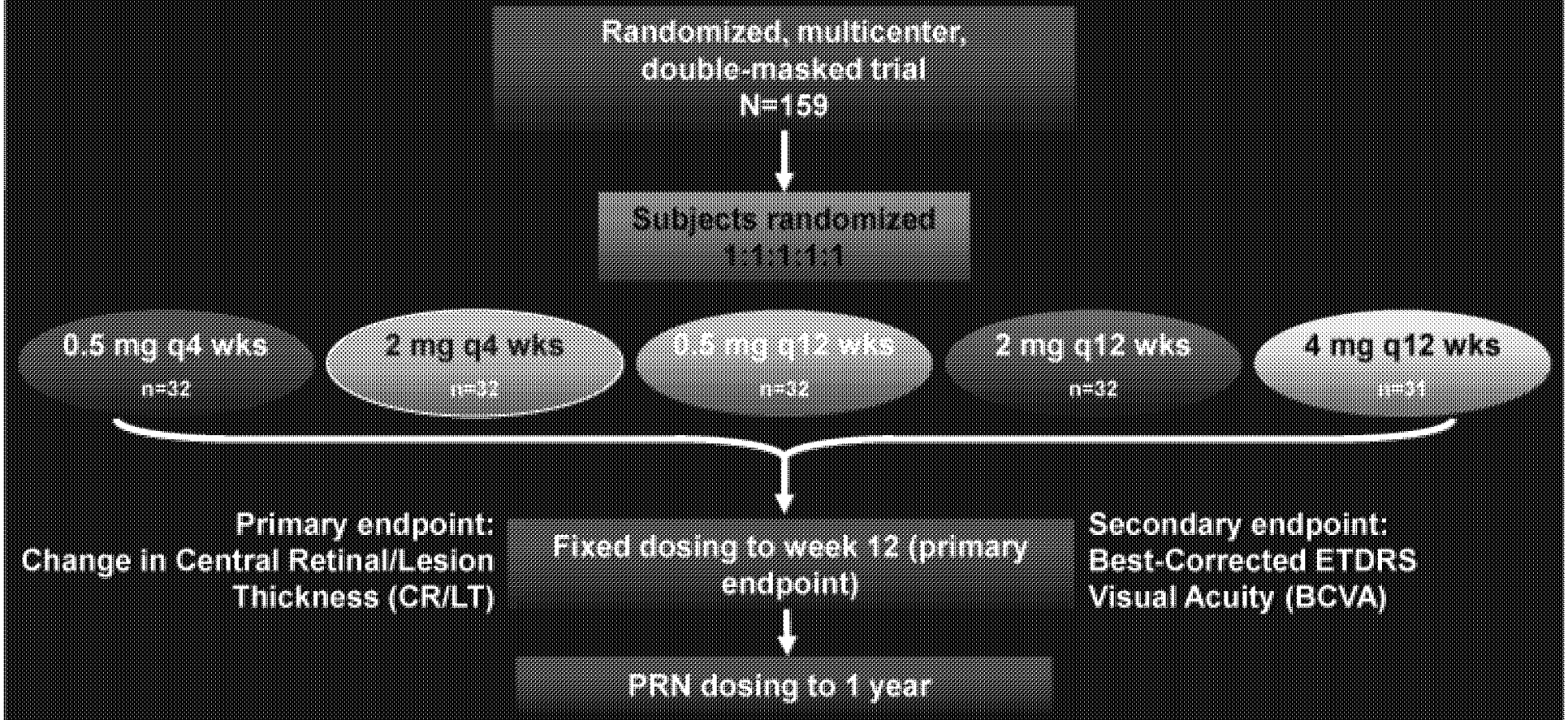
VEGF Trap-Eye in Wet AMD CLEAR-IT 2: One-Year OCT and FA Outcomes

OCT and Fluorescein Angiographic Outcomes

Allen C. Ho, MD

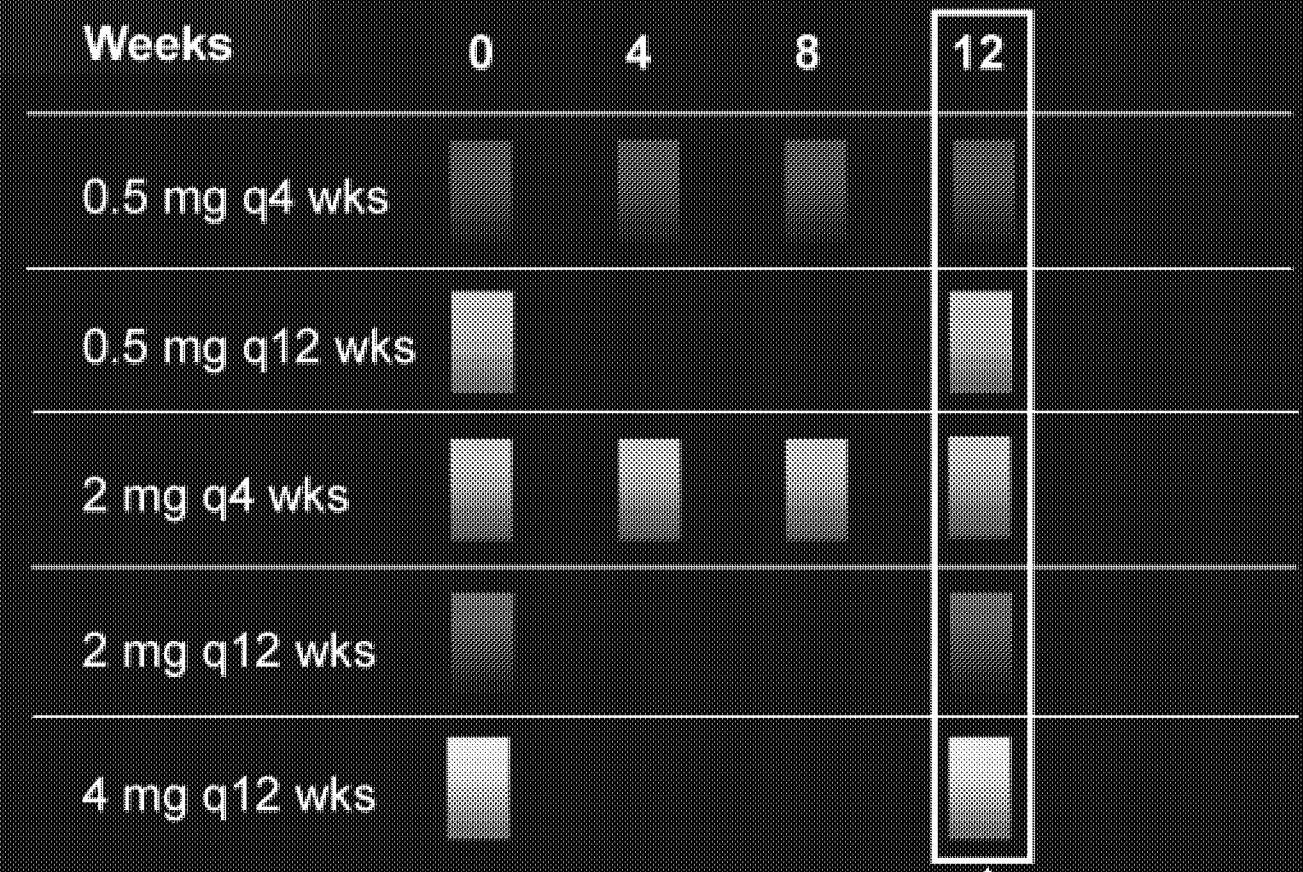
for the CLEAR-IT 2 Study Group

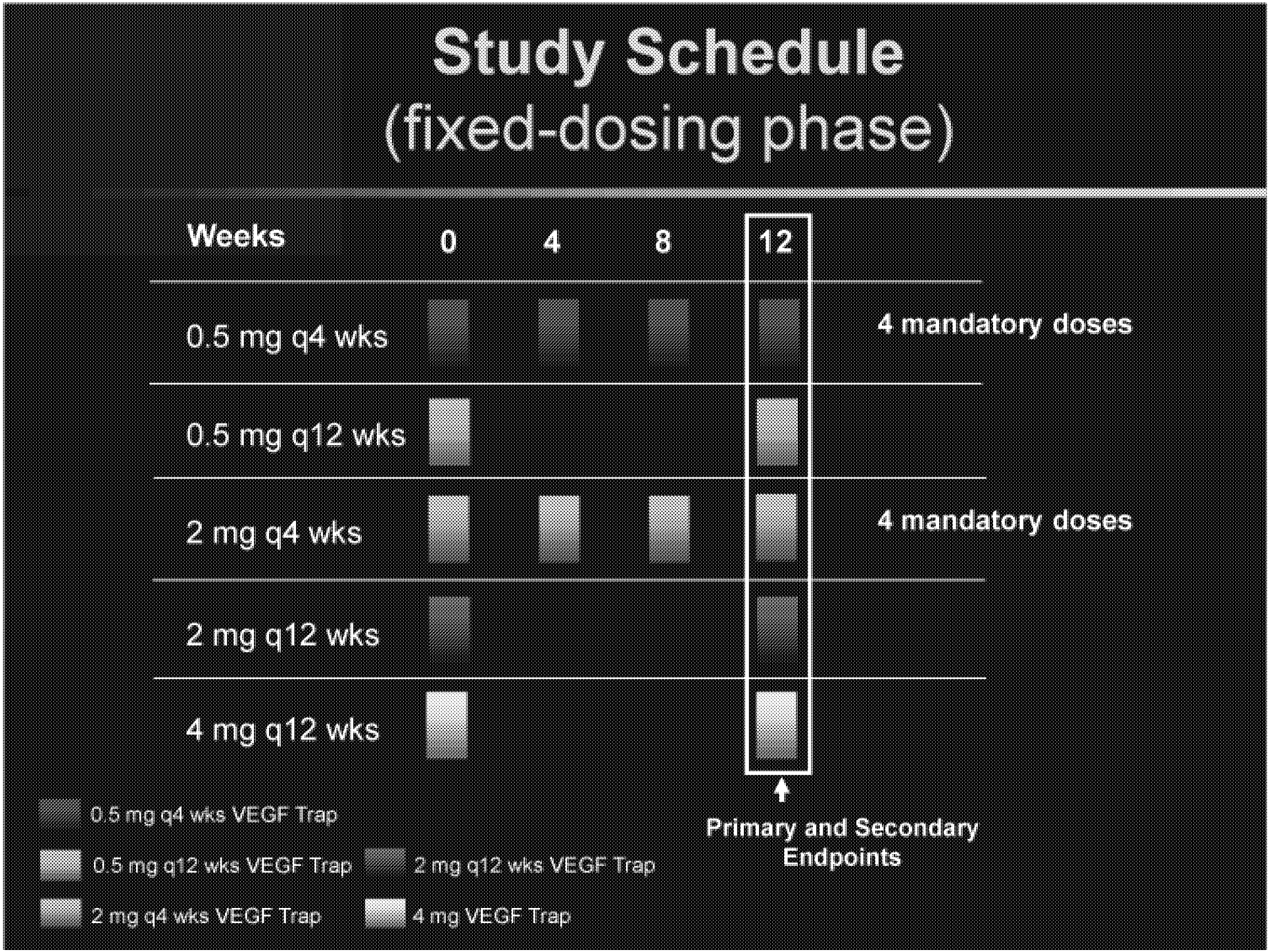
CLEAR-IT 2: Study Design

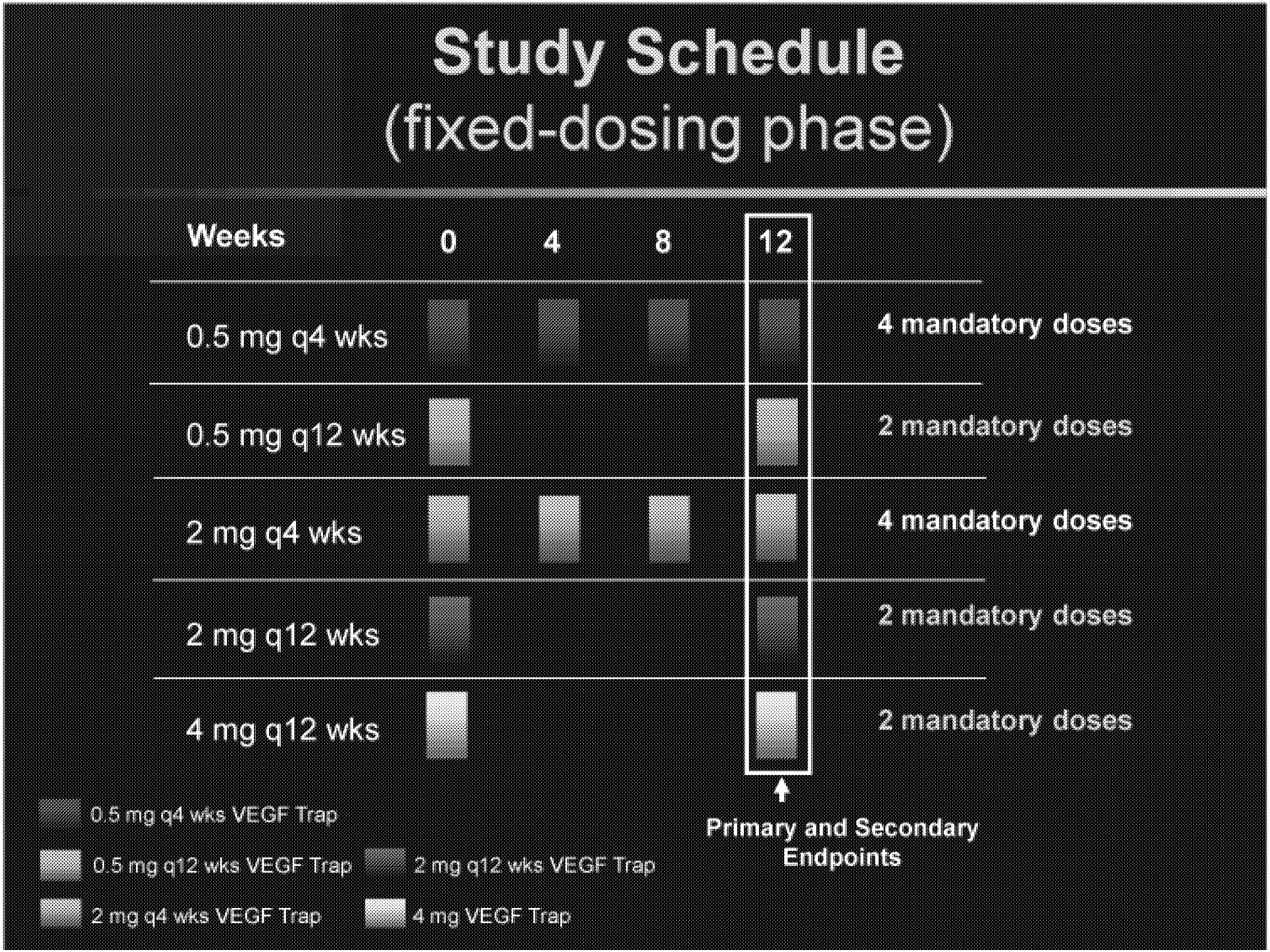


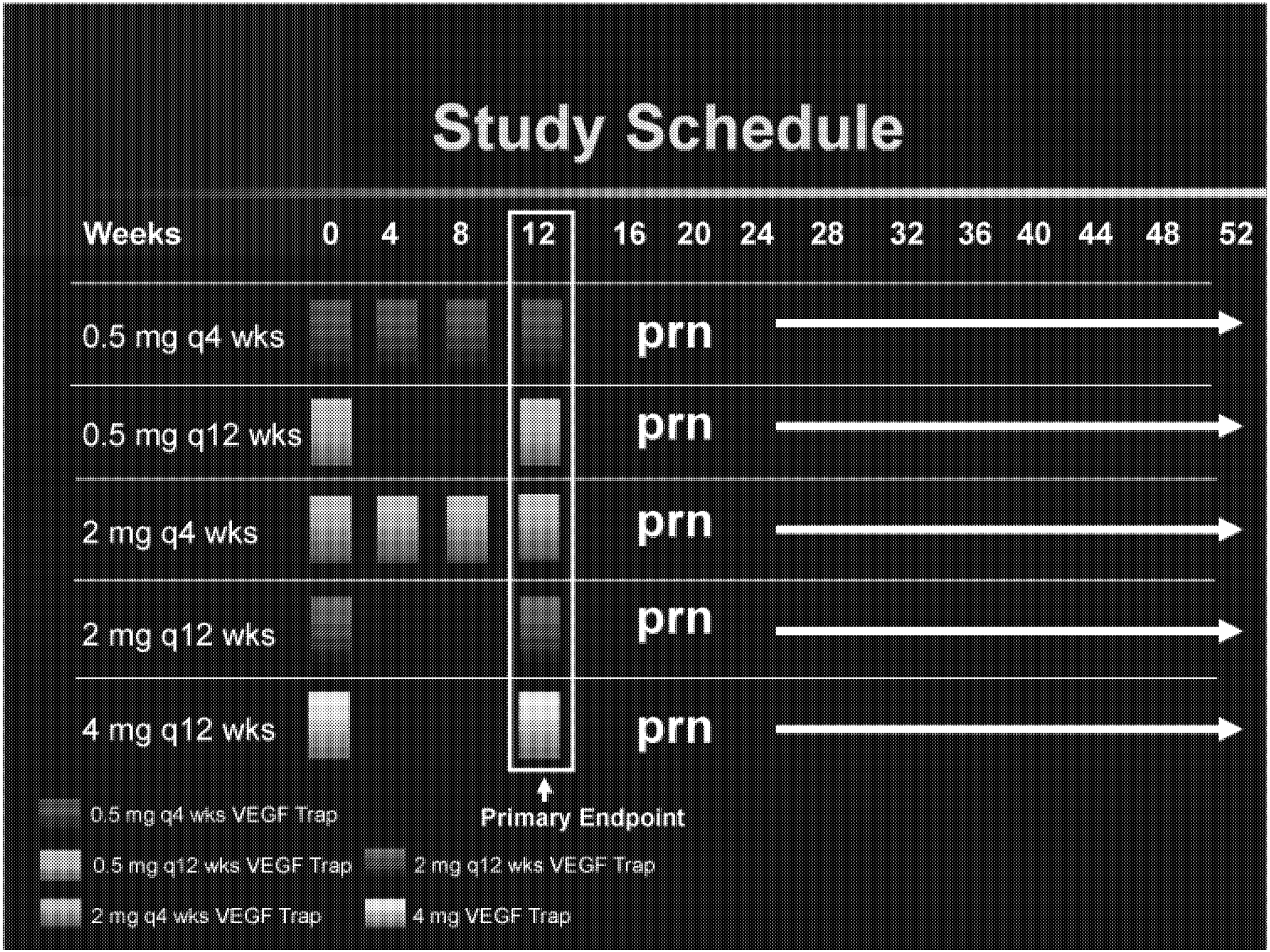
Patients were re-dosed at week 12; PRN dosing started at week 16

Study Schedule
(fixed-dosing phase)







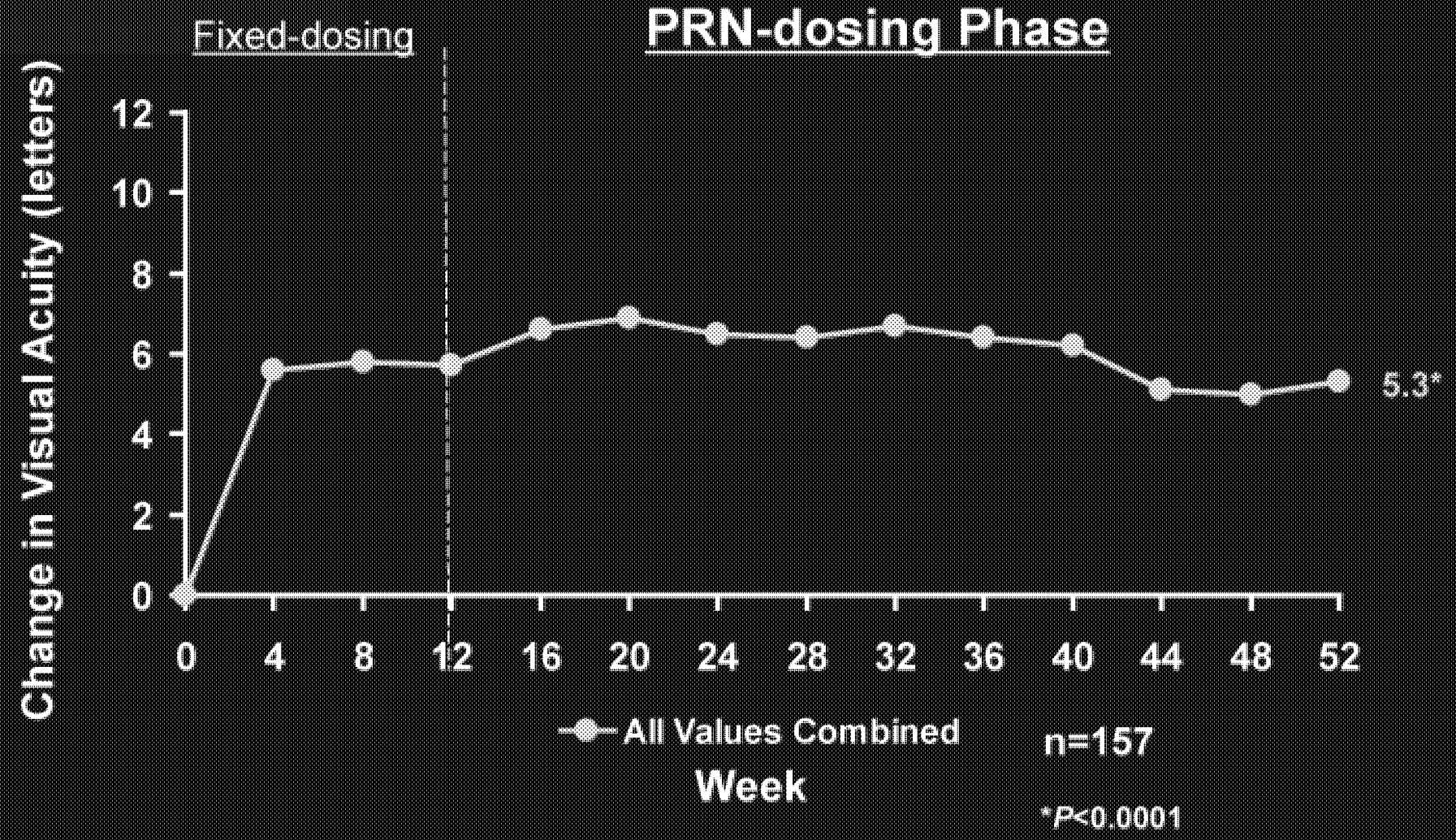


Baseline Characteristics

(n=157*)	Mean	Range
Age (years)	78.2	53-94
Gender (% M:% F)	38:62	
Disease Duration (months)	3.9	0-67
Lesion Size (mean±SD) in disc areas	3.11±2.12	
Lesion Type: number (%)		
Classic	30 (19.1)	
Predominantly Classic	30 (19.1)	
Minimally Classic	37 (23.6)	
Occult Lesions	60 (38.2)	
Disease Status		
Central Retinal/Lesion Thickness	456 µm	186-1316 µm
Foveal Thickness	327 µm	116-1081 µm
ETDRS BCVA (letters)	56	27-83
Baseline Total Lesion Size		

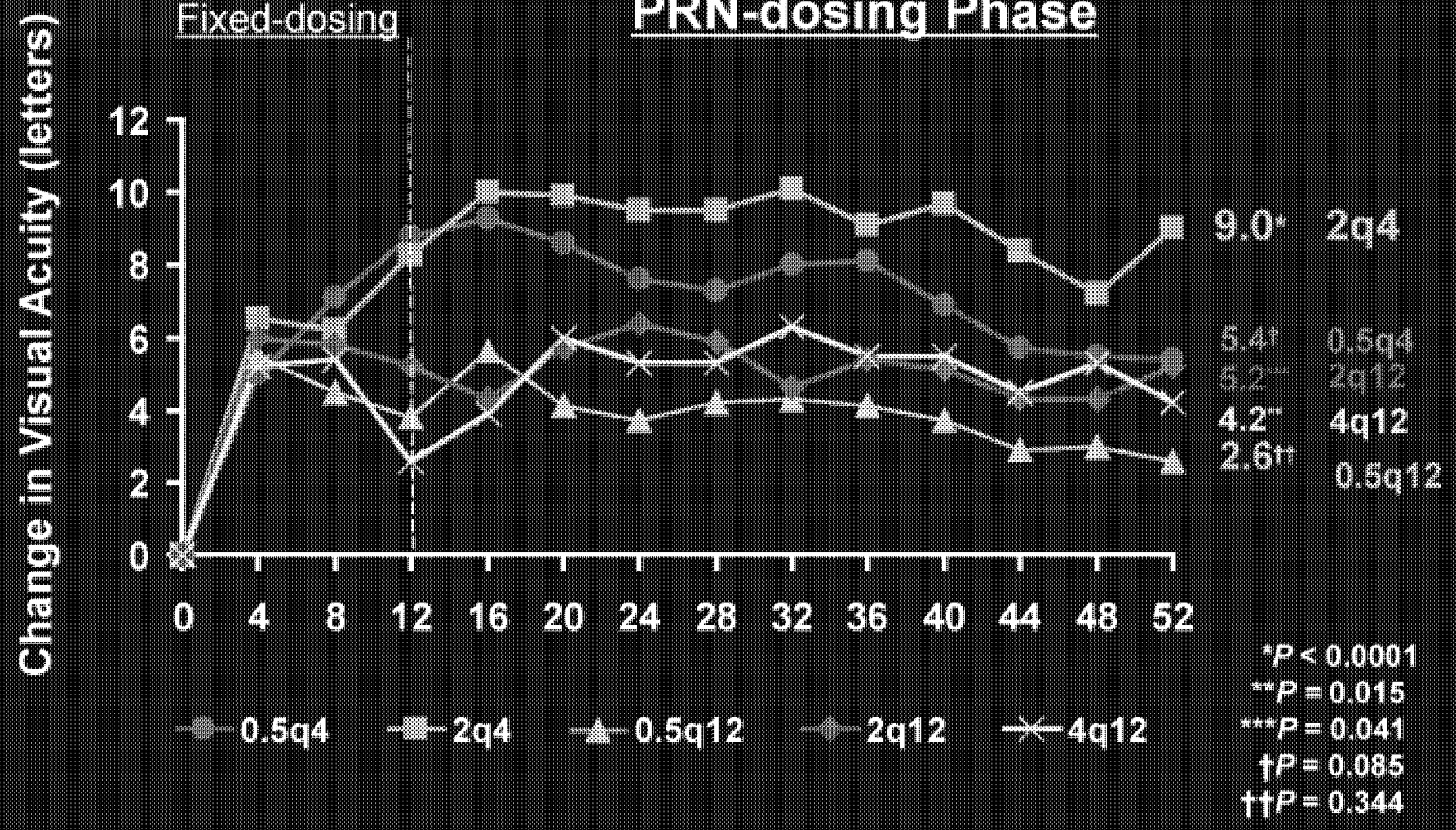
*N=159 randomized; n=157 treated

Mean Change in Visual Acuity



LOCF analysis; paired t-test

Mean Change in Visual Acuity



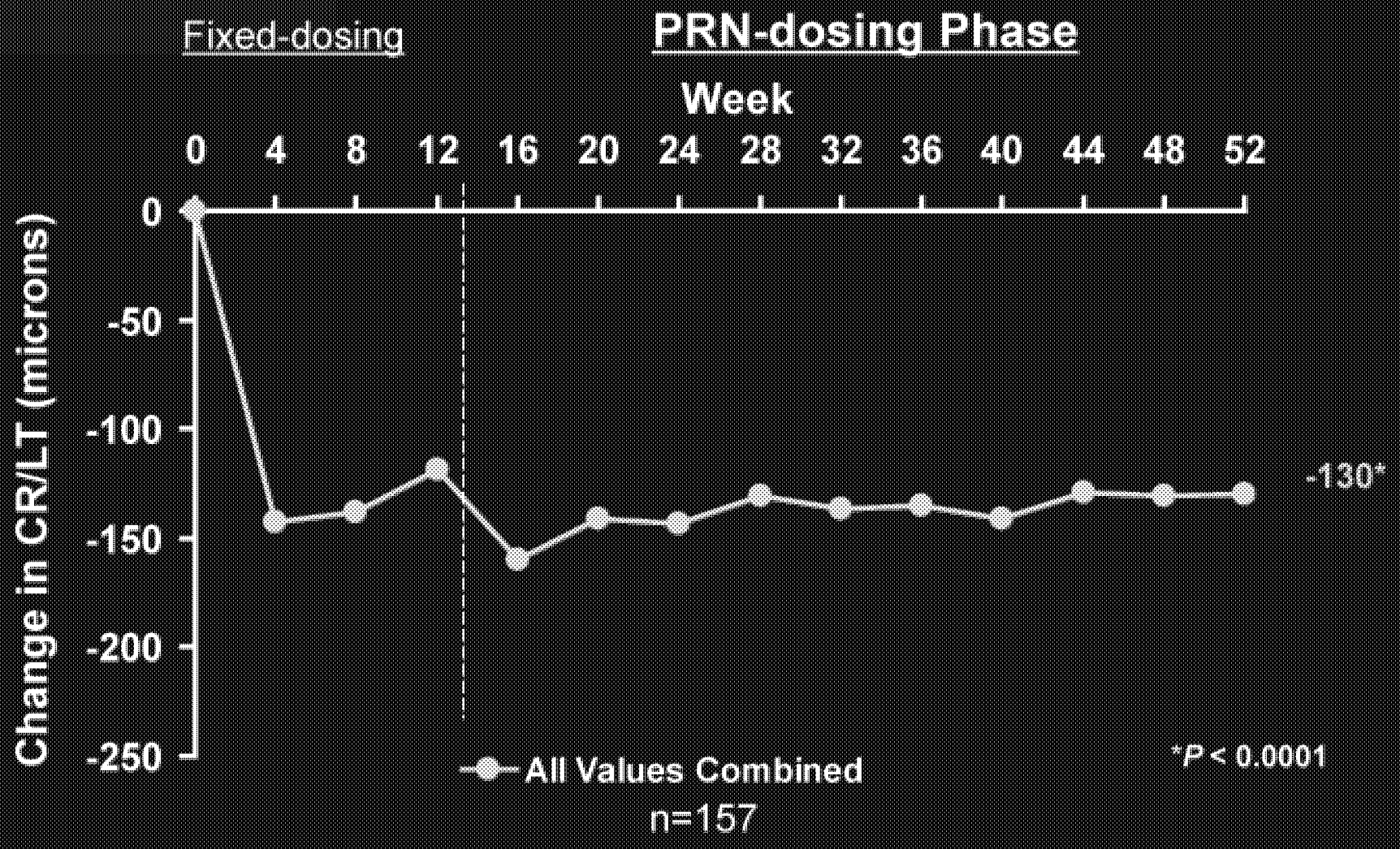
LOCF analysis; paired t-test. 0.5q4 and 0.5q12: n=32; 2q4, 2q12, and 4q12: n=31

Re-Treatment Outcome

VEGF Trap-Eye	Mean number of injections over PRN phase (week 12- 52)	Mean number of days to first injection over PRN phase (week 12 – 52)	Median number of days to first injection over PRN phase (week 12-52)
0.5 mg q4	2.52	102	85
2 mg q4	1.55	160	150
0.5 mg q12	1.84	133	86
2 mg q12	2.48	113	86
4 mg q12	1.7	138	111
All	**2.01**	**129**	**110**

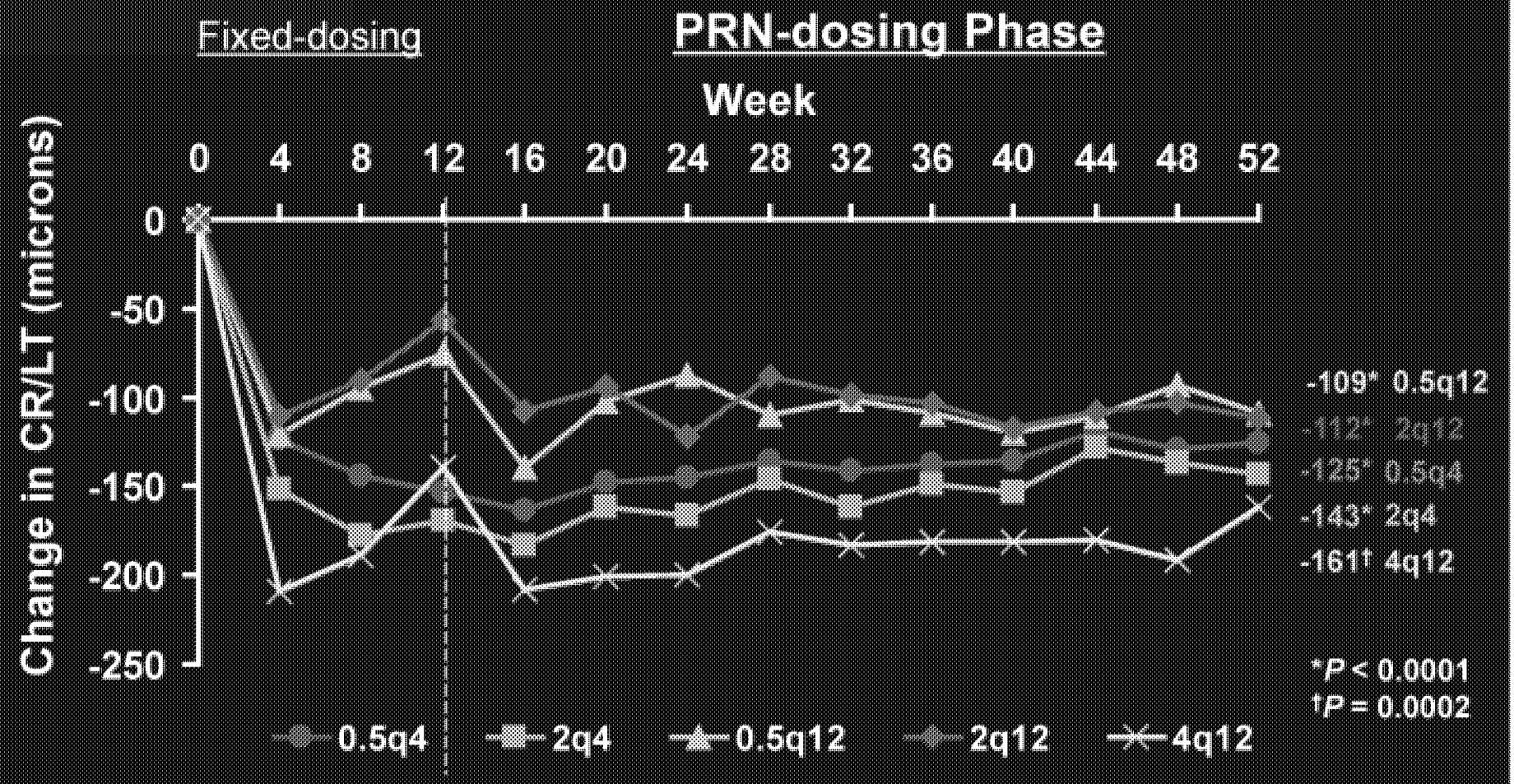
Optical Coherence Tomography

Mean Change in Central Retinal/ Lesion Thickness



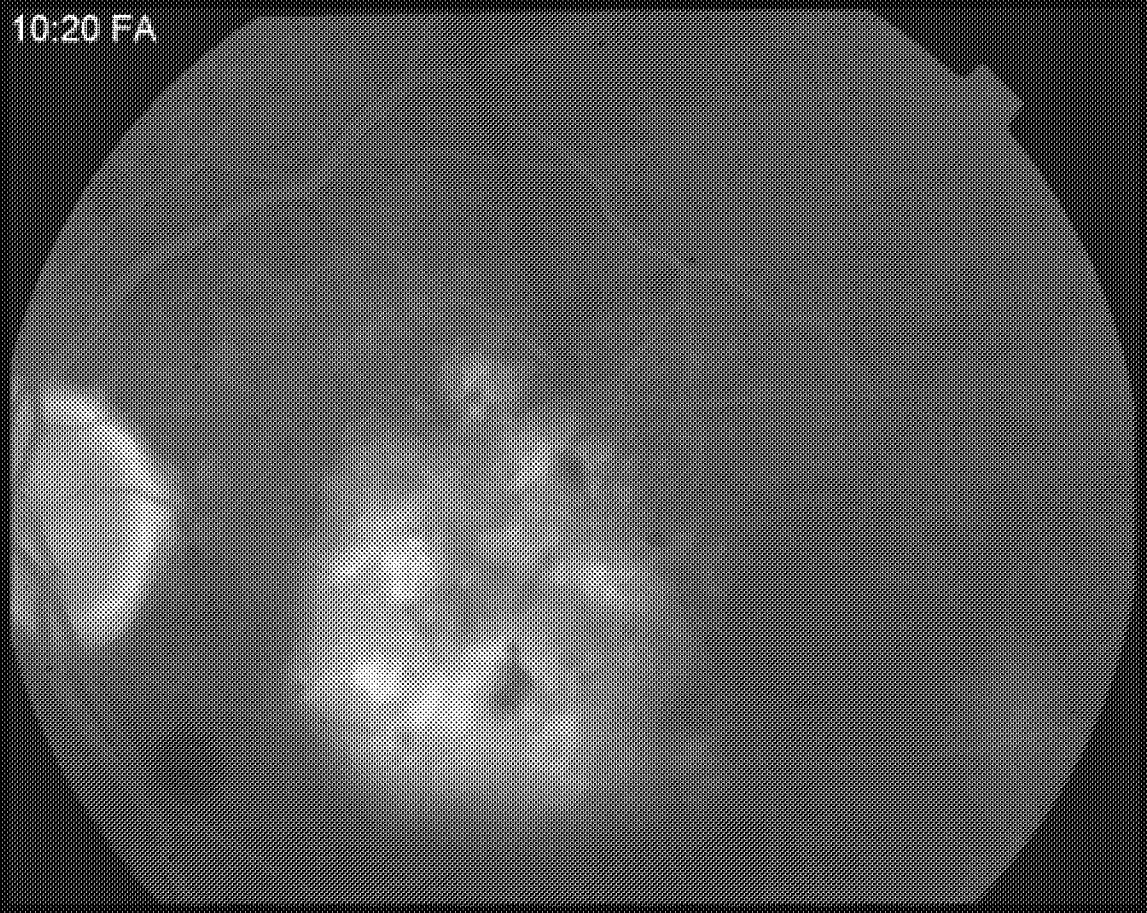
Posterior Pole Scans; LOCF analysis; paired t-test

Mean Change in Central Retinal/ Lesion Thickness



Posterior Pole Scans; LOCF analysis; 0.5q4 and 0.5q12: n=32; 2q4, 2q12, and 4q12: n=31

Fluorescein Angiography
1-Year Outcomes



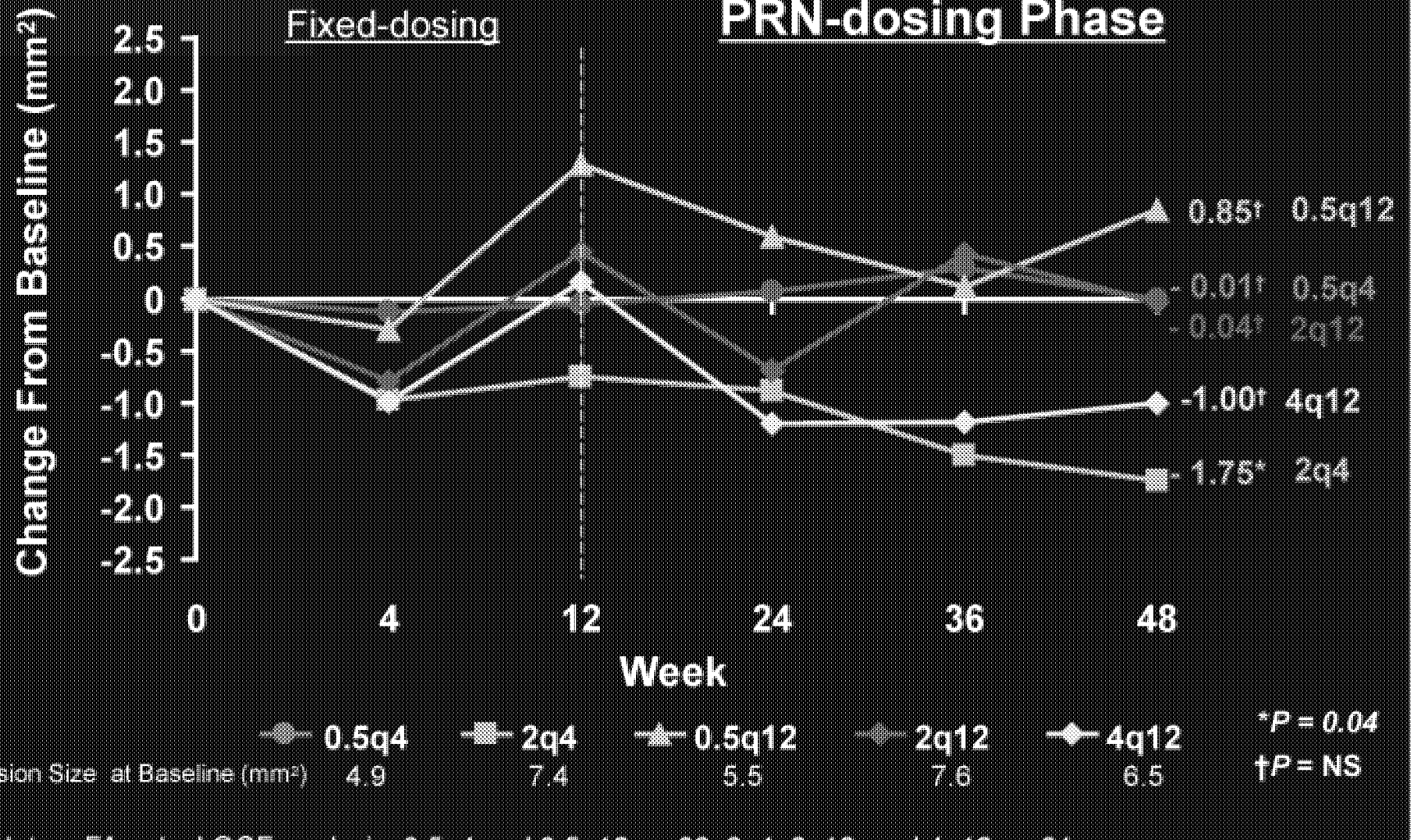
DARC Reading Center: Definitions

- Total lesion size
  - Classic and occult neovascular component, contiguous areas of blood, blocked fluorescence, serous pigment epithelial detachment (PED), and/or fibrosis/scar
- Active CNV
  - Area of visible CNV (classic and/or occult) which demonstrates angiographic evidence of late leakage or pooling of dye
- Classic CNV
  - Area of bright, well-demarcated hyperfluorescence in early phase, with progressive dye leakage into overlying subsensory retinal space in late phase of angiogram (not a measurement of area of leakage, but rather extent of the classic neovascular complex)
- Occult CNV
  - Angiogram shows staining or leakage from fibrovascular PED or hyperfluorescent leakage at level of RPE that represents late leakage of undetermined source (leakage in late phase without classic CNV or fibrovascular PED to account for leakage)

Mean Change in Total Lesion Size by Fluorescein Angiography

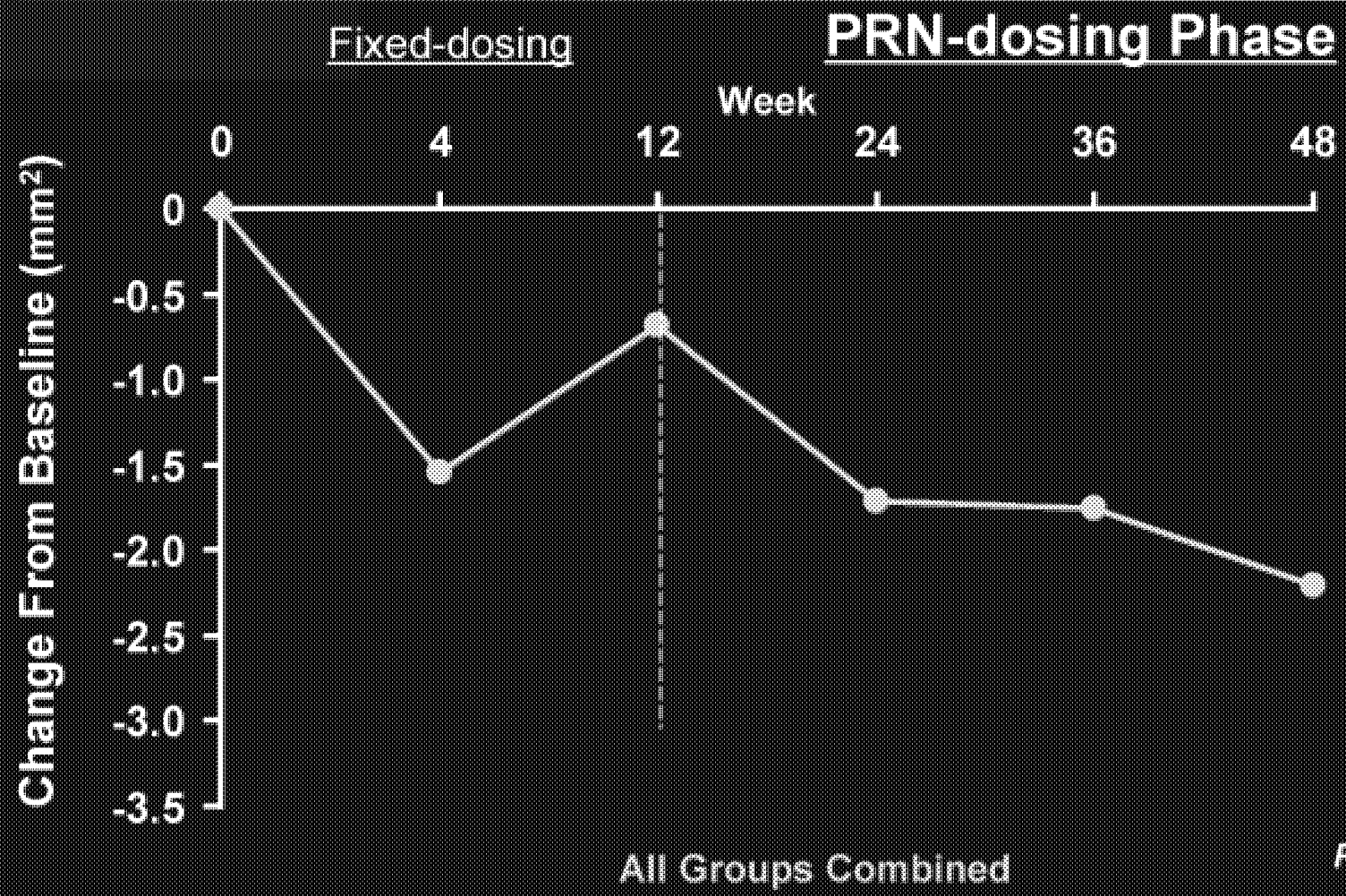
Mandatory FA only; LOCF analysis;

Mean Change in Total Lesion Size by Fluorescein Angiography



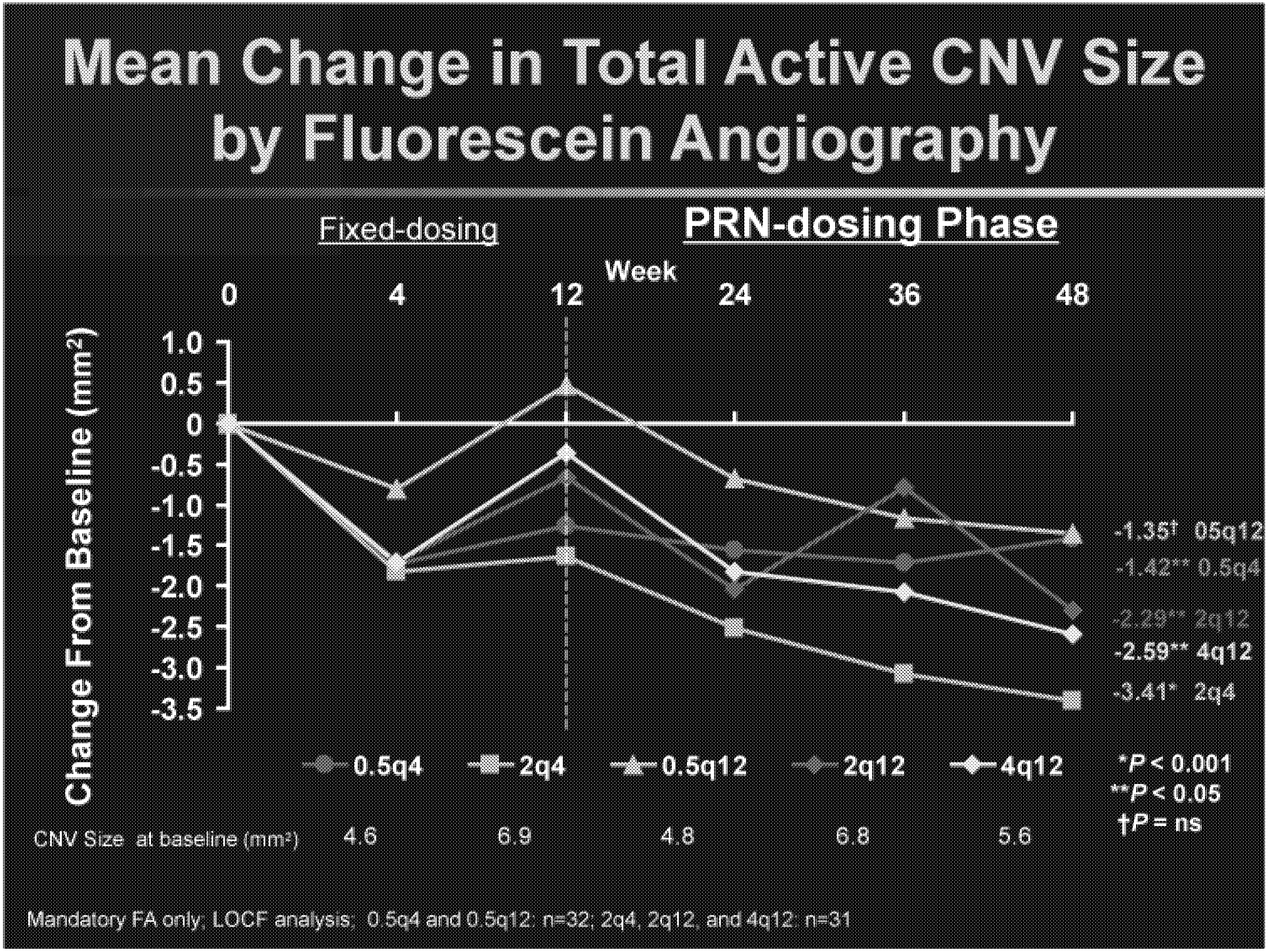
Mandatory FA only; LOCF analysis; 0.5q4 and 0.5q12: n=32; 2q4, 2q12, and 4q12: n=31

Mean Change in Total Active CNV Size by Fluorescein Angiography

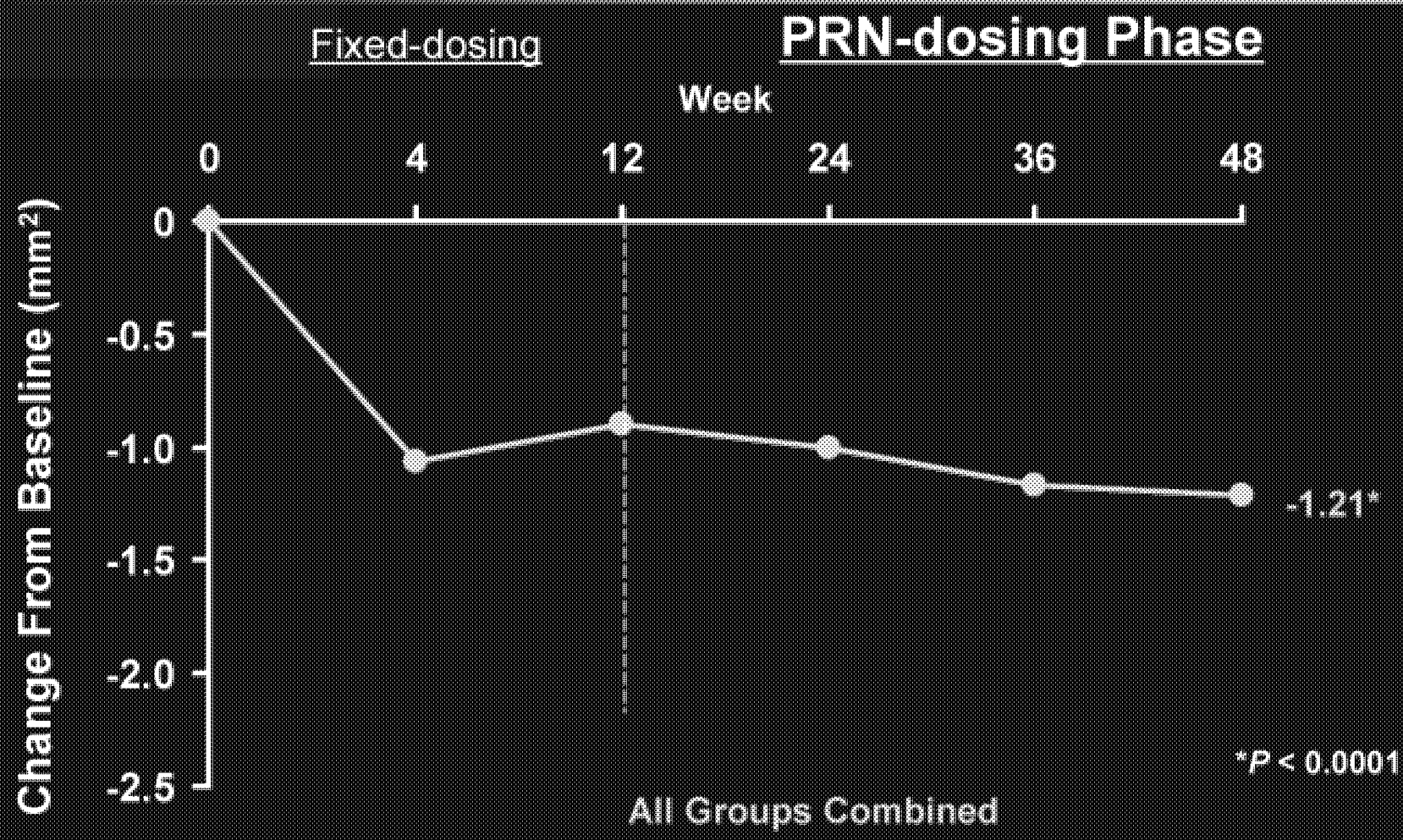


LOCF analysis; Mandatory FA only; CNV=choroidal neovascularization

Mean Change in Total Active CNV Size by Fluorescein Angiography

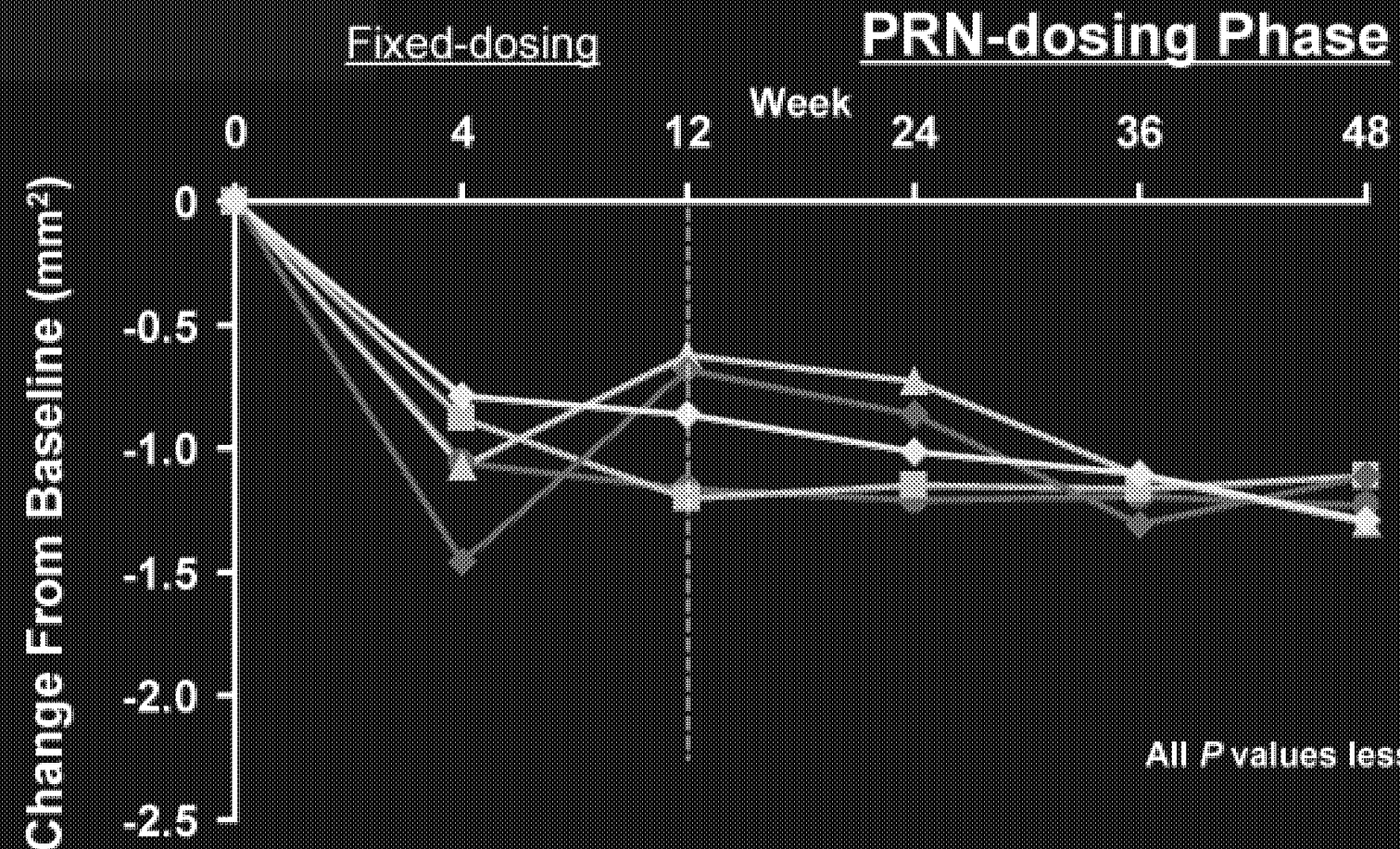


Mean Change in Classic CNV Size by Fluorescein Angiography



LOCF analysis; Mandatory FA only;

Mean Change in Classic CNV Size by Fluorescein Angiography



Mandatory FA only; LOCF analysis; 0.5q4 and 0.5q12: n=32; 2q4, 2q12, and 4q12: n=31

Safety: Serious Adverse Events

Ocular Serious Adverse Events in the Study Eye:

- 1 case of culture-negative endophthalmitis / uveitis (deemed not related to study drug)

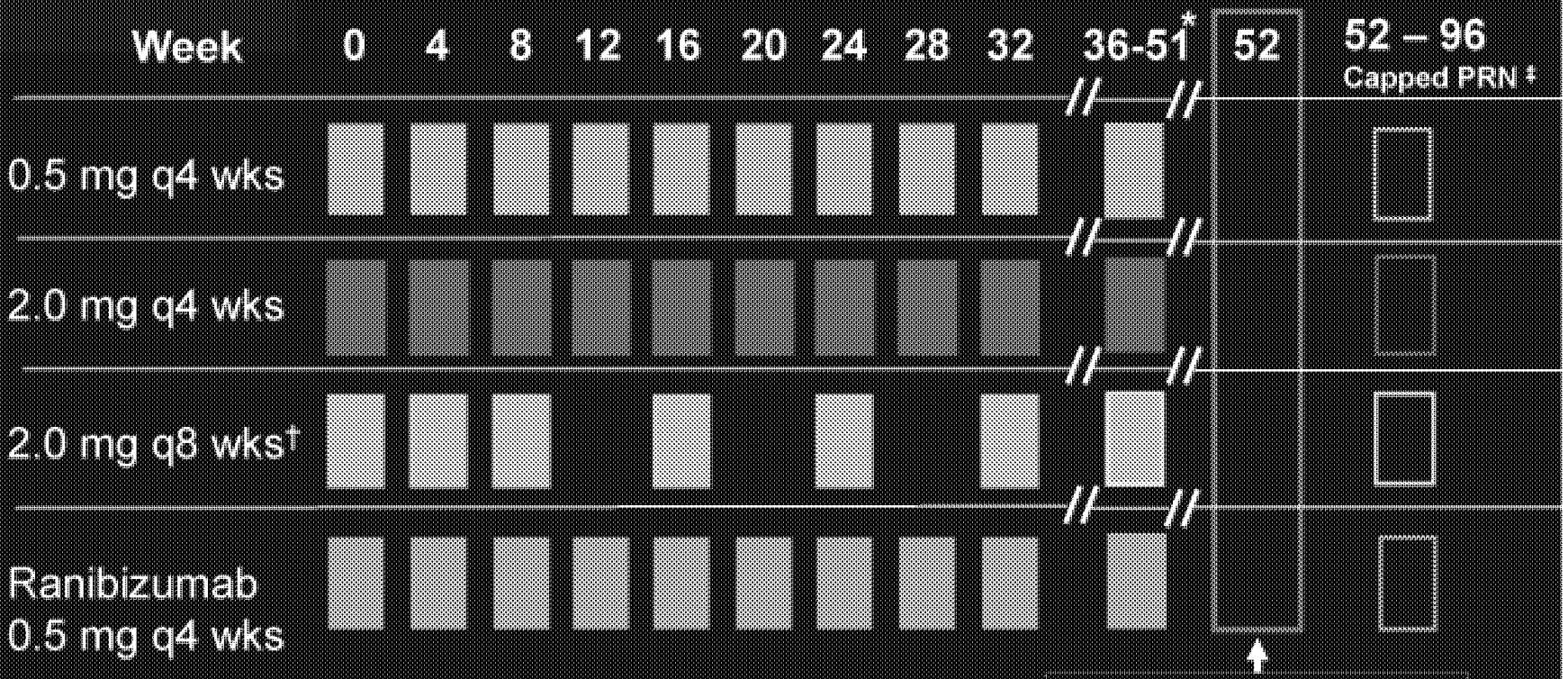
Systemic Serious Adverse Events:

- None deemed to be drug-related
- 2 deaths
  - Pulmonary hypertension (pre-existing condition)
  - Pancreatic carcinoma
- Arterial Thromboembolic Events (ATE's): 1 case of hemorrhagic stroke
  - subject had a history of prior stroke

Conclusions

- Significant reduction in retinal thickness over 1 year
  - Up to -161microns reduction on OCT as measured by CR/LT at wk 52
- Consistent improvement in lesion characteristics on fluorescein angiography
  - Up to -1.75 mm^2 change in total lesion size from baseline at wk 48
  - Up to -3.41 mm^2 change in CNV size from baseline at wk 48
- VEGF Trap-Eye achieved clinically meaningful and durable vision improvement
  - Up to +9.0 mean letters gained at wk 52
  - Average of only two additional injections over 40-week PRN-dosing phase (after a 12-week fixed dosing period)
- Generally well tolerated with no drug-related serious adverse events
  - Most common AE's typical of intravitreal injection

VIEW 1 & VIEW 2 Phase 3 Studies
(Dosing schedule-years 1 and 2)



*Continued dosing at their respective intervals

†After a loading dose period of 3-months

‡ Capped PRN: minimum injection frequency is q12 weeks

0.5 mg VEGF Trap | 2 mg q4 wks VEGF Trap | 2 mg q8 wks VEGF Trap | 0.5 mg Ranibizumab

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Vascular Endothelial Growth Factor and Other Signaling Pathways in Developmental and Pathologic Angiogenesis

Gavin Thurston, Nicholas W. Gale

Regeneron Pharmaceuticals, Tarrytown, New York, USA

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Abstract

The field of angiogenesis received a huge boost in 2003 with the announcement of positive results in a phase III clinical trial using a vascular endothelial growth factor (VEGF)-blocking antibody for the treatment of cancer. Although the VEGF pathway has emerged as a central signaling pathway in normal and pathologic angiogenesis, several other pathways are also now recognized as playing essential roles. This review focuses on 2 specific areas. First, we summarize some of the work on newly discovered angiogenic signaling pathways by primarily describing the molecular biology of the pathways and the evidence for their involvement in vascular development. Second, we describe progress in therapeutic antiangiogenesis in cancer, particularly with agents that block the VEGF pathway.

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Key words: Vascular endothelial growth factor; Inhibitors; Notch receptor; Robo receptor; Delta-like ligand

1. Introduction

The field of angiogenesis is in an exciting phase. The past several years have witnessed increasingly powerful experiments and insights into the biology of vascular endothelial growth factor (VEGF), which has emerged as the central signaling pathway in angiogenesis. Associated with these developments in our knowledge of the VEGF pathway have been several key clinical trials with positive results, which have energized the field and have provided new opportunities for treating various diseases. At the basic research end of the spectrum, we have also witnessed a proliferation in our understanding of additional signaling pathways that are believed to be involved in angiogenesis. Much of the data on this front come from developmental biology. Some of these new pathways appear to be upstream or downstream of the VEGF pathway, but others are clearly not and can function separately. The precise roles of these new pathways in the angiogenic process have not yet been defined. These pathways include Delta/Notch, Slit/Robo4, S1P/edg1, ephrin/Eph, and angiopoietin/Tie.

This review focuses on 2 specific areas. First, we summarize some of the work on new angiogenic signaling pathways by primarily describing the molecular biology of the pathways and the evidence for their involvement in vascular development (Table 1). Second, we describe progress in the use of therapeutic antiangiogenesis in treating cancer, particularly with agents that block the VEGF pathway. This review is not meant to be a comprehensive review of the fields of angiogenesis and antiangiogenesis but rather is intended to provide a very selective sampling of some of the hot areas of basic and clinical research.

Correspondence and reprint requests: Gavin Thurston, Regeneron Pharmaceuticals, 777 Old Saw Mill River Rd, Tarrytown, NY 10591, USA (e-mail: Gavin.Thurston@Regeneron.com; Nicholas.Gale@Regeneron.com).

2. New Angiogenic Pathways in Vascular Development

In the past several years, several newly discovered ligand/receptor signaling pathways have been implicated in angiogenesis. In many cases, the direct evidence for a protein's involvement in angiogenesis has come from genetic studies with mice (Table 1). Here, we briefly review vascular development in the mouse and then focus on 2 specific signaling pathways, Delta/Notch and Slit/Robo, that have emerged through a set of genetic experiments as important players in the angiogenic program.

2.1. Overview of Vascular Development

Embryonic vascular development can be divided into 2 major phases, termed vasculogenesis and angiogenesis. Established in the initial vasculogenic phase is a primitive vascular system that includes the principle arteries and veins, a beating heart, and a poorly structured plexus of peripheral

Table 1.
Some of the Vascular Receptors and Ligands Discussed in This Review Summarized with Respect to Expression Patterns, Phenotype of Genetic Deletion, and Human Diseases Associated with Genetic Alterations*

Receptor/Ligand	Expression Pattern	Knock-out/Transgenic Vascular Phenotype	Human Phenotype
Notch1	Arterial endothelial and smooth muscle cells [135]	Defective vascular remodeling (angiogenesis) and somewhat defective vasculogenesis resulting in embryonic death at E11.5 [33]	Acute T-cell lymphoblastic leukemia
Notch2	During development, the Notch2 gene is expressed in a wide variety of tissues, including neuroepithelia, somites, optic vesicles, otic vesicles, and branchial arches, but not the heart [136]. Notch2 is also reportedly expressed in vascular smooth muscle cell and pericytes [135].	Two knock-outs are reported, 1 with embryonic death at E11.5 [136]. This report did not evaluate vasculature, but embryonic death may have been due to vascular defects. Another report described a hypomorphic allele with perinatal lethality due to kidney defects and a variety of developmental vascular defects, including pericardial and general edema and defective hyloid angiogenesis in the eye [137].	
Notch3	Vascular smooth muscle cells and pericytes [135]	No vascular phenotype reported [149]	CADASIL
Notch4	Arterial and venous endothelium [135]	No obvious vascular phenotype but synergistic with Notch1 knock-out with early angiogenic defects and death at E9.5 [34]	
Jag1	Arterial and venous endothelium and smooth muscle cells [135]	Failure/defects in angiogenesis resulting in embryonic death at E11.5 [31]	Alagille syndrome
Jag2	Jag expression observed in developing limb [138] and reportedly expressed in arteries [135]	Perinatal death due to defects in craniofacial morphogenesis with cleft palate and fusion of the tongue with the palatal shelves. Mutant mice also exhibit syndactyly of the forelimbs and hindlimbs, defects in thymic development with altered thymic morphology, and impaired differentiation of certain T-cell populations [138]. However, no vascular phenotype was described [138].	
Dll1		Hemorrhage beginning at E10.0, death by E12 [139]. Knock-out embryos also have left-right asymmetry defects, including improper formation of the heart and other midline structures [140], as well as defects in somitogenesis [139] and in subsequent neural crest cell migration through somites. Interestingly, Dll1 appears to be critical in regulating somitic expression of ephrins [141], as has also been suggested to occur in the vasculature.	
Dll3		No vascular defects. Dll3 was found to be mutated in the Pudgy line, which exhibits severe vertebra and rib abnormalities due to somite polarity defects [142].	Mutations cause spondylocostal dysostosis, a group of vertebral malsegmentation syndromes with reduced stature resulting from axial skeletal defects [143].
Dll4	Arterial (embryo) and capillary endothelium (adult) and sites of adult angiogenesis [135]	Haploinsufficiency leads to death at E10 due to failure in angiogenesis (unpublished data).	
O-Fucosyl-transferase 1	Ubiquitously expressed [23]	Embryonic death at midgestation with severe defects in somitogenesis, angiogenesis (and possibly vasculogenesis), cardiogenesis, and neurogenesis. The phenotype is similar to that of Notch1/Notch4 double–knock-out embryos but is more severe. The knock-out phenotype may be similar to that of embryos lacking downstream effectors of all Notch signaling pathways [23].	

Continued

Table 1. Continued

Slit2	Central nervous system	Most Slit2 knock-out mice die within a few days of birth, although they appear grossly normal just before birth and are present at normal Mendelian ratios [47]. Knock-out mice have subtle defects in retinal axon guidance but no apparent vascular defects.	
Slit1/Slit2 double knock-out	Central nervous system (Slit1)	Slit2 knock-out mice have subtle retinal axon guidance defects but otherwise appear normal and have normal survival rates. However, Slit1/Slit2 double–knock-out animals all die on the day of birth, although they appear grossly normal just before birth and are present at normal Mendelian ratios [47]. Knock-out mice have severe defects in retinal axon guidance and formation of the optic chiasm but have no vascular defects.	
Slit3	Expression is observed in the diaphragm, central nervous system, retina, exocrine pancreas, hair follicles, the anterior region of the limb buds, and blood vessels in the lung and kidney [48].	Knock-out animals are born at normal frequencies, but many develop congenital diaphragmatic hernia leading to death [48,49]. Herniation is caused by a defective central tendon of the diaphragm. In addition, the hearts of Slit3-deficient mice have an enlarged right ventricle [48,49].	Similar to very common spontaneous congenital defect known as congenital diaphragmatic hernia. Mutations associated with Slit3 have not been reported in this human condition.
Robo1	Broadly expressed	Knock-out mice frequently die at birth from . respiratory failure due to delayed lung maturation. Lungs from these mice have reduced air spaces and increased mesenchyme. Survivors acquire extensive bronchial epithelial abnormalities, including hyperplasia [144]	
Robo4	Expressed in vascular endothelium during development but down-regulated in quiescent adult vessels. Expression is reactivated in pathologic angiogenesis.		
Neuropilin 1	Arterial endothelium	Embryonic death is at E10.5 to E13.5 with axon guidance defects as well as impaired angiogenesis into neural tissues, aortic and brachial arch artery formation defects, and defective yolk sac vascularization [13].	
Neuropilin 2	Expression is initially in veins and becomes primarily restricted to lymphatics by E13 [145].	Mice are viable but are born at reduced frequency with axon guidance defects. Although knock-out mice develop arteries, veins, and large collecting lymphatics, they show an absence of or severe reduction in small lymphatic vessels and capillaries [145-147].	
Neuropilin 1/2 double knock-out	Arterial, venous, and lymphatic endothelium	Neuropilin 1/2 double–knock-out mice die at E8.5 because of major defects in yolk sac vasculogenesis and angiogenesis. Mice lacking 3 of 4 alleles were also embryonically lethal and survived to E10 to E10.5 with yolk sacs that failed to remodel the primitive vascular plexus. This abnormal vascular phenotype resembled that of VEGF and VEGF-R2 knock-outs [148].	

*E11.5 indicates embryonic day 11.5; CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; VEGF, vascular endothelial growth factor; VEGF-R2, VEGF receptor 2.

connecting vessels. In the second phase, angiogenesis, the primitive plexus of vessels undergoes sprouting, pruning, and remodeling events that refine it into a hierarchy of larger and smaller vessels, including arterioles, capillaries, and venules, with defined structural characteristics.

Although several of the key molecular players in the process have been elucidated by genetic means, how they actually function in vasculogenesis and angiogenesis is somewhat mysterious. In vasculogenesis, the principle players include VEGF-A and VEGF receptor 1 (VEGF-R1) through

VEGF-R3, and these critical players are also required throughout the subsequent steps of angiogenesis. Without the precise regulation of expression of these factors, the earliest steps of vascular development go awry. For example, VEGF-A and its receptor VEGF-R2 are clearly required for the proliferation and migration of the endothelial cells that constitute the first blood vessels. However, the molecular mechanisms involved in regulating the expression of VEGF and VEGF-R2 in the appropriate locations at the appropriate times, as well as the key factors involved in instructing endothelial cells where to go and how to form interconnected tubules, are very poorly understood.

Many molecular players in the phase of angiogenesis have also been elucidated by genetic methods. However, developmental angiogenesis is a delicate and essential process. Without fairly successful initiation and progression of angiogenesis, embryonic development cannot progress much beyond 10.5 days postconception, thus limiting the utility of standard genetic techniques in higher vertebrates for understanding how these factors work.

Unfortunately for those of us trying to understand the process by using gene-targeted mice, defects in the angiogenesis phase appear to elicit a programmed abortive process in development that leads to a fairly generic phenotype. This phenotype, most commonly described in the yolk sac or in the plexus of vessels in the head, manifests as arrested development at approximately embryonic day 9.5 with a relatively normal but primitive vasculature plexus that has failed to remodel into a vascular hierarchy. Pericardial edema and failed cardiac trabeculation are also frequent observations. Sometimes, subtle differences in this generic phenotype are exploited to rationalize a specific role for a particular factor. However, because the phenotype is quite generic, further data, such as expression patterns and/or data from in vitro models, are needed to claim more than that the factor does not appear to be critical for development leading up to the point of failure, in this case vasculogenesis, and is required to complete the phase of angiogenesis. Mutant mice for a large number of factors and their receptors fall into this broad phenotypic class and include the following factors and receptors: angiopoietin-1 [1] and Tie2 [2,3]; transforming growth factor β1 (TGF-β1) [4], TGF-(R1 [5], alkaline receptor–like kinase 1 (Alk1) [6,7], and endoglin [8,9]; ephrin-B2 [10] and EphB4 [11]; Sema3A [12]; and neuropilin 1 [13-15].

Because of the inherent difficulties in studying vascular development in mice beyond this critical point, more progress has been made in some cases by using the zebrafish as a model. Zebrafish embryos have several key advantages over mice. A critical advantage is that embryos can survive and develop without a functional cardiovascular system. This advantage, coupled with the ease of doing genetic screens and manipulations, has made the zebrafish an invaluable tool in elucidating mechanisms and molecules involved in vascular development. However, critical differences in the molecules involved and the manner in which the zebrafish vascular system forms make it difficult to translate all of the findings from zebrafish experiments to mammals. One example in zebrafish is Gridlock, a gene that when mutated was discovered to lead to defects in arterial and venous specification. Elegant studies with zebrafish demonstrated that Gridlock acts downstream of Notch and upstream of ephrin-B2 and EphB4 to specify the major arteries. However, knock-outs of Hey2, the Gridlock homolog in mice, have ventricular septal defects and cardiomyopathy with varying penetrance [16,17].

2.2. Delta/Notch Pathway

The Notch signaling pathway was first identified in *Drosophila*. In this species, a single Notch receptor and 2 ligands, Delta and Serrate, play key roles in development. Initially, hypomorphic mutations in Notch were described to lead to wing-patterning defects, but follow-up studies have shown that virtually all tissues examined are affected by Notch deficiency. Using a variety of approaches, investigators have shown that a loss of Notch signaling results in abnormalities in tissues derived from all 3 germ cell layers. Postembryonically, Notch signaling is needed for the elaboration of the central and peripheral nervous systems as well as for spermatogenesis, oogenesis, myogenesis, heart formation, and imaginal disc development (see [18]).

The mammalian Notch family, an expanded version of the fly system, comprises 4 receptors (Notch1 through Notch4) and 5 ligands, 3 of which are related to the *Drosophila* Delta ligands (called Delta-like ligands [Dlls] 1, 2, and 4) and 2 Serrate-related ligands (called Jagged1 [Jag1] and Jag2). The extracellular domains of the Notch family receptors contain between 29 and 36 epidermal growth factor (EGF) repeats, which are involved in ligand interaction, and 3 cysteine-rich LIN12 repeats that prevent signaling in the absence of ligand (see Figure 1). The cytoplasmic regions of the Notch receptors are more variable but contain several domains involved in protein-protein interactions, including a RAM (recombination signal sequence–binding protein for Jκ genes–associated molecule) domain, ankyrin repeats, several nuclear localization signals and a transactivating domain, and, in the case of Notch1 but not Notch3 or Notch4, a PEST degradation sequence (see Figure 1). Notch proteins are produced as a single polypeptide chain that is subsequently cleaved near the transmembrane domain and then reassembled at the cell surface, resulting in a heterodimeric receptor.

The Dlls and the Jags share an N-terminal domain, referred to as the Delta/Serrate domain or DSL, plus 8 or 16 EGF repeats (for Delta and Serrate relatives, respectively) and, in the case of the Jags, a cysteine-rich domain proximal to a transmembrane domain (see Figure 1). Thus far, no significant domain homology that might suggest a function in ligand regulation or signaling has been attributed to the cytoplasmic domains of Dlls or Jags. The ligand and the extracellular domain of Notch have been suggested to be internalized into the signal-sending cell, where they are eventually degraded. The cleavage and release of the Notch extracellular domain appear to be critical for the subsequent cleavage events described below that lead to Notch signaling [19]. However, at present it is not clear whether degradation of the ligand and the Notch extracellular domain have a role in preventing further activation of signaling or in preventing

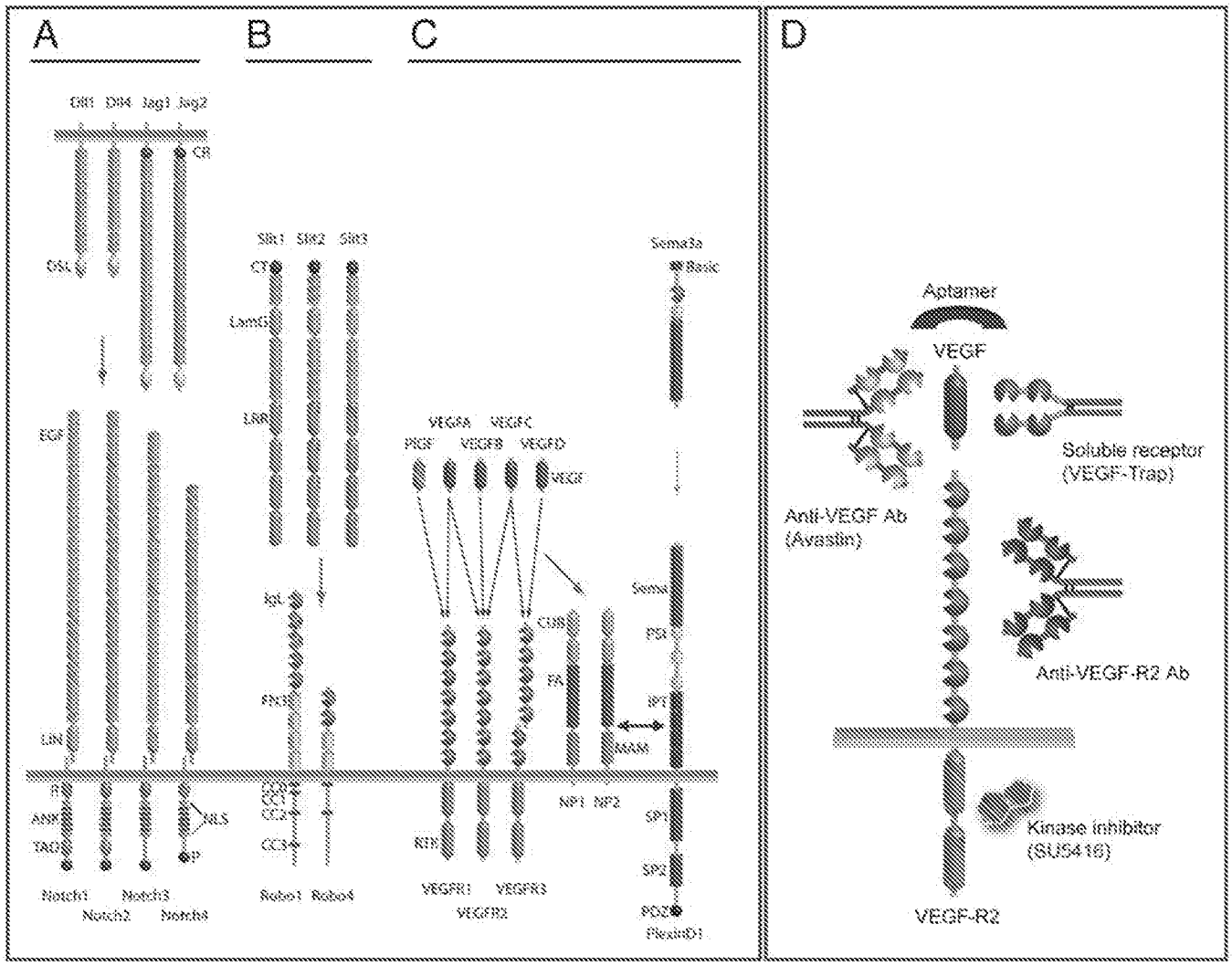

Figure 1. Diagram of 3 of the ligand/receptor systems implicated in angiogenesis. A, Shown is the Delta/Notch system with the membrane-bound ligands Delta-like 1 (Dll1), Dll4, Jagged-1 (Jag1), and Jag2 and the 3 endothelial cell Notch receptors. B, The Slit/Robo system is shown with 2 of the 3 known mammalian Slit ligands and 2 of the endothelial cell Robo receptors. C, Shown is the vascular endothelial growth factor (VEGF) system with VEGF ligands (VEGF-A, -B, -C, -D, and placental growth factor [PlGF]) and the endothelial cell receptors VEGF-R1, -R2, and -R3. Shown are neuropilin receptors NP1 and NP2, which can bind to specific VEGF ligands. Also shown is a member of the Semaphorin3 class (Sema3a), which can bind to the neuropilins. In the nervous system, neuropilins signal through coreceptors of the plexin family. Recently, PlexinD1 (shown) has been shown to be expressed in vascular endothelial cells, raising the possibility of similar signaling in these cells. D, Different approaches currently being pursued in the clinic to inhibit the VEGF signaling pathway. Approaches include the use of blocking antibodies (Ab) to VEGF such as Genentech's Avastin, blocking antibodies to VEGF-R2, small-molecule tyrosine kinase inhibitors of VEGF-R2 such as Sugen Pharmaceuticals' SU5416, soluble receptors such as Regeneron Pharmaceuticals' VEGF-Trap, and synthetic RNA molecules (aptamers) that bind to and block VEGF. CR indicates cysteine-rich domain; DSL, Delta, Serrate, and Lag domain; CT, C-terminal cysteine knot–like domain; LamG, laminin G–like domain; EGF, epidermal growth factor–like domain; LRR, leucine-rich–repeat domain; IgL, immunoglobulin-like domain; Sema, semaphorin domain; CUB, complement-binding domain; PSI, plexin, semaphorin, integrin domain; FA, factor V/factor VII coagulation factor homology domains; FN3, fibronectin type III domain; LIN, cysteine-rich Notch/LIN12 domain; IPT, cell surface receptor IPT/TIG domain; MAM, Meprin A5; R, RAM23 domain; CC, conserved Robo cytoplasmic domains; ANK, ankyrin repeat; NLS, nuclear localizing sequence; SP, semaphorin, plexin domain; TAD, transactivation domain; P, PEST domain; RTK, receptor tyrosine kinase domain; PDZ, PDZ domain.

the liberation of soluble Notch extracellular domain, which could act as an inhibitor of Notch signaling [20].

Notch receptors use a unique and remarkably direct mechanism of signal transduction. On ligand binding, a series of proteolytic cleavage steps leads to the liberation of the intracellular domain of Notch, which then translocates to the nucleus. Once in the nucleus, Notch acts as a potent transcriptional coactivator through its interaction with the CSL transcriptional factor, converting CSL from a transcriptional repressor to a transcriptional activator. Major targets

of this transcriptional complex include several helix-loop-helix–type transcription factors known as hairy and enhancers of split (HESs) (eg, HES1, HES5, and HES7) and HES-related repressor proteins (HERPs) (eg, HERP1, HERP2, and HERP3), among other genes.

As mentioned above, several proteolytic events and post-translational modifications are involved in the tight regulation of Notch signaling and activity. A key event is the cleavage of Notch by γ-secretase, a multicomponent protein complex that liberates the Notch intracellular domain, the active signaling component. Additional events include ubiquitinization, the liberation of soluble ligands and extracellular domains of receptors, and regulated endocytosis. We refer the reader to an excellent recent review [21] for a more thorough description of these processes.

Adding to the complexity of the system is the fact that Notch signaling appears to be highly regulated by glycosylation. In particular, Notch receptors and ligands undergo a very rare modification by *O*-fucosyltransferase, which adds fucose to threonine and serine residues within Notch's EGF repeats [22,23]. This type of modification is known to occur on only a few other proteins, eg, urokinase plasminogen activator, thrombospondin, and the locus PMP-C (for review see [24]). Notch function in *Drosophila* requires the addition of this *O*-fucose [22]. Similarly in mice, deletion of the *O*-fucosyltransferase-1 gene appears to largely phenocopy deletion of other components of the Notch pathway [23].

Further modifications occur on the *O*-fucose residues on Notch. In particular, fringe family proteins catalyze acetylglucosaminylation on the *O*-fucose. Three vertebrate fringe family members, lunatic fringe, maniac fringe, and radical fringe, have expression patterns that overlap with Notch family components. When fringe is coexpressed in cells that also express Notch, it is capable of glycosylating Notch [25,26]. Mutations in lunatic fringe in mice result in a phenotype similar to that of mice in which the Notch signaling pathway is deleted, supporting the notion that glycosylation by fringe is also essential for Notch function [23]. Thus, when Notch signaling is considered, a combinatorial expression of many regulating factors in the particular cells can induce positive or negative regulation of the pathway, depending on the combination of the factors that are coexpressed. The strict requirement of Notch to be glycosylated and the relative uniqueness of Notch glycosylation suggest that the enzymes mediating the posttranslational modifications are potential therapeutic targets for antiangiogenic therapy.

The mammalian Notch pathway has been implicated in a vast array of developmental and adult processes in a wide variety of tissues. For example, Notch is involved in induction of left-right asymmetry, limb bud development, somitogenesis, neurogenesis in the brain, cell fate decisions in the inner ear, inhibition of myogenesis and cardiogenesis, lymphopoiesis, and kidney development (see [27] for a review). Recently, Notch has become interesting to vascular biologists, in part because of expression studies clearly showing Notch in the vasculature. A variety of studies have evaluated the patterns of expression of Notch receptors in vascular cells as well as in different types of blood vessels. These expression patterns have been summarized in several recent reviews (see [28,29]). Even more recently, expression data for the Notch ligand Dll4 have become available. Dll4 is expressed early in mouse development (eg, at embryonic day 8), beginning in the dorsal aorta and the major arteries [30]. Subsequently, Dll4 appears to be down-regulated in the major blood vessels, and Dll4 expression instead shifts to capillaries. Dll4 expression is relatively low in the adult vasculature [30] but is dramatically up-regulated in tumor angiogenesis. Accordingly, Dll4 expression is up-regulated by hypoxia in cultured endothelial cells [30]. As noted above, there are inherent difficulties in trying to predict from surveys of expression the function or even the involvement of the Notch pathway in a tissue compartment. Nonetheless, such analyses have been a starting point in the quest to understand the role of Notch signaling in vascular development.

In addition to the expression data, functional genetic data have also begun to implicate the Notch pathway as a critical regulator of vascular development. The deletion of the ligand Jag1 [31,32] or Dll4 (unpublished data) or the deletion of their receptor Notch1 [33] results in embryonic lethality, apparently with the somewhat generic phenotype described above. Although Notch4 mutant mice do not appear to have a vascular phenotype, double-mutant mice lacking both Notch1 and Notch4 appear to have a more severe phenotype than those lacking Notch1 alone [34]. Mice with mutations in Notch3, which is expressed in vascular smooth muscle cells, apparently do not have vascular defects [149]. However, in humans Notch3 mutations are associated with CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), an adult-onset disease of small arteries characterized by cerebrovascular fragility [35]. These results suggest that the Delta/Notch pathway is not required for vasculogenesis but is required for essential but unspecified role(s) in angiogenesis.

2.3. Regulation of Dll4 and Notch1 by VEGF

Several lines of evidence suggest that the Notch pathway may act as a critical effector of the VEGF pathway. In cultured human iliac arterial endothelial cells, Dll4 as well as its receptor Notch1 can be up-regulated by VEGF but not by fibroblast growth factor [36], indicating that Dll4 and Notch1 are targets and possibly downstream effectors of VEGF. These results also suggest that the observed hypoxia-mediated regulation of Dll4 may be indirect via the up-regulation of VEGF, which is a well-known hypoxia-regulated gene. Interestingly, the response of other cultured endothelial cells was somewhat distinct from that of iliac artery endothelial cells: femoral artery endothelial cells also up-regulated Dll4 and Notch1 via VEGF, but Dll4 was also up-regulated by fibroblast growth factor. In venous or microvascular endothelial cells, Notch and Dll4 did not appear to be regulated by VEGF [36]. Furthermore, VEGF-mediated tube formation in cultured endothelial cells could be partially blocked by blocking Notch signals [36], thus bolstering the concept that Dll4/Notch1 signals may be critical effectors of VEGF signaling.

Because of the apparent specificity of Dll4 expression in embryonic arteries in mammals and lower vertebrates and because of the regulation of Dll4 and Notch in cultured

endothelial cells, VEGF has been suggested to be a key regulator of arteriogenesis via the Notch pathway (reviewed in [29]). In zebrafish, the connection between Notch signaling and arterial specification is well established [37-39]. The transcription factor Gridlock is a key effector of Notch signaling, and mutations in Gridlock in zebrafish result in failure to properly specify arteries and veins [38]. More recently, VEGF has been shown to act upstream of the Notch signals that specify arterial formation in zebrafish [40,41].

2.4. Slit/Robo Pathway

The Slit/Robo pathway has also recently been added to the list of angiogenic pathways. Slit proteins are secreted factors that bind to and signal through the roundabout (Robo) receptors. This signaling pathway has been best studied in axonal guidance, in which Slit ligands provide chemorepulsive cues to axons and neuronal cells that express Robo receptors. In *Drosophila* and zebrafish, Slit and Robo genes are highly expressed in the developing nervous system and function in establishing the complex network of connections within the brain by helping to guide axons and migrating neural cells to their appropriate locations. Slit and Robo genes have also been shown to be involved in muscle development, in which they provide chemotactic signals to muscle cells. In higher vertebrates, the family comprises 3 secreted factors (Slit1, Slit2, and Slit3) that contain a variety of protein domains, including leucine-rich repeats, 9 EGF-like repeats, a laminin G domain, and a C-terminal cysteine knot. Vertebrates have 4 Robo receptors (Robo1, Robo2, Robo3 (Rig-1), and Robo4). Robo1 and Robo3 contain 5 immunoglobulin domains and 3 fibronectin domains in the extracellular region. Although Robo1, Robo2, and Robo3 are largely neural specific, Robo1 may be expressed in cultured endothelial cells [42].

Robo4, a more recently discovered family member, appears to be quite specific to the vascular system. It was initially identified by means of a bioinformatic data-mining strategy to discover novel vascular-specific molecules [43]. The extracellular region of Robo4 contains only 3 of the 5 immunoglobulin domains and 2 of the 3 fibronectin domains found in other Robo genes. Robo4 was independently identified in differential gene expression experiments when genes expressed in normal and Alk1 knock-out embryos, which die during development with vascular defects [7,44], were compared [6]. In vitro studies suggest that Robo4 shares ligands with the neuronal Robo receptors, and it appears to mediate analogous functions. For example, Robo4 appears to inhibit endothelial cell migration.

Although the functional role in the vasculature has yet to be determined, Robo4 has a striking expression pattern in the vasculature. Robo4 is expressed in vascular endothelium during development [43,44]. It appears to be down-regulated in the adult vasculature but is reactivated in endothelial cells at sites of active angiogenesis [43].

Although Robo4 has been shown to bind various Slits [44], it is not entirely clear which Slit ligand(s) may be interacting with the vascular Robo4 receptors in vivo. One candidate for Robo4 binding is Slit2, which has been shown to induce network formation in cultured endothelial cells. Slit2 is highly expressed in a broad range of cancers, and its expression has been correlated with the extent of tumor growth and vascularity [42,45]. Furthermore, inhibition of Robo signaling with Robo blocking agents was reported to prevent tumor growth [42,45]. However, the function of Slit2 in tumors is still unresolved; some reports suggest that Slit2 is antiangiogenic [46].

Gene-targeting studies with the Slit/Robo pathway in mice have not been very informative to date for elucidating potential vascular involvement. Most mice deficient for Slit2 die at birth, although a small percentage of the mice can survive for several days. Mice that are deficient for both Slit1 and Slit2 are born in the expected Mendelian ratios; however, these mice also die at birth. Slit2 and Slit1/2 knock-out mice apparently do not have any vascular defects [47], suggesting that Slit1 and Slit2 are not required for normal cardiovascular development. Unfortunately, the perinatal death of the Slit2 and Slit1/2 knock-out mice prevents analysis of postnatal angiogenesis, such as in the neonatal retina and in tumors.

One possibility is that Slit3 is able to compensate for the loss of Slit1 and Slit2. Several groups have generated Slit3-deficient mice [48,49]; however, these mice also do not have obvious vascular defects, thus casting some doubt as to whether the 3 Slit family members are important for vascular development. The most obvious phenotype in the Slit3 knock-out was congenital diaphragmatic hernia [49].

Robo4 gene–targeted mice have not yet been described, and these mice will certainly be very informative regarding whether Robo signaling is required in vascular development. It is possible that combinatorial deletion of all 3 Slit family members will be required to determine if Slit ligands are important in vascular development, and conditional alleles of Slit2 may be valuable in assessing its suggested roles in tumor angiogenesis. However, it also is possible that novel ligands for the vasculature-specific Robo4 will be identified. Finally, it will be very interesting to see how Robo and Slit family members fit into the angiogenic pathway with other more well-established pathways, particularly the VEGF pathway.

3. Therapeutic Antiangiogenesis

More than 30 years ago, Judah Folkman proposed that blocking tumor angiogenesis could be an approach to treat cancer [50]. At that time, certain unique features of tumor endothelial cells, such as increased rates of proliferation relative to endothelial cells in normal tissues [51-54], were beginning to emerge, but agents to specifically block tumor angiogenesis had not yet become available. The past 25 years have seen a dramatic increase in our knowledge of the molecular pathways involved in angiogenesis and have accordingly increased opportunities to specifically interfere with tumor angiogenesis. In the past few years, the agents to rigorously pursue Folkman's proposal have been developed and have begun to be applied in the clinic.

Although numerous molecular pathways have been explored, the VEGF pathway has emerged as perhaps the key angiogenic growth–signaling system in initiating normal and pathologic angiogenesis. Now that several potent and

specific inhibitors are available (see below), a wealth of accumulated data shows that VEGF is essential in such physiological angiogenic processes as ovarian cycling [55,56], postnatal bone growth [57], and angiogenesis-dependent wound healing. In addition, VEGF is now well documented to be necessary for many situations of pathologic angiogenesis, including ischemia-induced retinopathy [58,59] and cancer.

3.1. Biology of VEGF

Various genes for VEGF (VEGF-A, -B, -C, -D, and the placental growth factor gene, PlGF), which encode distinct gene products, are evolutionally conserved in mammals (Figure 1C). VEGF-A appears to be the key initiator of blood vessel growth. Each of the VEGFs has various isoforms as a result of alternative splicing, the major ones of VEGF-A being 121 kD, 165 kD, and 189 kD [60-62]. A recent report suggests that alternative splicing of the last exon of VEGF can produce yet another family of isoforms; however, these isoforms appear to be down-regulated in cancer and appear to block many of the traditional actions of VEGF [63]. The larger isoforms of VEGF, particularly VEGF-189, contain heparin-binding regions and bind strongly to components of the extracellular matrix. In contrast, VEGF-121 is relatively freely diffusible.

VEGF expression is regulated by a number of factors and stimuli, including hypoxia. VEGF-A is crucial for vascular development; loss of even a single allele results in embryonic lethality in mice, with an almost complete failure to form blood islands and a profound lack of primitive vessels [64,65]. VEGF continues to be required throughout embryonic and perinatal development. Inhibition of VEGF in mice at a postnatal age of 2 weeks resulted in the loss of blood vessels in some organs and in death [66]. However, the need for VEGF is apparently diminished as mice mature; inhibition of VEGF in adult mice has much less of an effect on vessels in most organs [66].

VEGF has at least 3 distinct but highly related receptors known as VEGF-R1, -R2, and -R3. In addition, other cell surface molecules (such as neuropilin) can bind VEGF molecules and appear to play important roles in cardiovascular development. The key receptor for VEGF-A on blood vascular endothelial cells appears to be VEGF-R2, and most therapies against VEGF receptors have targeted this receptor. VEGF receptors are intrinsic tyrosine kinases that, once they are dimerized by homodimers of VEGF, autophosphorylate on multiple tyrosine residues and thereby transmit signals into the cell [67-69]. Activation of VEGF-R2 on cultured endothelial cells can increase cell survival, proliferation, and migration [67,70-72].

3.2. Clinical Approaches to Block VEGF

At the present time, several different approaches are being pursued to block the VEGF signaling pathway (see Figures 1D).

VEGF-blocking antibodies are being developed by Genentech. The agent currently used in the clinic is known as bevacizumab (Avastin) [62]. Bevacizumab is a humanized monoclonal antibody with approximately 13% residual mouse sequence. It binds to human VEGF-A (all isoforms) and inhibits the binding of VEGF-A to VEGF-R1 and VEGF-R2. Although bevacizumab and its parental mouse monoclonal antibody (A4.6.1) do not effectively block murine VEGF, preclinical studies have shown that bevacizumab given systemically is effective at blocking the growth of many human tumors in nude mice [73-75]. Studies with monkeys have shown that bevacizumab and related antibodies are effective at blocking ovarian cycling in the mature female reproductive cycle and bone growth in postnatal development [56,76].

Another approach to inhibit the VEGF pathway is being taken by Regeneron Pharmaceuticals with the development of a soluble receptor known as VEGF-Trap [77]. This molecule is a fusion of portions of both VEGF-R1 and VEGF-R2 with the Fc domain of human immunoglobulin G1. VEGF-Trap has an affinity for VEGF-A in the range of 1 pM and has a half-life in humans that is similar to that of bevacizumab. Preclinical studies have shown that VEGF-Trap can block vessel growth in human and murine cancers grown in mice [77,78] and can inhibit ascites produced by human ovarian cancer cell lines grown in mice [79].

A third approach, which is being pursued by Imclone Systems, is to use antibodies to VEGF-R2 that block binding of VEGF to the receptor [80]. Fully human antibodies generated by phage display were shown to block binding of VEGF to human VEGF-R2 and to block the downstream effects of VEGF on human endothelial cells in culture [81]. Antibodies to VEGF-R2 were also potent inhibitors of tumor growth in mice [82]. Subsequent generations of antibodies, made by focusing on a particular heavy-chain variable sequence and testing various variable light-chain sequences, have resulted in antibodies to VEGF-R2 with affinities of approximately 100 pM and improved potencies at inhibiting downstream cellular signaling of VEGF [83].

Another approach, which is being pursued by Eyetech with its drug Macugen, is to use synthetic RNA molecules (aptamers) that bind to VEGF and block interaction with VEGF-R2 [84,85]. Macugen binds specifically to the VEGF-165 isoform but not to other VEGF isoforms [86]. Macugen is being used primarily in treating ocular diseases that involve angiogenesis and/or edema in the retina or choroidal vasculature. In clinical studies to date, Macugen has been delivered by local intraocular injection.

Several pharmaceutical companies have developed small molecules that inhibit the tyrosine kinase activity of VEGF-R2. An early leader was Sugen Pharmaceuticals, and now Pfizer, AstraZeneca, and Novartis Pharmaceuticals are all pursuing this approach. One example of a drug from this class is the orally available compound PTK787 from Novartis Pharmaceuticals. PTK787 inhibits the tyrosine kinase activity of all 3 VEGF receptors with an IC_{50} of <100 nM and inhibits related receptor tyrosine kinases, such as platelet-derived growth factor receptor β (PDGF-R() and c-Kit, at somewhat higher concentrations [87]. PTK787 was shown to have antiangiogenic and antitumor activities against several types of transplanted tumors, including thyroid [88], pancreatic [89], and renal cell carcinomas [90].

3.3. Insights into Actions of VEGF in Tumor Angiogenesis

With the advent of potent and specific blockers of VEGF, we have gained new insight into the precise actions of the VEGF pathway in tumor angiogenesis. However, even before VEGF blockers were widely available, one study turned off VEGF expression in an androgen-dependent tumor and monitored changes in tumor vessels by in vivo microscopy [91]. This study found that the tumor vasculature changed rapidly on the removal of androgen, with vessel regression and endothelial cell apoptosis occurring within 1 day of decreased VEGF expression. At 1 week after decreased VEGF expression, the tumor vessels were less tortuous and less leaky, suggesting a normalization of vessel structure and function [91,92].

The unexpected finding that inhibition of VEGF signaling could lead to a "normalization" of tumor vessels has provoked much follow-up study. In addition, the abnormalities of tumor vessels have been described in the past several years in increasingly elegant studies. These more recent studies have greatly extended and clarified a body of earlier work that described abnormal tumor vessels [93-97]. In most of the experimental models used to date, tumor vessels are generally disorganized and are irregular in diameter and spacing. The normal hierarchy of arterioles, capillaries, and veins is typically not observed in tumor tissue. Instead, the vessels form a multiplicity of interconnections. Despite a dense vascularity, tumors often contain regions of hypoxia [98-103]. Another important and consistent physiological abnormality of experimental and human solid tumors is their increased interstitial pressure [104-108].

On a cellular level, the endothelial cells of tumor vessels can form a defective monolayer. Instead of a continuous layer of tightly apposed endothelial cells, the endothelium of tumor vessels contains large pores or gaps between endothelial cells [109] that permit free leakage of macromolecules across the vessel wall and into the tumor tissue [110,111]. The luminal surface charge on the tumor endothelium also appears to be different from that found on normal endothelium, thus facilitating enhanced cell surface binding of circulating cationic liposomes [112,113] and the delivery of complexed chemotherapeutic agents [114,115]. On the abluminal surface, the endothelial cells extend numerous sprouts into the surrounding tumor tissue [109,116]. Instead of the normal tight association between pericytes and endothelial cells, the pericytes of tumor vessels are often poorly associated with the endothelial cells and extend away from the vessels [116,117]. Furthermore, the basement membrane of tumor vessels is abnormal, with holes and poor association with endothelial cells and pericytes [118].

Remarkably, inhibition of the VEGF pathway with specific VEGF blockers rapidly results in tumor vessels that are more regularly spaced and of more uniform size [91,119]. Most of the endothelial cell sprouts disappear, and the endothelium forms a better monolayer. In addition, tumor vessel leakiness is decreased, and even the pericyte–endothelial cell association is tightened. Treatment of tumor-bearing mice with VEGF-blocking antibody also resulted in a significant decrease in tumor interstitial pressure [120]. In a recent clinical study [121], the interstitial pressure and the size of colorectal tumors were rapidly reduced after treatment with bevacizumab.

Despite expectations to the contrary, inhibition of VEGF and the subsequent pruning and normalization of tumor vessels appear to increase the delivery of oxygen and chemotherapeutics to tumor tissue [122] and to improve the responses of some tumors to radiation [120], at least for some duration after VEGF blockade [119]. Clearly, blockade of VEGF can cause major changes in tumor physiology, and these changes have important implications in the actions of other tumor therapies such as chemotherapy and radiation therapy. Our understanding of the changes in tumor physiology with VEGF blockade is still very incomplete, and our understanding of how to exploit these changes with cotherapies is even more rudimentary.

3.4. Positive Clinical Results

A key development in the field of antiangiogenesis was the release at the 2003 meeting of the American Society of Clinical Oncology of positive clinical results with an anti-VEGF antibody (bevacizumab, Avastin). In a previous phase III trial for breast cancer, Avastin had failed to significantly increase patient survival times, although the time to progression was increased. However, in a phase III trial for advanced colorectal cancer that involved more than 900 patients, Avastin produced positive results [123] (see also the Genentech web site for a summary). Approximately 800 of the patients either received standard chemotherapy with IFL (5-fluorouracil, leucovorin, and CPT-11) plus placebo or received IFL plus Avastin. Avastin was given at 5 mg/kg every 2 weeks. Patients in the Avastin group had significantly improved overall survival times (20.3 months versus 15.6 months). In addition, patients who received Avastin had an increased time to disease progression (10.6 months versus 6.2 months) and an increased duration of response. The Avastin group tended to have increased hypertension as a side effect, although it was generally mild. The positive results of this trial led to US Food and Drug Administration approval of Avastin in February 2004 for first-line treatment of patients with metastatic colorectal cancer. Thus, the field of antiangiogenesis appears to be on its feet.

Avastin study is continuing with trials for several other cancers. Interestingly, the use of Avastin alone in a more recent trial did not seem to provide colorectal patients with a benefit better than that of standard chemotherapy, suggesting that antiangiogenic therapy is best used in combination with other forms of therapy. In addition to the studies with Avastin, several of the agents described above are also in clinical trials. For example, PTK787 is being tested in a large phase III trial for colorectal cancer. In addition to providing benefit for patients, these wide-ranging trials should provide a wealth of new data on the role of VEGF in various cancers and on the optimal dosing and timing for antiangiogenic therapy.

3.5. Other Approaches to Antiangiogenic Therapy

Blockade of the VEGF pathway is currently the most fruitful clinical approach for inhibiting tumor angiogenesis, but it is by no means the only approach. Indeed, numerous other antiangiogenic approaches have been or are being tested in preclinical models and in clinical trials. These agents have been divided based on their site of action into "direct" or "indirect" antiangiogenic agents [124]. Some of these potential antiangiogenic agents, such as those that target the PDGF signaling system, have a well-defined mechanism of antiangiogenesis, whereas other agents, such as endostatin, are still being characterized in terms of their signaling pathways and mechanisms of antiangiogenesis [125]. These other antiangiogenic agents may be useful in combination with agents that inhibit VEGF signaling. For example, one study found a more potent antitumor effect by blocking both the PDGF and VEGF signaling pathways than by blocking either pathway alone [126]. The conclusion from this study was that it may be necessary to target multiple vascular cell types in late-stage tumors to achieve maximal antiangiogenic effects, perhaps by targeting both established and newly formed tumor vessels.

Another approach to antiangiogenic therapy is to alter the dosing and timing of standard chemotherapy agents and thereby shift the site of action from the tumor cells to the endothelial cells [127,128]. This approach, often called metronomic therapy, has used reduced amounts of chemotherapeutic drugs given on a more frequent dosing schedule [129-131]. The result is less overall toxicity and a different mechanism of anticancer action. The protracted dosing appears to selectively target proliferating endothelial cells [132] and not tumor or stromal cells. Strikingly, this change in dosing appears to bypass previously induced chemoresistance. In one study, low doses of cyclophosphamide given daily to mice with transplanted human prostatic cancer cells produced substantial growth delays, but the tumors eventually grew progressively [133]. However, these regrowing tumors remained sensitive to low-dose cyclophosphamide when transplanted to new hosts, suggesting that the tumor cells had not acquired chemoresistance [133]. Clinical trials are currently under way to evaluate the utility of metronomic chemotherapy in cancer treatment [134].

4. Summary and Perspectives

With the variety of potent and specific agents now available to inhibit the VEGF signaling pathway, we are likely to learn much more about the role of VEGF in a variety of diseases, with the potential to extend antiangiogenic therapy beyond the field of cancer and into other diseases, including many inflammatory diseases. In addition, we are likely to learn whether other signaling pathways can compensate in any way for VEGF blockade. Such compensation may be in the form of inducing vessel growth in the absence of VEGF, making tumors more invasive and thereby more able to co-opt existing vessels, or adapting the tumor cells to grow in a more hypoxic environment. Because of the potent effects of VEGF inhibitors on tumor physiology, we are also likely to gain great insight into how to manipulate the vascular physiology of tumors and normal tissues for therapeutic advantage. For example, blocking VEGF can profoundly alter vascular perfusion in tumors, with corresponding alterations in tumor oxygenation. These changes in the tumor environment offer new windows of opportunity for applying existing or novel chemotherapeutic agents. Many years of creative research will be required to explore all of the therapeutic possibilities now open in this area.

Although VEGF has emerged as a central angiogenic pathway, several other pathways are also now recognized as essential for normal angiogenesis, and the list of such pathways is likely to grow. The recognition and characterization of other angiogenic signaling pathways provides new therapeutic targets. Some of the pathways appear to lie upstream or downstream of the VEGF pathway, whereas others appear to be parallel and separate. Much work is needed to integrate these various pathways into a rigorous developmental program. At the next level of complexity, we are just beginning to study variations on the basic angiogenic program, such as tissue-specific and vascular segment–specific angiogenic factors. Ongoing angiogenesis research will continue to rely heavily on studies with gene-targeted mice, coupled with careful analyses of gene expression. Thorough expression studies are increasingly important as we learn that some of the factors can be regulated on multiple levels. Future research will place increased reliance on conditional gene knock-outs to avoid the early embryonic lethality and the generic vascular phenotype described above. Transgenic mice and other in vivo overexpression systems (such as transduced tumor cells or myoblasts) are another very valuable research approach.

Finally, we have not mentioned the large amount of basic and clinical research that has been done in the area of therapeutic proangiogenesis. Much of this work has involved VEGF as an angiogenic factor, but other angiogenic factors have also been tested. As we learn more about the precise spatial and temporal orchestration of the different angiogenic pathways, we may be better able to provide a sequential cocktail of factors and thereby mimic the tissue's endogenous proangiogenic program. Research on angiogenesis is likely to only accelerate in the coming years.

References

1. Suri C, Jones PF, Patan S, et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell*. 1996;87:1171-1180.
2. Dumont DJ, Gradwohl G, Fong GH, et al. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev*. 1994;8:1897-1909.
3. Sato TN, Tozawa Y, Deutsch U, et al. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature*. 1995;376:70-74.
4. Tang Y, McKinnon ML, Leong LM, Rusholme SA, Wang S, Akhurst RJ. Genetic modifiers interact with maternal determinants in vascular development of *Tgfb1*$^{-/-}$ mice. *Hum Mol Genet*. 2003;12:1579-1589.
5. Larsson J, Goumans MJ, Sjostrand LJ, et al. Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *EMBO J*. 2001;20:1663-1673.
6. Oh SP, Seki T, Goss KA, et al. Activin receptor-like kinase 1 mod-

ulates transforming growth factor-β 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A*. 2000;97:2626-2631.
7. Urness LD, Sorensen LK, Li DY. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat Genet*. 2000;26: 328-331.
8. Li DY, Sorensen LK, Brooke BS, et al. Defective angiogenesis in mice lacking endoglin. *Science*. 1999;284:1534-1537.
9. Arthur HM, Ure J, Smith AJ, et al. Endoglin, an ancillary TGFβ receptor, is required for extraembryonic angiogenesis and plays a key role in heart development. *Dev Biol*. 2000;217:42-53.
10. Wang HU, Chen ZF, Anderson DJ. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell*. 1998;93:741-753.
11. Gerety SS, Wang HU, Chen ZF, Anderson DJ. Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol Cell*. 1999;4: 403-414.
12. Serini G, Valdembri D, Zanivan S, et al. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature*. 2003;424:391-397.
13. Kawasaki T, Kitsukawa T, Bekku Y, et al. A requirement for neuropilin-1 in embryonic vessel formation. *Development*. 1999;126: 4895-4902.
14. Kitsukawa T, Shimizu M, Sanbo M, et al. Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. *Neuron*. 1997;19:995-1005.
15. Gu C, Rodriguez ER, Reimert DV, et al. Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev Cell*. 2003;5:45-57.
16. Gessler M, Knobeloch KP, Helisch A, et al. Mouse gridlock: no aortic coarctation or deficiency, but fatal cardiac defects in Hey2 –/– mice. *Curr Biol*. 2002;12:1601-1604.
17. Donovan J, Kordylewska A, Jan YN, Utset MF. Tetralogy of Fallot and other congenital heart defects in Hey2 mutant mice. *Curr Biol*. 2002;12:1605-1610.
18. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science*. 1999; 284:770-776.
19. Parks AL, Klueg KM, Stout JR, Muskavitch MA. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development*. 2000;127:1373-1385.
20. Kramer H. RIPping Notch apart: a new role for endocytosis in signal transduction? *Sci STKE*. 2000;2000:PE1.
21. Schweisguth F. Notch signaling activity. *Curr Biol*. 2004;14:R129-R138.
22. Okajima T, Irvine KD. Regulation of Notch signaling by O-linked fucose. *Cell*. 2002;111:893-904.
23. Shi S, Stanley P. Protein *O*-fucosyltransferase 1 is an essential component of Notch signaling pathways. *Proc Natl Acad Sci U S A*. 2003;100:5234-5239.
24. Haltiwanger RS. Regulation of signal transduction pathways in development by glycosylation. *Curr Opin Struct Biol*. 2002;12: 593-598.
25. Bruckner K, Perez L, Clausen H, Cohen S. Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature*. 2000;406:411-415.
26. Moloney DJ, Panin VM, Johnston SH, et al. Fringe is a glycosyltransferase that modifies Notch. *Nature*. 2000;406:369-375.
27. Lai EC. Notch signaling: control of cell communication and cell fate. *Development*. 2004;131:965-973.
28. Iso T, Hamamori Y, Kedes L. Notch signaling in vascular development. *Arterioscler Thromb Vasc Biol*. 2003;23:543-553.
29. Shawber CJ, Kitajewski J. Notch function in the vasculature: insights from zebrafish, mouse and man. *Bioessays*. 2004;26: 225-234.
30. Mailhos C, Modlich U, Lewis J, Harris A, Bicknell R, Ish-Horowicz D. Delta4, an endothelial specific Notch ligand expressed at sites of physiological and tumor angiogenesis. *Differentiation*. 2001;69: 135-144.
31. Xue Y, Gao X, Lindsell CE, et al. Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet*. 1999;8:723-730.
32. McCright B, Lozier J, Gridley T. A mouse model of Alagille syndrome: *Notch2* as a genetic modifier of *Jag1* haploinsufficiency. *Development*. 2002;129:1075-1082.
33. Swiatek PJ, Lindsell CE, del Amo FF, Weinmaster G, Gridley T. Notch1 is essential for postimplantation development in mice. *Genes Dev*. 1994;8:707-719.
34. Krebs LT, Xue Y, Norton CR, et al. Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev*. 2000;14:1343-1352.
35. Joutel A, Corpechot C, Ducros A, et al. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *Nature*. 1996;383:707-710.
36. Liu ZJ, Shirakawa T, Li Y, et al. Regulation of *Notch1* and *Dll4* by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. *Mol Cell Biol*. 2003;23:14-25.
37. Lawson ND, Scheer N, Pham VN, et al. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development*. 2001;128:3675-3683.
38. Zhong TP, Childs S, Leu JP, Fishman MC. Gridlock signalling pathway fashions the first embryonic artery. *Nature*. 2001;414:216-220.
39. Thurston G, Yancopoulos GD. Gridlock in the blood. *Nature*. 2001; 414:163-164.
40. Weinstein BM, Lawson ND. Arteries, veins, Notch, and VEGF. *Cold Spring Harb Symp Quant Biol*. 2002;67:155-162.
41. Lawson ND, Vogel AM, Weinstein BM. *sonic hedgehog* and *vascular endothelial growth factor* act upstream of the Notch pathway during arterial endothelial differentiation. *Dev Cell*. 2002;3: 127-136.
42. Wang B, Xiao Y, Ding BB, et al. Induction of tumor angiogenesis by Slit-Robo signaling and inhibition of cancer growth by blocking Robo activity. *Cancer Cell*. 2003;4:19-29.
43. Huminiecki L, Gorn M, Suchting S, Poulsom R, Bicknell R. Magic roundabout is a new member of the roundabout receptor family that is endothelial specific and expressed at sites of active angiogenesis. *Genomics*. 2002;79:547-552.
44. Park KW, Morrison CM, Sorensen LK, et al. Robo4 is a vascular-specific receptor that inhibits endothelial migration. *Dev Biol*. 2003;261:251-267.
45. Liu ZJ, Herlyn M. Slit-Robo: neuronal guides signal in tumor angiogenesis. *Cancer Cell*. 2003;4:1-2.
46. Dallol A, Krex D, Hesson L, Eng C, Maher ER, Latif F. Frequent epigenetic inactivation of the *SLIT2* gene in gliomas. *Oncogene*. 2003;22:4611-4616.
47. Plump AS, Erskine L, Sabatier C, et al. Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. *Neuron*. 2002;33:219-232.
48. Yuan W, Rao Y, Babiuk RP, Greer JJ, Wu JY, Ornitz DM. A genetic model for a central (septum transversum) congenital diaphragmatic hernia in mice lacking *Slit3*. *Proc Natl Acad Sci U S A*. 2003; 100:5217-5222.
49. Liu J, Zhang L, Wang D, et al. Congenital diaphragmatic hernia, kidney agenesis and cardiac defects associated with Slit3-deficiency in mice. *Mech Dev*. 2003;120:1059-1070.
50. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971;285:1182-1186.
51. Tannock IF. The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. *Br J Cancer*. 1968;22:258-273.
52. Tannock IF, Hayashi S. The proliferation of capillary endothelial cells. *Cancer Res*. 1972;32:77-82.
53. Denekamp J. Endothelial cell proliferation as a novel approach to targeting tumour therapy. *Br J Cancer*. 1982;45:136-139.
54. Denekamp J, Hobson B. Endothelial-cell proliferation in experimental tumours. *Br J Cancer*. 1982;46:711-720.
55. Ferrara N, Chen H, Davis-Smyth T, et al. Vascular endothelial

growth factor is essential for corpus luteum angiogenesis. *Nat Med.* 1998;4:336-340.
56. Fraser HM, Dickson SE, Lunn SF, et al. Suppression of luteal angiogenesis in the primate after neutralization of vascular endothelial growth factor. *Endocrinology.* 2000;141:995-1000.
57. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med.* 1999; 5:623-628.
58. Adamis AP, Shima DT, Tolentino MJ, et al. Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. *Arch Ophthalmol.* 1996;114:66-71.
59. Aiello LP, Pierce EA, Foley ED, et al. Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. *Proc Natl Acad Sci U S A.* 1995;92:10457-10461.
60. Ferrara N, Houck KA, Jakeman LB, Winer J, Leung DW. The vascular endothelial growth factor family of polypeptides. *J Cell Biochem.* 1991;47:211-218.
61. Connolly DT, Heuvelman DM, Nelson R, et al. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest.* 1989;84:1470-1478.
62. Ferrara N. Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. *Semin Oncol.* 2002;29(suppl 16):10-14.
63. Bates DO, Cui TG, Doughty JM, et al. VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Res.* 2002;62:4123-4131.
64. Ferrara N, Carver-Moore K, Chen H, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature.* 1996;380:439-442.
65. Carmeliet P, Ferreira V, Breier G, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature.* 1996;380:435-439.
66. Gerber HP, Hillan KJ, Ryan AM, et al. VEGF is required for growth and survival in neonatal mice. *Development.* 1999;126:1149-1159.
67. Quinn TP, Peters KG, de Vries C, Ferrara N, Williams LT. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci U S A.* 1993;90:7533-7537.
68. Shen H, Clauss M, Ryan J, et al. Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes. *Blood.* 1993;81:2767-2773.
69. Guo D, Jia Q, Song HY, Warren RS, Donner DB. Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains: association with endothelial cell proliferation. *J Biol Chem.* 1995;270: 6729-6733.
70. Millauer B, Wizigmann-Voos S, Schnurch H, et al. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell.* 1993;72:835-846.
71. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science.* 1992;255:989-991.
72. Gerber HP, McMurtrey A, Kowalski J, et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3(-kinase/Akt signal transduction pathway: requirement for Flk-1/KDR activation. *J Biol Chem.* 1998;273: 30336-30343.
73. Kim KJ, Li B, Winer J, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature.* 1993;362:841-844.
74. Mesiano S, Ferrara N, Jaffe RB. Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. *Am J Pathol.* 1998;153:1249-1256.
75. Mordenti J, Thomsen K, Licko V, Chen H, Meng YG, Ferrara N. Efficacy and concentration-response of murine anti-VEGF monoclonal antibody in tumor-bearing mice and extrapolation to humans. *Toxicol Pathol.* 1999;27:14-21.
76. Ryan AM, Eppler DB, Hagler KE, et al. Preclinical safety evaluation of rhuMAbVEGF, an antiangiogenic humanized monoclonal antibody. *Toxicol Pathol.* 1999;27:78-86.
77. Holash J, Davis S, Papadopoulos N, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci U S A.* 2002;99:11393-11398.
78. Huang J, Frischer JS, Serur A, et al. Regression of established tumors and metastases by potent vascular endothelial growth factor blockade. *Proc Natl Acad Sci U S A.* 2003;100:7785-7790.
79. Byrne AT, Ross L, Holash J, et al. Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. *Clin Cancer Res.* 2003;9:5721-5728.
80. Witte L, Hicklin DJ, Zhu Z, et al. Monoclonal antibodies targeting the VEGF receptor-2 (Flk1/KDR) as an anti-angiogenic therapeutic strategy. *Cancer Metastasis Rev.* 1998;17:155-161.
81. Zhu Z, Witte L. Inhibition of tumor growth and metastasis by targeting tumor-associated angiogenesis with antagonists to the receptors of vascular endothelial growth factor. *Invest New Drugs.* 1999;17:195-212.
82. Prewett M, Huber J, Li Y, et al. Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. *Cancer Res.* 1999;59:5209-5218.
83. Lu D, Shen J, Vil MD, et al. Tailoring in vitro selection for a picomolar affinity human antibody directed against vascular endothelial growth factor receptor 2 for enhanced neutralizing activity. *J Biol Chem.* 2003;278:43496-43507.
84. Eyetech Study Group. Anti-vascular endothelial growth factor therapy for subfoveal choroidal neovascularization secondary to age-related macular degeneration: phase II study results. *Ophthalmology.* 2003;110:979-986.
85. Eyetech Study Group. Preclinical and phase 1A clinical evaluation of an anti-VEGF pegylated aptamer (EYE001) for the treatment of exudative age-related macular degeneration. *Retina.* 2002;22: 143-152.
86. Ishida S, Usui T, Yamashiro K, et al. VEGF164-mediated inflammation is required for pathological, but not physiological, ischemia-induced retinal neovascularization. *J Exp Med.* 2003;198: 483-489.
87. Wood JM, Bold G, Buchdunger E, et al. PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res.* 2000;60:2178-2189.
88. Schoenberger J, Grimm D, Kossmehl P, Infanger M, Kurth E, Eilles C. Effects of PTK787/ZK222584, a tyrosine kinase inhibitor, on the growth of a poorly differentiated thyroid carcinoma: an animal study. *Endocrinology.* 2004;145:1031-1038.
89. Solorzano CC, Baker CH, Bruns CJ, et al. Inhibition of growth and metastasis of human pancreatic cancer growing in nude mice by PTK 787/ZK222584, an inhibitor of the vascular endothelial growth factor receptor tyrosine kinases. *Cancer Biother Radiopharm.* 2001;16:359-370.
90. Drevs J, Hofmann I, Hugenschmidt H, et al. Effects of PTK787/ZK 222584, a specific inhibitor of vascular endothelial growth factor receptor tyrosine kinases, on primary tumor, metastasis, vessel density, and blood flow in a murine renal cell carcinoma model. *Cancer Res.* 2000;60:4819-4824.
91. Jain RK, Safabakhsh N, Sckell A, et al. Endothelial cell death, angiogenesis, and microvascular function after castration in an androgen-dependent tumor: role of vascular endothelial growth factor. *Proc Natl Acad Sci U S A.* 1998;95:10820-10825.
92. Hansen-Algenstaedt N, Stoll BR, Padera TP, et al. Tumor oxygenation in hormone-dependent tumors during vascular endothelial growth factor receptor-2 blockade, hormone ablation, and chemotherapy. *Cancer Res.* 2000;60:4556-4560.

93. Warren BA. In vivo and electron microscopic study of vessels in two transplantable tumours in the hamster. *Bibl Anat.* 1967;9:412-417.
94. Warren BA. The ultrastructure of the microcirculation at the advancing edge of Walker 256 carcinoma. *Microvasc Res.* 1970;2:443-453.
95. Papadimitrou JM, Woods AE. Structural and functional characteristics of the microcirculation in neoplasms. *J Pathol.* 1975;116:65-72.
96. Steinberg F, Konerding MA, Streffer C. The vascular architecture of human xenotransplanted tumors: histological, morphometrical, and ultrastructural studies. *J Cancer Res Clin Oncol.* 1990;116:517-524.
97. Konerding MA, Steinberg F. Scanning and transmission electron microscopic studies on the vascular system of xenotransplanted human tumors on nude mice. *Prog Clin Biol Res.* 1989;295:475-480.
98. Vaupel P. Hypoxia in neoplastic tissue. *Microvasc Res.* 1977;13: 399-408.
99. Brown JM, Giaccia AJ. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res.* 1998; 58:1408-1416.
100. Chaplin DJ, Hill SA, Bell KM, Tozer GM. Modification of tumor blood flow: current status and future directions. *Semin Radiat Oncol.* 1998;8:151-163.
101. Feldmann HJ, Molls M, Vaupel P. Blood flow and oxygenation status of human tumors: clinical investigations. *Strahlenther Onkol.* 1999;175:1-9.
102. Semenza GL. Angiogenesis in ischemic and neoplastic disorders. *Annu Rev Med.* 2003;54:17-28.
103. Vaupel P, Briest S, Hockel M. Hypoxia in breast cancer: pathogenesis, characterization and biological/therapeutic implications. *Wien Med Wochenschr.* 2002;152:334-342.
104. Wiig H, Tveit E, Hultborn R, Reed RK, Weiss L. Interstitial fluid pressure in DMBA-induced rat mammary tumours. *Scand J Clin Lab Invest.* 1982;42:159-164.
105. Hori K, Suzuki M, Abe I, Saito S. Increased tumor tissue pressure in association with the growth of rat tumors. *Jpn J Cancer Res.* 1986;77:65-73.
106. Boucher Y, Kirkwood JM, Opacic D, Desantis M, Jain RK. Interstitial hypertension in superficial metastatic melanomas in humans. *Cancer Res.* 1991;51:6691-6694.
107. Less JR, Posner MC, Boucher Y, Borochovitz D, Wolmark N, Jain RK. Interstitial hypertension in human breast and colorectal tumors. *Cancer Res.* 1992;52:6371-6374.
108. Gutmann R, Leunig M, Feyh J, et al. Interstitial hypertension in head and neck tumors in patients: correlation with tumor size. *Cancer Res.* 1992;52:1993-1995.
109. Hashizume H, Baluk P, Morikawa S, et al. Openings between defective endothelial cells explain tumor vessel leakiness. *Am J Pathol.* 2000;156:1363-1380.
110. Yuan F, Dellian M, Fukumura D, et al. Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. *Cancer Res.* 1995;55:3752-3756.
111. Jain RK. Transport of molecules across tumor vasculature. *Cancer Metastasis Rev.* 1987;6:559-593.
112. Thurston G, McLean JW, Rizen M, et al. Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice. *J Clin Invest.* 1998;101:1401-1413.
113. Campbell RB, Fukumura D, Brown EB, et al. Cationic charge determines the distribution of liposomes between the vascular and extravascular compartments of tumors. *Cancer Res.* 2002;62: 6831-6836.
114. Kunstfeld R, Wickenhauser G, Michaelis U, et al. Paclitaxel encapsulated in cationic liposomes diminishes tumor angiogenesis and melanoma growth in a "humanized" SCID mouse model. *J Invest Dermatol.* 2003;120:476-482.
115. Krasnici S, Werner A, Eichhorn ME, et al. Effect of the surface charge of liposomes on their uptake by angiogenic tumor vessels. *Int J Cancer.* 2003;105:561-567.
116. Abramsson A, Berlin O, Papayan H, Paulin D, Shani M, Betsholtz C. Analysis of mural cell recruitment to tumor vessels. *Circulation.* 2002;105:112-117.
117. Morikawa S, Baluk P, Kaidoh T, Haskell A, Jain RK, McDonald DM. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am J Pathol.* 2002;160:985-1000.
118. Baluk P, Morikawa S, Haskell A, Mancuso M, McDonald DM. Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. *Am J Pathol.* 2003;163:1801-1815.
119. Jain RK. Molecular regulation of vessel maturation. *Nat Med.* 2003; 9:685-693.
120. Lee CG, Heijn M, di Tomaso E, et al. Anti-vascular endothelial growth factor treatment augments tumor radiation response under normoxic or hypoxic conditions. *Cancer Res.* 2000;60:5565-5570.
121. Willett CG, Boucher Y, di Tomaso E, et al. Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med.* 2004;10:145-147.
122. Jain RK, Munn LL, Fukumura D. Dissecting tumour pathophysiology using intravital microscopy. *Nat Rev Cancer.* 2002;2:266-276.
123. Fernando NH, Hurwitz HI. Inhibition of vascular endothelial growth factor in the treatment of colorectal cancer. *Semin Oncol.* 2003;30(suppl 6):39-50.
124. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer.* 2002;2:727-739.
125. Abdollahi A, Hahnfeldt P, Maercker C, et al. Endostatin's antiangiogenic signaling network. *Mol Cell.* 2004;13:649-663.
126. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest.* 2003;111:1287-1295.
127. Browder T, Butterfield CE, Kraling BM, et al. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res.* 2000;60:1878-1886.
128. Klement G, Baruchel S, Rak J, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest.* 2000;105: R15-R24.
129. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. *J Clin Invest.* 2000;105:1045-1047.
130. Kerbel RS, Viloria-Petit A, Klement G, Rak J. 'Accidental' antiangiogenic drugs: anti-oncogene directed signal transduction inhibitors and conventional chemotherapeutic agents as examples. *Eur J Cancer.* 2000;36:1248-1257.
131. Kerbel RS, Klement G, Pritchard KI, Kamen B. Continuous low-dose anti-angiogenic/ metronomic chemotherapy: from the research laboratory into the oncology clinic. *Ann Oncol.* 2002;13:12-15.
132. Bocci G, Nicolaou KC, Kerbel RS. Protracted low-dose effects on human endothelial cell proliferation and survival in vitro reveal a selective antiangiogenic window for various chemotherapeutic drugs. *Cancer Res.* 2002;62:6938-6943.
133. Man S, Bocci G, Francia G, et al. Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. *Cancer Res.* 2002;62:2731-2735.
134. Kaur H, Budd GT. Metronomic therapy for breast cancer. *Curr Oncol Rep.* 2004;6:49-52.
135. Villa N, Walker L, Lindsell CE, Gasson J, Iruela-Arispe ML, Weinmaster G. Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. *Mech Dev.* 2001;108:161-164.
136. Hamada Y, Kadokawa Y, Okabe M, Ikawa M, Coleman JR, Tsujimoto Y. Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. *Development.* 1999;126: 3415-3424.
137. McCright B, Gao X, Shen L, et al. Defects in development of the kidney, heart and eye vasculature in mice homozygous for a hypomorphic Notch2 mutation. *Development.* 2001;128:491-502.
138. Jiang R, Lan Y, Chapman HD, et al. Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev.* 1998; 12:1046-1057.
139. Hrabe de Angelis M, McIntyre J 2nd, Gossler A. Maintenance of somite borders in mice requires the Delta homologue DII1. *Nature.* 1997;386:717-721.

140. Przemeck GK, Heinzmann U, Beckers J, Hrabe de Angelis M. Node and midline defects are associated with left-right development in *Delta1* mutant embryos. *Development*. 2003;130:3-13.

141. De Bellard ME, Ching W, Gossler A, Bronner-Fraser M. Disruption of segmental neural crest migration and ephrin expression in Delta-1 null mice. *Dev Biol*. 2002;249:121-130.

142. Kusumi K, Dunwoodie SL, Krumlauf R. Dynamic expression patterns of the pudgy/spondylocostal dysostosis gene *Dll3* in the developing nervous system. *Mech Dev*. 2001;100:141-144.

143. Bulman MP, Kusumi K, Frayling TM, et al. Mutations in the human *Delta* homologue, *DLL3*, cause axial skeletal defects in spondylocostal dysostosis. *Nat Genet*. 2000;24:438-441.

144. Xian J, Clark KJ, Fordham R, Pannell R, Rabbitts TH, Rabbitts PH. Inadequate lung development and bronchial hyperplasia in mice with a targeted deletion in the *Dutt1/Robo1* gene. *Proc Natl Acad Sci U S A*. 2001;98:15062-15066.

145. Yuan L, Moyon D, Pardanaud L, et al. Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development*. 2002;129:4797-4806.

146. Chen H, Bagri A, Zupicich JA, et al. Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. *Neuron*. 2000;25:43-56.

147. Giger RJ, Cloutier JF, Sahay A, et al. Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. *Neuron*. 2000;25:29-41.

148. Takashima S, Kitakaze M, Asakura M, et al. Targeting of both mouse neuropilin-1 and neuropilin-2 genes severely impairs developmental yolk sac and embryonic angiogenesis. *Proc Natl Acad Sci U S A*. 2002;99:3657-3662.

149. Krebs LT, Xue Y, Norton CR, et al. Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. *Genesis*. 2003;37(3):139-143.

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BIOLOGY CONTRIBUTION

VEGF TRAP IN COMBINATION WITH RADIOTHERAPY IMPROVES TUMOR CONTROL IN U87 GLIOBLASTOMA

PHYLLIS R. WACHSBERGER, PH.D.,* RANDY BURD, PH.D.,§ CHRIS CARDI, M.S.,†
MATHEW THAKUR, PH.D.,† CONSTANTINE DASKALAKIS, SC.D.,‡ JOCELYN HOLASH, PH.D.,¶
GEORGE D. YANCOPOULOS, PH.D.,¶ AND ADAM P. DICKER, M.D., PH.D.,*

Departments of *Radiation Oncology, †Radiology, ‡Pharmacology and Experimental Therapeutics, Thomas Jefferson University, Philadelphia, PA; §Department of Nutritional Sciences, University of Arizona, Tucson, AZ; and ¶Novartis, Emeryville, CA

Purpose: To determine the effect of vascular endothelial growth factor VEGF Trap (Regeneron Pharmaceuticals, Tarrytown, NY), a humanized soluble vascular endothelial growth factor (VEGF) receptor protein, and radiation (RT) on tumor growth in U87 glioblastoma xenografts in nude mice.

Methods and Materials: U87 cell suspensions were implanted subcutaneously into hind limbs of nude mice. VEGF Trap (2.5–25 mg/kg) was administered every 3 days for 3 weeks alone or in combination with a single dose of 10 Gy or fractionated RT (3 × 5 Gy). In addition, three scheduling protocols for VEGF Trap plus fractionated RT were examined.

Results: Improved tumor control was seen when RT (either single dose or fractionated doses) was combined with the lowest dose of VEGF Trap (2.5 mg/kg). Scheduling did not significantly affect the efficacy of combined therapy. Although high-dose VEGF Trap (10 mg/kg or 25 mg/kg) significantly reduced tumor growth over that of RT alone, there was no additional benefit to combining high-dose VEGF Trap with RT.

Conclusions: Vascular endothelial growth factor Trap plus radiation is clearly better than radiation alone in a U87 subcutaneous xenograft model. Although high doses of VEGF Trap alone are highly efficacious, it is unclear whether such high doses can be used clinically without incurring normal tissue toxicities. Thus, information on lower doses of VEGF Trap and ionizing radiation is of clinical relevance. © 2007 Elsevier Inc.

Vascular endothelial growth factor Trap, Radiotherapy, Anti-angiogenic, U87 glioblastoma.

INTRODUCTION

Radiation (RT) therapy is an important treatment modality for many cancers; however, its therapeutic success is impeded by dose-limiting normal tissue toxicities and the development of radioresistance. Recent studies emphasize the importance of the tumor microvascular response in addition to the tumor cell response in determining tumor radioresistance (1, 2). Ionizing radiation can directly induce endothelial cell apoptosis (1, 3), which can inhibit tumor growth and lead to radiosensitization. However, in opposition to endothelial cell damage, radiation also induces signal transduction cascades, which contribute to radiation resistance through upregulation of proliferative, survival, and angiogenic pathways (4). In particular, radiation induces vascular cytokines, such as vascular endothelial growth factor (VEGF) (5, 6), one of the most potent endothelial cell survival factors (7), which functions as a powerful antiapoptotic factor for endothelial cells in new blood vessels (8, 9). Radiation-induced VEGF results in tumor radioresistance through vascular radioprotection (2, 10).

Inhibition of VEGF activity or disabling the function of VEGF receptors is therefore a potential strategy for improving radiation outcome. The VEGF blockade alone has been shown to inhibit both tumor growth and metastasis in a variety of animal tumor models (11). Currently, three approaches are in clinical development to target the VEGF/VEGFR-signaling pathway: *(1)* monoclonal antibodies directed against VEGF or its receptors (12–15), *(2)* small molecule inhibitors of the VEGFR-2 tyrosine kinase enzyme (16–19), and *(3)* soluble decoy receptors created from the VEGFR1 receptor which selectively inhibit VEGF (20, 21). The relative benefits of these strategies have yet to be determined clinically.

Tumor cures are rare when VEGF blockers are used as the sole method of treatment; in general, antiangiogenics

Reprint requests to: Phyllis R. Wachsberger, Ph.D., Department of Radiation Oncology, Bodine Center for Cancer Treatment, Thomas Jefferson University Hospital, 111 South 11th Street, Philadelphia, PA 19107; Fax: (215) 955-5825, E-mail: Phyllis.Wachsberger@mail.tju.edu

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appear to work best in combination with cytotoxic therapies (22). A number of preclinical studies suggest that radiotherapy in combination with VEGF targeting agents enhances the radiotherapeutic ratio (see reviews; 23, 24). The best way to incorporate VEGF inhibition strategies into current radiotherapy regimens remains unknown.

Because of the role that angiogenesis plays in the radiation response, the objective of this study was to determine whether VEGF Trap (Regeneron Pharmaceuticals, Tarrytown, NY), a potent anti-VEGF angiogenesis inhibitor that traps circulating VEGF in the bloodstream and in the extracellular space, would enhance radiation therapy in the human U87 glioblastoma (GBM) tumor model. Because GBM tumors are among the most radioresistant and vascular of neoplasms and are known to secrete high levels of VEGF (25), U87 GBM was deemed an appropriate model to assess the effects of VEGF Trap and radiation. It was hypothesized that inhibition of VEGF signaling by VEGF Trap would improve the human U87 glioblastoma model response to radiotherapy.

The administration of decoy soluble VEGF receptors has been found to be a very effective way to block the VEGF signaling pathway (26–29). VEGF Trap is a unique human fusion protein comprising portions of human VEGF receptor 1 (VEGFR1) and human VEGF receptor 2 (VEGFR2) extracellular domains fused to the constant region (Fc) of human IgG1 (21). VEGF Trap has greater affinity for the VEGF ligand than anti-VEGF monoclonal antibodies (mab) do (dissociation constant <1 pMol/L for VEGF Trap vs. 0.1–10 nMol/L for mab) (30). VEGF Trap has been shown to inhibit neoangiogenesis and tumor growth in tumor xenografts and metastases, as well as reduce the formation of malignant ascites (14, 21, 31).

METHODS AND MATERIALS

Analysis of VEGF levels in U87 tumor cells in culture

U87 glioblastoma cells (American Type Culture Collection) were maintained in alpha MEM (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). U87 cells were irradiated at doses between 2 and 20 Gy in the presence or absence of 40 nM VEGF Trap and incubated for 48 h. Using a commercially available human VEGF immunoassay kit (R&D Systems, Minneapolis, MN), VEGF was assayed from culture supernatants.

Animal and tumor model

U87 cell suspensions (5×10^5 cells in 100 μL phosphate buffered saline) were implanted subcutaneouly (SC) into the right hind limbs of athymic NCR NUM mice (Taconic Farms, Hudson, NY). A SC xenograft model was chosen to facilitate radiation dosing and ease of tumor measurements in the more than 200 mice measured in this study. Mice were not pretreated before tumor implantation. U87 tumors were allowed to grow for approximately 14 to 18 days until reaching an approximate diameter of 4 to 5 mm before treatment.

Drug and irradiation treatment

In an initial pilot study, VEGF Trap was administered at two doses, a high dose (25 mg/kg) or low dose (2.5 mg/kg), every 3 days, up to 3 weeks, with or without a single dose of radiation (10 Gy) given on Day 0. VEGF Trap was administered every 3 days because it has a half-life of 72 h in mouse serum (drug pharmacokinetics communicated by Regeneron). Drug was administered 2 h before radiation. When fractionated radiotherapy was used, VEGF Trap was combined at 2.5 mg/kg (low dose) or 10 mg/kg (intermediate dose) with fractionated radiotherapy (three fractions of 5 Gy each) on Days 0, 1, and 2. Scheduling of VEGF Trap was either 1 week before fractionated radiation and continuing for a period of 3 weeks, concurrent with radiation and continuing for a period of 3 weeks, or 3 days postradiation treatment and continuing for a period of 3 weeks. Thus, the total number of drug doses was constant for each schedule (see Fig. 1 for dose and irradiation scheduling protocol).

Irradiation was performed on anesthetized mice using an X-ray machine (Gulmay Medical, Bethel, CT) operating at 250 kV, 10 mA, with a 2-mm aluminum filtration. The effective photon energy was $\approx$90 keV. Mice were anesthetized with a combination of ketamine and acepromazine at a concentration of 75 mg/kg and 0.35 mg/kg, respectively. Each mouse was confined in a lead casing with its tumor-bearing leg extended through an opening on the side to allow the tumor to be irradiated locally. Radiation was administered as three daily fractions of 5 Gy each as described earlier.

Tumor size was measured 4 to 5 times per week after treatment by direct measurement with calipers and calculated by the formula [(smallest diameter (2) $\times$ widest diameter) / 2]. Tumors were not allowed to grow beyond 2,000 mm^3 in accordance with Institutional Animal Care and Use Committee regulations.

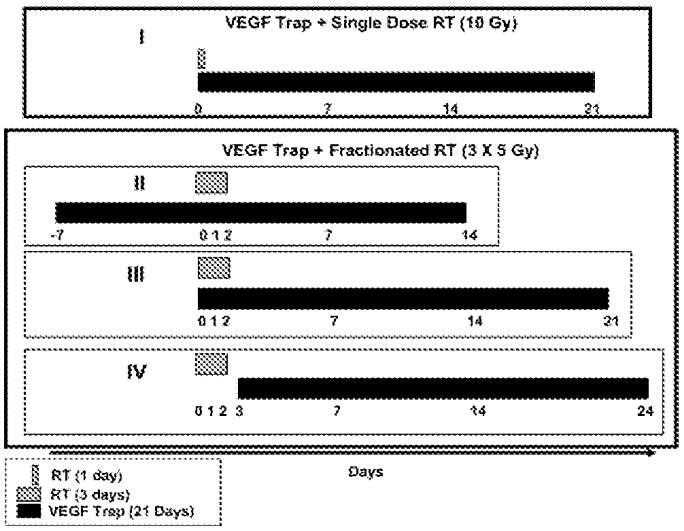

Fig. 1. Scheduling protocols for vascular endothelial growth factor (VEGF) Trap administration in combination with radiation (RT). VEGF Trap was given at 2.5, 10, or 25 mg/kg every 3 days in four schedules: (I) VEGF Trap given on Day 0 concurrent with a single dose of RT (10 Gy) and continued up to 3 weeks; (II) VEGF Trap given on day -7 before RT (3×5 Gy) and continued for 3 weeks; (III) VEGF Trap given on Day 0 concurrent with RT (3×5 Gy) and continued up to 3 weeks; (IV) VEGF Trap given on Day 3 post RT (3×5 Gy) and continued up to 3 weeks. All three protocols received the same number of drug doses. Day 0 was always the start of radiation.

Positron emission tomography imaging

The MOSAIC PET scanner (Philips Medical Systems, Brisbane, CA) was used for PET studies. Before imaging, mice were anesthetized with ketamine (75 mg/kg) and acepromazine (0.35 mg/kg) via a SC injection. Once anesthetized 0.3 to 0.5 μCi of 18-fluorodeoxyglucose (FDG) was administered intravenously. Sixty to seventy min were allowed for uptake of the tracer. Mice were placed in a 50-mL specimen tube to facilitate multimodality stereotactic positioning. The PET data were acquired in a single position for 15 min. Volumes of interest (VOIs) were defined by drawing multislice regions of interest (ROIs) on the PET images using 50% of the full-width-at-half-maximum (FWHM) of the tumor to determine the tumor boundary. In the case of tumors with a core lacking FDG uptake, the tumor and core boundaries were defined by 50% FWHM of each wall adjacent to the core. Mice were divided into three groups (n = 3–6 animals per group): untreated; low-dose VEGF Trap–treated (2.5 mg/kg), and high-dose VEGF Trap–treated (10 mg/kg).

Immunohistochemistry

Platelet–endothelial cell adhesion molecule 1 (PECAM-1) immunostaining for microvessel density (MVD): control, radiation-treated, VEGF Trap–treated tumors, and VEGF Trap plus radiation–treated tumors were immunostained with a rat antimouse PECAM-1 mAb (BD Biosciences, Boston, MA) and a rabbit antirat biotinylated secondary antibody (Vector Labs, Burlingame, CA). Enhanced horseradish peroxidase–conjugated streptavidin and a substrate chromogen, AEC (3-amino-9-ethyl carbazole), were used to visualize the signal. (HISTOSTAIN-*PLUS* kit, Invitrogen, Carlsbad, CA); slides were examined with a Nikon Eclipse E600 microscope to calculate MVD, the area occupied by the PECAM-1-positive microvessels, and total tissue area per section were quantified using National Institute of Health Image J software. Microvessel density was expressed as percent area of blood vessels stained per tissue section. Areas of necrosis were excluded from calculations. Four or five high-power fields were identified on each section with three to four sections per tumor and two tumors per endpoint.

Statistical analysis of tumor growth

Tumor size measurements over time were obtained from the following groups: control; radiation alone; VEGF Trap, low dose (2.5 mg/kg), intermediate dose (10 mg/kg), or high dose (25 mg/kg); and the corresponding two radiation plus VEGF Trap combinations (n = 10–14 animals per group). Tumor growth over the entire study follow-up period was modeled via mixed-effects linear regression. This approach fits a "random" growth curve to each animal's data and then statistically "averages" these curves within each treatment group to estimate an overall "fixed effect" for each group. It also properly handles unbalanced data (*i.e.*, different number of measurements for different animals) and takes into account the correlation of each animal's measurements over time. Because tumors typically grow exponentially, the base-10 logarithm of tumor volume was modeled as a function of time and treatment. The interpretation of the linear model for the log of tumor volume is in terms of geometric means and geometric mean ratios (while the usual interpretation of a regression model for an untransformed outcome is in terms of arithmetic means and mean differences). The fitted linear growth curves fitted the data well. In addition, an allowance was made for the variance of the random effects to differ across groups to account for the larger variability of measurements in certain treated groups. All statistical analyses were conducted in SAS 8.2 (SAS Institute, Cary, NC, 1999–2001).

The mixed-effects regression has multiple advantages over analyses of tumor growth delay that typically compare groups with respect to the average time it takes tumors to reach some arbitrary size (*e.g.*, 2,000 mm^3). First, mixed-effects regression yields more general parameters of interest, such as average daily tumor growth rate and doubling time. Second, it can investigate (if necessary) treatment interactions and nonlinear patterns of tumor growth. Finally, it is more efficient because it used the repeated tumor size measurements obtained over the entire study period.

RESULTS

Effect of VEGF Trap and radiation on VEGF secretion in U87 cells in culture

Levels of VEGF increased in U87 culture supernatants in a dose-dependent manner following irradiation (Fig. 2). The addition of VEGF Trap (40 nM) reduced free VEGF in the supernatant to undetectable levels.

Effect of VEGF Trap and radiation on U87 tumor growth inhibition

The linear models for the log-transformed tumor growth fitted the data quite well in all groups. The raw data for all treatment groups with regression lines are plotted in Figs. 3 through 6 with corresponding Tables 1 through 4. The average daily percent increase in tumor volume for the untreated control group was consistent across all protocols and ranged between 27% and 31%, corresponding to a tumor doubling time between 2.5 and 3.0 days (Tables 1–4). Radiation alone (both single or fractionated doses) or VEGF Trap alone (all doses) significantly reduced the tumor growth rate compared with control ($p < 0.001$, Figs. 3–6, Tables 1–4). Results with VEGF Trap in combination with single dose or fractionated radiotherapy are now summarized.

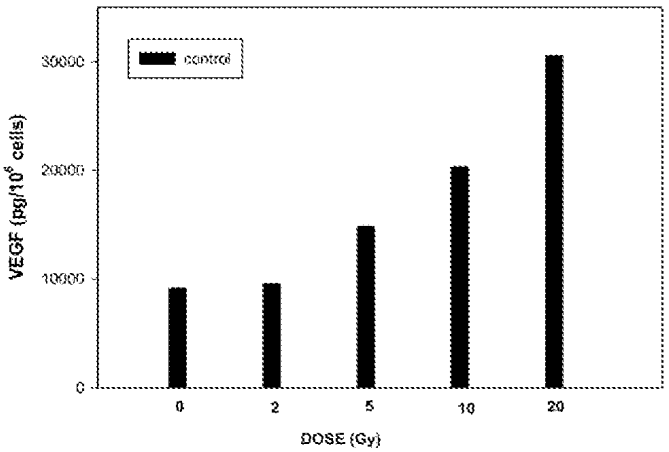

Fig. 2. Effect of vascular endothelial growth factor (VEGF) Trap and radiation on VEGF secretion in U87 cells in culture. U87 cells were irradiated at doses between 2 and 20 Gy in the presence or absence of 40 nM VEGF Trap. Cell culture supernatants were assayed for VEGF secretion 48 h following treatment. VEGF secretion was undetectable in presence of 40 nM VEGF Trap.

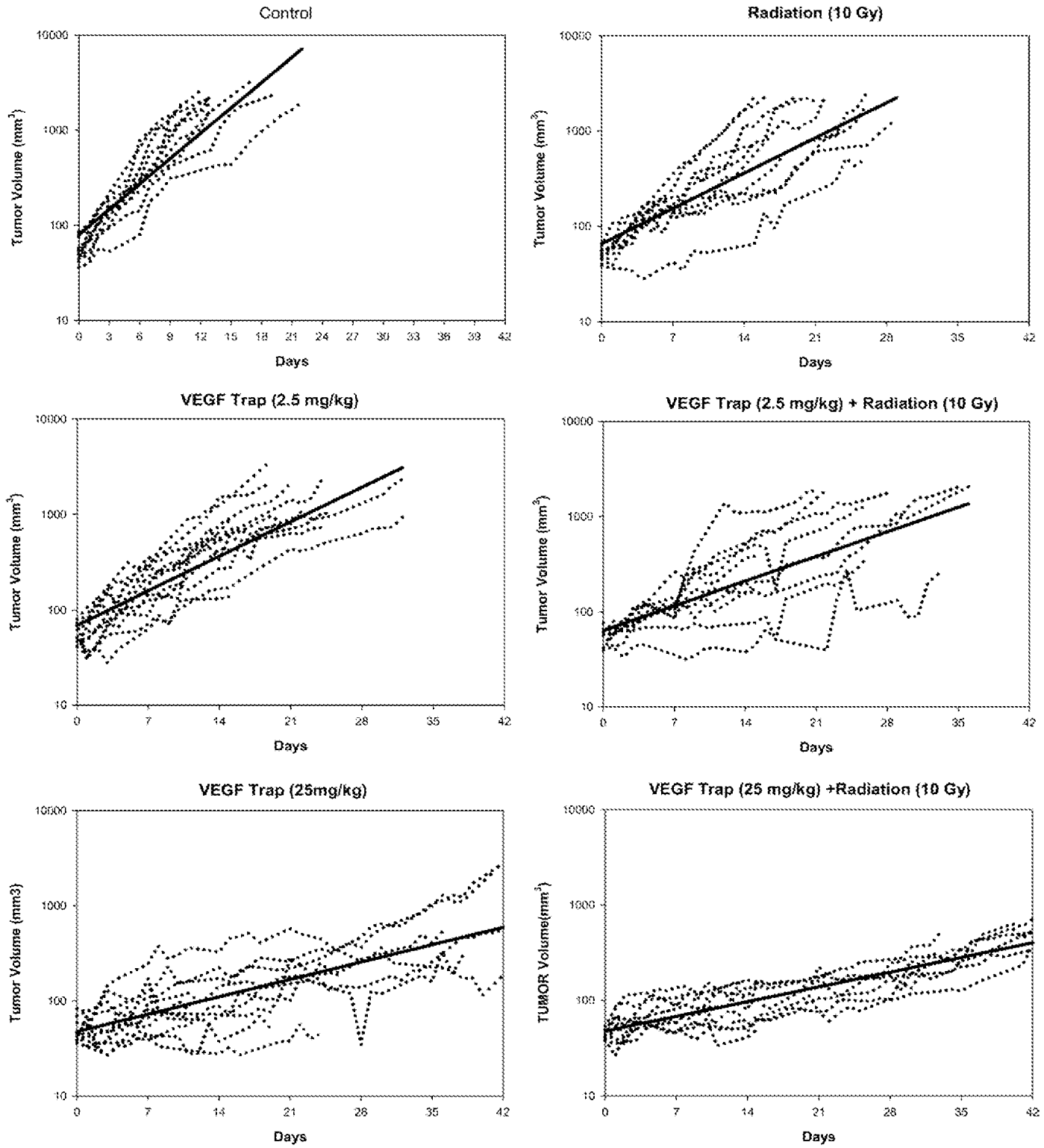

Fig. 3. Effect of vascular endothelial growth factor (VEGF) Trap combined with single-dose radiation (10 Gy) on tumor growth in U87GBM. Individual mouse data for six treatment groups (n = 10–12 animals per group). VEGF Trap was given at 2.5 or 25 mg/kg starting on Day 0, concurrent with radiation and continuing every 3 days for 3 weeks (see schedule I, Fig. 1).

Effect of VEGF Trap and single dose radiation (10 Gy) on U87 tumor growth inhibition

Table 1 presents tumor growth data based on the mixed-effects linear regression analysis described in Methods and Materials, and Fig. 3 presents the original animal data. In this experiment, a low dose of VEGF Trap (2.5 mg/kg) initiated concurrently with a single dose of 10 Gy was compared with a 10× higher dose of VEGF Trap (25 mg/kg) plus 10 Gy. The

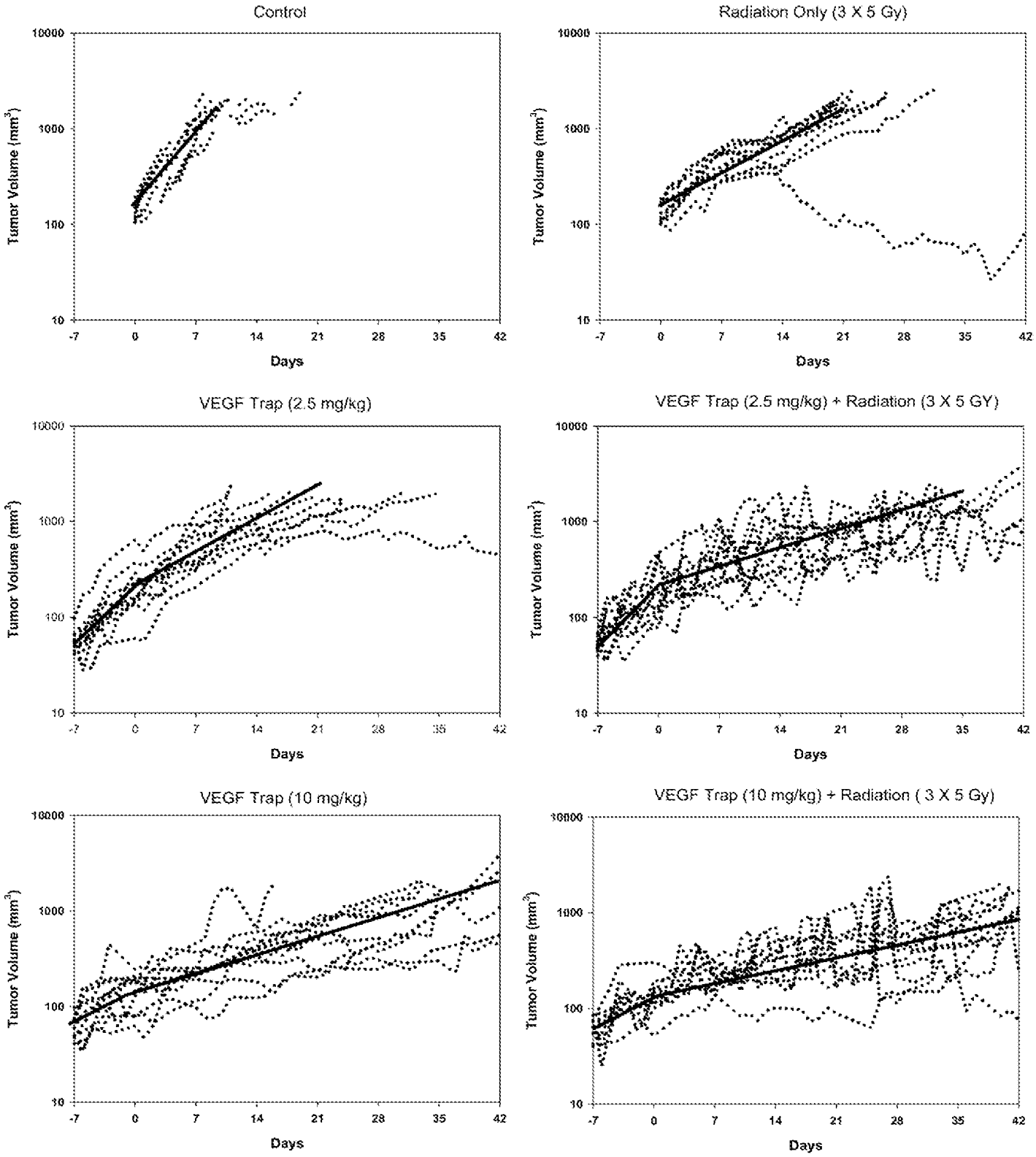

Fig. 4. Effect of vascular endothelial growth factor (VEGF) Trap initiated before fractionated radiation (3 × 5 Gy) on tumor growth in U87 GBM. Individual mouse data for 6 treatment groups (n = 10–14 animals/group). VEGF Trap was given at 2.5 or 10 mg/kg starting on Day −7 and continuing every 3 days for 3 weeks (see schedule H, Fig. 2).

six groups are compared in terms of average daily tumor growth and doubling time. It can be seen from Table 1 and Fig. 3 that both low-dose and high-dose VEGF Trap were effective inhibitors of daily percent increase in tumor volume (%Δ = 15% and 5%, respectively, vs. 31% for controls, p = 0.001). Although low-dose VEGF Trap was not significantly better than 10-Gy treatment alone, the combination of low-dose VEGF Trap and 10 Gy slowed daily tumor growth (%Δ =

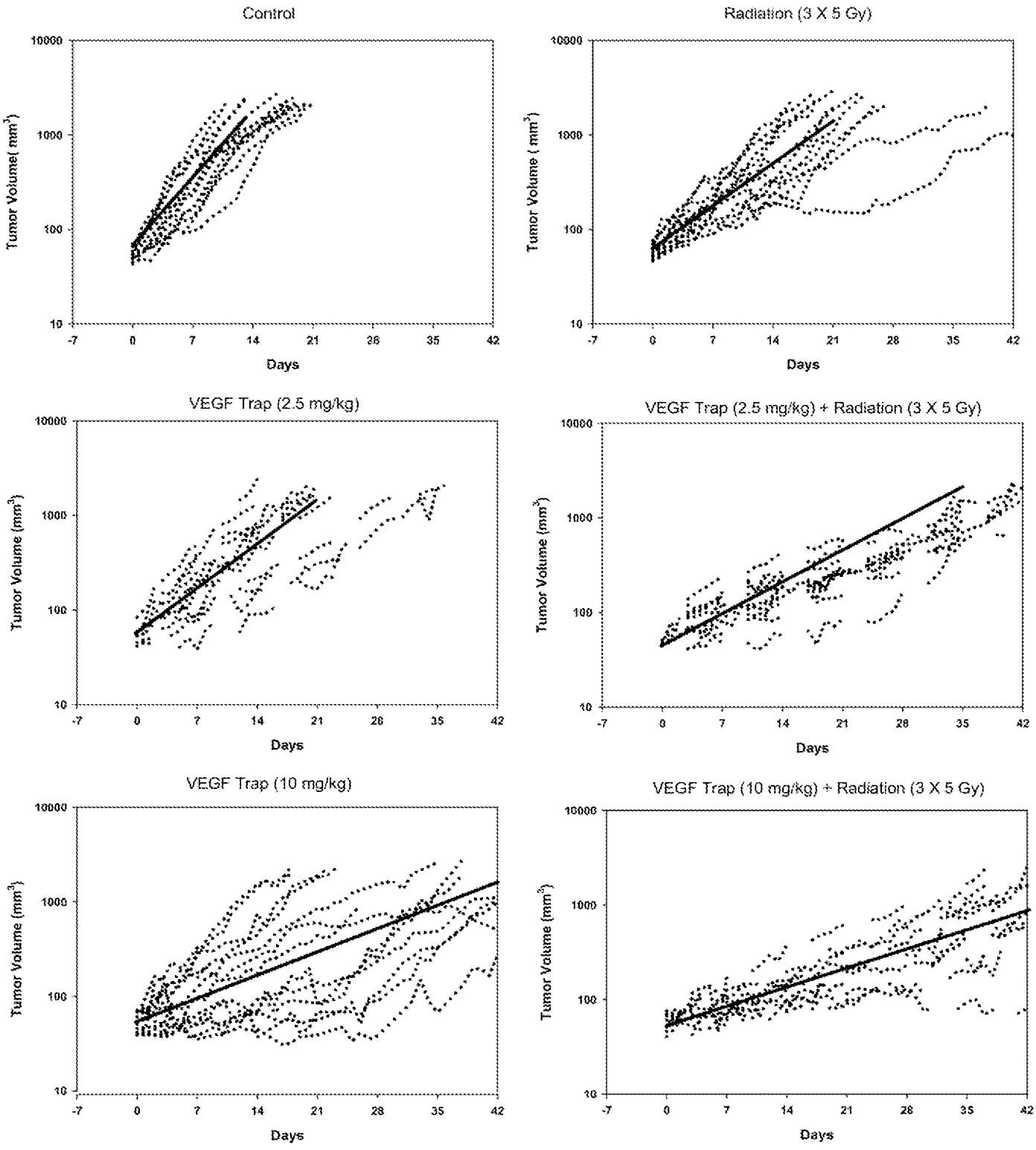

Fig. 5. Effect of vascular endothelial growth factor (VEGF) Trap sequenced concurrent with fractionated radiation (3 × 5 Gy) on tumor Growth in U87 GBM. Individual mouse data for six treatment groups (n = 10–14 animals per group). VEGF Trap was given at 2.5 or 10 mg/kg starting on Day 0 and continuing every 3 days for 3 weeks (see schedule III, Fig. 3).

12% vs. 18% for 10 Gy alone and 15% for low VEGF Trap alone). Thus, a less than additive enhancement in tumor control over either modality alone was observed. High-dose VEGF Trap, as a single treatment modality, was highly effective in slowing daily percent increase in tumor volume (5% vs. 18% for 10 Gy). Its efficacy was not improved by the addition of 10 Gy. This study suggests that low-dose VEGF Trap in combination with single-dose radiation has an enhanced effect on

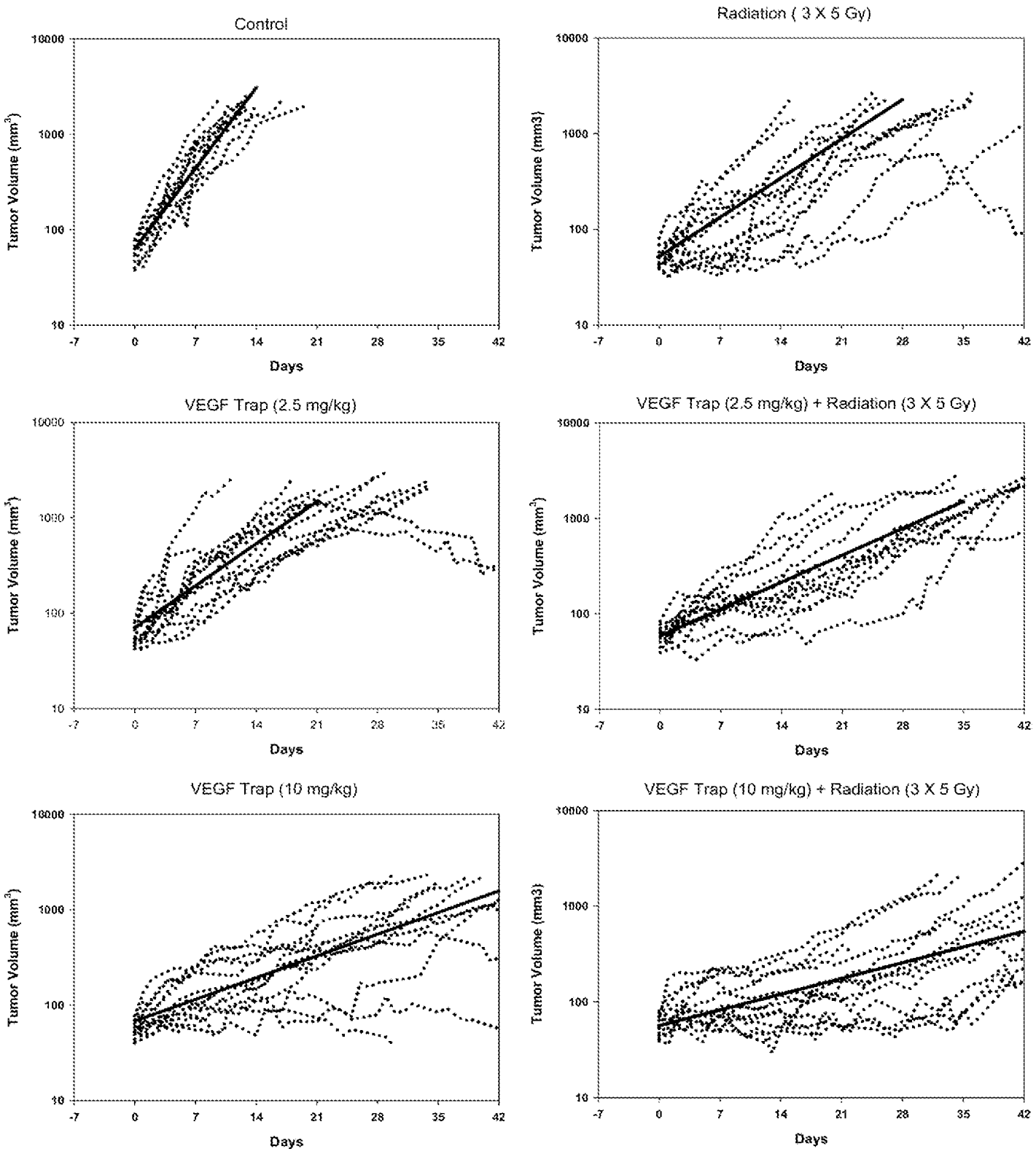

Fig. 6. Effect of vascular endothelial growth factor (VEGF) Trap sequenced post–fractionated radiation (3 × 5 Gy) on tumor Growth in U87 GBM. Individual mouse data for six treatment groups (n = 10–14 animals/group). VEGF Trap was given at 2.5 or 10 mg/kg starting on Day 3 and continuing every 3 days for 3 weeks (see schedule IV, Fig. 4).

tumor cell kill. It was thought that this enhancement might be improved by varying dose and scheduling protocol. Additional studies were carried out in which low-dose VEGF Trap at 2.5 mg/kg was compared with an intermediate dose of 10 mg/kg (because VEGF Trap at 25 mg/kg appeared to have masked any additional benefit of radiation in enhancing tumor control) in combination with a more clinically relevant fractionated radiotherapy protocol. The

Table 1. Effect of VEGF Trap combined with single-dose radiation: Summary of tumor growth (Schedule I)

Treatment	%Δ	(95% CI)	T2x	*p* values
Control (human FC protein)	31.0	(27–35)	2.6	
RT (10 Gy)	18.0	(15–21)	4.2	0.001 vs. control, 0.19 vs. VEGF Trap (low)
VEGF Trap (2.5 mg/kg	15.0	(13–28)	4.9	0.001 vs. control, 0.19 vs. RT alone
VEGF Trap (25 mg/kg)	5.0	(2–7)	15.2	0.001 vs. control, 0.001 vs. RT alone, 0.001 vs. VEGT Trap (low)
VEGF Trap (2.5mg/kg) + RT	12.0	(9–14)	6.3	0.003 vs. RT, 0.06 vs. VEGF Trap (low)
VEGF Trap (25 mg/kg) + RT	5.0	(2–7)	15.5	0.001 vs. RT, 0.417 vs. VEGF Trap (high), 0.96 vs. VEGF Trap (low) + RT

Abbreviations: %Δ = daily% increase in tumor volume; CI = confidence interval; RT = radiation; VEGF = vascular endothelial growth factor; T2x = average doubling time for tumor volume (in days).

results of these studies are reported in the following sections.

Effect of VEGF Trap and fractionated radiation on U87 tumor growth inhibition

VEGF Trap given before fractionated radiation: in this protocol, VEGF Trap was administered 7 days before radiation. The analyses allowed for separate tumor growth rates in the first and second periods (preradiation: Days −7 to 0; postradiation: Days 0+) for the groups that received radiation. The study's main aim was to compare tumor growth rates across treatment groups in the latter period, when all treatments had been applied. Table 2 summarizes the results of the tumor growth modeling analyses during this main study phase, and Fig. 4 presents the original animal data. The low-dose VEGF Trap group (2.5 mg/kg every third day, starting at Day −7) demonstrated a reduction in daily percent increase in tumor volume (12% vs. 27% for control; $p = 0.001$) that was similar to the first single-dose radiation study, whereas the high-dose VEGF Trap group (10 mg/kg every third day, starting at Day −7) had an even stronger effect (7%) that, again, was similar in trend to the first study. In the radiation only group, tumor daily growth was slowed to 11% ($p < 0.001$ vs. control). Although low-dose VEGF Trap was comparable to radiation alone ($p = 0.59$), the combination of low-dose VEGF Trap with radiation (7% average daily percent increase in tumor volume, Table 1) was significantly better than either radiation alone ($p = 0.036$) or low-dose VEGF Trap alone ($p < 0.005$). The combination of high-dose VEGF Trap with radiation (5% average percent daily increase in tumor volume) was also significantly better than radiation alone ($p = 0.002$) but not significantly better than high dose VEGF Trap alone ($p = 0.33$).

VEGF Trap given concurrently with fractionated radiation: Table 3 summarizes the results of the tumor growth modeling analyses based on original animal data shown in Fig. 5. High-dose VEGF Trap was significantly better than radiation in reducing daily percent increase in tumor volume (8.5% vs. 16.1% for radiation, $p = 0.001$). The combination of low-dose VEGF Trap with radiation (12% average daily increase in tumor volume) was significantly better than either radiation alone ($p = 0.029$) or low-dose VEGF Trap alone ($p = 0.012$). The combination of high-dose VEGF Trap (10 mg/kg) with radiation (7% average daily increase in tumor volume) was also significantly better than radiation alone ($p = 0.001$) but not high-dose VEGF Trap alone ($p = 0.417$).

VEGF Trap given postradiation: Table 4 summarizes the results of the tumor growth modeling analyses based on original animal data shown in Fig. 6. The results of this schedule followed the same pattern as seen in the previous two schedules with fractionated radiation as well as the first experiment with single-dose radiation. The benefit of combining VEGF Trap with radiation compared with single-modality treatments was once again seen with low-dose VEGF Trap plus radiation. High-dose VEGF Trap at 10 mg/kg plus radiation significantly reduced percent daily increase in tumor volume when compared with radiation alone but was not significantly different from VEGF Trap alone ($p = 0.187$).

In summary, improved tumor control was seen when radiation (either single dose or fractionated doses) were combined with the lowest dose of VEGF Trap (2.5 mg/kg)

Table 2. VEGF Trap initiated before fractionated radiation: Summary of tumor growth (Schedule II)

Treatment	%Δ	(95% CI)	T2x	*p* values
Control (human FC protein)	27.0	(23–31)	3.0	
RT (3 × 5 Gy)	11.0	(8–15)	6.5	0.001 vs. control, 0.59 vs. VEGF Trap (low), 0.027 vs. VEGF Trap (high)
VEGF Trap (2.5 mg/kg)	12.0	(10–15)	5.9	0.001 vs. control, 0.59 vs. RT
VEGF Trap (10 mg/kg)	7.0	(4–9)	11	0.001 vs. control, 0.027 vs. RT, 0.001 vs. VEGF Trap (low)
VEGF Trap (low) + RT	7.0	(4–9)	10.6	0.034 vs. RT, 0.004 vs. VEGF Trap (low)
VEGF Trap (high) + RT	5.0	(2–7)	15.3	0.002 vs. RT, 0.33 vs. VEGF Trap (high)

Abbreviations: %Δ = daily% increase in tumor volume; CI = confidence interval; RT = radiation; VEGF = vascular endothelial growth factor; T2x = average doubling time for tumor volume (in days).

Table 3. VEGF Trap sequenced concurrently with radiation: Summary of tumor growth (Schedule III)

Treatment	%Δ	(95% CI)	T2x	*p* values
Control (human FC protein)	27.0	(24–30)	2.9	
RT (3 × 5 Gy)	16.0	(13–19)	4.6	0.001 vs. control, 0.729 vs. VEGF Trap (low), 0.001 vs. VEGF Trap (high)
VEGF Trap (2.5 mg/kg)	17.0	(14–19)	4.5	0.001 vs. control, 0.729 vs. RT alone
VEGF Trap (10 mg/kg)	8.5	(6–11)	8.5	0.001 vs. control, 0.001 vs. RT alone, 0.001 vs. VEGT Trap (low)
VEGF Trap (low) + RT	12.0	(9–14)	6.3	0.020 vs. RT, 0.008 vs. VEGF Trap (low)
VEGF Trap (high) + RT	7.0	(5–9)	10.3	0.001 vs. RAD, 0.392 vs. VEGF Trap (high), 0.014 vs. VEGF Trap (low) + RT

Abbreviations: %Δ = daily% increase in tumor volume; CI = confidence interval; RT = radiation; VEGF = vascular endothelial growth factor; T2x = average doubling time for tumor volume (in days).

used in these studies. Scheduling did not significantly affect the efficacy of combined therapy. The relative benefits of combined low-dose VEGF Trap plus fractionated radiation relative to radiation as judged by percent reduction in average daily increase in tumor volume were 36% for VEGF Trap given before radiation, 27% for concurrent treatment, and 32% for drug given postradiation treatment. Although high-dose VEGF Trap (either 10 mg/kg or 25 mg/kg) significantly reduced tumor growth over that of radiation alone, there was no added benefit to combining high dose VEGF Trap with radiation.

Effect of VEGF Trap and radiation on microvessel density

Immunoassaying for endothelial cells with PECAM-1 revealed an inhibition of tumor angiogenesis 3 weeks after treatment with VEGF Trap or VEGF Trap and radiation. Tumor MVD was similar in the control and radiation-treated tumors. Tumor MVD in the VEGF Trap treated tumors was decreased to between 43% to 57% of control or radiation-treated tumors ($p = 0.06$). Tumor MVD in VEGF and radiation-treated groups decreased to between 15% and 30% of control or radiation-treated groups ($p = 0.001$) (Fig. 7). There was no significant difference in MVD between high-dose VEGF Trap–treated with radiation vs. high dose VEGF Trap alone ($p = 0.29$). However, there was a significant difference in MVD between low-dose VEGF Trap-treated with radiation and low-dose VEGF Trap alone ($p = 0.01$, Fig. 8).

18-fluorodeoxyglucose–PET imaging of VEGF Trap–treated tumors

Figure 9a illustrates a series of images from a representative, untreated mouse. Figure 9b represents a series of images from a representative mouse treated with VEGF Trap dosed at or 10 mg/kg every 3 days (starting at Day 0) for 3 weeks. Tumor volume (mm^3) and days following start of treatment are indicated. Because of the difficulty in matching tumor volumes and time after treatment, the percent of metabolically inactive tumor volume (as measured by FDG uptake) was measured as a function of tumor volume and averaged over a range of tumor volumes between 900 and 1,600 mm^3. The percent of metabolically inactive tumor was significantly less in untreated tumors (2.46% ± 0.18%) than in tumors treated with 10 mg/kg VEGF Trap (8.7 ± 1.26%, $p = 0.01$) but not significantly different from tumors treated with 2.5 mg/kg VEGF Trap (3.36 ± 0.36%, $p = 0.13$).

DISCUSSION

This work demonstrated that VEGF Trap alone is an effective dose-dependent inhibitor of tumor growth in U87GBM. These findings agreed with previous studies of VEGF Trap in other preclinical animal models demonstrating efficacy in halting angiogenesis and shrinking tumors (30). Because VEGF Trap was very potent by itself and could have potentially masked any additional benefits of radiation, both low-dose and high-dose scheduling of the drug were used with radiotherapy. In all scheduling protocols that were investigated, the combination of low-dose VEGF Trap with radiation was significantly better than either treatment modality alone. On the other hand, high-dose VEGF Trap was significantly better than radiation alone and therefore masked any additional benefit that may have resulted from combination therapy.

The benefit of combined treatment with low VEGF Trap

Table 4. VEGF Trap sequenced post–fractionated radiation: Summary of tumor growth (Schedule IV)

Treatment	%Δ	(95% CI)	T2x	*p* values
Control (human FC protein)	31.5	(28–35)	2.5	
RT (3 × 5 Gy)	15.0	(13–17)	5.1	0.001 vs. control, 0.460 vs. VEGF Trap (low), 0.001 vs. VEGF Trap (high)
VEGF Trap (2.5 mg/kg)	16.0	(13–19)	4.7	0.001 vs. control, 0.460 vs. RT alone
VEGF Trap (10 mg/kg)	8.0	(5–10)	9.2	0.001 vs. control, 0.001 vs. RT alone, 0.001 vs. VEGT Trap (low)
VEGF Trap (low) + RT	10.0	(7–12)	7.4	0.011 vs. RT, 0.001 vs. VEGF Trap (low)
VEGF Trap (high) + RT	5.5	(3–8)	12.8	0.001 vs. RT, 0.187 vs. VEGF Trap (high), 0.013 vs. VEGF Trap (low) + RT

Abbreviations: %Δ = daily% increase in tumor volume; CI = confidence interval; RT = radiation; VEGF = vascular endothelial growth factor; T2x = average doubling time for tumor volume (in days).

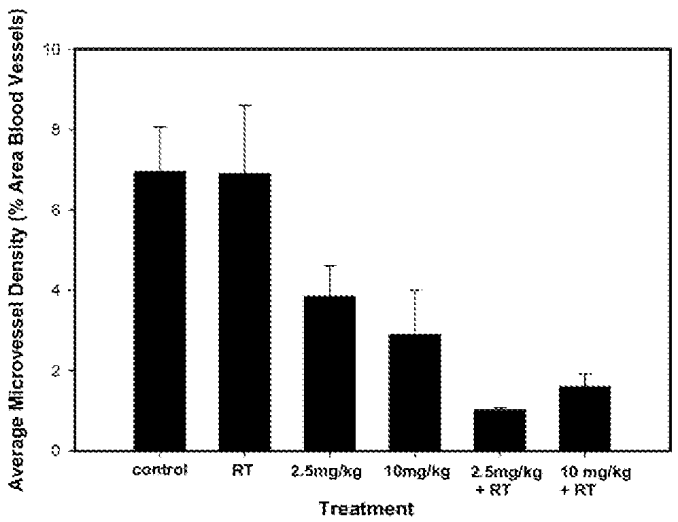

Fig. 7. Effect of vascular endothelial growth factor (VEGF) Trap and radiation (RT) (Schedule II) on microvessel density (MVD). Tumor MVD in VEGF Trap–treated tumors was decreased to between 43% and 57% of control or RT-treated tumors ($p = 0.06$). Tumor MVD in VEGF Trap and RT-treated groups decreased to between 15% and 30% of control or RT-treated groups ($p = 0.001$). There was no significant difference in MVD between VEGF Trap–treated (high dose) + radiation vs. VEGF Trap (high dose) alone ($p = 0.29$). However, there was a significant difference in MVD between VEGF Trap (low dose) + radiation and VEGF Trap (low dose) alone ($p = 0.01$).

and radiation relative to radiation alone was not influenced by scheduling protocol. This result was in contrast to earlier work demonstrating improved radiation response when a VEGFR2 blocker, DC101, was given 4 to 6 days before radiotherapy (32). This earlier work suggested that tumor vasculature normalization occurred during pretreatment with the VEGFR2 blocker, a process in which pruning of immature and inefficient blood vessels occurs leading to improved tumor perfusion and oxygenation and improved radiation response. The current observations may reflect the absence of a normalization effect by VEGF Trap on U87 GBM vasculature or a missed window of opportunity for normalization because of the particular protocols used in this work. Because it is not known how tumor oxygenation levels may have varied throughout the course of combined treatment with VEGF Trap and radiation, additional studies are warranted to resolve the issue of normalization.

The observation that scheduling did not have an impact on efficacy of combined treatment with VEGF Trap and radiation in this study is also in contrast to recent studies in which VEGF blockade was obtained either by a VEGF receptor2 tyrosine kinase inhibitor, ZD6474, or indirectly by HIF-1 alpha blockade of VEGF secretion. In both these studies, optimal antitumor efficacy was obtained when VEGF blockers were sequenced following radiation (2, 33). These studies suggested that prolonged suppression of radiation-induced angiogenesis account for enhanced efficacy of combined treatments with angiogenesis blockade and radiation. However, it is not clearly understood why there is a difference in the impact of scheduling among these agents.

This work is encouraging in that it demonstrates for the first time a benefit in combining VEGF Trap with ionizing radiation in a highly resistant GBM tumor model. VEGF Trap is a unique human fusion protein with very potent binding affinity for VEGF A isoforms as well as placental growth factor (PIGF) and is currently in clinical trials. Its affinity for VEGF is potentially 100- to 1,000-fold higher than existing VEGF monoclonal antibodies such as bevacizumab (34). This high-affinity blockade of VEGF differentiates VEGF Trap from other anti-VEGF strategies and therefore gives this drug the potential to enhance combination modality treatment with lower dosing.

Mechanisms of enhanced U87 tumor control by com-

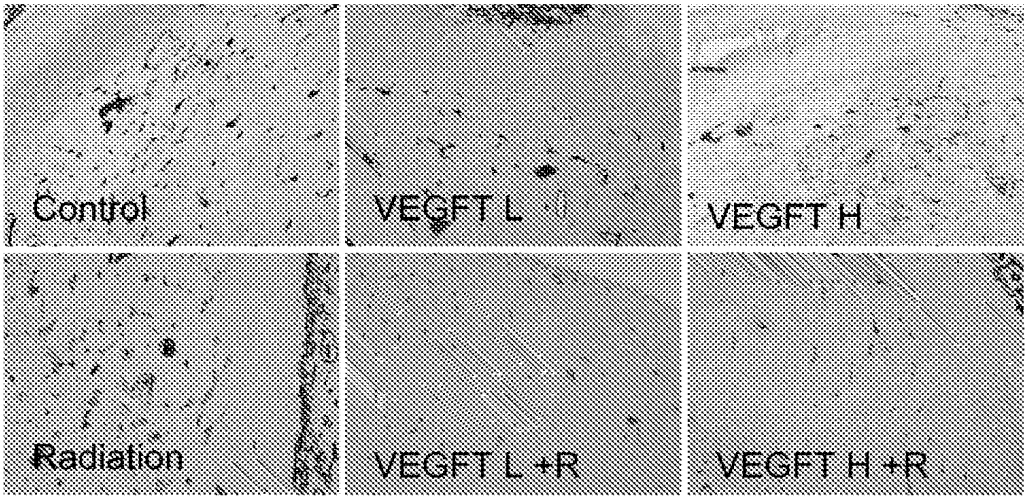

Fig. 8. Platelet–endothelial cell adhesion molecule 1 (PECAM-1) staining in subcutaneous U87 glioblastoma xenografts treated with vascular endothelial growth factor (VEGF) Trap with and without radiation therapy (Schedule II). Lower microvessel density (MVD) and altered vessel morphology were observed in treated tumors. (VEGFT L = VEGF Trap low dose; VEGFT H = VEGF Trap high dose; *R* = radiation) Original magnification: ×100.

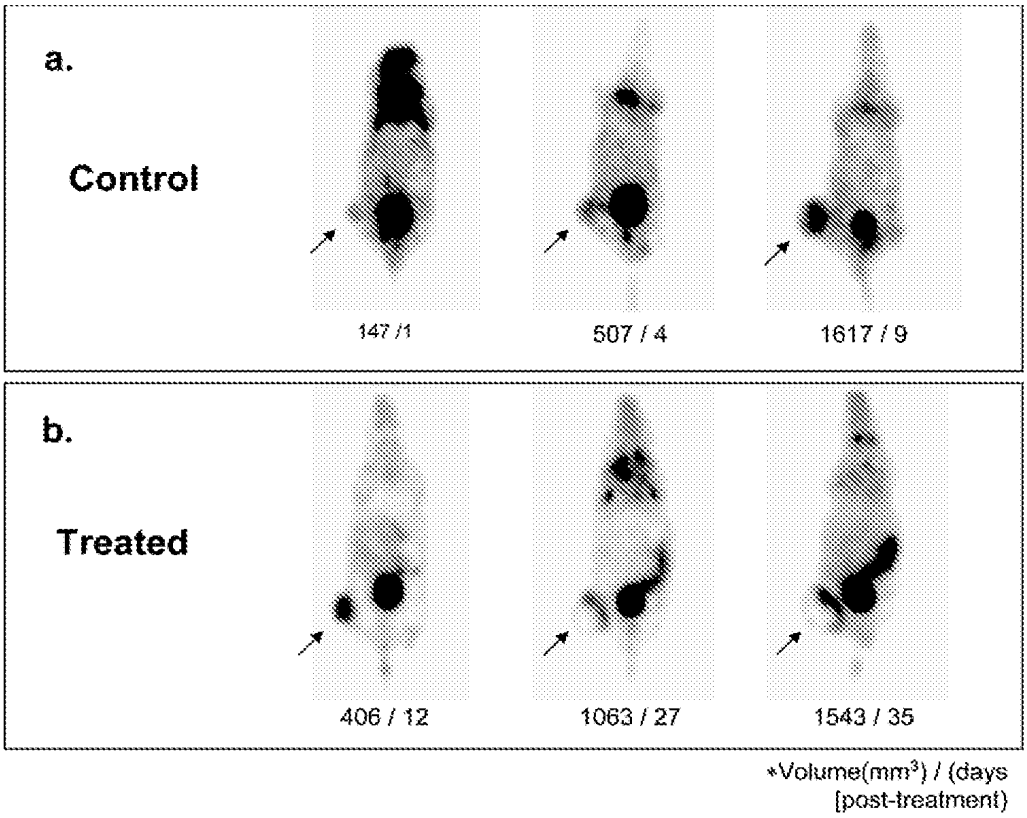

Fig. 9. 18-fluorodeoxyglucose (FDG)–PET imaging of human U87 glioblastoma xenografts in nude mice. (a) A series of typical images from an untreated mouse. (b) A series of images from a mouse treated with vascular endothelial growth factor (VEGF) Trap dosed at 10 mg/kg every 3 days (starting at Day 0) for 3 weeks. Tumor volume (mm^3) and days following start of treatment are indicated. Imaging was performed as described in Methods and Materials. The percent of metabolically inactive tumor (as measured by FDG uptake) was significantly less in untreated tumors than in tumors treated with 10 mg/kg VEGF Trap.

bined therapy with VEGF Trap and radiation most likely include inhibition of radiation-induced angiogenesis by VEGF Trap sequestration of circulating VEGF in the bloodstream and in the extracellular tumor space resulting from radiation-induced secretion. Indeed, in this study, a radiation-dose-dependent increase in VEGF secretion by U87 glioblastoma cells was observed and excess VEGF was bound in the presence of VEGF Trap. In addition, immunohistochemical findings indicated a reduction in MVD 3 weeks following treatment with VEGF Trap and radiation. Inhibition of radiation-induced angiogenesis was also observed indirectly through FDG–PET imaging, which revealed an increase in metabolically inactive tumor tissue after VEGF Trap treatment, possibly arising from the induction of tumor necrosis or apoptosis in the presence of angiogenesis inhibition. It is also of interest that in this study, a brief period of fractionated radiotherapy with VEGF Trap resulted in tumor growth retardation but not remission. The lack of remission is probably related to continued production of VEGF after removal of drug and radiation and points to the need for chronic therapy with VEGF Trap, which is in agreement with what has been observed for the transient effects of other antiangiogenic agents on tumor control (23, 35).

In conclusion, these studies demonstrate that the combination of low-dose VEGF Trap and radiation is clearly better than radiation alone in a U87 subcutaneous xenograft model. Although high doses of VEGF Trap alone are highly efficacious, it is unclear whether such high doses can be used clinically without incurring normal tissue toxicities. Thus, information on lower doses of VEGF Trap and ionizing radiation are of clinical relevance.

It is understood that the SC xenograft model used in this study has shortcomings in that ectopic tumors implanted SC in the hind limb of animals do not duplicate the vascular microenvironment of orthotopic brain implants (36). However, the use of hind limb injection is the standard approach for xenograft studies with radiation. In addition, human xenografts in immunocompromised nude mice, whether they be ectopic or orthotopic, both have deficiencies in that they can only approximate the human patient situation and seldom reflect accurately the glioblastoma multiforme histopathology seen in patients. This study is encouraging in that it demonstrates for the first time a benefit in combining VEGF Trap with ionizing radiation and warrants further investigations both preclinically and clinically.

REFERENCES

1. Garcia-Barros M, Paris F, Cordon-Cardo C, *et al.* Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science* 2003;300:1155–1159.
2. Moeller BJ, Dreher MR, Rabbani ZN, *et al.* Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity. *Cancer Cell* 2005;8:99–110.
3. Paris F, Fuks Z, Kang A, *et al.* Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. *Science* 2001;293:293–297.
4. Dent P, Yacoub A, Contessa J, *et al.* Stress and radiation-induced activation of multiple intracellular signaling pathways. *Radiat Res* 2003;159:283–300.
5. Lanza-Jacoby S, Dicker AP, Miller S, *et al.* Cyclooxygenase (COX)-2-dependent effects of the inhibitor SC236 when combined with ionizing radiation in mammary tumor cells derived from HER-2/neu mice. *Mol Cancer Ther* 2004;3:417–424.
6. Wachsberger P, Burd Marreron R, Rossetti D, *et al.* Improvement of fractionated radiation therapy by combination with a VEGF blocker, VEGF Trap. 96th Annual Meeting of the American Association of Cancer Research, Anaheim, CA, 2005.
7. Ferrara N. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 2001;280:C1358–C1366.
8. Benjamin LE, Golijanin D, Itin A, *et al.* Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* 1999;103:159–165.
9. Tran J, Master Z, Yu JL, *et al.*A role for survivin in chemoresistance of endothelial cells mediated by VEGF. *Proc Natl Acad Sci U S A* 2002;99:4349–4354.
10. Moeller BJ, Cao Y, Li CY, *et al.* Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. *Cancer Cell* 2004;5: 429–441.
11. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. *J Clin Oncol* 2005;23:1011–1027.
12. Asano M, Yukita A, Matsumoto T, *et al.*An anti-human VEGF monoclonal antibody, MV833, that exhibits potent anti-tumor activity in vivo. *Hybridoma* 1998;17:185–190.
13. Kamiya K, Konno H, Tanaka T, *et al.* Antitumor effect on human gastric cancer and induction of apoptosis by vascular endothelial growth factor neutralizing antibody. *Jpn J Cancer Res* 1999;90:794–800.
14. Kim ES, Serur A, Huang J, *et al.* Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma.*Proc Natl Acad Sci U.S.A* 2002;99:11399–11404.
15. Willett CG, Boucher Y, di Tomaso E, *et al.* Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med* 2004;10:145–147.
16. Abdollahi A, Lipson KE, Han X, *et al.* SU5416 and SU6668 attenuate the angiogenic effects of radiation-induced tumor cell growth factor production and amplify the direct anti-endothelial action of radiation in vitro. *Cancer Res* 2003;63: 3755–3763.
17. Drevs J, Hofmann I, Hugenschmidt H, *et al.* Effects of PTK787/ZK 222584, a specific inhibitor of vascular endothelial growth factor receptor tyrosine kinases, on primary tumor, metastasis, vessel density, and blood flow in a murine renal cell carcinoma model. *Cancer Res* 2000;60:4819–4824.
18. Fong TA, Shawver LK, Sun L, *et al.* SU5416 is a potent and selective inhibitor of the vascular endothelial growth factor receptor (Flk-1/KDR) that inhibits tyrosine kinase catalysis, tumor vascularization, and growth of multiple tumor types. *Cancer Res* 1999;59:99–106.
19. Wedge SR, Ogilvie DJ, Dukes M, *et al.* ZD4190: an orally active inhibitor of vascular endothelial growth factor signaling with broad-spectrum antitumor efficacy. *Cancer Res* 2000;60: 970–975
20. Hasumi Y, Mizukami H, Urabe M, *et al.* Soluble FLT-1 expression suppresses carcinomatous ascites in nude mice bearing ovarian cancer. *Cancer Res* 2002;62:2019–2023.
21. Holash J, Davis S, Papadopoulos N, *et al.* VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci U S A* 2002;99:11393–11398.
22. Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2002;2:727–739.
23. Wachsberger P, Burd R, Dicker AP. Tumor response to ionizing radiation combined with antiangiogenesis or vascular targeting agents: exploring mechanisms of interaction. *Clin Cancer Res* 2003;9:1957–1971.
24. Wachsberger P, Burd R, Dicker AP. Improving tumor response to radiotherapy by targeting angiogenesis signaling pathways. *Hematol Oncol Clin North Am* 2004;18: 1039–1057, viii.
25. Vajkoczy P, Schilling L, Ullrich A, *et al.* Characterization of angiogenesis and microcirculation of high-grade glioma: an intravital multifluorescence microscopic approach in the athymic nude mouse. *J Cereb Blood Flow Metab* 1998;18:510–520.
26. Ferrara N, Chen H, Davis-Smyth T, *et al.* Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nat Med* 1998;4:336–340.
27. Gerber HP, Kowalski J, Sherman D, *et al.* Complete inhibition of rhabdomyosarcoma xenograft growth and neovascularization requires blockade of both tumor and host vascular endothelial growth factor. *Cancer Res* 2000;60:6253–6258.
28. Gerber HP, Hillan KJ, Ryan AM, *et al.* VEGF is required for growth and survival in neonatal mice. *Development* 1999;126: 1149–1159.
29. Kuo CJ, Farnebo F, Yu EY, *et al.* Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer. *Proc Natl Acad Sci U S A* 2001;98:4605–4610.
30. Konner J, Dupont J. Use of soluble recombinant decoy receptor vascular endothelial growth factor trap (VEGF Trap) to inhibit vascular endothelial growth factor activity. *Clin Colorectal Cancer* 2004;4(Suppl. 2):S81–S85.
31. Huang J, Frischer JS, Serur A, *et al.* Regression of established tumors and metastases by potent vascular endothelial growth factor blockade. *Proc Natl Acad Sci U S A* 2003;100:7785–7790.
32. Winkler F, Kozin SV, Tong RT, *et al.* Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. *Cancer Cell* 2004;6:553–563.
33. Williams KJ, Telfer BA, Brave S, *et al.* ZD6474, a potent inhibitor of vascular endothelial growth factor signaling, combined with radiotherapy: schedule-dependent enhancement of antitumor activity. *Clin Cancer Res* 2004;10:8587–8593.
34. Bergsland EK. Update on clinical trials targeting vascular endothelial growth factor in cancer. *Am J Health Syst Pharm* 2004;61:S12–S20.
35. Klement G, Baruchel S, Rak J, *et al.* Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest* 2000;105:R15–R24.
36. Blouw B, Song H, Tihan T, *et al.* The hypoxic response of tumors is dependent on their microenvironment. *Cancer Cell* 2003;4:133–146.

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1174-5886/08/0004-0261/$48.00/0

Aflibercept

AVE 0005, AVE 005, AVE0005, VEGF Trap – Regeneron, VEGF Trap (R1R2), VEGF Trap-Eye

Abstract

Aflibercept is a fully human recombinant fusion protein composed of the second Ig domain of VEGFR1 and the third Ig domain of VEGFR2, fused to the Fc region of human IgG_1. Aflibercept is in clinical development with Regeneron Pharmaceuticals and sanofi-aventis for the treatment of cancer, while Regeneron and Bayer are developing the agent for eye disorders. Aflibercept binds to all VEGF-A isoforms as well as placental growth factor (PlGF), thereby preventing these factors from stimulating angiogenesis. Blockade of VEGF can also prevent blood vessel formation and vasuclar leakage associated with wet age-related macular degeneration (AMD). Aflibercept is a member of Regeneron's proprietary family of 'Trap' product candidates that catch, hold and block (i.e. trap) certain harmful cytokines or growth factors.

Regeneron and Bayer HealthCare entered into a collaboration agreement in October 2006 to develop and commercialize aflibercept for the treatment of eye disorders outside the US. The companies will share equally in profits from this market, while Regeneron will retain exclusive commercialization rights and profits from sales in the US.[1]

Regeneron and sanofi-aventis amended their aflibercept collaboration agreement to include Japan. Under the terms of the amended agreement, reported in December 2005, the two companies will jointly develop and commercialize aflibercept worldwide in all indications, except for intraocular delivery to the eye. sanofi-aventis paid $US25 million to Regeneron for the inclusion of Japan and will pay milestone payments linked to Japanese regulatory approvals, plus royalties on Japanese sales. sanofi-aventis will lead Japanese development and will pay all development costs; however, Regeneron will repay 50% of these expenses out of profits generated through the commercialization of aflibercept.[2]

sanofi-aventis reaffirmed its commitment to the aflibercept programme in oncology in January 2005, while the exclusive rights to develop and commercialize the agent for eye diseases through local delivery systems reverted to Regeneron. A $US25 million clinical development milestone payment to Regeneron was also triggered in connection with this agreement.[3]

Aventis (now sanofi-aventis) and Regeneron entered into a global (excluding Japan) agreement in September 2003 to jointly develop and commercialize aflibercept. Under the terms of the agreement, Aventis was to pay Regeneron $US125 million and fund development costs. An additional early clinical milestone payment of $US25 million was also outlined in the agreement. The two companies will share promotional rights equally, and profits globally. Aventis will also pay Regeneron up to $US360 million at identified milestones related to the receipt of marketing approvals for up to eight indications in Europe and the

US. The companies initially agreed to jointly develop aflibercept in oncology, ophthalmology and possibly in other indications.[4]

Originally, aflibercept was being developed under a research and development alliance between Regeneron and Procter & Gamble. However, in 2000 this agreement was restructured and Regeneron regained all rights.

An NCI-sponsored phase II trial (NCT00407654) of aflibercept, involving 80 patients with previously treated metastatic colorectal cancer, is also underway in the US and Canada. The trial was initiated in October 2006 and is evaluating the efficacy of aflibercept in this patient group, as measured by objective tumour response and progression-free survival at 4 months.

In September 2006, a phase II trial in 82 patients with locally advanced, unresectable or metastatic gynaecological soft tissue sarcoma was initiated by NCI and Regeneron in the US and Canada. This ongoing trial (NCT00390234) will evaluate the efficacy of aflibercept, as measured by progression-free survival and tumour response rate.

Regeneron and sanofi-aventis are conducting a phase II trial of intravenously (IV) administered aflibercept in patients with advanced ovarian cancer who have recurrent symptomatic malignant ascites (SMA). The trial (NCT00327444) began in July 2006 and was continuing to recruit a total of 54 patients at centres in the US, Canada, India and the EU (Austria, Belgium, Hungary, Spain and the UK) in April 2007.

In October 2006, the companies initiated a second small phase II trial of aflibercept (NCT00396591) in 15 patients with malignant ascites associated with ovarian cancer. The study will assess the efficacy, safety, pharmacokinetics and immunogenicity of aflibercept IV given every 2 weeks in the US and EU (Italy and Sweden) and was recruiting patients in May 2007.

Regeneron and sanofi-aventis are also conducting a single-agent phase II study of aflibercept in non-small-cell lung adenocarcinoma (NSCLA). The open-label, single-arm study (NCT00284141) has completed enrolment of approximately 100 patients with platinum- and erlotinib-resistant, locally advanced or metastatic NSCLA to receive aflibercept (4.0 mg/kg IV) in the US, France and Canada. Results from the first 37 evaluable patients have been reported showing aflibercept was generally well tolerated and two partial reponses were noted.[5,6]

Regeneron has completed an open-label phase I trial in patients with solid tumours and non-Hodgkin's lymphoma (NHL) at three sites in the US. The study enrolled 38 patients with incurable, relapsed or refractory solid tumours who received subcutaneous injections. In total, the trial enrolled patients with 15 different types of cancer who were treated with seven subcutaneous doses of aflibercept over 10 weeks. In June 2004, Regeneron presented results from this study showing that the aflibercept was well tolerated and had a good safety profile. The maximum tolerated dose was not established. The company has not conducted any further trials in this indication with aflibercept as a monotherapy, although the NCI has ongoing trials of aflibercept in patients with solid tumours and NHL (e.g. NCT0008283).[7]

In May 2005, Regeneron announced initiation of a phase I safety and tolerability study with aflibercept in combination with the FOLFOX-4 regimen (oxaliplatin, 5-fluorouracil and leucovorin) in patients with advanced solid tumours. As at

August 2006, the maximum tolerated dose had not been reached and dose-escalation was continuing in this study.[8,9]

The NCI/Regeneron trial in patients with metastatic or unresectable kidney cancer began in September 2007 with continued recruitment in April 2008. This trial (NCT00357760) is anticipated to recruit 120 patients in the US to evaluate the efficacy of two doses of aflibercept.

Regeneron and Bayer inititiated a phase III trial of aflibercept in approximately 1200 patients with the neovascular form of wet AMD in August 2007. The non-inferiority, VIEW 1 (VEGF Trap: Investigation of Efficacy and safety in Wet age-related macular degeneration) study will evaluate the safety and efficacy of intravitreal aflibercept at doses of 0.5 mg and 2.0 mg administered at 4-week dosing intervals, and 2.0 mg at an 8-week dosing interval, compared with 0.5 mg ranibizumab administered every 4 weeks. The randomized, double-blind trial will be conducted at more than 200 centres throughout the US and Canada, pursuant to a Special Protocol Assessment (SPA) issued by the the US FDA. Patients will continue to be treated and followed for an additional year, after the first year of treatment. The VIEW 1 study is the first in a phase III global development programme in wet AMD, which is expected to be conducted in the US, Europe and other nations. Regeneron received a $US20 million milestone payment from Bayer HealthCare in August 2007 following dosing of the first patient.[10,11]

A second phase III trial (VIEW 2) in wet AMD began with the first patient dosed in May 2008. The VIEW 2 trial will enrol approximately 1200 patients from the EU, Asia Pacific, Japan and Latin America. This study will evaluate the safety and efficacy of aflibercept at 0.5 mg and 2.0 mg administered at 4-week intervals and 2.0 mg at an 8-week dosing interval, including one additional 2.0 mg dose at week 4. Patients randomized to the ranibizumab arm of the trial will receive a 0.5 mg dose every 4 weeks. The primary endpoint will be the proportion of patients treated with aflibercept who maintain vision at the end of 1 year compared with ranibizumab patients.[12,13]

Regeneron has completed a 12-week, phase II trial in patients with wet AMD, to evaluate the safety and efficacy of intravitreal aflibercept using different doses and dose regimens. Two patient groups received monthly doses of 0.5 or 2.0 mg, and three groups received quarterly doses of 0.5, 2.0 or 4.0 mg (baseline and week 12). Analysis of data demonstrated that all five doses of aflibercept met the primary study endpoint of a statistically significant reduction in retinal thickness after 12 weeks and 32 weeks of treatment compared with baseline. The study commenced in April 2006 and enrolled 157 patients at sites in the US. Preliminary phase I trial results in 21 patients have also been presented.[14-16]

Additionally, Regeneron has conducted a phase I trial of aflibercept in five patients with diabetic macular oedema (DME) in the US. Results presented in May 2007 indicated that a single 4 mg injection resulted in a marked decrease in mean central retinal thickness and mean macular volume throughout the 6-week observation period. The VEGF Trap-Eye was generally well tolerated, and there were no drug-related serious adverse events.[17] Regeneron plans to conduct advanced studies of the VEGF Trap-Eye in DME.

Previously, sanofi-aventis and Regeneron had been collaborating on the development of aflibercept for eye diseases through local delivery systems. However, the exclusive rights to develop and commercialize aflibercept for eye diseases

through local delivery systems reverted to Regeneron in January 2005. Additionally, Regeneron chose to pursue intravitreal inection as a route of administration, instead of systemic delivery.[18]

Results from an earlier phase I trial assessing the safety and tolerability of intravenous infusions of aflibercept in patients with wet AMD have been reported. Preliminary results from the trial showed that the efficacy endpoint was met. Furthermore, systemic delivery of aflibercept was associated with a dose-dependent increase in blood pressure.[19]

Table I. Features and properties

CAS number	862111-32-8
WHO ATC code	A10X (Other Drugs Used in Diabetes) S01X (Other Ophthalmologicals) L01 (Antineoplastic Agents)
EphMRA ATC code	A10X (Other Drugs Used in Diabetes) S1X (Other Ophthalmologicals) L1 (Antineoplastics)
Originator	Regeneron Pharmaceuticals: USA
Licensee companies	Bayer HealthCare: world; sanofi-aventis: world
Highest development phase	Phase III (World)
Properties	
Mechanism of action	Vascular endothelial growth factor A antagonists
Pharmacodynamics	Halts new blood vessel growth and stopped leakage from existing blood vessels in mice; inhibits VEGF and abolishes mature, pre-existing vasculature of tumours in mice; inhibits development of ascites and decreases tumour burden in animal models of ovarian cancer
Route	IV

1. Profile

1.1 Pharmacokinetics

Clinical studies: Preliminary results of an open-label, phase I trial of a single dose of subcutaneous VEGF Trap (25, 50, 100 or 200 μg/kg) followed 4 weeks later by six weekly doses in patients with solid tumours or lymphoma showed that VEGF Trap binds to VEGF in plasma and has an apparent elimination half-life ($t_{1/2}$) of ≈17 days.[20]

Results of a phase I, open-label, dose-escalation trial of 38 patients with relapsed or refractory solid tumours showed that VEGF Trap has a long $t_{1/2}$ and binds to both VEGF 121 and VEGF 165 in patient plasma. Plasma VEGF Trap levels that were associated with antitumour activity in animal models were approached in patients receiving the two highest dose groups or 800 μg/kg once or twice weekly. In the trial patients received one or two initial doses of VEGF Trap followed 4 weeks later by six weekly or twice-weekly doses. Seven dose groups were evaluated in the trial ranging from 25 to 800 μg/kg weekly or 800 μg/kg twice weekly. Values for t_{max}, C_{max}, $t_{1/2}$, AUC_{28} and CL/F were 84 ± 60 hours, 3 ± 1 μg/mL, 25.3 ± 9.3 days, 1304 ± 256 μg • h/mL and 0.4 ± 0.1 mL/h/kg, respectively.[21]

1.2 Adverse Events

Solid tumours: Results of a phase I, open-label, dose-escalation trial of VEGF Trap in 38 patients with relapsed or refractory solid tumours showed that the drug had a good safety profile and was well tolerated overall. The maximum tolerated dose was not reached in the study, which reached the highest planned dose level of 800 μg/kg twice weekly. The majority of adverse events reported were grade 1 or

Table II. Drug development history

May 2000	Preclinical development for Cancer in the US (Unknown route)
Nov 2001	Phase-I for Non-Hodgkin's lymphoma in the US (Unknown route)
Nov 2001	Phase-I for Solid tumours in the US (Unknown route)
Jun 2003	Prein Age-related macular degeneration in the US (IV)
Jun 2003	Prein Eye disorders in the US (Intravitreous)
Jun 2003	Prein Wilms' tumour in the US (Intraperitoneal)
Sep 2003	Aflibercept has been licensed to Aventis worldwide (excluding Japan)
Mar 2004	Phase-I in Age-related macular degeneration in the US (IV)
Apr 2004	Regeneron has initiated enrolment in a phase I trial for cancer in the US
Aug 2004	Aventis has merged with Sanofi-Synthelabo to form sanofi-aventis
Feb 2005	Aflibercept received Fast Track designation for Malignant ascites [IV] in the US
Feb 2005	Discontinued – Phase-I for Age-related macular degeneration in the US (IV-infusion)
May 2005	Regeneron has initiated the safety and tolerability study with VEGF Trap in combination with the FOLFOX-4 regimen (oxaliplatin, 5-fluorouracil and folinic acid) in patients with advanced tumours
May 2005	Phase-I in Solid tumours in the US (IV)
Jul 2005	Phase-I in Age-related macular degeneration in the US (Intravitreous)
Jul 2005	Prein Eye disorders in the US (Intravitreous)
Dec 2005	Regeneron has licensed aflibercept to sanofi-aventis in Japan
Dec 2005	Phase-II in Non-small cell lung cancer in France (IV)
Dec 2005	Phase-II in Non-small cell lung cancer in Canada (IV)
Dec 2005	Phase-II in Non-small cell lung cancer in the US (IV)
May 2006	Phase-I in Diabetic macular oedema in the US (Intravitreous)
May 2006	Phase-II in Age-related macular degeneration in the US (Intravitreous)
Jun 2006	Phase-II in Ovarian cancer in the US (IV)
Jun 2006	Phase-II in Ovarian cancer in Australia (IV)
Jun 2006	Phase-II in Ovarian cancer in Canada (IV)
Jun 2006	Phase-II in Ovarian cancer in Europe (IV)
Jul 2006	Phase-II/III in Malignant ascites in India (IV)
Jul 2006	Phase-II/III in Malignant ascites in the US (IV)
Jul 2006	Phase-II/III in Malignant ascites in Canada (IV)
Jul 2006	Phase-II/III in Malignant ascites in Europe (IV)
Aug 2006	Phase-II in Glioma in the US (IV)
Sep 2006	Phase-II in Sarcoma in Canada (IV)
Sep 2006	Phase-II in Sarcoma in the US (IV)
Oct 2006	Aflibercept has been licensed to Bayer HealthCare for the treatment of eye disorders
Oct 2006	Phase-II in Colorectal cancer in Canada (IV)
Oct 2006	Regeneron and sanofi-aventis initiate enrolment in a second phase II trial in Malignant ascites in the EU and US
Oct 2006	Phase-II in Colorectal cancer in the US (IV)
Nov 2006	Phase-II in Bladder cancer in the USA (IV)
Dec 2006	Phase-II in Multiple myeloma in the US (IV)
Jan 2007	Phase-II in Gynaecological cancer in the US (IV)
Jan 2007	Phase-II in Breast cancer in the US (IV)

Continued next page

Table II. Contd

Mar 2007	Interim results from a phase II clinical trial in wet Age-related macular degeneration added to the Eye Disorders therapeutic trials section
Mar 2007	Phase-I in Cancer in Japan (IV)
Jun 2007	Final results from a phase I clinical trial in patients with diabetic macular oedema added to the adverse events and Eye Disorders therapeutic trials sections
Jun 2007	Data presented at the 43rd Annual Meeting of the American Society of Clinical Oncology (ASCO-2007) added to the adverse events and Cancer therapeutic trials sections
Jun 2007	Phase-II in Malignant melanoma in the US (IV)
Jul 2007	Suspended – Phase-II for Colorectal cancer in Canada (IV)
Jul 2007	Suspended – Phase-II for Colorectal cancer in the US (IV)
Aug 2007	Phase-III in Age-related macular degeneration in the US (Intravitreous)
Aug 2007	Regeneron initiates patient dosing in a phase III trial for Age-related macular degeneration in the US
Aug 2007	Phase-III in Prostate cancer in the US (IV)
Aug 2007	Phase-III in Prostate cancer in Canada (IV)
Aug 2007	Phase-III in Prostate cancer in European Union (IV)
Aug 2007	Phase-III in Prostate cancer in Switzerland (IV)
Aug 2007	Phase-III in Prostate cancer in South Africa (IV)
Aug 2007	Phase-III in Prostate cancer in South America (IV)
Aug 2007	Phase-III in Prostate cancer in Asia (IV)
Aug 2007	Phase-III in Non-small cell lung cancer in the US (IV)
Aug 2007	Phase-III in Non-small cell lung cancer in France (IV)
Aug 2007	Phase-III in Prostate cancer in Australia (IV)
Oct 2007	Results from a phase II clinical trial in age-related macular degeneration added to the Eye Disorders therapeutic trials section
Dec 2007	Phase-III in Pancreatic cancer in World (IV)
Dec 2007	Phase-III in Colorectal cancer in World (IV)
Dec 2007	Phase-III in Non-small cell lung cancer in World (IV)
Dec 2007	Phase-III in Prostate cancer in World (IV)
Dec 2007	Suspended – Phase-II for Breast cancer in the US (IV)
Apr 2008	Interim efficacy data from a phase II trial in wet AMD released by Regeneron
May 2008	Bayer and Regeneron initiates enrolment in the VIEW 2 trial for Age-related macular degeneration in EU, Asia Pacific, Japan, and Latin America

2, including fatigue, nausea and vomiting. Observed grade 3 and 4 adverse events that were potentially drug related were grade 3 leukopenia, afebrile neutropenia and proteinuria, and grade 3 and 4 thromboembolic events including a transient cerebral ischaemia and a pulmonary embolism. Dose-related adverse events included hypertension and grade 1 hoarseness and anorexia. All patients who discontinued participation in the extension study withdrew due to disease progression, except one patient who developed grade 3 hypertension and proteinuria and was withdrawn after 22 weeks.[21]

The VEGF Trap administered intravenously every 2 weeks was generally well tolerated in a phase I, open-label, dose-ascending study in 27 patients with advanced solid tumours. The maximum tolerated dose has not been reached. The most frequently reported adverse events included fatigue, pain and constipation. The majority of adverse events were mild to moderate by nature. Occasional adverse events, including hypertension, were manageable and reversible. There were no anti-VEGF Trap antibodies detected.[9]

1.3 Pharmacodynamics

1.3.1 Cancer

Preclinical studies: VEGF Trap inhibited VEGF and destroyed mature, pre-existing vasculature in nude mice bearing established Wilms' tumour (SK-NEP-1) xenografts. This could provide an alternative therapeutic option for patients with bulky, metastatic cancers. Destruction of blood vessels was followed by marked tumour regression that included regression of lung micrometastases. The size of pulmonary metastases was significantly smaller in the lungs of VEGF Trap-treated animals compared with controls. These observations indicated that VEGF inhibition by VEGF Trap had interrupted cell signalling of the endothelial-vascular wall essential for the protection of tumours from apoptosis. Thus, it was concluded that even low levels of VEGF could be critical to the integrity of blood vessels and the maintenance of even the smallest tumour masses.[22]

VEGF Trap inhibited the development of ascites and decreased tumour burden in animal models of ovarian cancer. Findings indicated that VEGF Trap's activity was facilitated by inhibition of tumour angiogenesis as well as reduction in vascular permeability. In the first model, SKOV-3 ovarian carcinoma cells were engineered to overexpress VEGF (SKOV-VEGF) and then injected into the peritoneum of female nude mice. The animals were then administered subcutaneous (SC) VEGF Trap 25 mg/kg or control solution twice weekly until they had lost >10% of bodyweight or had persistent ascites. In the second model, OVCAR-3 ovarian cancer cells were injected into the peritoneum of athymic Balb/C nude mice. Fourteen days later, twice-weekly treatment with subcutaneous VEGF Trap 25 mg/kg or control solution was initiated and continued for 5 weeks. Ascites developed considerably earlier in control animals injected with SKOV-VEGF cells, compared with those injected with unaltered SKOV-3 cells. In contrast, the majority of mice administered VEGF Trap did not develop ascites, and those that did develop ascites had much lower volumes of fluid, compared with controls, according to the researchers. The mean volume of ascites in the OVCAR-3 model was also significantly lower in the VEGF Trap group, compared with controls. In fact, VEGF Trap completely inhibited the development of measurable ascites. Furthermore, tumour burden was reduced by 56% in the VEGF Trap group, compared with the controls.[23]

1.3.2 Eye Disorders

Preclinical studies: Administration of VEGF Trap (either subcutaneously or directly into the eye) resulted in significantly less new blood vessel growth, and also blocked leaking of blood vessels usually caused by VEGF, in two groups of mice. In the first group, mice with laser-induced rupture of Bruch's membrane received a single intravitreous injection of VEGF Trap. In the second group, mice genetically engineered to express VEGF in the retina received subcutaneous injections of VEGF Trap. No adverse effects were observed in these studies.[24]

1.4 Therapeutic Trials

1.4.1 Cancer

Ovarian cancer: Preliminary results of an open-label, phase I trial of a single dose of subcutaneous aflibercept (25, 50, 100 or 200 μg/kg) followed 4 weeks later by six weekly doses in patients with solid tumours or lymphoma (n = 14 treated to date) showed stable disease in patients with renal cell carcinoma (up to 15 weeks) and colon cancer.[20]

Preliminary efficacy data of the aflibercept administered intravenously every 2 weeks showed the reduction of tumour size and prolonged stable disease in some patients. A partial response with disappearance of ascites has been achieved in one patient, two patients had minor responses, and a stable disease was maintained in one patient for more than 11 months.[9]

1.4.2 Eye Disorders

Age-related macular degeneration (AMD): At 32 weeks, the 157 patients receiving either 0.5 or 2.0 mg followed by as-needed (PRN) dosing achieved mean improvements in visual acuity of 8.0 and 10.1 letters, respectively, and mean decreases in retinal thickness of 141 and 162 microns, respectively.

While PRN dosing also maintained the improvements versus baseline following a fixed dosing regimen (quarterly dosing at baseline and week 12), the results achieved were generally not as robust as those achieved with initial fixed monthly dosing. After the last fixed-dose administration at week 12, patients from all dose groups required on average only one additional injection over the following 20 weeks to maintain visual acuity gain achieved. Fifty-five percent of patients receiving 2.0 mg monthly for 12 weeks did not require any additional treatment throughout the next 20-week PRN dosing period.[14]

Preliminary results from a phase I trial in 20 patients with wet AMD have shown rapid, substantial and prolonged (≥4 weeks) reductions in retinal thickness with single-dose intravitreal injections of VEGF Trap. Ninety-five percent of patients had stabilization or improvement in visual acuity.[16,25]

Preliminary results from a phase I trial in 25 patients with advanced AMD showed a statistically significant decrease in excess retinal thickness with VEGF Trap (0.3, 1.0 and 3.0 mg/kg) compared with placebo.[19]

References

1. Bayer HealthCare, Regeneron Pharmaceuticals Inc. Bayer HealthCare and Regeneron to Collaborate on VEGF Trap for the Treatment of Eye Diseases. Media Release: 18 Oct 2006. Available from URL: http://www.bayer.com
2. Regeneron Pharmaceuticals Inc. Sanofi-Aventis and Regeneron Pharmaceuticals Expand Their VEGF Trap Oncology Collaboration to Japan. Media Release: 22 Dec 2005. Available from URL: http://www.sanofi-aventis.com
3. sanofi-aventis. Sanofi-aventis and Regeneron Pharmaceuticals Reaffirm Development Commitment. Media Release: 10 Jan 2005. Available from URL: http://www.sanofi-aventis.com
4. Aventis, Regeneron Pharmaceuticals Inc. Aventis and Regeneron Enter Global Partnership to Develop and Commercialize the VEGF Trap. Media Release: 8 Sep 2003. Available from URL: http://www.aventis.com
5. Massarelli E, Miller VA, Leighl NB, et al. Phase II study of the efficacy and safety of intravenous AVE0005 (VEGF Trap) given every 2 weeks in patients with platinum- and erlotinib-resistant adenocarcinoma of the lung. Journal of Clinical Oncology 25 (Suppl.): 416 (plus poster) abstr. 7627, No. 18, Part 1, 20 Jun 2007
6. Regeneron Pharmaceuticals Inc. Regeneron Reports Fourth Quarter and Full Year 2005 Financial and Operating Results. Media Release: 24 Feb 2006. Available from URL: http://www.regeneron.com
7. Regeneron Pharmaceuticals Inc. Regeneron Reports Positive Preliminary Results from Phase 1 Study of the VEGF Trap in Patients with Advanced Solid Tumors. Media Release: 6 Jun 2004. Available from URL: http://www.regn.com
8. Regeneron Pharmaceuticals Inc. Regeneron Reports Second Quarter Financial and Operating Results; BLA Filing for Auto-Inflammatory Diseases Planned for Early 2007; Two Antibody Candidates from VelocImmune(R) Program to Enter Clinical Trials Each Year Beginning in 2007. Media Release: 3 Aug 2006. Available from URL: http://www.regeneron.com
9. Regeneron Pharmaceuticals Inc. Regeneron's VEGF Trap Demonstrates Positive Preliminary Results from Single-Agent Phase 1 Trial in Patients with Advanced Cancer; Results Presented at ASCO Annual Meeting on May 16, 2005. Media Release: 16 May 2005. Available from URL: http://www.regeneron.com
10. Regeneron Pharmaceuticals Inc. Regeneron Receives $20 Million Milestone Payment for Initiation of Phase 3 Study of VEGF Trap-Eye in Wet AMD. Media Release: 14 Aug 2007. Available from URL: http://www.regeneron.com
11. Regeneron Pharmaceuticals Inc, Bayer HealthCare. Regeneron and Bayer HealthCare Initiate Phase 3 Global Development Program For VEGF Trap-Eye In Wet Age-Related Macular Degeneration (AMD). Media Release: 3 Aug 2007. Available from URL: http://www.regeneron.com
12. Bayer HealthCare AG. Bayer and Regeneron start additional Phase 3 Study for VEGF Trap-Eye in Wet Age-related Macular Degeneration. Media Release: 8 May 2008. Available from URL: http://www.bayerscheringpharma.de
13. Bayer HealthCare AG, Regeneron Pharmaceuticals Inc. Bayer and Regeneron Dose First Patient in Second Phase 3 Study for VEGF Trap-Eye in Wet Age-Related Macular Degeneration. Media Release: 8 May 2008. Available from URL: http://www.bayerhealthcare.com
14. Regeneron Pharmaceuticals Inc, Bayer HealthCare AG. Regeneron and Bayer HealthCare Announce Encouraging 32-Week Follow-Up Results from a Phase 2 Study of VEGF Trap-Eye in Age-Related Macular Degeneration. Media Release: 29 Apr 2008. Available from URL: http://www.regeneron.com
15. Regeneron Pharmaceuticals Inc. Regeneron Announces Positive Primary Endpoint Results from a Phase 2 Study of VEGF Trap-Eye in Age-related Macular Degeneration. Media Release: 1 Oct 2007. Available from URL: http://www.regeneron.com
16. Regeneron Pharmaceuticals Inc. Regeneron Reports Positive Phase 1 Data for the VEGF Trap in Age-Related Macular Degeneration; Preliminary Results Show Improvements in Vision and Retinal Swelling; VEGF Trap Was Well Tolerated at All Dose Levels. Media Release: 1 May 2006. Available from URL: http://www.regeneron.com
17. Regeneron Pharmaceuticals Inc. VEGF Trap-Eye Phase 2 Wet AMD Results Reported at ARVO Annual Meeting. Media Release: 10 May 2007. Available from URL: http://www.regeneron.com
18. Regeneron Pharmaceuticals Inc. Regeneron Reports Fourth Quarter and Full Year 2004 Financial and Operating Results. Media Release: 22 Feb 2005. Available from URL: http://www.regeneron.com
19. Regeneron Pharmaceuticals Inc. Clinical and Pre-Clinical Studies of the VEGF Trap Show Potential for the Treatment of Neovascular Eye Diseases. Media Release: 3 May 2005. Available from URL: http://www.regeneron.com
20. Dupont J, Camastra D, Gordon MS, et al. Phase 1 study of VEGF Trap in patients with solid tumors and lymphoma. 39th

Annual Meeting of the American Society of Clinical Oncology: 194, 31 May 2003
21. Dupont J, Schwartz L, Koutcher J, et al. A phase I and pharmacokinetic clinical trial of subcutaneous VEGF Trap in advanced solid tumor patients. EJC Supplements 2: 43 (plus poster) abstr. 132, 29 Sep 2004
22. Huang J, Frischer JS, Serur A, et al. Regression of established tumors and metastases by potent vascular endothelial growth factor blockade. Proceedings of the National Academy of Sciences of the United States of America 100: 7785-7790, 24 Jun 2003
23. Byrne AT, Ross L, Holash J, et al. Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. Clinical Cancer Research 9: 5721-5728, No. 15, 15 Nov 2003
24. Johns Hopkins' Wilmer Eye Institute. Injection Prevents Blinding Blood Vessel Growth in Mice. Media Release: 17 Jun 2003. Available from URL: http://www.hopkinsmedicine.org
25. Regeneron Pharmaceuticals Inc. Regeneron's VEGF Trap Demonstrates Positive Preliminary Results in Patients with Age-Related Macular Degeneration; Phase 1 Preliminary Results to Be Presented at the Angiogenesis 2006 Meeting Sponsored by Bascom Palmer Eye Institute. Media Release: 2 Feb 2006. Available from URL: http://www.regeneron.com

June 17, 2011: Dermatologic and Ophthalmic Drugs Advisory Committee Meeting Announcement

Center	Date	Time	Location
CDER	June 17, 2011	8:00 a.m. - 4:30 p.m.	The Inn & Conference Center University Maryland University College (UMUC) Marriott Conference Centers 3501 University Blvd. East Adelphi, Maryland Telephone: 301-985-7300

Agenda

On June 17, 2011, the committee will discuss biologic license application (BLA) 125387, aflibercept ophthalmic solution, proposed trade name EYLEA, sponsored by Regeneron Pharmaceuticals, Inc., indicated for the treatment of neovascular age-related macular degeneration (wet AMD).

Meeting Materials

FDA intends to make background material available to the public no later than 2 business days before the meeting. If FDA is unable to post the background material on its Web site prior to the meeting, the background material will be made publicly available at the location of the advisory committee meeting, and the background material will be posted on FDA's Web site after the meeting.

- **2011 Meeting Materials, Dermatologic and Ophthalmic Drugs Advisory Committee (/7993/20170112101438/http://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/DermatologicandOphthalmicDrugsAdvisoryCommittee/ucm256601.htm)**

Public Participation Information

** Deadlines for Open Public Hearing Registration and Written Submissions Deadlines Extended – see below **

Interested persons may present data, information, or views, orally or in writing, on issues pending before the committee.

- Written submissions may be made to the contact person on or before June 10, 2010.
- Oral presentations from the public will be scheduled between approximately 1 p.m. and 2 p.m. Those desiring to make formal oral presentations should notify the contact person and submit a brief statement of the general nature of the evidence or arguments they wish to present, the names and addresses of proposed participants, and an indication of the approximate time requested to make their presentation on or before June 8, 2011.

Time allotted for each presentation may be limited. If the number of registrants requesting to speak is greater than can be reasonably accommodated during the scheduled open public hearing session, FDA may conduct a lottery to determine the speakers for the scheduled open public hearing session. The contact person will notify interested persons regarding their request to speak by June 9, 2011.

Contact Information

- Yvette Waples, Pharm.D.
  Center for Drug Evaluation and Research
  Food and Drug Administration
  10903 New Hampshire Avenue
  WO31-2417
  Silver Spring, Maryland 20993-0002
  Phone: 301-796-9001
  Fax: 301-847-8533
  E-mail: **DODAC@fda.hhs.gov (mailto:DODAC@fda.hhs.gov)**

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Division of Transplant and Ophthalmology Products Advisory Committee Meeting

Aflibercept injection

Sonal D. Wadhwa, MD
U.S. Food and Drug Administration
Medical Officer

June 17, 2011

Applicant Information

- Applicant: Regeneron Pharmaceuticals, Inc.
  777 Old Saw Mill River Road
  Tarrytown, NY 10591

Introduction and Background

- VEGF Trap-Eye (aflibercept) is a recombinant protein.
- VEGF Trap-Eye is a specific antagonist that binds and inactivates circulating VEGF and PlGF.

Drug Information

- Proposed Proprietary Name: Eylea
- Established name: aflibercept injection
- Pharmacologic Category: VEGF inhibitor
- Dosage Form and Route of Administration: intravitreal injection

Applicant Proposed Indication

- Treatment of patients with neovascular (wet) age-related macular degeneration (AMD)

Currently Available Treatments for Proposed Indication

Drug	Approval
Photodynamic therapy (PDT)/Verteporfin	April 2000
Macugen (pegaptanib injection)	December 2004
Lucentis (ranibizumab injection)	June 2006

Introduction and Background

- In comparison:
  - Pegaptanib (Macugen) is an inhibitor of the VEGF165 isomer.
  - Ranibizumab (Lucentis) is an inhibitor of all VEGF-A isomers.

Table of Clinical Trials

Study	Title	Type of Study	Number of Patients
VIEW #1 (VGFT-OD-0605)	A Randomized, Double-Masked Active Controlled Phase 3 Study of the Efficacy, Safety, and Tolerability of Repeated Doses of Intravitreal VEGF Trap-Eye in Subjects With Neovascular AMD	Safety and Efficacy	1217
VIEW #2 (311523)	A Randomized, Double-Masked, Active Controlled, Phase 3 Study of the Efficacy, Safety, and Tolerability of Repeated Doses of Intravitreal VEGF Trap-Eye in Subjects With Neovascular AMD	Safety and Efficacy	1240

Primary Objective of VIEW #1 and VIEW #2

- To assess the efficacy of intravitreally administered aflibercept compared to ranibizumab (in a non-inferiority paradigm) in preventing moderate vision loss in subjects with all sub-types of neovascular AMD.

- Moderate vision loss is defined as loss of fewer than 15 letters in ETDRS letter score compared to Baseline.

VIEW #1 and VIEW #2

- On day 1, subjects were randomly assigned in a 1:1:1:1 ratio to 1 of 4 dosing regimens:
  - 2 mg aflibercept administered every 4 weeks (2Q4)
  - 0.5 mg aflibercept administered every 4 weeks (0.5Q4)
  - 2 mg aflibercept administered every 8 weeks (2Q8) plus a sham injection at interim 4-week visits (when study drug was not administered) following 3 initial monthly doses
  - 0.5 mg ranibizumab administered every 4 weeks (RQ4)

VIEW #1 and VIEW #2

- The study consists of a 21-day screening period followed by clinic visits and IVT injections of study drug administered every 4 or 8 weeks (including sham injections at interim study visits when study drug was not administered) for 52 weeks (total of 16 visits) during the first year of the study.

VIEW #1 and VIEW #2

- The entire study duration is approximately 2 years (96 weeks plus the recruitment period).
- During the second year of treatment, sham injections will not be given.

VIEW #1 and VIEW #2

- During the second year of treatment, subjects will be evaluated every 4 weeks and will receive IVT injections of study drug at intervals determined by specific dosing criteria, but at least every 12 weeks. Therefore, patients will get drug every 4-12 weeks.

VIEW #1 and VIEW #2

- The pre-specified dosing criteria are:
  - Increase in central retinal thickness >=100 microns compared to lowest previous value as measured by OCT
  - A loss from the best previous letter score of >=5 ETDRS letters in conjunction with recurrent fluid as indicated by OCT
  - New or persistent fluid as indicated by OCT
  - New onset classic neovascularization
  - New or persistent leak on FA
  - New macular hemorrhage
  - 12 weeks has elapsed since the previous injection

Inclusion Criteria

- Signed informed consent
- Men and women ≥ 50 years of age
- Active primary subfoveal CNV lesion secondary to AMD, including juxtafoveal lesions that affected the fovea as evidenced by FA in the study eye
- CNV must be at least 50% of total lesion size
- ETDRS BCVA: 20/40-20/320 in the study eye
- Willing, committed, and able to return for all clinic visits and completed all study-related procedures
- Understand and willing to sign the ICF

Protocol Defined Analysis Populations

- Safety set: All subjects who received any study drug.
- Full analysis set: All randomized subjects who received any study drug and had a Baseline and at least one post-Baseline BCVA assessment.

Protocol Defined Analysis Populations

- Per protocol:
  - All subjects in the FAS who received at least 9 injections of study drug or sham and attended at least 9 scheduled visits during the first year, except for those who were excluded because of major protocol violations.
  - A major protocol violation was one that may affect the interpretation of study results (i.e. missing two consecutive injections before administration of the 9th injection).

Treatment Failure

- A treatment failure was a subject who had a decrease from Baseline in BCVA of at least 15 letters at two consecutive assessments, 4 weeks apart, during the first 52 weeks of the study.

VIEW #1 : Patient Disposition

	RQ4	2Q4	0.5Q4	2Q8
Randomized	306	304	304	303
Safety Set (SAF)	304	304	304	303
Full Analysis Set (FAS)	304	304	301	301
Per Protocol (PP)	269	285	270	265

VIEW #2 : Patient Disposition

	RQ4	2Q4	0.5Q4	2Q8
Randomized	303	313	311	313
Safety Set (SAF)	291	309	297	307
Full Analysis Set (FAS)	291	309	296	306
Per Protocol (PP)	269	274	268	270

Primary Efficacy Endpoint

- Primary efficacy variable:
  - The proportion of subjects who maintained vision at Week 52

Primary Efficacy Endpoint

- The primary analysis is an evaluation of the non-inferiority of aflibercept to ranibizumab and includes the following conditional sequence of calculations of the confidence intervals for the difference between treatments in proportion of subjects maintaining vision at Week 52:
  - Comparison 1: aflibercept 2mg q4 weeks versus ranibizumab
  - Comparison 2: aflibercept 0.5mg q4 weeks versus ranibizumab
  - Comparison 3: aflibercept 2mg q8 weeks versus ranibizumab

Primary Efficacy Endpoint

- The non-inferiority margin in individual VIEW #1 and VIEW #2 studies was 10%.
- Aflibercept was to be considered non-inferior to ranibizumab if the confidence interval of the difference lay entirely below 10%, where a positive difference favors ranibizumab.

Primary Efficacy Endpoint

- Once the non-inferiority was demonstrated, the superiority of aflibercept to ranibizumab was examined. Aflibercept was considered to be superior to ranibizumab if the confidence interval of the difference entirely lay below 0.

- A subject who withdrew from the study before Week 36 due to treatment failure was considered a non-responder; otherwise the last observation carried forward (LOCF) approach was used to impute missing data in this primary efficacy analysis.

VIEW #1: Primary Efficacy Analysis (FAS Population with LOCF)

	RQ4 N=304	2Q4 N=304	0.5Q4 N=301	2Q8 N=301
Subjects with maintained vision at Week 52	285 (93.8%)	289 (95.1%)	286 (95.0%)	284 (94.4%)
Difference (%) (95.1% CI)		-1.3 (-5.0, 2.4)	-1.3 (-4.9, 2.4)	-0.6 (-4.4, 3.2)

VIEW #1: Primary Efficacy Analysis (PP Population with observed cases)

	RQ4 N=269	2Q4 N=285	0.5Q4 N=270	2Q8 N=265
Subjects With Maintained vision at Week 52	243 (94.9%)	260 (94.9%)	241 (96.4%)	237 (96.3%)
Difference (%) (95.1% CI)		0.0 (-3.7, 3.8)	-1.5 (-5.0, 2.1)	-1.4 (-5.0, 2.2)

VIEW #2: Primary Efficacy Analysis (FAS Population with LOCF)

	RQ4 N=291	2Q4 N=309	0.5Q4 N=296	2Q8 N=306
Subjects With Maintained vision at Week 52	276 (94.9%)	292 (94.5%)	282 (95.3%)	292 (95.4%)
Difference (%) (95.1% CI)		0.4 (-3.3, 4.0)	-0.4 (-4.0, 3.1)	-0.6 (-4.1, 2.9)

VIEW #2: Primary Efficacy Analysis (PP Population with observed cases)

	RQ4 N=261	2Q4 N=263	0.5Q4 N=257	2Q8 N=264
Subjects With Maintained vision at Week 52	246 (94.3%)	251 (95.4%)	248 (96.5%)	253 (95.8%)
Difference (%) (95.1% CI)		-1.2 (-5.0, 2.6)	-2.3 (-5.9, 1.4)	-1.6 (-5.3, 2.2)

Primary Efficacy Endpoint

- The two studies found all dosing regimens of aflibercept to be non-inferior to ranibizumab.
- Neither study found any of the aflibercept doses to be superior to ranibizumab.

Secondary Endpoint: VIEW #1 Mean Change From Baseline to Week 52 in ETDRS Letter Score in the Study Eye (Full Analysis Set with LOCF)

	R0.5Q4 N=304	2Q4 N=304	0.5Q4 N=301	2Q8 N=301
Baseline: Mean ETDRS letter score (sd)	54.0 (13.4)	55.2 (13.2)	55.6 (13.1)	55.7 (12.8)
Week 52: Mean ETDRS letter score (sd)	62.1 (17.7)	66.1 (16.2)	62.4 (16.5)	63.6 (16.9)
Mean change from baseline at Week 52 (sd)	8.1 (15.3)	10.9 (13.8)	6.9 (13.4)	7.9 (15.0)

Secondary Endpoint: VIEW #2 Mean Change From Baseline to Week 52 in ETDRS Letter Score in the Study Eye (Full Analysis Set with LOCF)

	R0.5Q4 N=291	2Q4 N=309	0.5Q4 N=296	2Q8N=306
Baseline: Mean ETDRS letter score (sd)	53.8 (13.5)	52.8 (13.9)	51.6 (14.2)	51.6 (13.9)
Week 52: Mean ETDRS letter score (sd)	63.1 (16.6)	60.4 (18.3)	61.3 (17.8)	60.5 (17.5)
Mean change from baseline at Week 52 (sd)	9.4 (13.5)	7.6 (12.6)	9.7 (14.1)	8.9 (14.4)

Safety

- The main support of safety for aflibercept came from VIEW #1 and VIEW #2.

VIEW #1: Treatment Exposure During the First Year (Safety Analysis Set)

	RQ4 N=304	2Q4 N=304	0.5Q4 N=304	2Q8 N=303
Mean Number of Injections During the First Year Including Sham (sd)	12.1 (2)	12.5 (1)	12.1 (2)	12.0 (2)
Mean Number of Injections During the First Year Excluding Sham (sd)	12.1 (2)	12.5 (1)	12.1 (2)	7.5 (1)
Mean Total Amount of Study Medication During the First Year in mg (sd)	6.0 (1)	24.9 (2)	6.0 (1)	14.9 (2)

VIEW #2: Treatment Exposure During the First Year (Safety Analysis Set)

	RQ4 N=291	2Q4 N=309	0.5Q4 N=297	2Q8 N=307
Mean Number of Injections During the First Year Including Sham (sd)	12.7 (1)	12.6 (1)	12.7 (1)	12.6 (1)
Mean Number of Injections During the First Year Excluding Sham (sd)	12.7 (1)	12.6 (1)	12.7 (1)	7.7 (1)
Mean Total Amount of Study Medication During the First Year in mg (sd)	6.2 (1)	24.4 (4)	6.2 (1)	15.1 (3)

VIEW #1: Deaths

- In VIEW #1 there were a total of 17 deaths (5 subjects in the RQ4 group, 2 subjects in the 2Q4 group, 2 subjects in the 0.5Q4 group, and 8 subjects in the 2Q8 group) during Year 1.

- In VIEW #2 there were a total of 9 deaths (2 subjects in the RQ4 group, 3 subjects in the 2Q4 group, 2 subjects in the 0.5Q4 group, and 2 subjects in the 2Q8 group) during Year 1.

VIEW #1: Nonfatal Serious Adverse Events

	RQ4 N=304	2Q4 N=304	0.5Q4 N=304	2Q8 N=303
Number of subjects with at least 1 ocular SAE in study eye	10 (3.3%)	7 (2.3%)	6 (2.0%)	3 (1.0%)
Number of subjects with at least 1 non-ocular SAE	57 (18.8%)	40 (13.2%)	50 (16.4%)	51 (16.8%)

VIEW #1: Nonfatal Serious Adverse Events Ocular

- The most common ocular SAEs were:
  - Endophthalmitis (6 patients)
  - Reduced visual acuity (5 patients)
  - Retinal hemorrhage (4 patients)

VIEW #1: Nonfatal Serious Adverse Events Non-Ocular

- The most common non-ocular SAEs were:
  - Infections (44 patients)
  - Cardiac (42 patients)
  - Neoplasms (38 patients)

VIEW #2: Nonfatal Serious Adverse Events

	RQ4 N=291	2Q4 N=309	0.5Q4 N=297	2Q8 N=307
Number of subjects with at least 1 ocular SAE in study eye	9 (3.1%)	6 (1.9%)	5 (1.7%)	9 (2.9%)
Number of subjects with at least 1 non-ocular SAE	26 (8.9%)	36 (11.7%)	37 (12.5%)	38 (12.2%)

VIEW #2: Nonfatal Serious Adverse Events Ocular

- The most common ocular SAEs were:
  - Visual acuity reduced (8 patients)
  - Retinal hemorrhage (5 patients)
  - Cataract (3 patients)
- Endophthalmitis (0 patients)

VIEW #2: Nonfatal Serious Adverse Events Non-Ocular

- The most common non-ocular SAEs were:
  - Cardiac disorders (31 patients)
  - Neoplasms (20 patients)
  - Infections (18 patients)

VIEW #1: Disposition

	RQ4	2Q4	0.5Q4	2Q8
Randomized	306	304	304	303
Completed first year of study	284 (92.8%)	293 (96.4%)	277 (91.1%)	276 (91.1%)
Discontinuation from study within first year	22	11	27	27
AE	4	3	5	4
Death	3	1	2	7
Withdrawal by subject	10	5	7	8
Protocol deviation	3	0	3	1
Lost to f/u	1	2	4	4
Treatment failure	0	0	2	2
Other	1	0	4	1

VIEW #2: Disposition

	RQ4	2Q4	0.5Q4	2Q8
Randomized	303	313	311	313
Completed first year of study	276 (91.1%)	281 (89.8%)	274 (88.1%)	284 (90.7%)
Discontinuation from study within first year	27	32	37	29
AE	2	6	8	9
Death	1	3	2	1
Withdrawal by subject	11	15	13	11
Protocol deviation	2	1	1	0
Lost to f/u	4	1	2	2
Treatment failure	0	0	1	1
Other	7	6	10	5

Adverse Events: VIEW #1

- The most common treatment emergent ocular AEs were:
  - Conjunctival hemorrhage
  - Vitreous floaters
  - Eye pain

Adverse Events: VIEW #2

- The most common treatment emergent ocular AEs were:
  - Visual acuity reduced
  - Conjunctival hemorrhage
  - Retinal hemorrhage

VIEW #2: Special Safety Study
Nasomucosal examination (ENT sub-study)

- A subset of 160 subjects in VIEW #2 were additionally examined by an ENT specialist and had a nasal endoscopy.

- The purpose of the ENT sub-study was to better define potential nasomucosal side effects which were reported as histopathologic findings in a toxicology study (VGFT-TX-0511 or COV7369-112).

VIEW #2: Special Safety Study
Nasomucosal examination (ENT sub-study)

- A careful endoscopy of the nasal airways with a standardized documentation of findings comprised the rhinological investigation at Visit 2 (baseline nasal endoscopy).

- At Visit 6 and Visit 16, the participants were re-evaluated by an ENT specialist. The ENT specialist had to ask for nose bleeds and new nasal symptoms since the last ENT visit, and repeat nasal endoscopy was performed.

VIEW #2: Special Safety Study
Nasomucosal examination (ENT sub-study)

	R0.5Q4 N=37	2Q4 N=42	0.5Q4 N=37	2Q8 N=44
Nasal septum deviation	4	2	0	5
Nasal mucosal disorder	1	1	2	4
Rhinorrhea	0	1	2	4
Epistaxis	1	1	1	3
Nasal polyps	1	1	1	2
Nasal turbinate hypertrophy	0	0	1	2
Nasal dryness	0	0	0	1

VIEW #2: Special Safety Study
Nasomucosal examination (ENT sub-study)

	R0.5Q4 N=37	2Q4 N=42	0.5Q4 N=37	2Q8 N=44
Nasal mucosal discoloration	0	0	1	1
Nasal edema	0	0	0	1
Paranasal cyst	0	0	1	1
Rhinitis hypertrophy	1	0	0	0
Nasopharyngitis	5	2	4	8
Upper respiratory infection	1	1	1	4
Rhinitis	2	0	1	1
Viral rhinitis	0	0	1	1
Acute tonsillitis	1	0	0	0

VIEW#1: Number of Subjects with APTC Arterial Thromboembolic Events Through Year 1 (Safety Analysis Set)

	R0.5Q4 N=304	2Q4 N=304	0.5Q4 N=304	2Q8 N=303
Any APTC event	5 (1.6%)	2 (0.7%)	7 (2.3%)	6 (2.0%)
Non-fatal myocardial infarctions	4	1	4	1
Non-fatal strokes	0	1	2	1
Vascular deaths	1	0	1	4

VIEW#2: Number of Subjects with APTC Arterial Thromboembolic Events Through Year 1 (Safety Analysis Set)

	R0.5Q4 N=291	2Q4 N=309	0.5Q4 N=297	2Q8 N=307
Any APTC event	5 (1.7%)	4 (1.3%)	5 (1.7%)	8 (2.6%)
Non-fatal myocardial infarctions	2	2	2	5
Non-fatal strokes	2	1	1	2
Vascular deaths	1	1	2	1

VIEW #1: Number of Subjects With An Absolute Value of IOP >= 35mmHg During the Study (Safety Analysis Set)

	R0.5Q4 N=304	2Q4 N=304	0.5Q4 N=304	2Q8 N=303
Any Visit	13	13	7	13

VIEW #2: Number of Subjects With An Absolute Value of IOP >= 35mmHg During the Study (Safety Analysis Set)

	R0.5Q4 N=291	2Q4 N=309	0.5Q4 N=297	2Q8 N=307
Any Visit	9	9	4	5

VIEW #1: Proportion of Subjects With >=10mmHg Increase in IOP From Baseline to Any Pre-Dose Measurement (Safety Analysis Set)

	R0.5Q4 N=304	2Q4 N=304	0.5Q4 N=304	2Q8 N=303
Pre-dose from baseline	12	5	6	7

VIEW #2: Proportion of Subjects With >=10mmHg Increase in IOP From Baseline to Any Pre-Dose Measurement (Safety Analysis Set)

	R0.5Q4 N=291	2Q4 N=309	0.5Q4 N=297	2Q8 N=307
Pre-dose from baseline	7	3	8	7

Post-marketing Experience

- Because aflibercept is not marketed in any country, no sources of AE information exist, except for clinical study reports of the trials that were conducted for its development.

Questions for the Advisory Committee

- Do you think adequate safety and efficacy for aflibercept injection has been demonstrated for the treatment of neovascular AMD?
  - If yes, on which study(ies) are you basing your decision?
  - If not, what additional study(ies) should be performed? Do you have any suggestions regarding trial design?

Questions for the Advisory Committee

- What dosing should be approved (0.5mg Q4, 2mg Q4, or 2mg Q8)?
- If recommend approving a Q8 schedule should patients be monitored Q4?

Questions for the Advisory Committee

- Elevations in IOP following repeated dosing of VEGF-inhibitors have been reported in the literature and are seen in low frequency in the trials of aflibercept. Do you have recommendations regarding ways to handle the issue?

Questions for the Advisory Committee

- Do you have any suggestions concerning the proposed draft labeling of the product?

Division of Transplant and Ophthalmology Products Advisory Committee Meeting

Aflibercept injection

Sonal D. Wadhwa, MD
US Food and Drug Administration
Medical Officer
June 17, 2011

[redacted]

[redacted]

Study VGFT-OD-0702

- Primary objectives:
  - Allow subjects previously enrolled in VGFT-OD-0502, -0508, and -0603 to continue to receive VEGF Trap-Eye after completion of dosing in those studies.
  - Assess the long-term safety and tolerability of repeated IVT administration of VEGF Trap-Eye in subjects with all sub-types of neovascular AMD for periods of up to 3 years.

Study VGFT-OD-0702

- Secondary objectives:
  - Assess the safety of using VEGF Trap-Eye in PFS syringes and Vials
  - Assess the frequency of re-treatment
  - Assess the effect of continued VEGF Trap-Eye treatment on best corrected visual acuity (BCVA)

Study VGFT-OD-0702

- VGFT-OD-0702 was a single-masked (to the subject), randomized, multi-center clinical study.

- Subjects were initially enrolled to receive VEGF Trap-Eye from a Vial. After 152 subjects had been enrolled, a PFS syringe was introduced as a result of Protocol Amendment 1. From that point, upon enrollment, subjects were randomly assigned in 2:1 ratio to receive:
  - 2 mg VEGF Trap-Eye PRN in a 50 µL injection volume from a PFS (Sealed, sterile 3 mL Vials of approximately 0.5 mL of VEGF Trap-Eye. The VEGF Trap-Eye was withdrawn into a 1 mL syringe using aseptic technique. A sterile 30-gauge needle was used for intravitreal injection).
  - 2 mg VEGF Trap-Eye PRN in a 50 µL injection volume from a Vial (Single-use, PFS glass syringes with Snap-off Tip Cap. A plastic plunger rod was attached to the rubber stopper inside the barrel of the syringe. After removing the syringe cap, a 30-gauge needle was attached for administration).

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Advisory Committees

Slides for the December 1, 2011 Joint Meeting of the Drug Safety and Risk Management Advisory Committee and the Dermatologic and Ophthalmic Drugs Advisory Committee

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Morning Session

FDA

FDA Presentation for the December 01, 2011 Joint Meeting of the Drug Safety and Risk Management Advisory Committee and the Dermatologic and Ophthalmic Drugs Advisory Committee (PDF - 689KB)[1]

Mylan Pharmaceuticals

Mylan Main Presentation for the December 01, 2011 Joint Meeting of the Drug Safety and Risk Management Advisory Committee and the Dermatologic and Ophthalmic Drugs Advisory Committee (PDF - 993KB)[2]

Mylan Back-up Presentation for the December 01, 2011 Joint Meeting of the Drug Safety and Risk Management Advisory Committee and the Dermatologic and Ophthalmic Drugs Advisory Committee (PDF - 877KB)[3]

Afternoon Session

FDA

FDA Presentation for the December 01, 2011 Joint Meeting of the Drug Safety and Risk Management Advisory Committee and the Dermatologic and Ophthalmic Drugs Advisory Committee (PDF - 1.50MB)[4]

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Opening Remarks for Drug Safety and Risk Management and Dermatologic and Ophthalmic Advisory Committees Meeting

Claudia Karwoski, Pharm.D., Director
Division of Risk Management
Office of Medication Error Prevention and Risk Management
Office of Surveillance and Epidemiology
Center for Drug Evaluation and Research

December 1, 2011

Background

- Food and Drug Administration Amendments Act (FDAAA) of 2007
  - provides FDA the authority to require a a Risk Evaluation and Mitigation Strategy (REMS) if the Agency determines that a REMS is necessary to ensure the benefits of the drug outweigh the risk.

Elements of a REMS

- A REMS can include:
  - A Medication Guide or patient package insert
  - A Communication Plan to healthcare providers
  - Elements to assure safe use (ETASU)
  - An implementation system
  - Timetable for submission of assessments

Elements to Assure Safe Use

- The following elements can be included:
  - Healthcare providers who prescribe the drug have particular training or experience, or are specially certified
  - Pharmacies, practitioner, or health care setting that dispense the drug are specially certified
  - The drug is to be dispensed to patients only in certain health care settings, such as hospitals
  - The drug is to be dispensed to patients with evidence or other documentation of safe use conditions, such as laboratory test results
  - Each patient using the drug is to be subject to certain monitoring
  - Each patient using the drug is to be enrolled in a registry

Additional FDAAA Requirement

- Requires the Agency, at least annually,
  - to bring at least one drug with a REMS with elements to assure safe use (ETASU) to the Drug Safety and Risk Management Advisory Committee (DSaRM)
  - solicit views of DSaRM on whether the elements
    - Assure safe use of the drug
    - Are not unduly burdensome on patient access to the drug
    - To the extent practicable, minimize the burden on the healthcare delivery system

Today's Meeting

- The morning meeting will focus on the evaluation of one drug that has a REMS with ETASU, the isotretinoin REMS, called iPLEDGE
- The afternoon meeting will be a general discussion of how REMS programs may be implemented to minimize the negative effects on patient access to drugs covered by REMS and to decrease the burdens of REMS on the healthcare system
  - The afternoon meeting will not include any drug-specific information

Conclusion

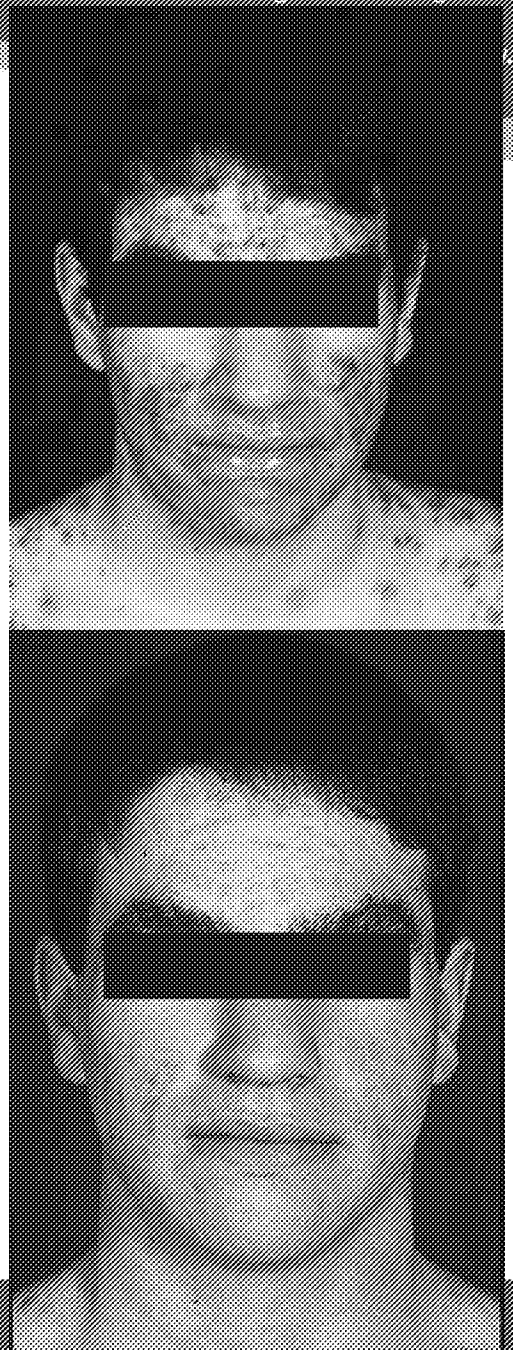
- The committee members will be asked to consider and discuss several points
- This meeting represents the first opportunity
  - for FDA to discuss an assessment of a REMS with ETASU
  - to discuss important REMS-related issues
- We look forward to the discussion

History of Pregnancy Exposure Risk Management for Isotretinoin

Jill Lindstrom, MD FAAD
Lead Medical Officer
Division of Dermatology and Dental Products
Office of New Drugs, ODE III
Center for Drug Evaluation and Research

Isotretinoin indication

- Severe recalcitrant nodular acne
- Sole drug approved
- Complete and prolonged disease remission in many patients after single 15-20 week course of therapy
- Off-label use includes
  - Scarring non-nodular acne
  - Neuroblastoma
  - Other cancers



Regulatory history

- Accutane approved 1982
- Generic products
  - Amnesteem 11/2002
  - Sotret 12/2002
  - Claravis 4/2003
- Accutane withdrawn 2010
  - Not for safety or efficacy

Teratogen

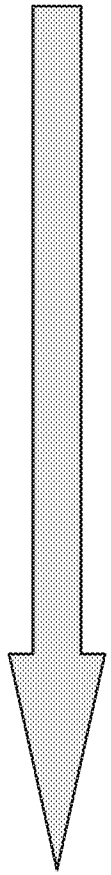
- Increased risk of spontaneous abortion and premature birth
- Major malformations: craniofacial, cardiac, thymus, CNS, functional
- High frequency of effect in exposed pregnancies
- No known safe dose/exposure window during gestation
- Long history of risk management

Risk management

- Risk assessment + risk minimization
- Four step process
  - Assessment of benefit-risk balance
  - Development and implementation of tools to minimize risks while preserving benefits
  - Evaluation of tool effectiveness and reassessment of benefit-risk balance
  - Adjustment of tools to further enhance benefit-risk balance

Risk minimization tool kit

- Routine measures
  - Rx status
  - Professional labeling
- Targeted education and outreach
  - Dear Health Care Provider letters
  - Medication Guides
- Reminder systems
  - Consent forms
  - Limitations on amounts dispensed/refills
  - Specialized product packaging
  - Prescription stickers
- Performance-linked access systems
  - Dispensing only with documentation of safe-use conditions



Four eras of isotretinoin risk management

1. 1982 – 1988: early marketing
2. 1988 – 2002: Accutane Pregnancy Prevention Program
3. 2002 – 2005: sticker-based programs
4. 2006 – present: iPLEDGE

Early marketing: 1982-1988

- Nonclinical signal for teratogenesis
  - Contraindications, Warnings, Precautions
  - Pregnancy category X
- Human data accrued (fetal exposure)
  - Labeling: Boxed warning 1984
  - Dear Doctor/Dear Pharmacist letters
  - Red warning stickers for pharmacist to apply to dispensed prescriptions
- Pregnancy exposures continued

Pregnancy Prevention Program: 1988-2002

- Sponsor proposed Pregnancy Prevention Program (PPP)
- PPP presented to the advisory committee in 1988

PPP Elements 1988-2002

- Revised labeling
- Targeted education and outreach tools
  - Dear Doctor letters
  - Patient brochures
- Reminder tools
  - Patient informed consent forms
  - Blister pack w/"avoid pregnancy" icon
  - Limitation of amount dispensed
- Assessment instruments: patient and prescriber surveys

PPP Assessment 1988-2002

- Substantial non-compliance with critical elements of PPP
- Low survey participation
- Pregnancy exposures continued
- Advisory committee convened in 2000

2000 Advisory Committee goals for isotretinoin risk management

- No woman begin isotretinoin therapy if she is pregnant
- No pregnancies occur while a woman is taking isotretinoin

Advisory Committee recommendations in 2000

- Augmentation of patient education
- Registration of all patients
- Registration of prescribers
- Implementation of pregnancy registry
- Linkage of prescription to adequate pregnancy testing

Sticker programs: 2002-2005

- Based on need for fetal exposure risk management, AC recommendations, and extensive discussions and negotiations between HLR and FDA

- Approved for innovator in October 2001
  - Accutane S.M.A.R.T.

- Generics' plans included essential elements
  - Amnesteem: S.P.I.R.I.T. November 2002
  - Sotret: I.M.P.A.R.T. December 2002
  - Claravis: A.L.E.R.T. April 2003

Sticker program tools

- Continued the content and tools of PPP
- Revised labeling
  - 2nd pregnancy test within first 5 days of menses
- Targeted education and outreach tool
  - Medication Guide dispensed with each prescription
- Reminder tools
  - Prescriber attestation
  - Yellow qualification sticker on prescription

Assessment of sticker program

- Hoffman-La Roche and FDA agreed to assess impact of sticker program after one year: Prescription Compliance Survey and Patient Survey

- HLR proposed metrics:
  - Increase patient survey enrollment to 60%
  - Demonstrate ≥90% Accutane Rxs had qualification sticker

- 2004: HLR, FDA, and Advisory Committee agreed that sticker program did not meet objectives

Sticker program impact

- No decline in number of exposed pregnancies in survey cohort
- Low participation in voluntary survey
- Sticker use an imperfect surrogate for pregnancy testing

February 2004 Advisory Committee DODAC/DSaRM

Recommended that isotretinoin risk management be strengthened and consolidated

- Registration of all patients, male and female
- Registration of all prescribers
- Registration of all pharmacies
- Tightly link pregnancy testing to dispensing of drug
- Establish a pregnancy registry for root-cause analysis

iPLEDGE: 2006 - present

- Technology-based pregnancy risk management program to prevent fetal exposure to isotretinoin

- Restricted distribution
  - Only registered wholesalers/distributors ship isotretinoin
  - Only registered and activated pharmacies receive/dispense isotretinoin
  - Only registered and activated prescribers prescribe isotretinoin

- Performance-linked access system
  - Registered and qualified patients receive isotretinoin

iPLEDGE: 2006 - present

- Approved August 12, 2005
- Stakeholder registration (wholesalers and pharmacies) began in September 2005
- Patient enrollment opened December 30, 2005
- Transition completed March 1, 2006

New Elements

- Single, consolidated program for innovator and generic firms
- Documentation monthly counseling for all patients
- Documentation monthly CLIA-certified laboratory pregnancy testing for FCBP
- Demonstration of comprehension by FCBPs: answering monthly questions
- Pregnancy registry for root cause analysis

Unique Aspects of iPLEDGE

- Single consolidated program for both innovator and generic products
- First performance-linked access system for widely prescribed drug
- Multi-source marketplace

Advisory Committee discussions

1988 2000 2004 2007 2011

	1982–1988 Early	1988–2002 PPP	2002–2006 Sticker programs	2006–present iPLEDGE
Routine Measures				
Rx status	X	X	X	X
Professional labeling	X	X	X	X
Targeted Education & Outreach				
HCP letters	X	X	X	X
Patient labeling	X	X	X	X
Medication Guide			X	X
Reminder Systems				
Consent forms		X	X	X
Limit amt dispensed		X	X	X
Specialized packaging		X	X	X
Prescription stickers			X	
Performance-linked access systems				
Stakeholder registration				X
Rx linked to safe-use conditions				X

iPLEDGE: Effects on Burden and Access

Marta Wosinska, PhD
Director for Analysis Staff
Office of Planning and Analysis
Center for Drug Evaluation and Research
Food and Drug Administration

Objectives for this presentation

- Discuss burden of iPLEDGE
  - Define burden in the context of REMS with elements to assure safe use*
  - Discuss challenges with measuring iPLEDGE-related burden
- Discuss patient access to therapy in iPLEDGE
  - Define patient access in the context of REMS
  - Present a framework for evaluating access under REMS
  - Review the analysis of iPLEDGE effects on patient access
  - Illustrate challenges with measuring iPLEDGE-related access

In this presentation, I will use the acronym REMS to refer to REMS with elements to assure safe use (ETASU)

Burden is inherent in risk mitigation

- Risk management by its nature imposes at least some level of burden on the healthcare delivery system
- REMS, including iPLEDGE, emphasize safe use practices for the drug in question, e.g.
  - Verifying that a patient about to receive isotretinoin is not pregnant
  - Counseling about the nature of the risk
  - Counseling what the patient needs to do to minimize risk
- REMS may also introduce new risk mitigation measures, e.g., pharmacists verify that safe use conditions are met
- Administrative checks support these risk mitigation efforts
  - Documenting and verifying that safe use practices are followed

Burden of REMS: What are we attempting to measure?

- FDAAA places emphasis on **minimizing burden**

"evaluate (…) whether the elements [to assure safe use], (…) to the extent practicable, minimize the burden on the health care delivery system"

- Describing burden alone falls short of FDAAA's evaluation goal
- Measuring whether burden could further be lowered requires identification of a process that is more efficient but does not compromise risk management goals

Is iPLEDGE overly burdensome?

- Burden-lowering enhancements have been implemented
- Not clear how an assessment can be done to identify the extent to which iPLEDGE currently minimizes the burden
  - Assessing this requires identifying a more efficient risk management mechanism/process, which does not compromise risk management goals
- Another way to look at whether iPLEDGE is overly burdensome is to consider its impact on patient access
  - Burden of REMS processes may translate into providers' unwillingness to prescribe a clinically appropriate therapy

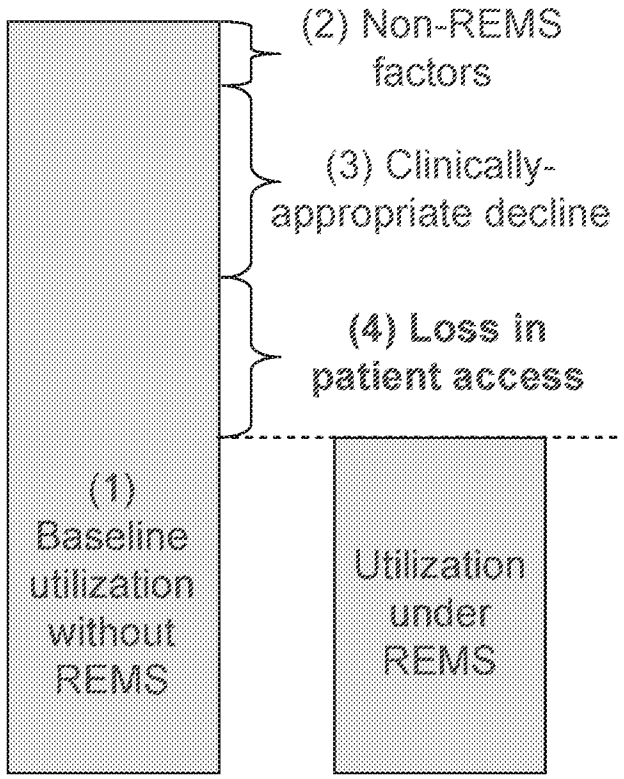
Impact on patient access: What are we attempting to measure?

- FDAAA requires an evaluation of whether the REMS are unduly burdensome on patient access to the drug
- Common definition of access-to-care* is the ability of a person to receive health care services, which is a function of:
  - Availability of personnel and supplies and
  - Ability to pay for those services
- In the context of REMS, patient access is the ability of a person to receive a drug under clinically appropriate conditions
  - Some REMS are designed to limit certain uses, e.g., use of a teratogen in pregnant women
  - REMS are generally designed to provide information about risks, which may lead to lower prescribing

**Source*: *McGraw-Hill Concise Dictionary of Modern Medicine*

Framework for analyzing patient access in the context of REMS

- Assessing impact on access requires:
  - (1) Defining an appropriate baseline
  - (2) Accounting for non-REMS factors
  - (3) Accounting for clinically-appropriate decline by:
    - (a) Isolating targeted inappropriate use
    - (b) Accounting for effects of education and/or new information
- Remaining difference in utilization can be used as surrogate for loss in patient access



- Goal of analysis is to estimate magnitude of components 2-4 above in the short term and long term

Analysis of patient access: Methods

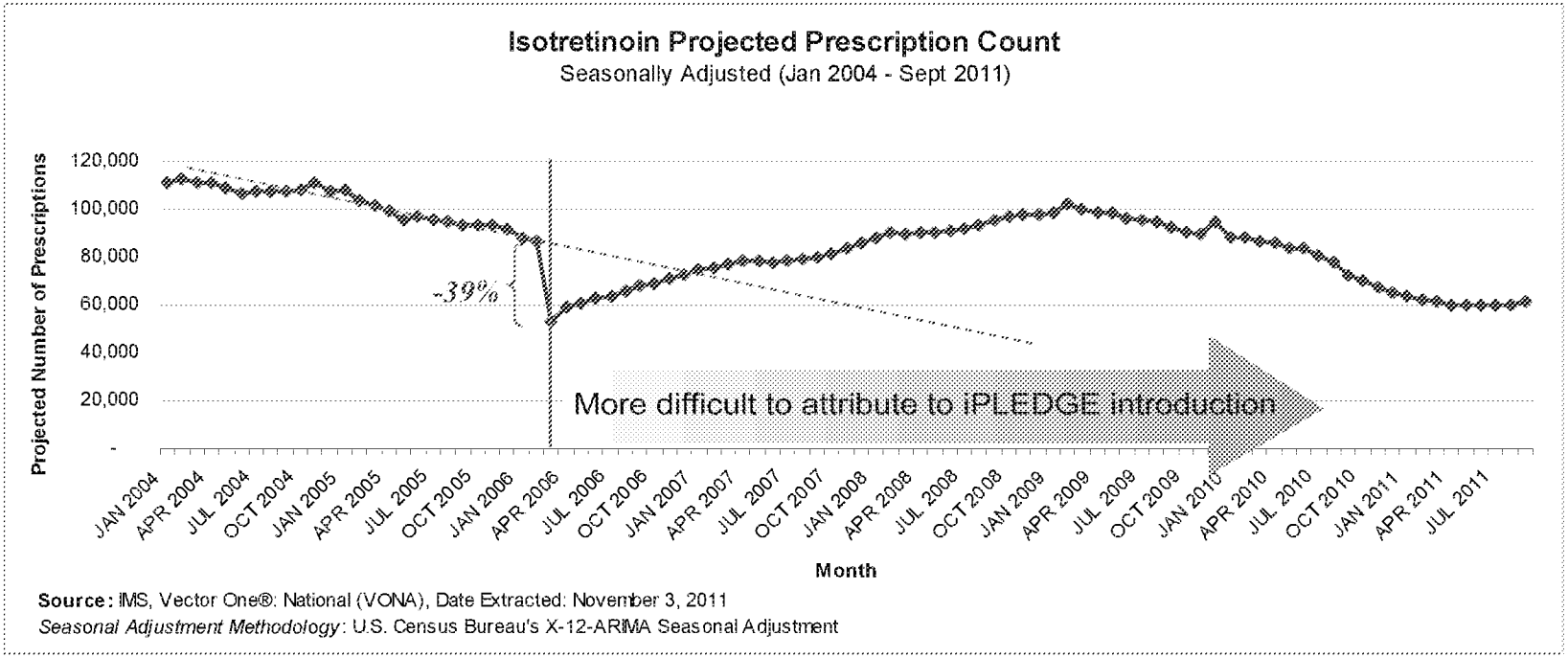
- Our analysis of access under iPLEDGE
  - Isolate non-REMS effects
  - Account for clinically-appropriate decrease in utilization
  - Focus on introduction of iPLEDGE
  - Study various metrics around patient utilization and provider participation
- We use several sources of data
  - Projected national Rx claims data (IMS, Vector One®: National VONA)
  - De-identified longitudinal sample of patients (Wolters Kluwer® CPA)
- We adjust for seasonality
  - Isotretinoin prescriptions exhibit strong seasonal patterns
  - Use Census Bureau ARIMA-12 seasonal adjustment software

(1) Establish baseline and (2) Isolate non-iPLEDGE factors

- Baseline: pre-iPLEDGE utilization
  - This baseline allows us to make inferences about iPLEDGE introduction, but inferences about today are not possible
  - Understanding the impact at introduction, including any short-term disruption, is important from perspective of future REMS

- Introduction of iPLEDGE did not coincide with other events that may affect utilization:
  - No new competing therapies were introduced
  - No discernable price or copayment changes took place
  - Media coverage was related to iPLEDGE
  - Time series were adjusted for seasonality

Introduction of iPLEDGE resulted in a 39% utilization drop with recovery in 10 months

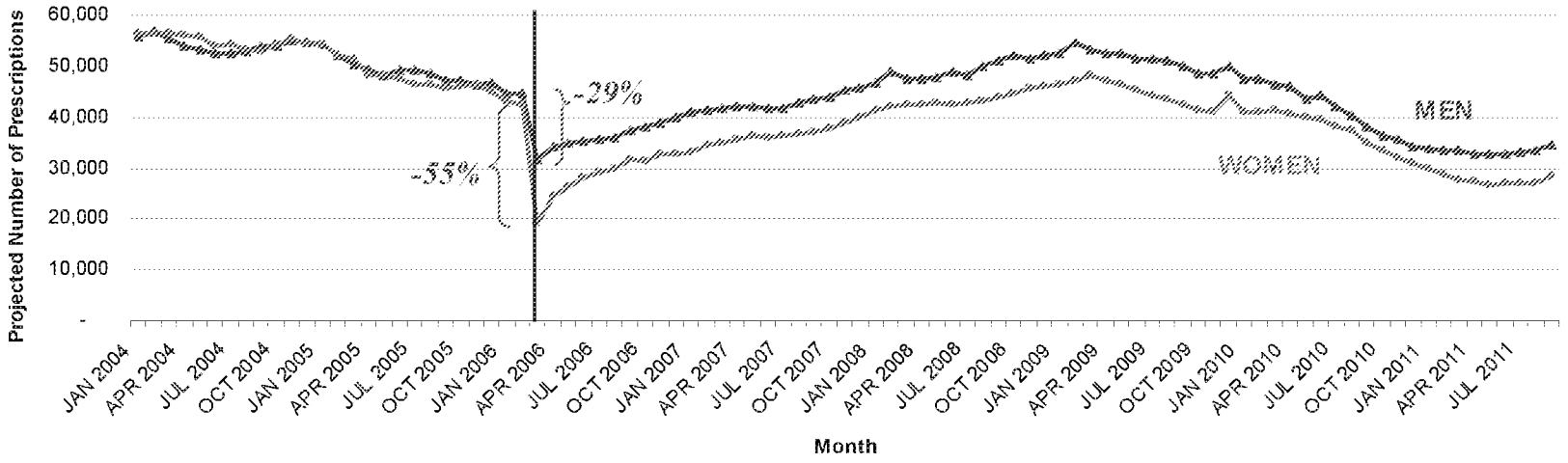


With no obvious utilization-lowering events unrelated to iPLEDGE, the 39% drop could be attributed to iPLEDGE becoming mandatory.

(3) Account for clinically-appropriate decrease in utilization

- iPLEDGE intends to prevent use by:
  - Pregnant women
  - Sexually active women not using appropriate contraception
- New information about risk may change a patient's or provider's assessment about the benefit-risk balance of isotretinoin
  - Part of iPLEDGE is an acknowledgement of risks and education about ways to mitigate the risk
  - Such clinically-appropriate reassessment may lead to a decrease in utilization
- Because of the focus on pregnancies, it is useful to consider iPLEDGE impact on utilization by gender

Introduction of iPLEDGE had a differential impact on use by men and women



Source: IMS, Vector One®: National (VONA), Date Extracted: November 3, 2011
Seasonal Adjustment Methodology: U.S. Census Bureau's X-12-ARIMA Seasonal Adjustment

Women face higher program requirements than men and the decline likely reflects that fact. But what about effect on clinically-appropriate use?

Short-term drop in utilization by men is most likely attributable to loss in access

- Introduction of iPLEDGE lead to a 29% decrease in utilization, which then returns to trend in 7-8 months
- iPLEDGE focuses on pregnancies so utilization by men should not have changed for clinical reasons
- The 29% initial drop could be interpreted as a decrease in patient access because
  - No identifiable factors unrelated to iPLEDGE took place in March'06
  - No clinically-appropriate decrease in prescribing to men is expected
- The return to pre-iPLEDGE utilization levels suggests that, on a broad population level, there may not have been an access problem for men past month 8

Changes in clinically-appropriate use and access are confounded for women

- Women's utilization initially decreased by 55% and returned to pre-iPLEDGE trend within a year
- Women face higher requirements than men and the decline likely reflects that. But what about clinically-appropriate use?
- iPLEDGE enhanced but did not introduce risk-mitigation efforts that may affect clinically-appropriate use:
  - Pregnancy prevention was the focus of education in prior program
  - Same uses were targeted by the prior program
- Nonetheless, disentangling the new requirements from potential changes in clinically-appropriate use is difficult

What are the possible explanations for the apparent initial access disruption?

- Technical impediments during implementation
  - Less than 10% of known prescribers registered their patients with iPLEDGE during phase-in
  - Calls initially exceeded call center capacity
- iPledge-related decrease in patient demand
  - Some patients may perceive the requirements as overly burdensome
- iPledge-related decrease in prescriber participation

Physician participation is a key component of patient access to therapy

- Decrease in patient access may occur when burden on providers translates into their unwillingness to prescribe a clinically appropriate therapy

- We studied the impact of iPLEDGE introduction on the number of prescribing physicians
  - Used a large longitudinal sample of isotretinoin patients from Wolters Kluwer® CPA
  - Identified the number of active prescribers in a given month by specialty and location
  - Adjusted time series for seasonality us Census ARIMA-12
  - Assessed the change in the number of prescribers at the time iPLEDGE became mandatory

Effect on patient access was in part driven by lower physician participation

- Effect of iPLEDGE differed by specialty rather than location
  - The number of prescribing dermatologists initially declined by a lower percentage than the number of non-dermatologists (15% dermatologists vs. 36% other)
  - This may reflect the fact that dermatologists are heavier prescribers
  - The initial percentage decrease following full iPLEDGE roll-out was similar by location (22% in rural vs. 20% in urban)
- In contrast to utilization, the number of active prescribers appears to have dropped permanently after iPLEDGE
  - Instead, the average number of patients per active prescriber rose

Takeaways about iPLEDGE impact on burden and patient access

- Risk management imposes burdens; goal is to minimize burden
- Assessment whether PLEDGE minimizes burden requires identifying a risk management process that is more efficient but does not compromise risk management goals
- Evidence that iPLEDGE initially adversely affected patient access but that it recovered within a year
  - Impact: combination of stakeholder response and implementation
- Methodology but not iPLEDGE experience extrapolates
  - Different REMS have different requirements
  - Same requirement may be implemented differently
  - Severity of condition and availability of treatment options may affect patients' and providers' willingness to embrace burden

FDA Perspective
iPLEDGE Assessments

Kathryn O'Connell, MD PhD
Medical Officer
Division of Risk Management
Office of Medication Error Prevention and Risk Management
Office of Surveillance and Epidemiology
Center for Drug Evaluation and Research

December 1, 2011

REMS assessments

- A ***R**isk **E**valuation and **M**itigation **S**trategy* is dynamic throughout the life cycle of its product
- Assessments are required periodically
- In each assessment review, we ask:
  - Is the REMS meeting the risk mitigation goal?
  - Is the REMS necessary to ensure the benefits outweigh the risks?

Is iPLEDGE…

- Preventing fetal exposure to isotretinoin?
- Necessary for prevention of fetal exposure to isotretinoin?

Is iPLEDGE preventing isotretinoin fetal exposure?

- The number of fetal exposures to isotretinoin (pregnancy rate) is an obvious direct metric to assess whether iPLEDGE is meeting this goal

- This metric presents two problems

To use fetal exposures as the metric for iPLEDGE 'success' we must…

- Pre-define 'how many exposed fetuses are too many?'
  - The aspirational goal is that there be no fetal exposures to isotretinoin at all
  - Problem: all complex factors that contribute to unplanned pregnancy cannot be eliminated

- Know the number of isotretinoin exposed pregnancies
  - Problem: complete ascertainment is unlikely due to lost-to follow up and under-reporting

Why is the fetal exposure number unknown?

- Severe nodular acne is a serious medical problem, but it is not life-threatening
- Patients who stop isotretinoin on discovering pregnancy have no medical need to return to the prescriber
  → lost to follow-up
- Stakeholders with privacy or compliance action concerns
  → under-reporting

Clinically meaningful metric alternative

- iPLEDGE processes are designed to prevent fetal exposure by:
  - eliminating *knowledge* deficits that can contribute to fetal exposure
  - eliminating *clinical practices* that can contribute to fetal exposure

- Use *knowledge* and *clinical practice* metrics to assess iPLEDGE

The question

'how-many-fetal exposures-are-too many?'

then becomes answerable

↓

Any that could be prevented by

knowledge and best *clinical practices*

Patients

- iPLEDGE data indicate that women across age groups understand the risk and the need to prevent pregnancy

- As shown by the pregnancy root cause analysis, consistent correct use of 2 forms of birth control is the challenge

Healthcare Providers

- iPLEDGE data indicate that most HCPs are performing clinical practices aimed at fetal exposure prevention
- Most identified problems are knowledge/practice errors amenable to iPLEDGE re-training protocols
- Prescribers and pharmacies found to be intentionally by-passing iPLEDGE requirements that they agreed to follow are subject to iPLEDGE deactivation

Most frequent serious HCP problem areas

- *Pharmacists*: dispensing without a risk management authorization (RMA#)
  - Why serious? The RMA# is the key to the 'closed loop' system; it signifies that all iPLEDGE requirements have been met for the patient's assigned risk category

- *Prescribers*: failure to correctly identify females of child-bearing potential
  - Why serious? Misclassification as FNCBP allows iPLEDGE to generate a RMA# without completion of requirements aimed at preventing/minimizing fetal exposure

RMA# importance

- Ensures that prescribers cannot treat with isotretinoin if unaware of iPLEDGE or choose not to enroll – they can write prescriptions, but the drug won't be dispensed

- Does not ensure patient won't become pregnant during 30-day supply, but does ensure to practicable extent that she is not already pregnant and understands risk/prevention

- iPLEDGE's 30-day supply limit, coupled with monthly pregnancy testing prior to the next RMA, may limit fetal exposure duration and time between conception and counseling

Because the RMA# is key to iPLEDGE integrity…

- All patients, regardless of risk category, need to be enrolled and have a RMA# for each dispensing
- The pharmacy then need verify only that the RMA# has been generated by iPLEDGE

By dispensing only with a RMA#

the pharmacist is the barrier between a potent teratogen and a pregnant and/or inadequately informed patient standing at the pharmacy's counter

Strategies to address identified problems

- Risk category classification errors

  new web 'wizard' will auto-assign based on approved labeling criteria for non-child bearing potential

- Dispensing without RMA#

  technology innovations are needed to integrate pharmacy systems with iPLEDGE's RMA#

↓

The afternoon session will address healthcare system integration in the larger context of REMS

FDA's perspective on iPLEDGE assessment

- iPLEDGE is ensuring delivery of needed knowledge and clinical practices for the majority of patients
- iPLEDGE does affect access and burden, but clinical practice burden is inherent to use of a potent teratogen
- No currently envisioned program can eliminate all fetal exposures
- This reality should not discourage robust efforts to achieve that goal, or justify denial of uniquely efficacious therapy

- iPLEDGE gives HCPs a powerful web-based tool to optimize the probability that all patients, under all healthcare delivery systems, benefit from systematized execution of best practices codified in isotretinoin's labeling

- Pending development of more advanced strategies for systematically managing use of potent teratogens, programs such as iPLEDGE are state-of-the art

- Programs such as iPLEDGE are required to ensure continued availability of needed drugs for eligible patients while minimizing the risk of fetal harm

Isotretinoin Pregnancy Risk Management Program

Electronic Acknowledgement Receipt

EFS ID:	36350358
Application Number:	16055847
International Application Number:	
Confirmation Number:	3451
Title of Invention:	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS
First Named Inventor/Applicant Name:	George D. Yancopoulos
Customer Number:	96387
Filer:	Karl Bozicevic/Kimberly Zuehlke
Filer Authorized By:	Karl Bozicevic
Attorney Docket Number:	REGN-008CIPCON3
Receipt Date:	19-JUN-2019
Filing Date:	06-AUG-2018
Time Stamp:	16:55:56
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	REGN-008CIPCON3_2019-06-19_Supp_IDS_trans.pdf	50922 8fa9c4e9ed123cc1febaaa0466934cf92ce81213	no	2

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21	Non Patent Literature	Thurston_2004.pdf	443408 31361c81ed78074a84bf68c00ea831eb7aa24e9b	no	14
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22	Non Patent Literature	Washsberger_2007.pdf	1418899 dfe7ec51c87c2c76806dc98ca964d4da53892d7d	no	12
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23	Non Patent Literature	Adis_RD_Profile_Aflibercept_2008.pdf	65282 3e86c5c0f2f98257cc95802232b7b573141d639a	no	10
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24	Non Patent Literature	FDA_meeting_June_17_2011.pdf	777577 ca0b27a585ab36772cc94cda41c9518f137c53c2	no	69
Warnings:					
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25	Non Patent Literature	FDA_meeting_December_1_2011.pdf	829178 57de3aa5273c92c1b9fd8b9c326e87e99dc2f78e	no	68
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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronically Filed 6/19/2019

INFORMATION DISCLOSURE STATEMENT	Attorney Docket No.	REGN-008CIPCON3
	Confirmation No.	3451
	First Named Inventor	George D. Yancopoulos
	Application Number	16/055,847
	Filing Date	August 6, 2018
	Group Art Unit	1647
Address to: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Examiner Name	Jon McClelland Lockard
	Title: "*Use of a VEGF Antagonist to Treat Angiogenic Eye Disorders*"	

Sir:

Applicants submit herewith documents which may be material to the examination of this application and in respect of which there may be a duty to disclose in accordance with 37 C.F.R. § 1.56. This submission is not intended to constitute an admission that any document referred to therein is "prior art" for this invention unless specifically designated as such. A listing of the documents is shown on enclosed Form PTO/SB/08A and copies of the foreign patents and non-patent literature are also enclosed.

The Examiner is requested to make the documents listed on the enclosed PTO/SB/08A of record in this application. Applicants would appreciate the Examiner initialing and returning the initialed copy of form PTO/SB/08A, indicating the documents cited therein have been considered and made of record herein.

Statements

☒ **No statement**

☐ **PTA Statement under 37 CFR § 1.704(d)(1):** Each item of information contained in the information disclosure statement filed herewith:

(i) Was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the information disclosure statement; or

(ii) Is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the information disclosure statement.

☐ **IDS Statement under 37 CFR § 1.97(e)(1):** Each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement; or

☐ **IDS Statement under 37 CFR § 1.97(e)(2):** No item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in § 1.56(c) more than three months prior to the filing of the information disclosure statement.

Fees

☒ No fee is believed to be due.

☐ The appropriate fee set forth in 37 C.F.R. §1.17(p) accompanies this information disclosure statement.

The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number REGN-008CIPCON3.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: June 19, 2019

By: /Karl Bozicevic, Reg. No. 28,807/
Karl Bozicevic
Reg. No. 28,807

BOZICEVIC, FIELD & FRANCIS LLP
201 Redwood Shores Parkway, Suite 200
Redwood City, CA 94065
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	16/065,847
				Filing Date	August 6, 2018
				First Named Inventor	Yancopoulos, George D.
				Art Unit	
				Examiner Name	
Sheet	1	of	3	Attorney Docket Number	REGN-008CIPCON3

U.S. PATENT DOCUMENTS

Examiner Initial*	Cite No.	Patent Number Number-Kind Code (*if known*)	Issue Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1				
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U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No.	Publication Number **Number-Kind Code** (*if known*)	Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1	**2008/0220004**	2008-09-11	Wiegand et al.	
	2				

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No.	Foreign Document Number Country Code-Number-Kind Code (*if known*)	Publication Date YYYY-MM-DD	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T
	1					
	2					

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T
	1	BARBAZETTO, "DOSING REGIMEN AND THE FREQUENCY OF MACULAR HEMORRHAGES IN NEOVASCULAR AGE-RELATED MACULAR DEGENERATION TREATED WITH RANIBIZUMAB." Retina, 30:9, 1376-85, 2010	
	2	Bayer Investor News, "Bayer and Regeneron Start additional Phase 3 Study for VEGF Trap=Eye in Wet Age-related Macular Degeneration." May 8, 2008	
	3	BOYER, "A Phase IIIb Study to Evaluate the Safety of Ranibizumab in Subjects with Neovascular Age-related Macular Degeneration." Opthalmology, 116:9, 1731-39, September 2009.	
	4	BROWN, "Ranibizumab versus Verteporfin for Neovascular Age-Related Macular Degeneration." N Engl J Med 355:14, 1432-44, October 5, 2006	
	5	BROWN, "Primary Endpoint Results of a Phase II Study of Vascular Endothelial Growth Factor Trap-Eye in Wet Age-related Macular Degeneration." Ophthalmology, 118:6, 1089-97, June 2011.	
	6	BROWN, "Long-term Outcomes of Ranibizumab Therapy for Diabetic Macular Edema: The 36-Month Results from Two phase III Trials." Ophthalmology, 2013.	
	7	CAMPOCHIARO, "Ranibizumab for Macular Edema following Branch Retinal Vein Occlusion." Ophthalmology, 2010.	
	8	CAMPOCHIARO, "Sustained Benefits from Ranibizumab for Macular Edema following Central Retinal Vein Occlusion: Twelve-Month Outcomes of a phase III Study." Ophthalmology, 188:10, 2041-49, October 2011.	
	9	CSAKY, "Safety Implications of Vascular Endothelial Growh Factor Blockade for Subjects Receiving Intravitreal Anti-Vascular Endothelial Growth Factor Therapies." Am. J. Ophthalmology, 148:5, 647-56, November 2009.	

Examiner Signature		Date Considered	

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				First Named Inventor	Yancopoulos, George D.
				Art Unit	
				Examiner Name	
Sheet	2	of	3	Attorney Docket Number	REGN-008CIPCON3

NON PATENT LITERATURE DOCUMENTS			
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	10	DO, "One-Year Outcomes of the DA VINCI Study of VEGF Trap-Eye in Eyes with Diabetic Macular Edema." Ophthalmology, 2012.	
	11	ENGELBERT, "The 'Treat and Extend' Dosing Regimen of Intravitreal Anti-Vascular Endothelial Growth Factor Therapy for Neovascular Age-Related Macular Degeneration." Ophthalmology Management, June 2010, available at http://www.visioncareprofessional.com/emails/amdupdate/index.asp?issue=42	
	12	ENGELBERT, "LONG-TERM FOLLOW-UP FOR TYPE 1 (SUBRETINAL PIGMENT EPITHELIUM) NEOVASCULARIZATION USING A MODIFIED 'TREAT AND EXTEND' DOSING REGIMENT OF INTRAVITREAL ANTIVASCULAR ENDOTHELIAL GROWTH FACTOR THERAPY." Retina, 30:9, 1368-75, 2010	
	13	ENGELBERT, "'TREAT AND EXTEND' DOSING OF INTRAVITREAL ANTIVASCULAR ENDOTHELIAL GROWTH FACTOR THERAPY FOR TYPE 3 NEOVASCULARIZATION/RETINAL ANGIOMATOUS PROLIFERATION." Retina, 29:10, 1424-31, 2009	
	14	Eylea®, Highlights of Prescribing Information, Revised 08/2018.	
	15	FUNG, "An Optical Coherence Tomography-Guided, Variable Dosing Regiment with Intravitreal Ranibizumab (Lucentis) for Neovascular Age-related Macular Degeneration." Am J Ophthalmology 143:4, 566-83, April 2007	
	16	GALE, "Complementary and Coordinated Roles of the VEGFs and Angiopoietins during Normal and Pathologic Vascular Formation." Cold Spring Harbor Symposia on Quantitative Biology, Volume LXVIL, pp. 267-73, 2002.	
	17	GARCIA-QUINTANILLA, "Pharmacokinetics of Intravitreal Anti-VEGF Drugs in Age-Related Macular Degeneration." Pharmaceutics, 11:365, 2019.	
	18	GOMEZ-MANZANO, "VEGF Trap induces antiglioma effect at different stages of disease." Society for Neuro-Oncology, December 2008.	
	19	GRAGOUDAS, "Pegaptanib for Neovascular Age-Related Macular Degeneration." N Engl J Med 351:27, 2805-16, December 30, 2004	
	20	HEIER, "Intravitreal Aflibercept for Diabetic Macular Edema." Ophthalmology, 2016.	
	21	Ho et al., Slides entitled CLEAR IT 2 One-Year Key Results, Retina Society 2008	
	22	KARIA, Niral, "Retinal vein occlusion: pathophysiology and treatment options" Clinical Ophthalmology, 4:809-816 (2010)	
	23	KOROBELNIK, "Intravitreal Aflibercept for Diabetic Macular Edema." Ophthalmology, 121:11, 2247-54, November 2014.	
	24	LALWANI, "All About PrONTO: Study Yielded Good Results in AMD With Treatment Guided by OCT." Retina Today, May 2007	
	25	LALWANI, 'A Variable-dosing Regimen with Intravitreal Ranibizumab for Neovascular Age-related Macular Degeneration: Year 2 of the PrONTO Study." Am J Ophthalmology, 148:1, 43-58, July 2009.	
	26	LEVINE, "MACULAR HEMORRHAGE IN NEOVASCULAR AGE-RELATED MACULAR DEGENERATION AFTER STABILIZATION WITH ANTIANGIOGENIC THERAPY." Retina, 29:8, 1074-79, 2009.	

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NON PATENT LITERATURE DOCUMENTS			
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	27	MARGOLIS, "HEMORRHAGIC RECURRENCE OF NEOVASCULAR AGE-RELATED MACULAR DEGENERATION NOT PREDICTED BY SPECTRAL DOMAIN OPTICAL COHERENCE TOMOGRAPHY." Retinal Cases & Brief Reports, 4:1, 2010	
	28	MASSIN, "Safety and Efficacy of Ranibizumab in Diabetic Macular Edema (RESOLVE Study*)." Diabetes Care, 33:11, 2399-405, November 2010.	
	29	MITCHELL, "The RESTORE Study, Ranibizumab Monotherapy or Combined with Laser versus Laser Monotherapy for Diabetic Macular Edema." Ophthalmology, 188:4, 615-25, April 2011.	
	30	NGUYEN, "Ranibizumab for Diabetic Macular Edema, Results from 2 Phase III Randomized Trials: RISE and RIDE." Ophthalmology, 119:4, 789-801, April 2012.	
	31	Regeneron Press Release, "Bayer and Regeneron Dose First Patient in Second Phase 3 Study for VEGF Trap-Eye in Wet Age-Related Macular Degeneration." May 8, 2008	
	32	Regeneron Press Release, "VEGF Trap-Eye Shows Positive Results in a Phase 2 Study in Patients With Diabetic Macular Edema." February 18, 2010	
	33	ROSENFELD, "Ranibizumab for Neovascular Age-Related Macular Degeneration." N Engl J Med, 355:14, 1419-31, October 5, 2006.	
	34	ROSENFELD, "Lessons Learned From Avastin and OCT-The Great, the Good, the Bad, and the Ugly: The LXXV Edward Jackson Memorial Lecture." Am. J. Ophthalmology, 204:26-45, August 2019.	
	35	SCHMIDT-ERFURTH, "Efficacy and Safety of Monthly versus Quarterly Ranibizumab Treatment in Neovascular Age-related Macular Degeneration." Ophthalmology, 2010.	
	36	SCHMIDT-ERFURTH, "Three-Year Outcomes of Individualized Ranibizumab Treatment in Patients with Diabetic Macular Edema." Ophthalmology, 121:5, 1045-53, May 2014	
	37	SPAIDE, "Ranibizumab According to Need: A Treatment for Age-related Macular Degeneration." Am J Ophthalmology, April 2007.	
	38	WOLFSON, "Regeneron Focuses on Age-Related Macular Degeneration." Chemistry & Biology 15:303-304, April 2008	
	39	YANCOPOULOS, "Vascular-specific growth factors and blood vessel formation." Nature 407:242-48, September 14, 2000.	
	40	YANCOPOULOS, "Clinical Application of Therapies Targeting VEGF." Cell 143, October 1, 2010.	
	41	YUNG, "moving Toward the Next Steps in Angiogenesis Therapy?" Society for Neuro-Oncology, 10:939, 2008	

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Vascular-specific growth factors and blood vessel formation

George D. Yancopoulos, Samuel Davis, Nicholas W. Gale, John S. Rudge, Stanley J. Wiegand & Jocelyn Holash

Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, New York 10591, USA

A recent explosion in newly discovered vascular growth factors has coincided with exploitation of powerful new genetic approaches for studying vascular development. An emerging rule is that all of these factors must be used in perfect harmony to form functional vessels. These new findings also demand re-evaluation of therapeutic efforts aimed at regulating blood vessel growth in ischaemia, cancer and other pathological settings.

Until recently, vascular endothelial growth factor (VEGF) was the only growth factor proven to be specific and critical for blood vessel formation[1–3]. Other long-known factors, such as the fibroblast growth factors (FGFs), had profound effects in various endothelial cell assays[4]. But such factors were also known to be nonspecific in that they could act on many other cell types, and it was questionable whether the assays used to evaluate them were physiologically relevant. For example, the most widely used assays involved adding putative angiogenic agents to cornea pocket models, or to chick chorioallantoic membranes[5,6]. In such assays, FGFs could robustly induce new vessel growth, but there was limited ability to evaluate the induced vessels functionally, or to determine the relevance of these inductions for normal vascular development.

A recent explosion of newly discovered growth factors acting on the vascular endothelium has coincided with application of powerful new genetic approaches to the problem of vascular development[7,8]. The vascular endothelium-specific growth factors now include five members of the VEGF family, four members of the angiopoietin family, and at least one member of the large ephrin family (Fig. 1). For almost all of these and their receptors, mouse models involving genetic disruption and/or transgenic misexpression have contributed to an understanding of their normal physiological roles, as well as of their pathological capabilities. A rule that is emerging is that all of these factors must be used in perfect harmony, in a complementary and coordinated manner, to form functional vessels[7]. In addition, many other growth factors that are not vascular endothelium-specific are also required for blood vessel formation, such as members of the platelet-derived growth factor or transforming growth factor-β families, although these factors also have critical roles for many other systems as well[8–10]. Furthermore, there are myriad other gene products — ranging from transcription factors to members of the Notch family — that have been shown crucial for vessel formation[8]. In an attempt to do justice to the topic, this review will focus only on the vascular endothelium-specific growth factors, and how they are involved in vessel formation.

The recent explosion in identifying and characterizing physiological regulators of blood vessel growth demands re-evaluation of therapeutic efforts aimed at regulating blood vessel growth — whether it be promoting vascular ingrowth to replenish ischaemic tissue, blocking vessel growth in order to blunt tumours, or repairing damaged and leaky vessels during inflammation or other pathological settings. The privilege of hindsight makes some of the bold, early therapeutic efforts directed towards ischaemic disease, based on random delivery of a single growth factor to grow an entirely new functional network of vessels, now appear somewhat naive and even misguided. On the other hand, recent insights continue to support the notion that blockade of even a single growth factor might limit disease-induced vascular growth, with the most compelling evidence supporting approaches based on blockade of VEGF. Furthermore, recent advances indicate previously unanticipated clinical applications for vascular growth factors, such as the use of angiopoietin-1 (Ang1) for the repair of damaged and leaky vessels.

Vasculogenesis and angiogenic remodelling

Vessel formation can occur by a number of different processes[4]. Early in development, vessel formation occurs by a process referred to as vasculogenesis (Fig. 2, stage A), in which endothelial cells differentiate and proliferate *in situ* within a previously avascular tissue, and then coalesce to form a primitive tubular network. This primary network includes some of the major vessels in the embryo, such as the aorta and major veins, as well as a honeycomb-like plexus connecting these major vessels. Angiogenic remodelling refers to the process by which this initial network is modified — through both pruning and vessel enlargement — to form the interconnecting branching patterns characteristic of the mature vasculature (Fig. 2, stage B). During this time, vessel walls also mature, as endothelial cells integrate tightly with supporting cells (such as smooth muscle cells and pericytes) and surrounding matrix (Fig. 2, stage C). A different process, referred to as angiogenic sprouting, involves the sprouting from existing vessels into a previously avascular tissue. In some cases, it seems as if mature vessels must first be destabilized to allow for subsequent sprouting (Fig. 2, stages D, F); once again, vessels formed by sprouting are initially immature and must further develop. Angiogenic sprouting is responsible for vascularizing certain structures during normal development, such as the neural tube or the retina, and for most new vessel formation in the adult. Destabilization of vessels can also apparently lead to vascular regression (Fig. 2, stage E), as described below.

Emerging model of vascular formation

Recent insights have led to a model of vascular formation that attempts to incorporate the known vascular-specific growth

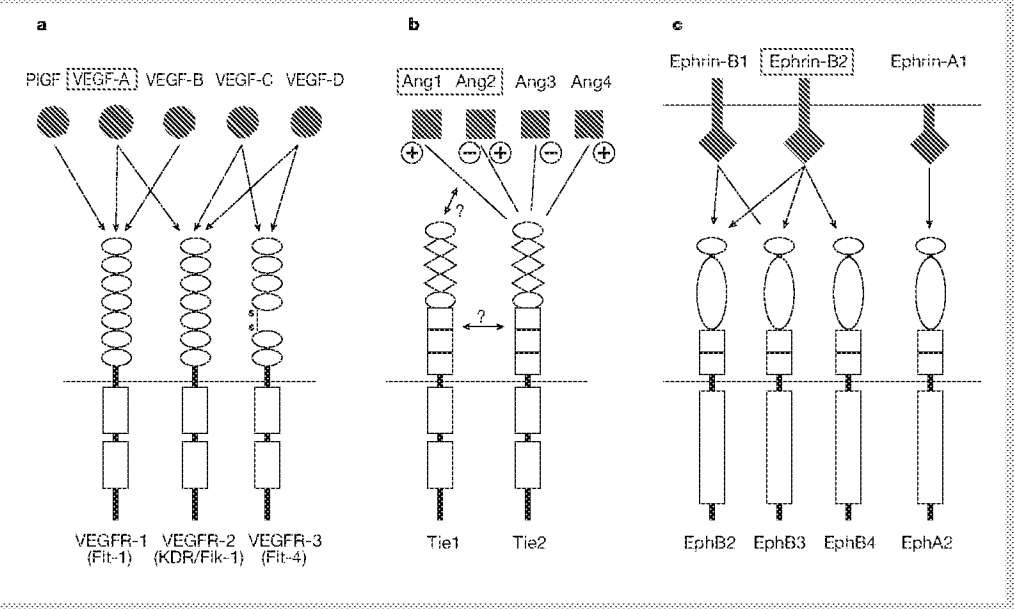

Figure 1 Schematic representation of three families of vascular growth factors and their receptor interactions. **a**, VEGFs; **b**, angiopoietins; **c**, ephrins. The four factors that are discussed in detail in this review are highlighted in red. In **b**, '+' or '−' indicates whether the particular angiopoietin activates or blocks the Tie2 receptor, whereas '?' indicates that a potential interaction has not yet been confirmed experimentally. In **c**, only those members of the large ephrin ligand family (and only their counterpart Eph receptors) that have been implicated in vascular growth are shown.

factors[7,11–14], and the details of this model will be a major subject of this review. According to this model, the first characterized vascular-specific growth factor, VEGF, maintains its position as the most critical driver of vascular formation, as it is required to initiate the formation of immature vessels by vasculogenesis or angiogenic sprouting (Fig. 2, stages A, F), during development as well as in the adult. Ang1 and ephrin-B2 are subsequently required for further remodelling and maturation of this initially immature vasculature (Fig. 2, stages B, C), with ephrin-B2 being particularly important in distinguishing developing arterial and venous vessels, as will be discussed in more detail below.

Following vessel maturation, Ang1 seems to continue to be important in maintaining the quiescence and stability of the mature vasculature (Fig. 2, stage C). Disruption of this stabilizing signal coincides with reinitiation of vascular remodelling in the adult — as occurs in the adult female reproductive system or in tumours (Fig. 2, stage D, and see below). Such de-stabilization seems to involve the autocrine induction — by the endothelium to be remodelled — of a natural antagonist of Ang1, termed Ang2 (Fig. 2, stage D). VEGFs, angiopoietins and ephrin-B2 apparently recapitulate their developmental roles during vascular remodelling in the adult, and administration of individual factors to the adult allows them to reprise these roles but not to trigger the entire process (see below). Thus VEGF administration can initiate vessel formation in adult animals, but by itself promotes formation of only leaky, immature and unstable vessels. In contrast, Ang1 administration seemingly further stabilizes and protects the adult vasculature, making it resistant to the damage and leak induced by VEGF or inflammatory challenges. Altogether, it is becoming clear that precise understanding of the normal developmental roles of the VEGFs, the angiopoietins and the ephrins will greatly aid in understanding how to manipulate these growth factor systems for therapeutic benefit.

VEGF, its relatives, and their receptors

VEGF was initially defined, characterized and purified for its ability to induce vascular leak and permeability, as well as for its ability to promote vascular endothelial cell proliferation[1,2]. Thus, it was originally termed vascular permeability factor as well as VEGF. Although most research efforts have focused on its growth-promoting ability, recent findings are once again highlighting its potent permeability-inducing effects, and in particular their role in disease. Other members of the VEGF family were identified based on their homology to VEGF[3]. The various members of the VEGF family have overlapping abilities to interact with a set of cell-surface receptors[3] that trigger responses to these factors (Fig. 1a). The main receptors that seem to be involved in initiating signal transduction cascades in response to the VEGFs comprise a family of closely related receptor tyrosine kinases consisting of three members now termed VEGFR-1 (previously known as Flt-1), VEGFR-2 (previously known as KDR or Flk-1) and VEGFR-3 (previously known as Flt-3). In addition, there are a number of accessory receptors such as the neuropilins[15] which seem to be involved primarily in modulating binding to the main receptors, although roles in signalling have not been ruled out.

VEGFR-2 seems to mediate the major growth and permeability actions of VEGF, whereas VEGFR-1 may have a negative role, either by acting as a decoy receptor or by suppressing signalling through VEGFR-2. Thus, mice engineered to lack VEGFR-2 fail to develop a vasculature and have very few endothelial cells[16], whereas mice lacking VEGFR-1 seem to have excess formation of endothelial cells which abnormally coalesce into disorganized tubules[17]. Mice engineered to express only a truncated form of VEGFR-1, lacking its kinase domain, appear rather normal, consistent with the notion that the primary role of VEGFR-1 may be that of a decoy receptor[18]. VEGFR-3 may be important during blood vessel development, but is most unique based on its expression on lymphatic vessels, for which it seems to be critical[19]. The first VEGF relative identified is known as placental growth factor (PlGF), and until recently little was known about its normal function, in part because mice engineered to lack PlGF were overtly normal[8,20]. Recent findings indicate that adult mice lacking PlGF exhibit deficiencies in certain models of adult vascular remodelling, raising the interesting possibility that the activity of PlGF may be limited to these settings[8]. VEGF-C, based on its ability to bind the lymphatic-specific VEGFR-3, seems to be important for lymphatic development, and transgenic overexpression of VEGF-C leads to lymphatic hyperplasia[21]. Mice lacking VEGF-B are overtly normal and fertile, but their hearts are reduced in size, suggesting that VEGF-B may have a role in coronary vascularization and growth[22]. Little is known about the normal physiological role of VEGF-D[3].

VEGF must be well regulated

Compared to its more recently discovered relatives, much more is known about VEGF. It is now quite clear that VEGF is such a potent and critical vascular regulator that its dosage must be exquisitely regulated in spatial, temporal and quantitative manner to avoid vascular disaster. Disruption of both VEGF alleles in mice mimicks

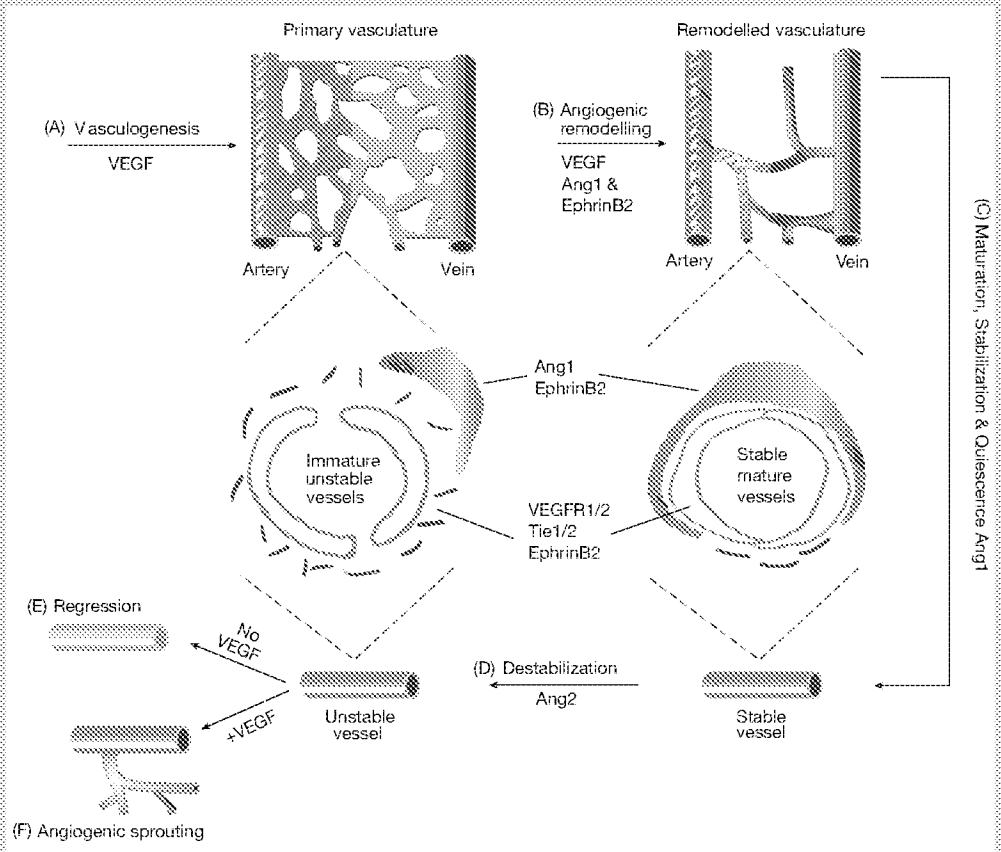

Figure 2 Schematic representation of the roles of VEGF, Ang1, Ang2 and ephrin-B2 during vessel formation. The processes include vasculogenesis (stage A), angiogenic remodelling (B), stabilization and maturation (C), destabilization (D), regression (E) and sprouting (F), as described in detail in the text. An attempt is made to assign the indicated vascular growth factors to the various processes, and to indicate their expression patterns. Although not noted in the figure, expression of ephrin-B2 marks arterial vessels from the earliest developmental times.

knockout of VEGFR-2, resulting in almost complete absence of a vasculature[23,24]. Disruption of even a single VEGF allele in mice leads to embryonic lethality due to severe vascular abnormalities, providing perhaps the only example of embryonic lethality due to a simple half-dosage effect[23,24]. Even more subtle alterations in VEGF expression during embryonic development result in profound abnormalities, leading to embryonic or early post-natal death[25,26]. VEGF continues to be critical during early post-natal growth and development, as evidenced by post-natal VEGF inactivation using Cre-loxP-mediated VEGF gene deletion, or by administration of a soluble VEGF receptor that effectively blocks VEGF action[27]. Although VEGF inactivation is lethal during the first few post-natal weeks, VEGF inactivation in older animals is much less traumatic, seemingly affecting only those structures that continue to undergo vascular remodelling, such as bone growth plates or ovarian corpus lutei[27–29]. Thus, VEGF does not seem to have a continuous maintenance function for much of the adult vasculature.

The most elegant demonstration of the need for exquisite VEGF regulation involves retinal vascularization, which occurs post-natally in rodents. Angiogenic sprouting into the initially avascular and hypoxic rodent retina depends upon its VEGF expression[30–32]. Any perturbation of normal VEGF expression patterns destroys retinal vascularization patterns, with dire results for retinal function; subsequent restoration of VEGF expression does not correct the problem, but rather exacerbates it. A simple way to perturb VEGF expression involves exposing post-natal rodents to a brief period of hyperoxia[31,33,34], which transiently suppresses retinal VEGF, resulting in cessation of vessel growth and even causing vascular regression[31,33,34]. When the rodents are returned to normoxia, the now undervascularized retina becomes hypoxic, causing an abnormal burst of VEGF, which promotes robust new angiogenesis, but of haemorrhagic and leaky vessels growing in totally abnormal patterns that wreak havoc upon the retina. This model reflects the ability of oxygen therapy in premature infants to cause retinopathy of prematurity, and shows the need for precise regulation of VEGF. Similarly, diabetic retinopathy initiates with damage and loss of healthy vessels, followed by retinal hypoxia and resulting VEGF induction, once again leading to an abnormal angiogenic response with leaky and haemorrhagic vessels[35,36]. These findings show that inappropriate induction of VEGF, in the absence of the entire angiogenic programme, leads to formation of immature and leaky vessels that cause disease. These findings also show that tissue hypoxia cannot necessarily induce a useful angiogenic response.

Consistent with the above findings concerning the devastating consequences of unregulated VEGF expression, several studies have delivered excess VEGF to adult tissues — to adult muscle using retrovirally engineered myoblasts[37], to skin using transgenic or adenoviral delivery[38–41], or to whole animals using acute adenoviral delivery[42] — and found that leaky and haemorrhagic vessels were formed, often associated with an inflammatory response, resulting in pronounced tissue swelling and oedema.

The angiopoietins and their Tie receptors

Despite its requisite role in vascular formation, VEGF must work in concert with other factors. The angiopoietins (Fig. 1b) seem to be some of VEGF's most important partners (Fig. 2). The angiopoietins were discovered as ligands for the Ties, a family of receptor tyrosine kinases that are as selectively expressed within the vascular endothelium (despite expression in some other cells, such as in the haemopoietic lineage) as are the VEGF receptors[43–47]. There are now four definitive members of the angiopoietin family, although Ang3 and Ang4 may represent widely diverged counterparts of the same gene locus in mouse

and man[12,48,49]. All of the known angiopoietins bind primarily to Tie2, and it is unclear whether there are independent ligands for the second Tie receptor, Tie1, or — as currently seems more likely — whether the known angiopoietins can in some way or under some circumstances also engage Tie1, perhaps as a second component in a heteromerized complex. The rest of this review will deal only with Ang1 and Ang2, since little more can be said at this time about Ang3 and Ang4.

Ang1 stabilizes vessel walls

The most important insights into the normal roles of Ang1 and its Tie2 receptor came from the analysis of mice engineered to lack these gene products[11,50,51]. Unlike mouse embryos lacking VEGF or VEGFR-2, embryos lacking Ang1 or Tie2 develop a rather normal primary vasculature. However, this vasculature fails to undergo normal further remodelling. The most prominent defects are in the heart, with problems in the associations between the endocardium and underlying myocardium as well as in trabeculae formation, and also in the remodelling of many vascular beds into large and small vessels. In these vascular beds, as in the heart, ultrastructural analysis indicates that endothelial cells fail to associate appropriately with underlying support cells, which are the cells that provide the Ang1 protein that acts on endothelial Tie2 receptors[11]. This finding led to the suggestion that Ang1 does not supply an instructive signal that actually directs specific vascular remodelling events, but rather has more of a permissive role by optimizing the manner in which endothelial cells integrate with supporting cells, thus allowing them to receive other critical signals from their environment[11].

Transgenic overexpression of Ang1 in skin results in pronounced hypervacularization[40,52]. Although there are modest increases in vessel number, the most marked increase is in vessel size. In contrast, VEGF in similar models primarily increases vessel number[38–40]. These findings indicate that Ang1 might promote circumferential as opposed to sproutive growth. Combining transgenic Ang1 and VEGF leads to unprecedented hypervascularity resulting from increases in both vessel size and number[40]. The vascular patterns induced by the combination are still obviously abnormal morphologically, suggesting that much must be learned about exploiting even this growth factor combination in therapeutic settings so as to grow normal vessels.

In addition to their effects on vascular morphology, transgenic overexpression of Ang1 and VEGF had distinct effects on vascular function and integrity. As had been expected, VEGF led to immature, leaky and haemorrhagic vessels[38–40]. On the other hand, Ang1 led to vessels that were actually resistant to leak, whether the leak was induced by VEGF or inflammatory agents[40]. This resistance seems related to the ability of Ang1 to maximize interactions between endothelial cells and their surrounding support cells and matrix, as the Ang1 vessels were resistant to treatments that normally created holes in the endothelial cell barrier[40]. These findings indicated that Ang1 might counter the effect of VEGF on permeability, raising multiple therapeutic possibilities[40]. There are numerous disease processes — ranging from diabetic retinopathy to inflammation to brain oedema following ischaemic stroke — in which vessels become damaged and leaky, and an agent that could repair the damage and prevent the leak could have enormous therapeutic benefit. Supporting the clinical potential of Ang1, acute adenoviral administration of Ang1 to adult animals showed that Ang1 can indeed protect the adult vasculature from vascular leak, without inducing immediate changes in vascular morphology[42].

Ang2: agonist and antagonist?

Ang2 was cloned based on its homology to Ang1, and displayed similarly high affinity for Tie2, but — depending on the cell examined — Ang2 could either activate or antagonize Tie2 (ref. 12). Transgenic overexpression of Ang2 in the embryonic endothelium resulted in embryonic death due to defects resembling those of Ang1 or Tie2 knockouts, demonstrating that Ang2 could act as a Tie2 antagonist *in vivo*, at least under some circumstances[12]. This possibility became even more intriguing when Ang2 expression profiles were examined. In adult animals, Ang2 was induced in the endothelium of vessels undergoing active remodelling, such as sprouting or regressing vessels in the ovary[12,53], or in tumours[13,14,54,55] (as will be discussed in detail below). These findings, together with the possibility that Ang2 could act as a Tie2 antagonist, led to the hypothesis that Ang2 might provide a key de-stabilizing signal involved in initiating angiogenic remodelling[12–14,55]. That is, based on previous evidence that Ang1 engagement of the Tie2 receptor was constitutive in the adult vasculature and indeed necessary to maintain its quiescence (Fig. 2, stage C), it was proposed that autocrine induction of Ang2 in endothelium blocked this constitutive stabilizing influence of paracrine Ang1, allowing the endothelial cells to revert to a more plastic and destabilized state reminiscent of developing vessels (Fig. 2, stage D). Such destabilized vessels could then be prone to two fates. On the one hand, these destabilized vessels would be prone to regression in the absence of associated growth factors, as also occurs with primitive vessels during development (Fig. 2, stage E). On the other hand, they would be more sensitive to angiogenic changes induced by simultaneously available angiogenic factors such as VEGF, essentially recapitulating an early embryonic situation in which VEGF acts prior to the involvement of Ang1 (Fig. 2, stage F).

This model of Ang2 as a destabilizing signal that reverts vessels to a more plastic and tenuous state, initially developed based on observations in the remodelling ovary[12], is consistent with more recent data in tumours (see below) as well as emerging data from knockout mice lacking Ang2. One of the best characterized settings of post-natal vascular regression and remodelling in mice involves the eye, in which regression of the hyaloid vasculature encasing the lens is coupled to angiogenic sprouting that leads to vascularization of the initially avascular retina, as described above. Neither regression of the hyaloid vasculature nor vascularization of the retina occur in mice lacking Ang2 (S.J.W., R. Tzekova, Q. Wong, N.W.G., C. Suri & G.D.Y., unpublished results). These data show that Ang2 is required for some post-natal vascular remodelling events, and support the notion that Ang2 provides a key role in destabilizing the vasculature in a manner that is necessary for its subsequent remodelling. However, other defects in the Ang2-knockout mice suggest that it may in some cases also have an agonistic role. That is, it is highly expressed in the developing aortic wall, which does not develop properly in mice lacking Ang2. Similarly, lymphatic development is perturbed in these mice.

The ephrins

The Eph receptor tyrosine kinases comprise the largest known family of growth factor receptors (Fig. 1c), and use the similarly numerous ephrins as their ligands[7,56]. The ephrins are unlike ligands for other receptor tyrosine kinases in that they must be tethered to the membrane to activate their Eph receptors[7,57]. Although initially characterized in the nervous system[7,56], recent knockout studies have suggested key roles for ephrin-B2 and its EphB4 receptor during vascular development[58–60]. Mouse embryos lacking ephrin-B2 and EphB4 suffer fatal defects in early angiogenic remodelling that are somewhat reminiscent of those seen in mice lacking Ang1 or Tie2[58–60]. Moreover, ephrin-B2 and EphB4 display remarkably reciprocal distribution patterns during vascular development, with ephrin-B2 marking the endothelium of primordial arterial vessels while EphB4 marks the endothelium of primordial venous vessels[58–60]. These distributions suggested that ephrin-B2 and EphB4 are involved in establishing arterial versus venous identity, perhaps in fusing arterial and venous vessels at their junctions, and that defects in these processes might account for the early lethality observed in mouse embryos lacking these proteins[58–60] (Fig. 2, stage A).

Ephrin-B2 continues to selectively mark arteries during later embryonic development as well as in the adult, although this expression extends progressively from the arterial endothelium to the

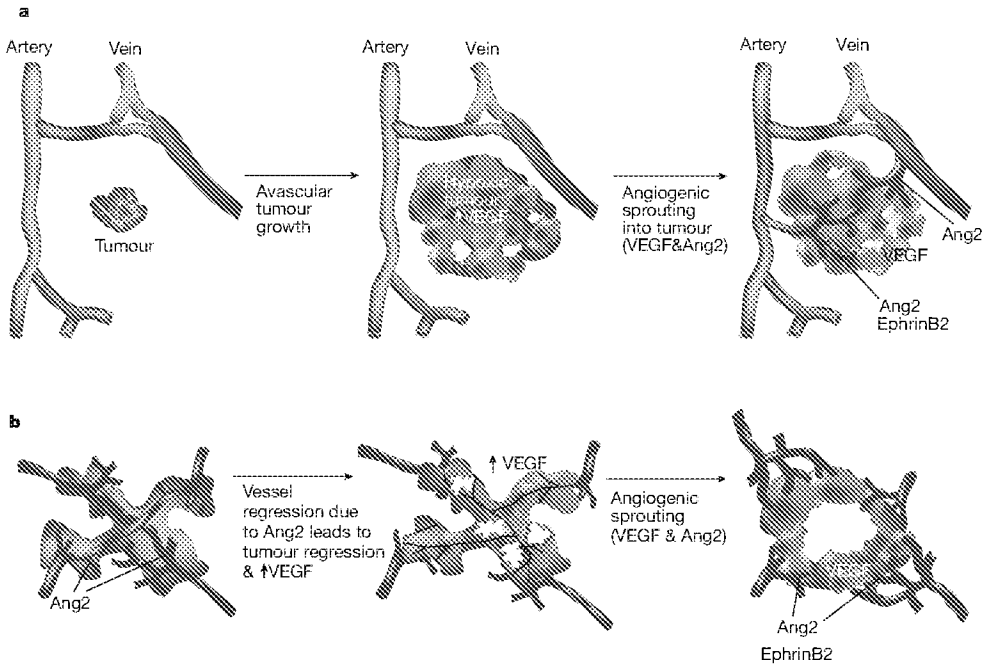

Figure 3 Models of tumour angiogenesis. **a**, Model of avascular tumour initiation contrasted with **b**, tumour initiation involving host vessel co-option. An attempt is made to assign the indicated vascular growth factors to roles in the various indicated steps in tumour development, and to indicate their expression patterns.

surrounding arterial smooth muscle and to pericytes (N.W.G. and G.D.Y., unpublished results; D. Shin and D. J. Anderson, unpublished results). Thus, ephrin-B2 is apparently not only required during the earliest stages of arterial/venous determination, but may continue to be important during the development of arteries, perhaps by regulating interactions between endothelial and smooth muscle cells involved in the formation of arterial muscular walls (Fig. 2, stage B). In adult settings of angiogenesis, as in tumours or in the female reproductive system, the endothelium of new vessels strongly re-expresses ephrin-B2 (N.W.G. and G.D.Y., unpublished results; D. Shin and D. J. Anderson, unpublished results) (Fig. 3a,b). The finding that angiogenic sprouting in the adult and in tumours involves re-expression of the ephrin-B2 arterial marker challenges existing dogma that such sprouting primarily involves venous or uncommitted vessels, and also suggests that ephrin-B2 may be important in these angiogenic settings.

VEGF and Ang2 in tumour angiogenesis

Much has been made of the notion that tumours and metastases initiate as small avascular masses, which only subsequently induce the angiogenic ingrowth that is required to allow further growth of the early tumour[61–63] (Fig. 3a). It is clear that many natural tumours initially arise in this manner, particularly primary epithelial tumours that are initially separated from underlying vessels by a basement membrane that must be broken before tumour cells can access the vasculature. In addition, many artificial model systems forcibly create initially avascular tumours by placing tumour cells in a space that is normally devoid of vessels — such as the subcutaneous space, the cornea pocket or the vitreous or the tumour window — thus requiring angiogenesis to get vessels to the tumour.

Despite all the attention directed towards avascular tumour growth, recent findings[14,55] have refocused attention on previous observations[64–66] that many tumours, and metastases in particular, do not initiate in an avascular manner (Fig. 3b). Rather, tumour cells can initially home in on and grow by co-opting existing host vessels, and thus start off as well-vascularized small tumours[13,14] (Fig. 3b, left). In response to co-option, the host vessels mount a defence — sensing inappropriate co-option, they regress, choking off the tumour and resulting in a secondarily avascular and hypoxic tumour (Fig. 3b, middle). However, successful tumours seem to overcome host vessel regression by inducing robust new angiogenesis (Fig. 3b, right). Ang2 and VEGF inductions correlate remarkably well with the above processes[13,14,55]. That is, soon after tumour co-option, host vessels start expressing high autocrine levels of Ang2; thus Ang2 is one of the earliest tumour markers described, and one of the most general because it marks co-opted vessels and not the tumour cells themselves (Fig. 3b, left). Consistent with the possibility that autocrine Ang2 expression can destabilize vessels (Fig. 2, stage D), the co-opted vessels begin to die by an apoptotic process shortly after expressing Ang2 (Fig. 3b, middle). As vessels die, the tumour becomes secondarily avascular and hypoxic, resulting in marked induction of tumour-derived VEGF (Fig. 3b, middle). These high levels of VEGF correlate with cessation of regression of the destabilized co-opted vessels, and onset of robust new angiogenesis sprouting from these vessels, allowing for tumour survival and further growth (Fig. 3b, right). Thus, in such settings, endothelial Ang2 expression seems to correlate with vessel destabilization, apparently leading to vessel regression in the absence of tumour-derived VEGF, or robust new angiogenesis following induction of tumour-derived VEGF (stage D in Fig. 2, and Fig. 3b). The possibility that tumour vessel Tie2 receptors are blocked continuously by Ang2 and thus have an imbalance towards VEGF may well explain long-standing observations that tumour vessels fail to mature, exhibit poor associations between endothelial cells and their supporting cells, and are characterized by their leaky and haemorrhagic state.

One practical prediction, which applies whether tumour growth initiates avascularly or through co-option, is that anti-VEGF therapy should ultimately blunt tumour growth. Early studies using an anti-VEGF antibody provided the first support for this notion[67]. This

has subsequently been confirmed in many laboratories using numerous approaches ranging from antibodies that bind and block VEGF, to those that bind and block VEGFR-2, to small molecules that block the activity of the VEGF-2 kinase domain, to genetic ablation of VEGF in tumour cells[68]. Thus, blockade of VEGF represents the best validated and most compelling anti-angiogenesis approach described so far.

Perspectives and therapeutic possibilities

There are many critical growth factors involved in the physiological regulation of blood vessel formation, and the actions of these molecular players must be very carefully orchestrated in terms of time, space and dose so as to form a functioning vascular network. The complexity of the process makes ongoing therapeutic efforts aimed at growing new vascular networks to treat ischaemic disease, using random delivery of single agents, appear somewhat naive with the potential to cause more harm (by forming malfunctioning vessels prone to leak and haemorrhage) than good. In their defence, these efforts were initiated years ago when much less was understood about the process of vascular formation. Recent failures of large, well-controlled clinical trials for cardiac ischaemia using delivery of single agents (either VEGF or FGF)[69,70] raises the question of why these trials failed despite claims of success in animal studies and earlier, smaller (and uncontrolled) human trials. As recently discussed[68], this may be due to the failure of animal models to correctly model the human disease, as well as the need for blind approaches in both animal and human studies to overcome investigator bias when measuring subjective endpoints, together with the requirement for placebo controls in settings where there is a marked placebo effect in subjective patient reports of their own condition.

Although the complexities of vascular formation create significant challenges for those trying to grow vessels for therapeutic use, these same complexities may work in favour of therapeutic approaches aimed at blocking vessel growth. That is, blockade of many different molecular players may all result in the blunting of vessel formation. There is no doubt that VEGF is the best-validated target for anti-angiogenesis therapies, based on overwhelming genetic, mechanistic and animal efficacy data. Despite the attention devoted to a number of other putative angiogenic antagonists for use in cancer (for example, endostatin, angiostatin and antithrombin)[71–73], most of these antagonists have yet to be characterized from a mechanistic and genetic point of view. Thus, they lack defined mechanisms of action, and cannot be placed within existing models of molecular angiogenesis using genetic approaches. Also troubling is that these agents seem to work whether they are delivered as properly folded proteins or as denatured aggregates[72].

Recent efforts also indicate as yet unimagined applications for vascular growth factors. For example, the possibility that Ang1 may help prevent or repair damaged and leaky vessels offers therapeutic hope for an assortment of unmet clinical needs, such as in diabetic retinopathy, acute macular degeneration, ischaemia/reperfusion injury (which can occur after strokes and in acute respiratory distress syndrome), or in inflammatory settings[40,42]. The continued discovery and characterization of the molecular factors that regulate vessel formation will lead to additional unexpected therapeutic opportunities, as well as to the refinement of current therapeutic approaches aimed at growing or blocking vessel formation. □

1. Ferrara, N. Vascular endothelial growth factor: molecular and biological aspects. *Curr. Top. Microbiol. Immunol.* **237**, 1–30 (1999).
2. Dvorak, H. F., Nagy, J. A., Feng, D., Brown, L. F. & Dvorak, A. M. Vascular permeability factor/vascular endothelial growth factor and the significance of microvascular hyperpermeability in angiogenesis. *Curr. Top. Microbiol. Immunol.* **237**, 97–132 (1999).
3. Eriksson, U. & Alitalo, K. Structure, expression and receptor-binding properties of novel vascular endothelial growth factors. *Curr. Top. Microbiol. Immunol.* **237**, 41–57 (1999).
4. Risau, W. Mechanisms of angiogenesis. *Nature* **386**, 671–674 (1997).
5. Kenyon, B. M. *et al.* A model of angiogenesis in the mouse cornea. *Invest. Ophthalmol. Vis. Sci.* **37**, 1625–1632 (1996).
6. Ribatti, D., Vacca, A., Roncali, L. & Dammacco, F. The chick embryo chorioallantoic membrane as a model for in vivo research on angiogenesis. *Int. J. Dev. Biol.* **40**, 1189–1197 (1996).
7. Gale, N. W. & Yancopoulos, G. D. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev.* **13**, 1055–1066 (1999).
8. Carmeliet, P. Mechanisms of angiogenesis and arteriogenesis. *Nature Med.* **6**, 389–395 (2000).
9. Hellstrom, M., Kaln, M., Lindahl, P., Abramsson, A. & Betsholtz, C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* **126**, 3047–3055 (1999).
10. Hirschi, K. K., Rohovsky, S. A., Beck, L. H., Smith, S. R. & D'Amore, P. A. Endothelial cells modulate the proliferation of mural cell precursors via platelet-derived growth factor-BB and heterotypic cell contact. *Circ. Res.* **84**, 298–305 (1999).
11. Suri, C. *et al.* Requisite role of Angiopoietin-1, a ligand for the Tie2 receptor, during embryonic angiogenesis. *Cell* **87**, 1171–1180 (1996).
12. Maisonpierre, P. C. *et al.* Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* **277**, 55–60 (1997).
13. Holash, J., Wiegand, S. J. & Yancopoulos, G. D. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* **18**, 5356–5362 (1999).
14. Holash, J. *et al.* Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science* **284**, 1994–1998 (1999).
15. Soker, S., Takashima, S., Miao, H., Neufeld, G. & Klagsbrun, M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92**, 735–745 (1998).
16. Shalaby, F. *et al.* Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376**, 62–66 (1995).
17. Fong, G. H., Rossant, J., Gertenstein, M. & Breitman, M. L. Role of the Flt-1 receptor tyrosine kinase in regulating assembly of vascular endothelium. *Nature* **376**, 66–70 (1995).
18. Hiratsuka, S., Minowa, O., Kuno, J., Noda, T. & Shibuya, M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl Acad. Sci. USA* **95**, 9349–9354 (1998).
19. Taipale, J. *et al.* Vascular endothelial growth factor receptor-3. *Curr. Top. Microbiol. Immunol.* **237**, 85–96 (1999).
20. Persico, M. G., Vincenti, V. & DiPalma, T. Structure, expression and receptor-binding properties of placenta growth factor (PlGF). *Curr. Top. Microbiol. Immunol.* **237**, 31–40 (1999).
21. Olofsson, B., Jeltsch, M., Eriksson, U. & Alitalo, K. Current biology of VEGF-B and VEGF-C. *Curr. Opin. Biotechnol.* **10**, 528–535 (1999).
22. Bellomo, D. *et al.* Mice lacking the vascular endothelial growth factor-B gene (Vegfb) have smaller hearts, dysfunctional coronary vasculature, and impaired recovery from cardiac ischemia. *Circ. Res.* **86**, E29–E35 (2000).
23. Carmeliet, P. *et al.* Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435–439 (1996).
24. Ferrara, N. *et al.* Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439–442 (1996).
25. Carmeliet, P. *et al.* Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. *Nature Med.* **5**, 495–502 (1999).
26. Miquerol, L., Langille, B. L. & Nagy, A. Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development* **127**, 3941–3946 (2000).
27. Gerber, H. P. *et al.* VEGF is required for growth and survival in neonatal mice. *Development* **126**, 1149–1159 (1999).
28. Ferrara, N. *et al.* Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nature Med.* **4**, 336–340 (1998).
29. Gerber, H. P. *et al.* VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nature Med.* **5**, 623–628 (1999).
30. Stone, J. *et al.* Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *J. Neurosci.* **15**, 4738–4747 (1995).
31. Alon, T. *et al.* Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nature Med.* **1**, 1024–1028 (1995).
32. Benjamin, L. E., Hemo, I. & Keshet, E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* **125**, 1591–1598 (1998).
33. Stone, J. *et al.* Roles of vascular endothelial growth factor and astrocyte degeneration in the genesis of retinopathy of prematurity. *Invest. Ophthalmol. Vis. Sci.* **37**, 290–299 (1996).
34. Pierce, E. A., Foley, E. D. & Smith, L. E. Regulation of vascular endothelial growth factor by oxygen in a model of retinopathy of prematurity. *Arch. Ophthalmol.* **114**, 1219–1228 (1996). [Published erratum appears in *Arch. Ophthalmol.* **115**, 427 (1997).]
35. Aiello, L. P. *et al.* Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N. Engl. J. Med.* **331**, 1480–1487 (1994).
36. Adamis, A. P. *et al.* Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. *Am. J. Ophthalmol.* **118**, 445–450 (1994).
37. Springer, M. L., Chen, A. S., Kraft, P. E., Bednarski, M. & Blau, H. M. VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol. Cell* **2**, 549–558 (1998).
38. Detmar, M. *et al.* Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. *J. Invest. Dermatol.* **111**, 1–6 (1998).
39. Larcher, F., Murillas, R., Bolontrade, M., Conti, C. J. & Jorcano, J. L. VEGF/VPF overexpression in skin of transgenic mice induces angiogenesis, vascular hyperpermeability and accelerated tumor development. *Oncogene* **17**, 303–311 (1998).
40. Thurston, G. *et al.* Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* **286**, 2511–2514 (1999).
41. Pettersson, A. *et al.* Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial growth factor. *Lab. Invest.* **80**, 99–115 (2000).
42. Thurston, G. *et al.* Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nature Med.* **6**, 460–463 (2000).
43. Korhonen, J. *et al.* Enhanced expression of the *tie* receptor tyrosine kinase in endothelial cells during

neovascularization. *Blood* **80,** 2548–2555 (1992).
44. Maisonpierre, P. C., Goldfarb, M., Yancopoulos, G. D. & Gao, G. Distinct rat genes with related profiles of expression define a TIE receptor tyrosine kinase family. *Oncogene* **8,** 1631–1637. (1993).
45. Sato, T. N., Qin, Y., Kozak, C. A. & Audus, K. L. *tie-1* and *tie-2* define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. *Proc. Natl Acad. Sci. USA* **90,** 9355–9358 (1993).
46. Dumont, D. J., Gradwohl, G. J., Fong, G.-H., Auerbach, R. & Breitman, M. L. The endothelial-specific receptor tyrosine kinase, *tek*, is a member of a new subfamily of receptors. *Oncogene* **8,** 1293–1301 (1993).
47. Iwama, A. *et al.* Molecular cloning and characterization of mouse *Tie* and *Tek* receptor tyrosine kinase genes and their expression in hematopoietic stem cells. *Biochem. Biophys. Res. Commun.* **195,** 301–309 (1993).
48. Davis, S. *et al.* Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* **87,** 1161–1169 (1996).
49. Valenzuela, D. *et al.* Angiopoietins 3 and 4: diverging gene counterparts in mouse and man. *Proc. Natl Acad. Sci. USA* **96,** 1904–1909 (1999).
50. Dumont, D. J. *et al.* Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev.* **8,** 1897–1909 (1994).
51. Sato, T. N. *et al.* Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376,** 70–74 (1995).
52. Suri, C. *et al.* Angiopoietin-1 promotes increased vascularization in vivo. *Science* **282,** 468–471 (1998).
53. Goede, V., Schmidt, T., Kimmina, S., Kozian, D. & Augustin, H. G. Analysis of blood vessel maturation processes during cyclic ovarian angiogenesis. *Lab. Invest.* **78,** 1385–1394 (1998).
54. Stratmann, A., Risau, W. & Plate, K. H. Cell type-specific expression of Angiopoietin-1 and Angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am. J. Pathol.* **153,** 1459–1466 (1998).
55. Zagzag, D. *et al.* In Situ expression of angiopoietins in astrocytomas identifies angiopoietin-2 as an early marker of tumor angiogenesis. *Exp. Neurol.* **159,** 391–400 (1999).
56. Flanagan, J. G. & Vanderhaeghen, P. The ephrins and Eph receptors in neural development. *Annu. Rev. Neurosci.* **21,** 309–345 (1998).
57. Davis, S. *et al.* Ligands for the EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science* **266,** 816–819 (1994).
58. Wang, H. U., Chen, Z. & Anderson, D. J. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93,** 741–753 (1998).
59. Adams, R. H. *et al.* Roles for ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev.* **13,** 295–306 (1999).
60. Gerety, S. S., Wang, H. U., Chen, Z. F. & Anderson, D. J. Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol. Cell* **4,** 403–414 (1999).
61. Folkman, J. Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.* **285,** 1182–1186 (1971).
62. Folkman, J. What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Instit.* **82,** 4–6 (1990).
63. Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86,** 353–364 (1996).
64. Wesseling, P., van der Laak, J. A., de Leeuw, H., Ruiter, D. J. & Burger, P. C. Quantitative immunohistological analysis of the microvasculature in untreated human glioblastoma multiforme. *J. Neurosurg.* **81,** 902–909 (1994).
65. Holmgren, L., O'Reilly, M. S. & Folkman, J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nature Med.* **1,** 149–153 (1995).
66. Pezzella, F. *et al.* Non-small-cell lung carcinoma tumor growth without morphological evidence of neo-angiogenesis. *Am. J. Pathol.* **151,** 1417–1423 (1997).
67. Kim, K. J. *et al.* Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* **362,** 841–844 (1993).
68. Ferrara, N. & Alitalo, K. Clinical applications of angiogenic growth factors and their inhibitors. *Nature Med.* **5,** 1359–1364 (1999).
69. Henry, T. D. *et al.* Results of intracoronary recombinant human vascular endothelial growth factor (RhVEGF) administration trial. *J. Am. Coll. Cardiol.* **31,** 65A (1998).
70. Chiron Corporation. Chiron announces preliminary findings from phase II clinical trial of FGF-2. <http://www.prnewswire.com/micro/CHIR> Company Press Release 12 March 2000.
71. O'Reilly, M. S. *et al.* Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis Lung Carcinoma. *Cell* **79,** 315–328 (1994).
72. O'Reilly, M. S. *et al.* Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* **88,** 277–285 (1997).
73. O'Reilly, M. S., Pirie-Shepherd, S., Lane, W. S. & Folkman, J. Antiangiogenic activity of the cleaved conformation of the serpin antithrombin. *Science* **285,** 1926–1928 (1999).

Electronic Acknowledgement Receipt

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Application Number:	16055847
International Application Number:	
Confirmation Number:	3451
Title of Invention:	USE OF A VEGF ANTAGONIST TO TREAT ANGIOGENIC EYE DISORDERS
First Named Inventor/Applicant Name:	George D. Yancopoulos
Customer Number:	96387
Filer:	Karl Bozicevic/Kimberly Zuehlke
Filer Authorized By:	Karl Bozicevic
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	REGN-008CIPCON3_2019-09-18_Supp_IDS_trans.pdf	50971 ffc0593fefab51e6be0793d986fdff55fa2ab9c4	no	2

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2	Non Patent Literature	Barbazetto_Retina_2010.pdf	1353701 d1b271008011f809f30c1145a8ebe6f1960fb06e	no	10
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13	Non Patent Literature	Engelbert_Treat_and_Extend_Article_on_Failed_PIER_Trial_2010.pdf	410243 a3dfe8b73f1483741876ee1134d489a29acb2959	no	2
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39	Non Patent Literature	Lalwani_Retina_Today_2007.pdf	145091 3435d73022ddbb15d91d95efcf25c6fbba0e9d34	no	1
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42	Information Disclosure Statement (IDS) Form (SB08)	REGN-008CIPCON3_2019-09-18_IDS_SB08A.pdf	48950 36dbfa176f4ea18692b9dde16a0218f67fa3c95e	no	3
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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronically Filed 9/18/2019

INFORMATION DISCLOSURE STATEMENT	Attorney Docket No.	REGN-008CIPCON3
	Confirmation No.	3451
	First Named Inventor	George D. Yancopoulos
	Application Number	16/055,847
	Filing Date	August 6, 2018
	Group Art Unit	1647
Address to: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Examiner Name	Jon McClelland Lockard
	Title: "*Use of a VEGF Antagonist to Treat Angiogenic Eye Disorders*"	

Sir:

Applicants submit herewith documents which may be material to the examination of this application and in respect of which there may be a duty to disclose in accordance with 37 C.F.R. § 1.56. This submission is not intended to constitute an admission that any document referred to therein is "prior art" for this invention unless specifically designated as such. A listing of the documents is shown on enclosed Form PTO/SB/08A and copies of the foreign patents and non-patent literature are also enclosed.

The Examiner is requested to make the documents listed on the enclosed PTO/SB/08A of record in this application. Applicants would appreciate the Examiner initialing and returning the initialed copy of form PTO/SB/08A, indicating the documents cited therein have been considered and made of record herein.

Statements

☒ **No statement**

☐ **PTA Statement under 37 CFR § 1.704(d)(1):** Each item of information contained in the information disclosure statement filed herewith:

(i) Was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the information disclosure statement; or

(ii) Is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office, and this communication was not received by any individual designated in § 1.56(c) more than thirty days prior to the filing of the information disclosure statement.

☐ **IDS Statement under 37 CFR § 1.97(e)(1):** Each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement; or

☐ **IDS Statement under 37 CFR § 1.97(e)(2):** No item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in § 1.56(c) more than three months prior to the filing of the information disclosure statement.

Fees

☒ No fee is believed to be due.

☐ The appropriate fee set forth in 37 C.F.R. §1.17(p) accompanies this information disclosure statement.

The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number REGN-008CIPCON3.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: September 18, 2019

By: /Karl Bozicevic, Reg. No. 28,807/
Karl Bozicevic
Reg. No. 28,807

BOZICEVIC, FIELD & FRANCIS LLP
201 Redwood Shores Parkway, Suite 200
Redwood City, CA 94065
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

DOSING REGIMEN AND THE FREQUENCY OF MACULAR HEMORRHAGES IN NEOVASCULAR AGE-RELATED MACULAR DEGENERATION TREATED WITH RANIBIZUMAB

IRENE BARBAZETTO, MD,* NAMRATA SAROJ, OD,† HOWARD SHAPIRO, PhD,† PAMELA WONG, MPH,† K. BAILEY FREUND, MD*

Purpose: The purpose of this study was to investigate if monthly intravitreal ranibizumab decreases risk of macular hemorrhages in patients with choroidal neovascularization secondary to age-related macular degeneration.

Methods: Incidences of macular hemorrhages in the control and ranibizumab groups from three, multicenter, randomized, clinical trials (MARINA, ANCHOR, and PIER) were compared. Two time intervals (Months 0–3 and 5–17) were evaluated to account for transition from monthly to quarterly injections in PIER. Time interval after Month 17 was excluded because of crossover from control to active treatment in all trials.

Results: Months 0–3: All trials showed higher incidence rates of hemorrhages in control compared with ranibizumab groups (ANCHOR: photodynamic therapy [27.3%], 0.3 mg [8.0%], 0.5 mg [8.6%]; MARINA: sham [18.6%], 0.3 mg [8.8%], 0.5 mg [8.8%]; and PIER: sham [16.1%], 0.3 mg [3.4%], 0.5 mg [3.3%]). In ANCHOR and MARINA, data of Months 5–17 showed higher incidence rates in control compared with monthly ranibizumab groups (ANCHOR: photodynamic therapy [47.8%], 0.3 mg [12.5%], 0.5 mg [12.3%]; and MARINA: sham [38.0%], 0.3 mg [13.2%], 0.5 mg [13.0%]), but this was not seen for quarterly ranibizumab groups in PIER (sham [22.4%], 0.3 mg [23.7%], 0.5 mg [28.3%]).

Conclusion: Treatment with monthly intravitreal ranibizumab was associated with reduced risk of new macular hemorrhages when compared with photodynamic therapy (ANCHOR) or sham (MARINA and PIER). There was no difference between PIER quarterly ranibizumab-treated and sham patients.

RETINA 30:1376–1385, 2010

Macular hemorrhages are considered to be a hallmark of neovascular age-related macular degeneration (AMD). Reading centers use the presence of subretinal hemorrhages or hemorrhagic pigment epithelial detachments as a criteria for the presence of choroidal neovascularization[1] when grading fundus photographs of patients with AMD in the absence of other imaging modalities such as fluorescein angiography or optical coherence tomography. Even intraretinal hemorrhages can be a sign of serious progression because they have been associated with the early stages of retinal angiomatous proliferation/type 3 neovascularization.[2] Overall, macular hemorrhages are considered to be a sign of disease activity and, when occupying larger areas or located in the subfoveal region, they are usually associated with a poor visual prognosis in a majority of cases.[3–5] Therefore, prevention or suppression of hemorrhagic incidences should help arrest vision loss.

Intravitreal antivascular endothelial growth factor therapy has become the new standard of care for treating neovascular AMD. This therapy has not only changed the management of neovascular AMD but also, for the first time, improved visual function and limited disease activity in the majority of patients for at least two years.[6,7] Thus, it seems reasonable to believe that

frequent treatment could also potentially limit the occurrence of macular hemorrhages in these patients.

The aim of this exploratory analysis of the data from three Phase 3 clinical trials was to investigate if monthly treatment with intravitreal ranibizumab (Lucentis; Genentech Inc., South San Francisco, CA) decreases the risk of new macular hemorrhages in patients with choroidal neovascularization secondary to AMD.

Material and Methods

An exploratory analysis was conducted using the 2-year safety data from patients enrolled in three, Phase 3, randomized, controlled, multicenter, clinical trials: MARINA,[6] ANCHOR,[7,8] and PIER.[9] Safety-evaluable population included all patients who received at least one study treatment.

Treatment and Follow-up

In MARINA, patients were randomized to sham control or monthly intravitreal ranibizumab injections of 0.3 mg or 0.5 mg. Patients in the ANCHOR study were assigned to verteporfin photodynamic therapy (PDT) (plus monthly sham injections) control or monthly intravitreal ranibizumab injections of 0.3 mg or 0.5 mg (plus sham PDT with saline infusion). In PIER, patients were randomized to sham control or intravitreal ranibizumab injections of 0.3 mg or 0.5 mg. Patients received 3 initial monthly treatments of their assigned dose followed by treatment every 3 months.

In all 3 studies, patients were examined at screening and Day 0. In MARINA and ANCHOR, patients were seen at Day 7 and then monthly from Month 1 through Month 24. In PIER, patients were examined monthly through Month 3 and quarterly starting at Month 5 through Month 23 with additional visits at Months 12 and 24. There was no Month 4 visit in the PIER study. At all study visits, patients were evaluated using Early Treatment Diabetic Retinopathy Study protocol–based best-corrected visual acuity, slit-lamp examination, intraocular pressure measurement, and dilated binocular indirect and high-magnification ophthalmoscopy. Adverse events were collected at every visit except at screening. Fluorescein angiography and fundus photography were performed at screening and at Months 3, 6, 12, and 24 in MARINA, every 3 months starting at screening up to 24 months in ANCHOR, and at screening and at Months 3, 5, 8, 12, and 24 in PIER. In MARINA and ANCHOR, optical coherence tomography was done at select sites at Days 0 and 7 as well as at Months 1 and 12. In PIER, optical coherence tomography was done at select sites at Day 0 and at Months 1, 2, 3, 5, 8, and 12.

Data Collection

The incidences of new macular hemorrhages detected during these studies were identified based on verbatim reports by the study investigators. All verbatim adverse event descriptions coded to the MEDDRA (Medical Dictionary of Regulatory Activities) preferred term: "RETINAL HEMORRHAGE" in the databases were reviewed by the authors (I.B., K.B.F., and N.S.) and reclassified to three categories ("Yes," "Maybe," and "No") on whether they were macular hemorrhages. Only events coded with "Yes" or "Maybe" were included in the final analysis (Table 1).

To account for the transition from monthly injections to quarterly injections in the PIER trial after 3 months, the number of events in all studies was evaluated for 2 time intervals: 0 to 3 months (during monthly injections in MARINA, ANCHOR, and PIER) and 5 to 17 months (during monthly injections in MARINA and ANCHOR and quarterly injections in PIER). The 5- to 17-month time interval was further broken down into quarterly intervals: 5 to <8 months, 8 to <11 months, 11 to <14 months, and 14 to 17 months. The time interval between 3 and 5 months

Table 1. Included cases for "macular hemorrhage" based on investigator verbatim report coded to the MedDRA (Medical Dictionary for Regulatory Activities) preferred term: "RETINAL HEMORRHAGE"

New subretinal hemorrhage
Punctate hemorrhage – subretinal
Hemorrhagic pigment epithelial detachment
Peripapillary subretinal hemorrhage
Recurrent subretinal hemorrhage
Worsening of subretinal macular hemorrhage
Macular dot-blot hemorrhage

From the *Vitreous Retina Macula Consultants of New York, New York, New York; and †Genentech Inc., South San Francisco, California.

This material was partially presented at the Retina Society Annual Meeting, Scottsdale, Arizona, September 2008.

K.B. Freund is a consultant for Genentech. P. Wong, N. Saroj and H. Sahpiro are employees of Genentech.

The study protocols of the ANCHOR, MARINA, and PIER trials (primary reports of safety and efficacy published previously[6-9]) were approved by the Institutional Review Board, National Competent Authority, or Ethics Committee at each participating clinical center before the start of the study. All US sites were compliant with the Health Insurance Portability and Accountability Act of 1996. The three studies are registered at ClinicalTrials.gov (ANCHOR ID No. = NCT0061594; MARINA ID No. = NCT00056836; PIER ID No. = NCT00090623). Before determination of their full eligibility for enrollment, all patients provided written informed consent for their study participation.

Reprint requests: K. Bailey Freund, MD, Vitreous Retina Macula Consultants of New York, 460 Park Avenue, 5th Floor, New York, NY 10022; e-mail: kbfnyf@aol.com

was excluded because there was no Month 4 study visit in the PIER trial. The time interval after Month 17 was excluded because control patients remaining in the studies were allowed to "crossover" to receive ranibizumab in all 3 studies, and many patients switched to monthly 0.5 mg during this period in PIER. However, all adverse events occurring after the crossover were excluded from the analysis.

Statistical Analysis

Incidences of macular hemorrhages were compared using Pearson chi-square or Fisher exact test (when expected cell counts <5) for treatment comparisons within each study as well as cross-study comparisons within each treatment group. These cross-study comparison tests were performed not for formal comparison but for hypothesis generation only. Statistical significance was defined as $P < 0.05$; although in an exploratory analysis, it is particularly important to consider the risks of false conclusions due to multiple comparisons. All statistical analyses were carried out using SAS software v9.1 (SAS Inc, Cary, NC).

Subgroup Analyses

The influence of selected variables was explored in three separate subgroup analyses comparing the incidences of macular hemorrhages: 1) by baseline angiographic lesion composition, presence of classic (either predominantly classic or minimally classic) versus occult lesions in MARINA and PIER; 2) by baseline presence or absence of anticoagulation/platelet inhibitors; and 3) by baseline presence or absence of blood on fluorescein angiography.

Results

A total of 1,315 patients receiving at least 1 study treatment were analyzed from the 3, randomized, controlled, clinical trials: ANCHOR (n = 420), MARINA (n = 713), and PIER (n = 182). The sample size of each study for the evaluated treatment intervals and study arms is shown in Figure 1.

Incidences of Macular Hemorrhages

Months 0–3: monthly ranibizumab or sham injection. In all 3 trials, a higher percentage of patients developed macular hemorrhages in the control group compared with both the ranibizumab-treated groups during Months 0 to 3. In the ANCHOR trial (Figure 1A), new macular hemorrhages were seen in 27.3% of PDT-treated eyes compared with 8.0% in the 0.3-mg ranibizumab-treated group ($P < 0.0001$) and 8.6% in the 0.5-mg ranibizumab-treated group ($P < 0.0001$).

The MARINA trial (Figure 1B) showed 18.6% in the sham group developing macular hemorrhages compared with 8.8% in the 0.3-mg ranibizumab-treated group ($P = 0.0019$) and 8.8% in the 0.5-mg ranibizumab-treated group ($P = 0.0018$).

In the PIER trial (Figure 1C), 16.1% in the sham group and 3.4% in the 0.3-mg ranibizumab-treated group ($P = 0.019$) and 3.3% in the 0.5-mg ranibizumab-treated group ($P = 0.016$) developed macular hemorrhages.

Months 5–17: monthly ranibizumab or sham injection (MARINA/ANCHOR); quarterly ranibizumab or sham injection (PIER). During Months 5 to 17, the incidence of macular hemorrhages was still higher in the control groups for ANCHOR and MARINA when compared with the ranibizumab-treated groups. The ANCHOR trial (Figure 1, Panel D) had 47.8% of PDT treated patients compared to 12.5% in the 0.3 mg ranibizumab-treated group ($P < 0.0001$) and 12.3% in the 0.5 mg ranibizumab-treated group ($P < 0.0001$) develop a macular hemorrhage. The MARINA trial (Figure 1E) also showed a higher rate of new macular hemorrhages in the sham group with 38.0% compared with lower rates of 13.2% in the 0.3-mg ranibizumab-treated group ($P < 0.0001$) and 13.0% in the 0.5-mg ranibizumab-treated group ($P < 0.0001$).

However, in the PIER trial (Figure 1F), the incidence rates were not lower in the ranibizumab-treated groups compared with the control group (in fact, they were slightly higher although the differences were not statistically significant). 22.4% of patients in the sham group developed new macular hemorrhages when compared with 23.7% in the 0.3-mg ranibizumab-treated group ($P = 0.87$) and 28.3% in the 0.5-mg ranibizumab-treated group ($P = 0.46$).

For quarterly incidences of new macular hemorrhages in the ANCHOR (Figure 2A) and MARINA (Figure 2B) studies after Month 5, the rate in the ranibizumab-treated groups appears stable between 1% and 7%, whereas the control groups (sham/PDT) range from 10% to 22%. In the PIER study (Figure 2C), the overall incidence of new macular hemorrhages ranged from 3% to 17% for the ranibizumab-treated eyes and from 4% to 10% for the control (sham) eyes.

Cross-Study Comparison Between Studies

Given the different patient populations; different control groups; and differences in sample size, follow-up, and crossover regimens, cross-study comparisons (Figure 1) are intended for hypothesis generation only and the data should be reviewed with caution. As a reference (not for formal comparisons), Pearson chi-square or Fisher exact test (when expected cell counts <5) yields the following P values for cross-study

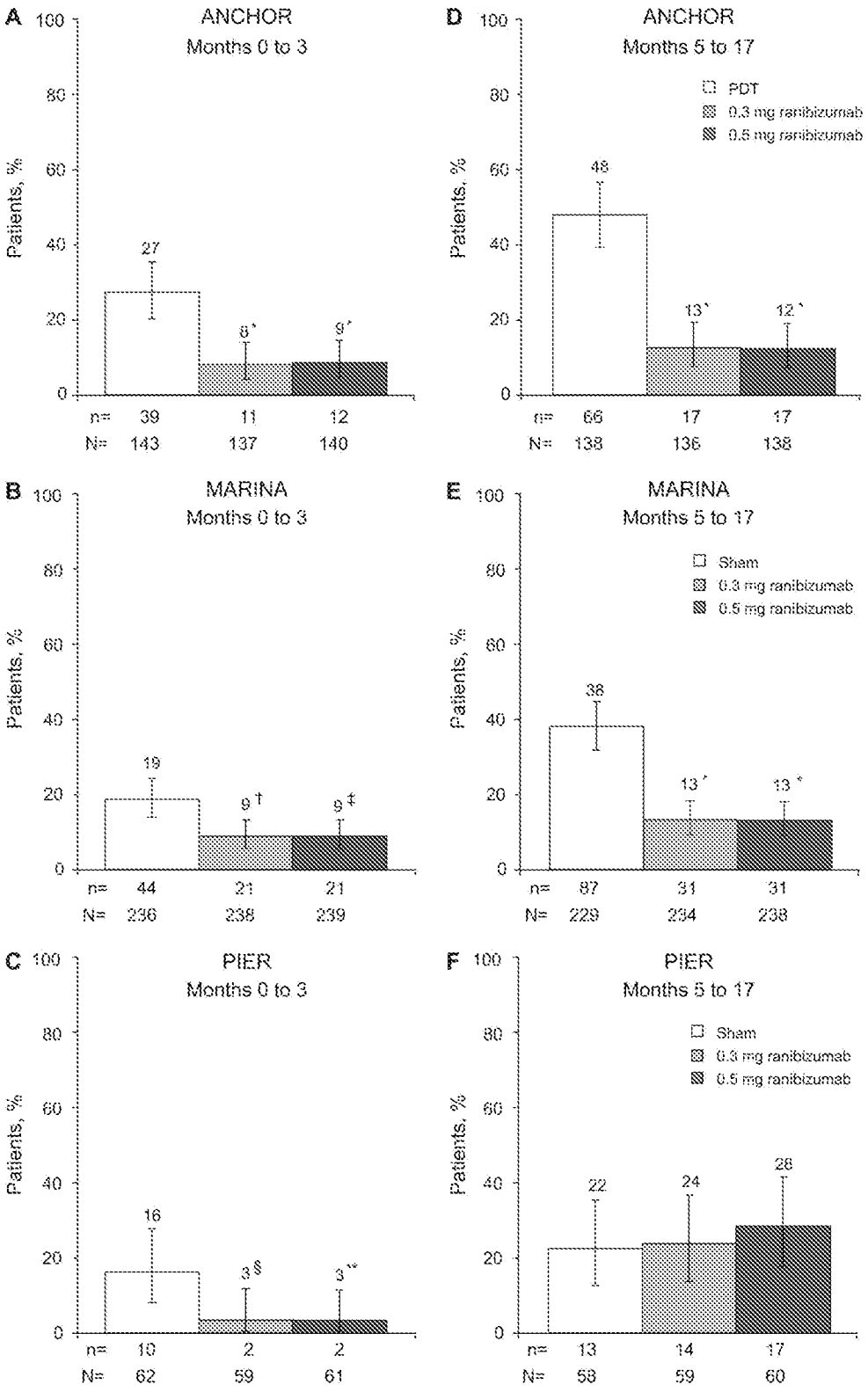

Fig. 1. Summary of the incidences of new macular hemorrhages in the study eye in the ANCHOR (**A** & **D**), MARINA (**B** & **E**) and PIER (**C** & **F**) studies subdivided for the 2 study periods (months 0–3 and months 5–17). *$P < 0.0001$, †$P = 0.0019$, ‡$P = 0.018$ §$P = 0.019$, **$P = 0.016$ vs. control (sham or PDT). Error bars are 95% exact confidence intervals.

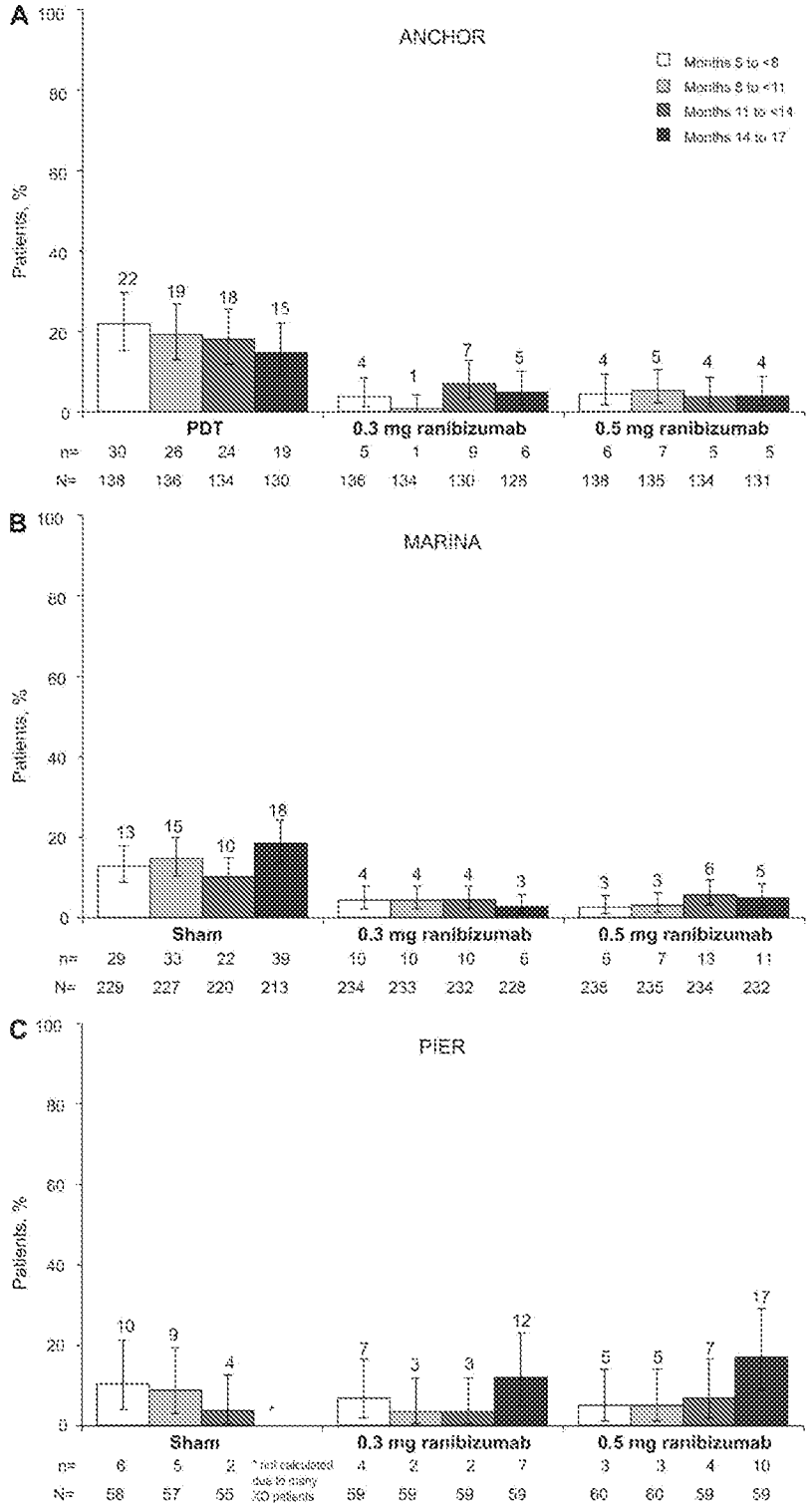

Fig. 2. Quarterly reported incidences of new macular hemorrhages in the ANCHOR (**A**), MARINA (**B**), and PIER (**C**) studies, Months 5 to 17. *Months 14 to 17 for the PIER sham group were excluded from the summary because most patients in sham crossed over to receive 0.5-mg ranibizumab at Month 14. Error bars are 95% exact confidence intervals.

comparison for the incidence of macular hemorrhages within each treatment group.

For the Months 0 to 3 interval, little difference was found between the incidence rates in the PIER and MARINA trials (control [$P = 0.65$], 0.3 mg [$P = 0.27$], and 0.5 mg [$P = 0.18$]). Similarly, little difference was found between the incidence rates in PIER and ANCHOR trials (control [$P = 0.09$], 0.3 mg [$P =$

0.35], and 0.5 mg [$P = 0.24$]). For the Months 0 to 3 interval, the incidence of macular hemorrhages was 27% in the ANCHOR PDT arm and 19% in the MARINA sham arm ($P = 0.049$) although there was little difference between ANCHOR and MARINA in the ranibizumab arms (0.3 mg [$P = 0.79$] and 0.5 mg [$P = 0.94$]).

However, at months 5 to 17 differences were observed between the rates in the PIER and MARINA trials for all the study arms including the control group (PIER vs MARINA: control [$P = 0.026$, rate in PIER less than MARINA], 0.3 mg [$P = 0.046$, rate in PIER greater than MARINA], and 0.5 mg [$P = 0.0039$, rate in PIER greater than MARINA]). The comparison of the PIER and the ANCHOR studies also showed similar results with differences between all groups (PIER versus ANCHOR: control [$P = 0.0009$, rate in PIER less than ANCHOR]), 0.3 mg ($P = 0.049$, rate in PIER greater than ANCHOR), and 0.5 mg ($P = 0.0060$, rate in PIER greater than ANCHOR). For the Months 5 to 17 interval, the incidence of macular hemorrhages differed little between ANCHOR and MARINA among the treatment groups (control [$P = 0.06$], 0.3 mg [$P = 0.84$], and 0.5 mg [$P = 0.84$]).

Lesion Composition at Baseline: Presence of Classic (Predominantly Classic or Minimally Classic) or Occult Without Classic

Baseline angiographic lesion composition, as determined by the reading center (Figure 3), did not reveal any significant influence on the subsequent incidences of macular hemorrhages in the MARINA

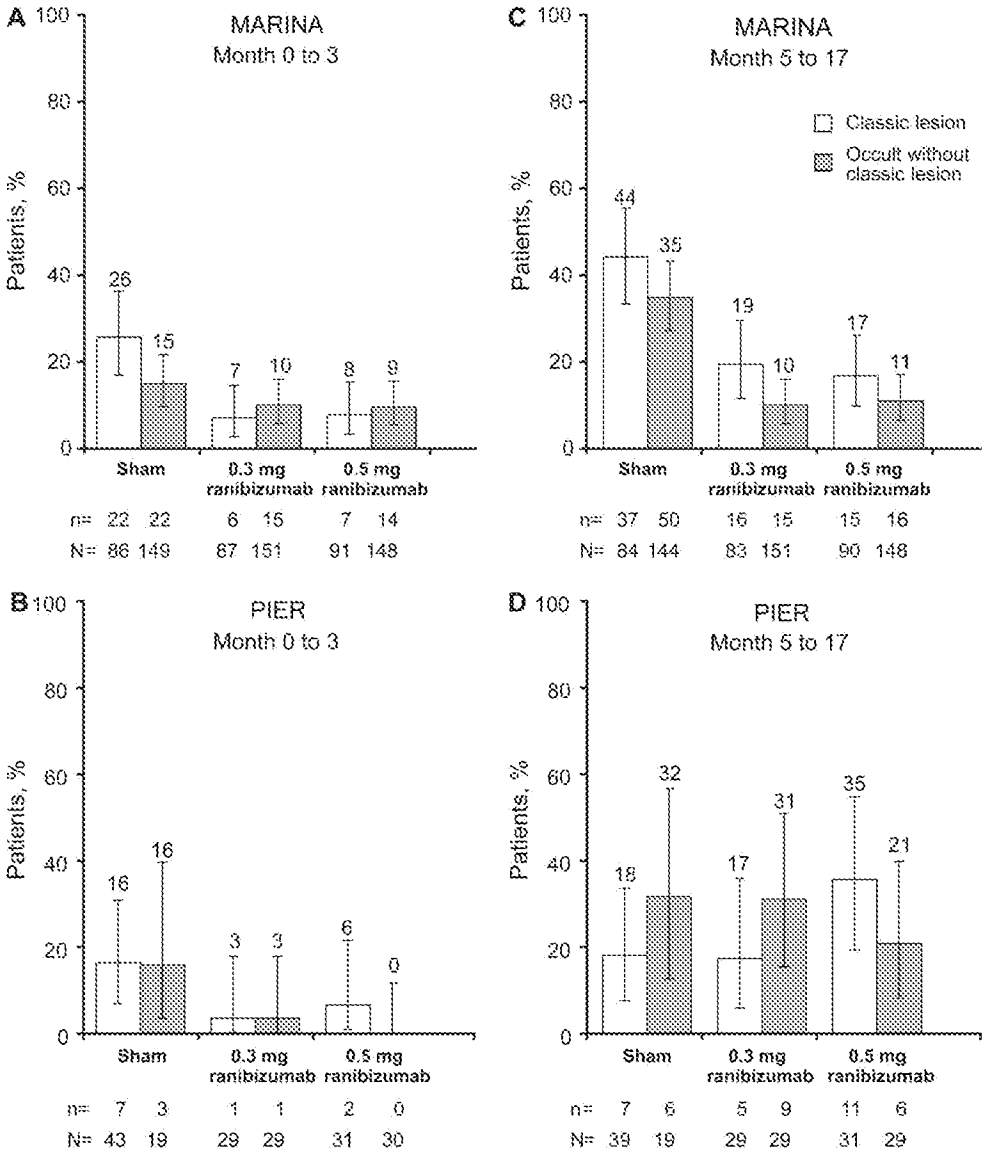

Fig. 3. Macular hemorrhages by baseline lesion type (classic vs occult lesions) in the MARINA (**A** & **C**) and PIER (**B** & **D**) studies subdivided for the 2 study periods (months 0–3 and months 5–17). There was only one safety-evaluable patient in ANCHOR with occult without classic lesion at baseline. Therefore, the subgroup analysis by lesion type was not done for ANCHOR. One PIER patient had a baseline lesion type that could not be classified and was excluded from the analysis. Error bars are 95% exact confidence intervals.

and PIER studies during the first 3 months, except for MARINA sham ($P = 0.041$, 26% classic versus 15% occult without classic). In the Months 5 to 17 interval, the MARINA study showed a trend toward lesions with a classic component to be more likely to develop hemorrhages than occult without classic lesions; the difference was statistically significant for the 0.3-mg group ($P = 0.044$, 19% classic versus 10% occult without classic). However, the PIER study showed a reversed indication during Months 5 to 17 (not statistically significant) where occult lesions were more likely to develop hemorrhages than classic lesions for the sham and the 0.3-mg ranibizumab–treated groups.

The subgroup analysis stratified by lesion type was not done for ANCHOR because of the inclusion criterion that allowed only patients with at least 50% classic lesions in the trial. However, there was one safety-evaluable patient in ANCHOR with occult without classic lesion at baseline.

Role of Anticoagulation/Platelet Inhibitors at Baseline

The incidence of macular hemorrhages was analyzed for patients taking anticoagulants (i.e., warfarin) or platelet aggregation inhibitors (i.e., acetylsalicylic acid [Aspirin], clopidogrel bisulfate [Plavix], etc.) versus patients taking none of these medications at baseline (Figure 4). No statistical differences between the groups were found in ANCHOR, MARINA, or PIER for each time interval.

Presence or Absence of Blood at Baseline

Blood at baseline was categorized as present, absent, questionable, or cannot be graded. For these analyses, present and questionable were combined as present at baseline and compared with absent at baseline (Figure 5).

In ANCHOR, the incidence of macular hemorrhage was higher with blood present at baseline compared with blood absent at baseline except for the control group at Months 0 to 3 and the 0.5-mg group at Months 5 to 17. However, none of the comparisons were statistically significant.

In MARINA, the incidence of macular hemorrhage was higher with blood present at baseline compared with blood absent at baseline in all instances although none were statistically significant.

In PIER, the incidence of macular hemorrhage was higher with blood present at baseline compared with blood absent at baseline except for the control group at Months 5 to 17 and 0.3 mg at Months 0 to 3 where the reverse was found but was not statistically significant. One statistically significant comparison was found, for the sham group at Months 0 to 3 ($P = 0.050$, 22.2% for present versus 0% for absent).

Discussion

Macular hemorrhages of varying degrees and extensions are known to occur as part of the natural course of AMD[3,4] and as complications after interventions for neovascular disease.[10] The outcome can be devastating for the patient and not only lead to visual impairment but also place a significant psychological burden on patients and families.[5]

Controlling the neovascular process with reduction of exudative activity is a logical approach to reduce the risk of developing new hemorrhages secondary to AMD. Sustained inhibition of vascular endothelial growth factor with intravitreal ranibizumab is a promising strategy for such a therapeutic/preventive concept.[6,11]

The analysis of the safety data of the three major controlled clinical trials (ANCHOR, MARINA, and PIER) presents the unique opportunity to evaluate large treated patient cohorts and controls for the incidence of macular hemorrhages in randomized double-blind studies with standardized treatment protocols. Patients on monthly ranibizumab in MARINA, ANCHOR, and PIER were significantly less likely to develop macular hemorrhages when compared with the control groups. In PIER, no benefit of ranibizumab over sham was observed after the patients were switched to the quarterly protocol. It should be noted that the evaluation of the PIER study data is complicated as a result of the study arms being significantly smaller and some patients of the sham group having crossed over (15 at Month 14 and another 17 at Month 17) to receive 0.5-mg ranibizumab. Baseline angiographic lesion composition as well as medications for anticoagulation and platelet aggregation inhibition did not influence the risk of developing macular hemorrhages in any of the study treatment groups. Baseline presence of blood was mostly consistent as to incidence of macular hemorrhages being higher in the group with blood present at baseline versus blood absent at baseline, but only 1 of the 18 comparisons among study treatment groups was statistically significant.

This study is also limited by several factors, which include but are not limited to the retrospective nature of the data review as well as the differences in follow-up, treatment protocols, and control group composition of the individual studies. In the PIER study, the number of patients is significantly smaller when compared with the MARINA and ANCHOR studies. In addition, the follow-up intervals after the initial 3 months were only quarterly in PIER, which may result in missed events and lead to an underestimation of the number of events. Finally, because none of the studies required monthly fundus photographic documentation, identification of macular hemorrhages is dependent on verbatim reports

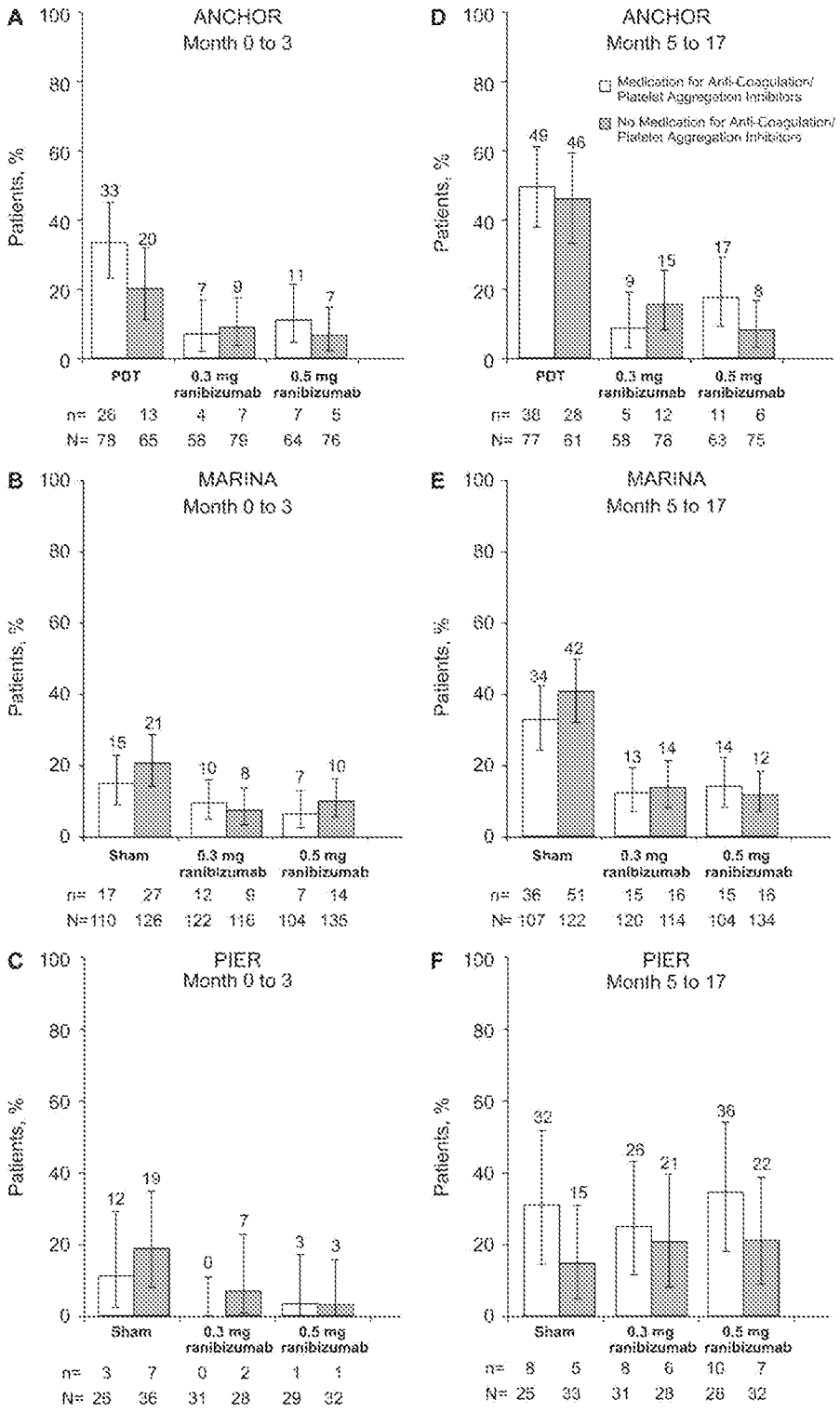

Fig. 4. Macular hemorrhages by baseline medication for anticoagulation/platelet inhibitors in the ANCHOR (**A** & **D**), MARINA (**B** & **E**) and PIER (**C** & **F**) studies subdivided for the 2 study periods (months 0–3 and months 5–17). Error bars are 95% exact confidence intervals.

of adverse events by the individual investigators who may have varying definitions/methods of identification. However, in spite of its limitations, the results of this analysis may give the treating ophthalmologists additional information on how to manage their patients with AMD and to achieve best functional results and visual preservation.

In conclusion, although none of the treatment arms in MARINA, ANCHOR, and PIER showed a complete absence of new macular hemorrhages during the study

period, monthly application of 0.3-mg or 0.5-mg ranibizumab significantly decreased the risk of developing new macular hemorrhages when compared with the control groups (sham or PDT). As seen in PIER, switching from monthly to quarterly injection intervals may not have the same beneficial effect and could put the patient at an increased risk for vision-threatening complications.

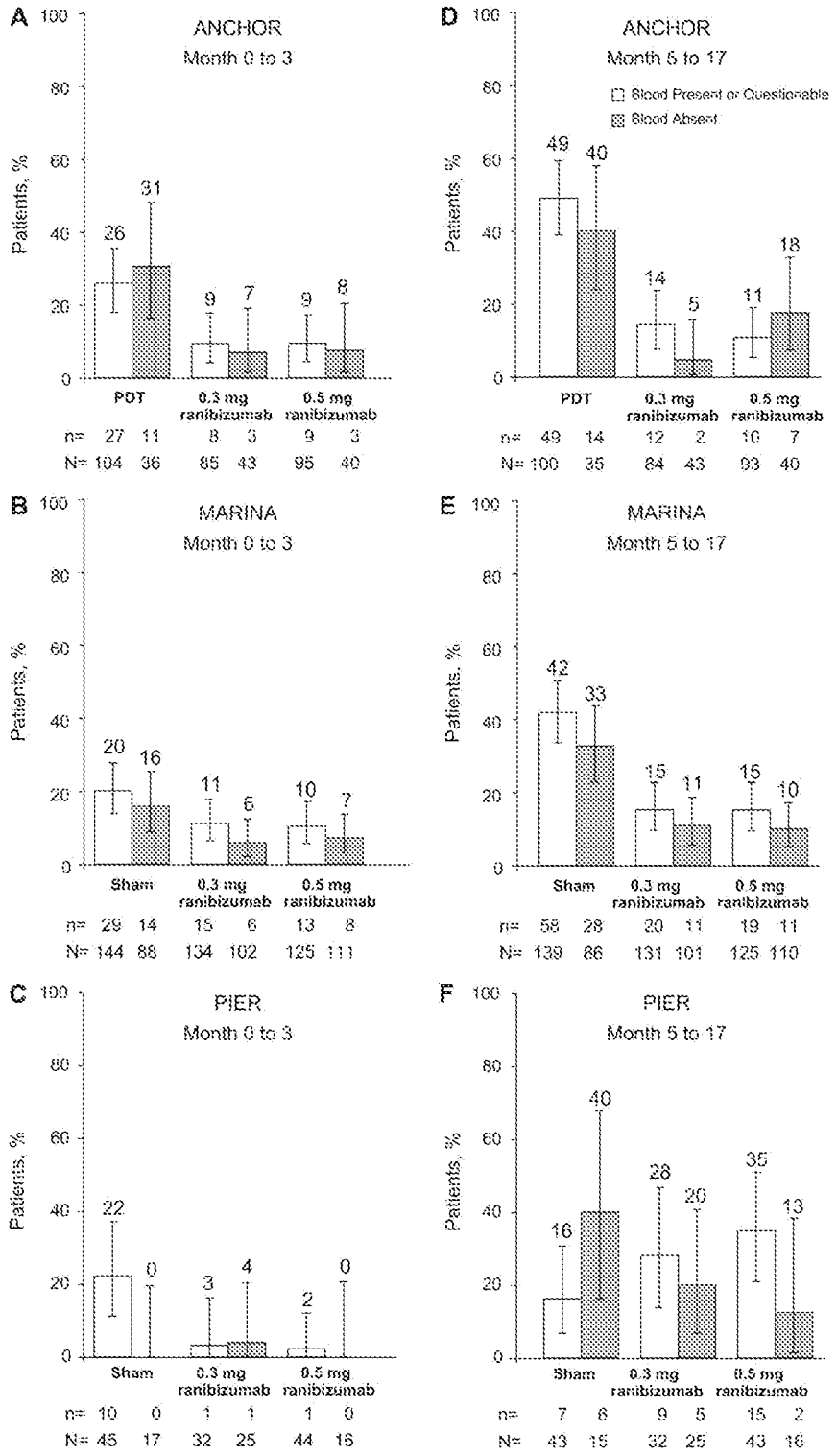

Fig. 5. Macular hemorrhages by baseline presence or absence of blood in the ANCHOR (**A** & **D**), MARINA (**B** & **E**) and PIER (**C** & **F**) studies subdivided for the 2 study periods (months 0–3 and months 5–17). Error bars are 95% exact confidence intervals.

Key words: age-related macular degeneration, choroidal neovascularization, ranibizumab, macular hemorrhage.

Acknowledgments

The ANCHOR, MARINA, and PIER studies were funded by Genentech, Inc., South San Francisco, CA, and Novartis Pharma, AG, Basel, Switzerland. Genentech designed and oversaw the conduct of the ANCHOR, MARINA, and PIER studies and managed and statistically analyzed the data.

References

1. Davis MD, Gangnon RE, Lee LY, et al. The Age-Related Eye Disease Study severity scale for age-related macular degeneration: AREDS Report No. 17. Arch Ophthalmol 2005; 123:1484–1498.
2. Yannuzzi LA, Negrao S, Iida T, et al. Retinal angiomatous proliferation in age-related macular degeneration. Retina 2001; 21:416–434.
3. Scupola A, Coscas G, Soubrane G, Balestrazzi E. Natural history of macular subretinal hemorrhage in age-related macular degeneration. Ophthalmologica 1999; 213:97–102.
4. Avery RL, Fekrat S, Hawkins BS, Bressler NM. Natural history of subfoveal subretinal hemorrhage in age-related macular degeneration. Retina 1996;16:183–189.
5. Mozaffarieh M, Sacu S, Benesch T, Wedrich A. Subretinal hemorrhages secondary to age-related macular degeneration: psychological and vision-related functional perspectives. Ophthalmologica 2008;222:199–204.
6. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–1431.
7. Brown DM, Michels M, Kaiser PK, Heier JS, Sy JP, Ianchulev T. Ranibizumab versus verteporfin photodynamic therapy for neovascular age-related macular degeneration: two-year results of the ANCHOR study. Ophthalmology 2009;116:57–65.e5.
8. Brown DM, Kaiser PK, Michels M, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N Engl J Med 2006;355:1432–1444.
9. Regillo CD, Brown DM, Abraham P, et al. Randomized, double-masked, sham-controlled trial of ranibizumab for neovascular age-related macular degeneration: PIER Study year 1. Am J Ophthalmol 2008;145:239–248.
10. Goverdhan SV, Lochhead J. Submacular haemorrhages after intravitreal bevacizumab for large occult choroidal neovascularisation in age-related macular degeneration. Br J Ophthalmol 2008;92:210–212.
11. Gaudreault J, Fei D, Beyer JC, et al. Pharmacokinetics and retinal distribution of ranibizumab, a humanized antibody fragment directed against VEGF-A, following intravitreal administration in rabbits. Retina 2007;27:1260–1266.

A Phase IIIb Study to Evaluate the Safety of Ranibizumab in Subjects with Neovascular Age-related Macular Degeneration

David S. Boyer, MD,[1] Jeffrey S. Heier, MD,[2] David M. Brown, MD,[3] Steven F. Francom, PhD,[4] Tsontcho Ianchulev, MD,[4] Roman G. Rubio, MD[4]

Objective: To evaluate the safety and efficacy of intravitreal ranibizumab in a large population of subjects with neovascular age-related macular degeneration (AMD).

Design: Twelve-month randomized (cohort 1) or open-label (cohort 2) multicenter clinical trial.

Participants: A total of 4300 subjects with angiographically determined subfoveal choroidal neovascularization (CNV) secondary to AMD.

Methods: Cohort 1 subjects were randomized 1:1 to receive 0.3 mg (n = 1169) or 0.5 mg (n = 1209) intravitreal ranibizumab for 3 monthly loading doses. Dose groups were stratified by AMD treatment history (treatment-naïve vs. previously treated). Cohort 1 subjects were retreated on the basis of optical coherence tomography (OCT) or visual acuity (VA) criteria. Cohort 2 subjects (n = 1922) received an initial intravitreal dose of 0.5 mg ranibizumab and were retreated at physician discretion. Safety was evaluated at all visits.

Main Outcome Measures: Safety outcomes included the incidence of ocular and nonocular adverse events (AEs) and serious adverse events (SAEs). Efficacy outcomes included changes in best-corrected VA over time.

Results: Some 81.7% of cohort 1 subjects and 49.9% of cohort 2 subjects completed the 12-month study. The average total number of ranibizumab injections was 4.9 for cohort 1 and 3.6 for cohort 2. The incidence of vascular and nonvascular deaths during the 12-month study was 0.9% and 0.7% in the cohort 1 0.3 mg group, 0.8% and 1.5% in the cohort 1 0.5 mg group, and 0.7% and 0.9% in cohort 2, respectively. The incidence of death due to unknown cause was 0.1% in both cohort 1 dose groups and cohort 2. The number of vascular deaths and deaths due to unknown cause did not differ across cohorts or dose groups. Stroke rates were 0.7%, 1.2%, and 0.6% in the 0.3 mg and 0.5 mg groups and cohort 2, respectively. At month 12, cohort 1 treatment-naïve subjects had gained an average of 0.5 (0.3 mg) and 2.3 (0.5 mg) VA letters and previously treated subjects had gained 1.7 (0.3 mg) and 2.3 (0.5 mg) VA letters.

Conclusions: Intravitreal ranibizumab was safe and well tolerated in a large population of subjects with neovascular AMD. Ranibizumab had a beneficial effect on VA. Future investigations will seek to establish optimal dosing regimens for persons with neovascular AMD.

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Neovascular age-related macular degeneration (AMD) is characterized by new vessel growth and leakage in the choroidal vascular network beneath the macula, with extension and leakage into the subretinal space. Although the pathologic events that precede choroidal neovascularization (CNV) are not clearly understood, disrupting the activity of vascular endothelial growth factor A (VEGF-A), a diffusible cytokine that promotes angiogenesis and vascular permeability, effectively treats CNV secondary to AMD.

Ranibizumab (LUCENTIS, Genentech, Inc., South San Francisco, CA) is a recombinant, humanized monoclonal antibody antigen-binding fragment (Fab) that neutralizes all active forms of VEGF-A. In 2 pivotal phase III trials—Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular Age-Related Macular Degeneration (MARINA)[1] and Anti-Vascular Endothelial Growth Factor (VEGF) Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization (CNV) in Age-related Macular Degeneration (ANCHOR)[2]—monthly intravitreal injections of 0.3 mg or 0.5 mg ranibizumab not only prevented vision loss but also improved visual acuity (VA) in patients with minimally classic or occult without classic and predominantly classic CNV, respectively. In those studies, ranibizumab treatment was associated with a low rate of serious adverse events (SAEs), including those attributable to systemic VEGF inhibition.

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The Safety Assessment of Intravitreous Lucentis for AMD (SAILOR) study was a phase IIIb follow-up study to the MARINA and ANCHOR studies to evaluate the long-term safety and efficacy of ranibizumab in a large population of subjects with all subtypes (minimally classic, occult without classic, and predominantly classic) of neovascular AMD. SAILOR included more than 5 times as many ranibizumab-treated subjects as the MARINA and ANCHOR studies combined. Thus, it is the largest multicenter randomized study to date to evaluate safety and efficacy outcomes of anti-VEGF treatment in wet AMD, and it is the only phase III study to examine individualized, criteria-based retreatment.

Materials and Methods

SAILOR was a 12-month, multicenter, phase IIIb study intended to further characterize the safety and efficacy profiles of intravitreal ranibizumab. Protocols were approved by the institutional review board at each study site, and the study was conducted according to the International Conference on Harmonisation E6 Guideline for Good Clinical Practice and any national requirements. All subjects provided informed consent before participation in the study. The SAILOR study is registered at www.clinicaltrials.gov (NCT00251459; accessed February 5, 2009).

Two study cohorts were enrolled. Cohort 1 subjects were randomized 1:1 to receive 0.3 mg or 0.5 mg intravitreal ranibizumab. Cohort 2 subjects received open-label 0.5 mg intravitreal ranibizumab. Eligible subjects were ≥50 years of age with 20/40 to 20/400 (Snellen equivalent) best-corrected VA in the study eye. Cohort 1 VA was assessed with the Early Treatment Diabetic Retinopathy Study (ETDRS) chart. In the interest of conserving time and resources, VA for cohort 2 (under a less rigorous treatment and assessment schedule) was assessed using Snellen charts. All subjects had angiographically determined subfoveal CNV (minimally classic, occult without classic, predominantly classic) secondary to AMD (as determined by the investigating physician), with evidence of recent disease progression defined by any of the following: loss of ≥5 ETDRS letters (or ≥1 Snellen line) within 6 months before study initiation (i.e., day 0); 10% increase in the CNV lesion area determined by comparing a fluorescein angiogram performed within 1 month before day 0 with an angiogram performed within 6 months before day 0; subretinal hemorrhage associated with CNV within 1 month before day 0; or classic CNV comprising >50% of the CNV lesion area.

Key exclusion criteria included verteporfin photodynamic therapy, pegaptanib sodium, or other AMD therapy within 30 days before day 0; previous submacular surgery or other surgical intervention for AMD in the study eye; participation in an investigational drug (except vitamins and minerals) study within 30 days before day 0; previous participation in a ranibizumab clinical trial; intravitreal administration of bevacizumab within 30 days before day 0; or current use of systemic anti-VEGF agents. Also excluded were subjects with fibrosis or atrophy involving the foveal center of the treated eye in the absence of a new lesion; CNV in either eye due to other causes, such as ocular histoplasmosis, trauma, or pathologic myopia; a tear in the retinal pigment epithelium of the study eye involving the macula; or any current intraocular condition in the study eye (e.g., cataract or diabetic retinopathy) that, in the investigating physician's opinion, would require medical or surgical intervention during the 12-month study period or, if allowed to progress untreated, would likely contribute to the loss of at least 2 Snellen equivalent lines of VA over the 12-month study period. Subjects with a history of cardiovascular disease were not excluded if their disease was controlled.

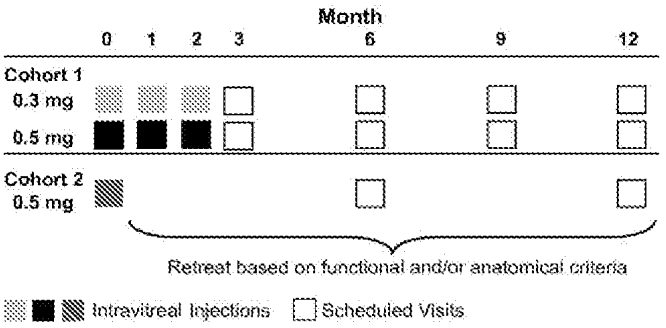

Figure 1. Study treatment and assessments. Cohort 1 subjects received 3 loading doses of ranibizumab and were retreated on the basis of VA (>5 letter decrease in VA from highest score at prior visits) or VA and/or OCT (>100 μm increase in CFT from the lowest measurement at prior visits) criteria. Cohort 2 subjects received 1 dose of ranibizumab on day 1 and were retreated at physician discretion. CFT = central foveal thickness; OCT = optical coherence tomography; VA = visual acuity.

Cohort 1 subjects were randomized 1:1 to receive 0.3 mg or 0.5 mg intravitreal ranibizumab. To prevent bias in reporting AEs, subjects were masked to treatment dose. (Because SAILOR was not designed with efficacy as an objective, physicians and study monitors were not masked.) Randomization was stratified according to treatment history. "Previously treated" subjects had previously received treatment AMD. "Treatment-naïve" subjects were newly diagnosed with neovascular AMD. Cohort 1 subjects received 3 monthly loading doses of intravitreal ranibizumab (day 0, month 1, and month 2) with scheduled follow-up visits at months 3, 6, 9, and 12 (Fig 1). If, at any time, the investigating physician believed that the between-visit interval was too long for a patient to go without being assessed, an unscheduled visit could occur. After the 3 loading doses, retreatment was based on (1) VA (a >5 ETDRS letter decrease in VA compared with the highest VA score at any prior scheduled visit) or (2) VA (same as above) and/or optical coherence tomography (OCT) (a >100-μm increase in central foveal thickness [CFT] compared with the lowest measurement at any previous scheduled study visit, with intraretinal or subretinal fluid present). Thus, OCT assessment was required only for retreatment option 2, in which case OCT data were consistently obtained at all study visits. Retreatment was to occur no more frequently than every 30 days. Before randomization, the investigating physician selected the retreatment criterion for each subject that was to be used throughout the study.

Cohort 1 subjects were evaluated with a full ocular examination and best-corrected VA (ETDRS chart at a distance of 4 m) and safety assessments on day 0 and at all scheduled (months 1, 2, 3, 6, 9, and 12) visits. Visual acuity assessments were required at unscheduled visits if a subject was being evaluated for retreatment. Safety assessments were required at all unscheduled visits.

Cohort 2 included both previously treated and treatment-naïve subjects. Subjects received 0.5 mg of ranibizumab, with an initial injection on day 0 and retreatment at the investigating physician's discretion, no more frequently than every 30 days. Cohort 2 subjects were evaluated for Snellen VA at day 0 and months 6 and 12. At unscheduled visits, VA was assessed at the investigating physician's discretion. Serious adverse events and adverse events (AEs) were assessed at scheduled and unscheduled visits, with formal safety assessments scheduled for months 6 and 12.

Adverse events included any unfavorable or unintended sign, symptom, or disease temporally associated with use of study drug or other protocol-imposed intervention. An AE was classified as an SAE if it caused or led to death, required or prolonged subject hospitalization, resulted in persistent or significant disability or incapacitation, or was considered to be a significant medical event by the investigating physician.

One eye per subject (i.e., the study eye) was treated. After thoroughly cleansing the lid, lashes, periorbital area, and conjunctiva with povidone iodine, local anesthesia and antimicrobials (ofloxacin ophthalmic solution, trimethoprim-polymyxin B ophthalmic solution, moxifloxacin ophthalmic solution, or gatifloxacin ophthalmic solution) were administered to the study eye. A 30-gauge, 0.5-inch needle attached to a low-volume syringe containing 50 μL of ranibizumab solution was inserted through the conjunctiva and sclera, 3.5 to 4.0 mm posterior to the limbus, avoiding the horizontal meridian and aiming toward the center of the globe. The injection volume was delivered slowly. The needle was slowly removed, ensuring that all drug solution was in the eye. Immediately after the injection, antimicrobial drops were administered, and the subject was instructed to self-administer antimicrobial drops 4 times daily for 3 days. The study eye was assessed with a finger count test and intraocular pressure within 15 and 70 minutes, respectively, of the ranibizumab injection.

The primary safety end point for cohort 1 was incidence of ocular and nonocular SAEs evaluated through month 12. A secondary safety end point was incidence of ocular and nonocular AEs evaluated through month 12. Efficacy end points for cohort 1 included change from baseline VA, proportion of subjects who gained ≥15 VA letters from baseline, and change from baseline CFT across the study period.

The primary safety end points for cohort 2 were the incidence of ocular and nonocular SAEs and AEs evaluated through month 12. Efficacy outcomes for cohort 2 included median change in Snellen VA from baseline and the proportion of subjects with Snellen 20/200 or worse at baseline compared with months 6 and 12.

Statistical Analysis

Safety and efficacy analyses included all subjects who received at least 1 injection of ranibizumab. Incidence of ocular and nonocular SAEs and AEs and 95% 2-sided confidence intervals for key SAEs were determined for both cohorts and each dose group. No formal hypothesis testing was conducted to compare cohorts, dose groups, or treatment-naïve and previously treated subjects. A sample of 2378 cohort 1 subjects and 1922 cohort 2 subjects was considered sufficient to estimate rates of uncommon SAEs and AEs.

Efficacy results for cohort 1 were stratified by dose group and treatment history. Estimated proportions were obtained for dichotomous end points. Continuous end points were evaluated using descriptive statistics, including mean, median, standard deviation, standard error, and range.

To further evaluate stroke rates across cohorts and dose groups, each subject's medical history was reviewed, and subjects were classified by preexisting conditions that may have been associated with the incidence of stroke during the 12-month study. These included prior stroke, myocardial infarction (MI), hypertension, transient ischemic attack, coronary artery disease, arrhythmias, valve malfunction, congestive heart failure, angioplasty, deep vein thrombosis, diabetes, endocardectomy, cardiac inflammation, prior stent, and use of aspirin, lipid-lowering drugs, anticoagulants, or platelet aggregation inhibitors. A univariate Cox proportional hazard regression model was used to identify which of those were significant (i.e., $P \leq 0.05$) risk factors for stroke in SAILOR. In addition, models that included the interaction of dose with each of the significant risk factors were fit separately.

Missing Data

Missing data were not imputed for safety end points. For cohort 1, missing values for efficacy end points were imputed using the last-observation-carried-forward method. For cohort 2, missing Snellen values were not imputed.

Results

From November 2005 to June 30, 2006 (when ranibizumab was approved for the treatment of neovascular AMD by the Food and Drug Administration), 2378 cohort 1 subjects were randomly assigned to receive 0.3 mg (n = 1169) or 0.5 mg (n = 1209) intravitreal ranibizumab at 105 US centers. Cohort 1 subjects had an average age of 79 years, and 59% were female (Table 1). Approximately 60% of cohort 1 subjects in each dose group had been previously treated for AMD. The types of previous treatment were similar across dose groups and included photodynamic therapy (33%), intravitreal pegaptanib sodium (30%), intravitreal triamcinolone acetonide (17%), and laser photocoagulation (10%). Investigating physicians elected to use the VA plus OCT retreatment criterion for approximately 81% of the subjects in each dose group.

Previously treated and treatment-naïve subjects had similar baseline ocular characteristics, with the exception that previously treated subjects had a longer time since first diagnosis and lower baseline VA (Table 2). Approximately 18% of cohort 1 subjects in each dose group discontinued the study before the month 12 visit (Table 3). Baseline ocular characteristics of subjects who com-

Table 1. Subject Baseline Characteristics

Characteristic	Cohort 1		Cohort 2
	0.3 mg (n = 1169)	*0.5 mg (n = 1209)*	*0.5 mg (n = 1922)*
Age (yrs)			
Mean ± SD	78.7±7.6	78.7±8.6	78.7±8.1
Range	51–97	52–101	45–99
Sex			
Female	59.9	58.1	61.6
Race			
Caucasian	96.6	97.1	96.2
AMD treatment history			
Treatment naïve	39.5	40.5	—
Previously treated	60.5	59.5	—
Retreatment criteria			
VA	19.3	18.4	—
VA plus OCT	80.7	81.6	—
Systolic BP			
Mean ± SD	137.4±17.3	137.8±18.0	—
Range	90–213	80–220	—
Diastolic BP			
Mean ± SD	76.2±9.7	77.0±9.7	—
Range	48–118	48–110	—

AMD = age-related macular degeneration; BP = blood pressure; OCT = optical coherence tomography; SD = standard deviation; VA = visual acuity.
Values are percentages except where otherwise noted.

Table 2. Baseline Ocular Characteristics

	Cohort 1				Cohort 2
	Treatment Naive		*Previously Treated*		
	0.3 mg (n = 462)	*0.5 mg (n = 490)*	*0.3 mg (n = 707)*	*0.5 mg (n = 719)*	*0.5 mg (n = 1922)*
Age at diagnosis (yrs)	79.9±7.9	75.8±8.0	79.9±7.5	79.9±7.5	—
Time since diagnosis (yrs)	0.3±1.4	0.3±0.7	1.4±2.0	1.3±1.7	—
CNV type (%)					
Predominantly classic	32.0	29.4	30.6	31.7	—
Minimally classic	19.7	20.2	26.2	23.5	—
Occult without classic	45.5	48.6	38.6	40.6	—
VA					
ETDRS letters	55.0±12.5	48.9±13.8	53.8±13.8	50.0±14.3	—
Snellen					
Median	20/80	20/80	20/100	20/100	20/100
20/200 or worse (%)	12.2	15.0	22.9	23.0	39
Central foveal thickness (μm)	312±104	322±116	315±113	310±113	—
Intraocular pressure (mmHg)	15.3±3.2	15.3±3.2	15.7±3.3	15.4±3.4	—

CNV = choroidal neovascularization; ETDRS = Early Treatment Diabetic Retinopathy Study; VA = visual acuity.
Values are mean ± standard deviation except where otherwise noted.

pleted the study and those who discontinued were similar. All cohort 1 subjects received their assigned dose of ranibizumab on day 0, and approximately 96% of cohort 1 subjects received their assigned dose at months 1 and 2 (Fig 2). Cohort 1 subjects received an average of 4.6 injections during the 12-month study (the protocol required 3 initial injections). The average number of visits was 8.8 (the protocol required 7 scheduled visits). During months that visits were not scheduled (i.e., months 4, 5, 7, 8, 10, and 11), approximately 40% of the subjects made unscheduled visits, and approximately 16% of those subjects received an injection of ranibizumab at the unscheduled visit (relative to the number of subjects remaining in the study that month) (Fig 2).

From March 2006 to June 30, 2006, 1922 cohort 2 subjects were enrolled at 104 US centers and received 0.5 mg intravitreal ranibizumab (Table 1). Approximately 50% of cohort 2 subjects discontinued the study before the month 12 visit (Table 3). All cohort 2 subjects received the protocol-required injection on day 0 and received an average of 3.6 injections during the 12-month study (the protocol required 1 injection). The average number of visits for cohort 2 subjects was 4.9 (the protocol required 3 scheduled visits). During months that visits were not required (i.e., all but months 6 and 12), the percentage of subjects who remained in the study that made unscheduled visits ranged from 65% at month 2 to 17.4% at month 11. The percentage of subjects receiving injections ranged from 64% at month 2 to 16.5% at month 11.

Table 3. Reasons for Discontinuation

	Cohort 1		Cohort 2
	0.3 mg (n = 1169)	*0.5 mg (n = 1209)*	*0.5 mg (n = 1922)*
Discontinued early (%)	18.6	18.0	50.1
Reason for early discontinuation (%)			
Death	1.7	2.3	1.5
Adverse event	2.6	2.2	1.8
Loss to follow-up	0.7	0.9	2.0
Subject decision	6.7	5.8	29.0
Physician decision	3.4	2.8	9.4
Sponsor decision	0.2	0.1	0.3
Subject noncompliance	0.6	0.9	0.9
Subject's condition mandated other therapeutic intervention	2.7	3.1	5.3
Reason not provided	0.1	0	0

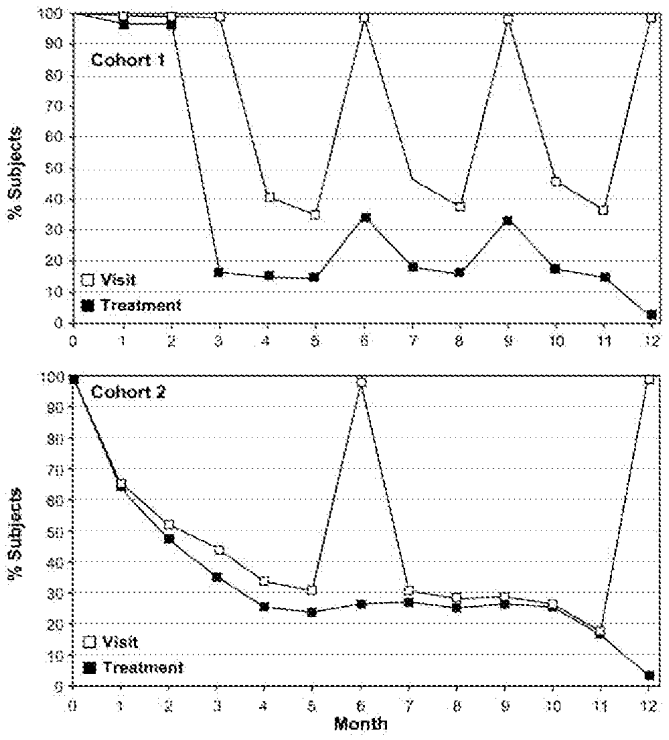

Figure 2. Visits and treatment. The percentage of cohort 1 (upper) and cohort 2 (lower) patients making visits and receiving ranibizumab treatment during each month of the 12-month study are shown. Cohort 1 visits were scheduled for day 0 and months 1, 2, 3, 6, 9, and 12. Cohort 2 visits were scheduled for day 0 and months 6 and 12. Data from cohort 1 0.3 and 0.5 mg dose groups are combined. Values are based on the percentage of subjects remaining in the study at each time point. Treatment received at month 12 was in violation of the protocol.

Table 4. Key Ocular Serious Adverse Events

Event, %	Cohort 1		Cohort 2
	0.3 mg (n = 1169)	0.5 mg (n = 1209)	0.5 mg (n = 1922)
Presumed endophthalmitis*	0.2	0.4	0.1
Uveitis	0.1	0.2	0
Retinal detachment	0.1	0	0.1
Retinal tear	0	0.1	0
Retinal hemorrhage	0.9	0.9	0.3
Detachment of retinal pigment epithelium	0	0.2	0.1
Vitreous hemorrhage	0.3	0.1	0.2
Cataract	0.1	0.1	0.1

*Includes 2 cases of uveitis and 1 case of iridocyclitis that were treated with antibiotics.

Safety

Ocular safety. The rates of individual key ocular SAEs in cohort 1 were <1% and similar across dose groups (Table 4). Two subjects (0.2%) in the 0.3 mg group and 5 subjects (0.4%) in the 0.5 mg group developed endophthalmitis or presumed endophthalmitis (i.e., ocular infection treated with antibiotics). One subject in each cohort 1 dose group had a serious cataract event. The rates of individual key ocular SAEs in cohort 2 were <1%. One cohort 2 subject developed endophthalmitis, and 1 subject had a serious cataract event (Table 4).

The incidence of ocular inflammation AEs, including iritis, uveitis, vitritis, and iridocyclitis, was 1.0% in the 0.3 mg group, 1.5% in the 0.5 mg group, and 0.5% in cohort 2. The overall incidence of cataract AEs was 5.4% in the 0.3 mg group, 6.0% in the 0.5 mg group, and 2.8% in cohort 2, and was similar when broken down by nuclear, subcapsular, and cortical subtypes.

Nonocular safety. The rates of key nonocular SAEs were similar across cohort 1 dose groups (Fig 3; Table 5). Nonvascular death, stroke, and hemorrhage rates were numerically higher in the 0.5 mg group. Eight subjects (0.7%) in the 0.3 mg group and 15 subjects (1.2%) in the 0.5 mg group had a stroke during the

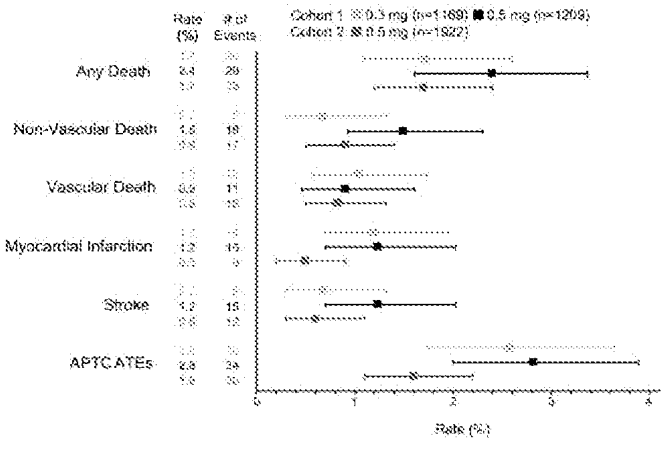

Figure 3. Key nonocular SAEs. The rates of individual events are depicted as point estimates with 2-sided Blyth-Still-Casella 95% confidence intervals. Antiplatelet Trialists' Collaboration ATEs include vascular deaths and deaths due to unknown cause, nonfatal MI, and nonfatal stroke. APTC = Antiplatelet Trialists' Collaboration; ATE = arterial thromboembolic event; SAE = serious adverse events.

Table 5. Nonocular Adverse Events Potentially Related to Anti-Vascular Endothelial Growth Factor Therapy

Classification, %	Cohort 1		Cohort 2
	0.3 mg (n = 1169)	0.5 mg (n = 1209)	0.5 mg (n = 1922)
Arterial thromboembolic events			
All	3.8	4.1	2.4
Serious	2.5	3.1	1.6
Hypertension			
All	9.0	10.3	3.0
Serious	0.1	0.1	0
Nonocular hemorrhage			
All	2.9	3.1	1.4
Serious	0.9	1.5	0.6
Proteinuria			
All	0.1	0	0
Serious	0	0	0
Other			
All	0.7	0.4	0.1
Serious	0.3	0.2	0.1

VEGF = vascular endothelial growth factor.

12-month study period. The incidence of MI and Antiplatelet Trialists' Collaboration (APTC)[3] arterial thromboembolic events (ATEs), which include vascular death and death of unknown cause, nonfatal MI, and nonfatal cardiovascular accidents, were similar across cohort 1 dose groups.

Rates of key nonocular SAEs in cohort 2 were generally lower than those in cohort 1, which may be a result of underreporting because of the large number of cohort 2 subjects who discontinued. The incidence of nonocular AEs potentially related to anti-VEGF therapy was low and comparable across cohorts and dose groups.

Prior stroke, history of arrhythmias, and history of congestive heart failure were significant risk factors for stroke (Fig 4). Although the numbers were small, there was a nonstatistically significant trend toward higher incidence of stroke in the cohort 1 0.5 mg group subjects with a history of stroke. Seven of the 73 subjects (9.6%) with a history of stroke in the 0.5 mg group experienced a stroke during the study compared with 2 of the 73 subjects (2.7%) with a history of stroke in the 0.3 mg group. None of the cohort 2 subjects with a history of stroke experienced a stroke during the study (Fig 4).

Twenty subjects (1.7%) in the cohort 1 0.3 mg group, 29 subjects (2.4%) in the cohort 1 0.5 mg group, and 33 subjects (1.7%) in cohort 2 died during the 12-month study (Table 6). The number of vascular deaths and deaths due to unknown cause did not differ across cohorts or dose groups.

Efficacy

Cohort 1 efficacy results were stratified by dose and previous treatment for AMD. For all groups, study eye VA increased with 3 loading doses of ranibizumab (day 0, month 1, month 3) (Fig 5). At month 3, treatment-naïve subjects in the 0.3 mg group had gained an average of 5.8 VA letters and those in the 0.5 mg group had gained an average of 7.0 VA letters. From months 3 to 12, with protocol-defined retreatment, VA tended to decrease. At month 12, treatment-naïve subjects in the 0.3 mg group had gained an average of 0.5 VA letters and those in the 0.5 mg group had gained an average of 2.3 letters. A similar pattern was observed for previ-

ously treated subjects. At month 3, previously treated subjects in the 0.3 mg group had gained an average of 4.6 VA letters and those in the 0.5 mg group had gained an average of 5.8 VA letters. At month 12, previously treated subjects in the 0.3 mg group had gained an average of 1.7 VA letters and those in the 0.5 mg group had gained an average of 2.3 letters.

In all cohort 1 groups, the proportion of subjects who gained ≥15 letters from baseline VA increased with 3 loading doses of ranibizumab (Fig 6). At month 3, 19.4% of treatment-naïve subjects in the 0.3 mg group and 20.1% in 0.5 mg group had gained ≥15 letters. The proportion of those who gained ≥15 letters tended to be maintained for the duration of the 12-month study, with 14.6% of 0.3 mg group subjects and 19.3% of 0.5 mg subjects gaining ≥15 VA letters at month 12. A similar pattern was observed for previously treated subjects. At month 3, 16.0% of previously treated subjects in the 0.3 mg group and 18.6% in the 0.5 mg group had gained ≥15 letters; and at month 12, 15.8% of 0.3 mg group subjects and 16.5% of 0.5 mg group subjects had gained ≥15 VA letters.

Study eye CFT of cohort 1 subjects for whom OCT data were available decreased with 3 loading doses of ranibizumab, increased from months 3 to 6, and remained stable from months 6 to 12 (Fig 7). For treatment-naïve subjects, CFT had decreased an average of 107.0 μm in the 0.3 mg group and 122.0 μm in the 0.5 mg group at month 3. At month 12, the average decrease from baseline CFT was 72.0 μm in the 0.3 mg group and 92.0 μm in the 0.5 mg group. For previously treated subjects, CFT had decreased an average of 98.0 μm in the 0.3 mg group and 108.0 μm in the 0.5 mg group at month 3. At month 12, the average decrease from baseline CFT was 71.0 μm in the 0.3 mg group and 76.0 μm in the 0.5 mg group.

Because of the large number of cohort 2 subjects who discontinued, the last-observation-carried-forward method was not used to impute missing efficacy values, and observed results are reported. This should be kept in mind when interpreting the results.

Table 6. Cause of Death

	Cohort 1		Cohort 2
	0.3 mg (n = 1169)	0.5 mg (n = 1209)	0.5 mg (n = 1922)
All deaths, %	1.7	2.4	1.7
Deaths due to unknown cause, %	0.1	0.1	0.1
Vascular deaths, %	0.9	0.8	0.7
Cardiovascular[a]	0.8	0.5	0.7
Stroke[b]	0.2	0.3	0.1
Nonvascular deaths, %	0.7	1.5	0.9
Respiratory: pneumonia, respiratory failure pulmonary failure pulmonary edema	0.3	0.6	0.5
Accident, injury, intracranial bleed secondary to fall	0	0.2	0.1
Renal failure	0	0.1	0
Cancer	0	0.4	0.3
Infection (septic shock, sepsis, urosepsis), liver failure due to hepatitis	0.3	0.2	0
Postoperative bowel obstruction	0.1	0	0
Vasculitis	0	0	0.1

[a]Includes ischemic cardiomyopathy, coronary heart disease, cardiac arrest, MI, saddle pulmonary embolism, and heart failure.
[b]Includes stroke, acute ischemic stroke, intracerebral hemorrhage, cerebrovascular disease, and brain hemorrhage secondary to fall. Three 0.5 mg subjects with preexisting cancer had previously received cancer treatment.

Snellen VA in cohort 2 subjects improved from a median of 20/100 at baseline to 20/80 at months 6 and 12. The proportion of subjects with a Snellen equivalent of 20/200 or worse decreased from approximately 39% at baseline to 31% at month 6 and 32% at month 12.

Discussion

SAILOR is the largest study to date to evaluate safety (primary objective) and efficacy (secondary objective) of

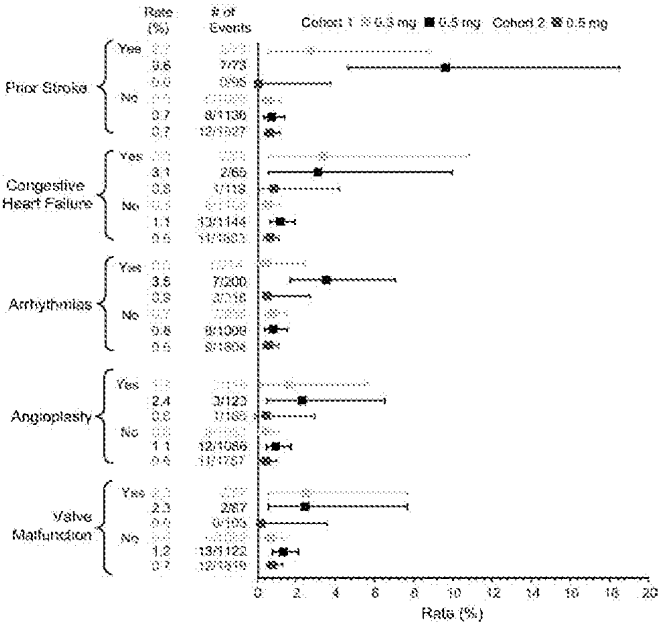

Figure 4. Stroke rate by risk factor. Point estimates and 2-sided Blyth-Still-Casella 95% confidence intervals for stroke rate when the risk factor was present or absent are shown. We evaluated the impact of 21 factors on the incidence of stroke. The 5 risk factors that had the greatest effect on stroke rates are presented.

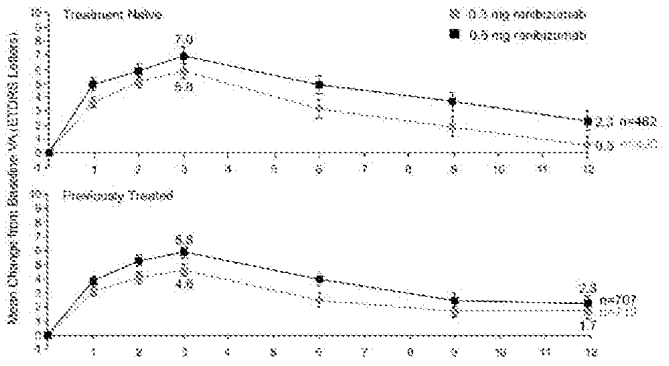

Figure 5. Change from baseline VA (cohort 1). For all groups, VA increased with 3 loading doses of ranibizumab (day 0, month 1, month 3). From months 3 to 12, with protocol-defined retreatment, VA tended to decrease. Error bars are ±1 standard error. ETDRS = Early Treatment Diabetic Retinopathy Study; VA = visual acuity.

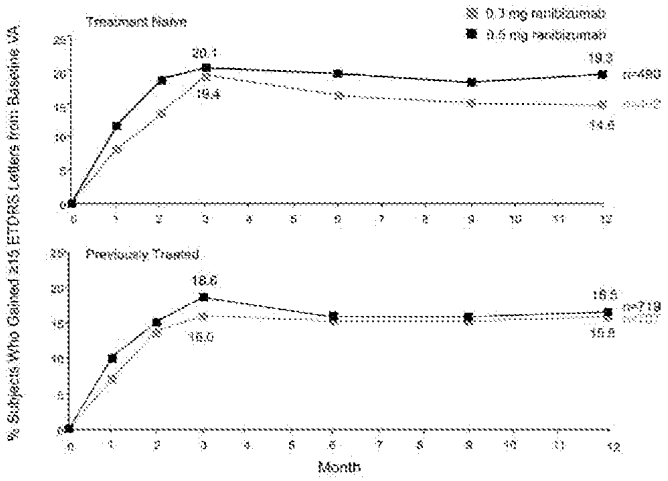

Figure 6. Subjects gaining ≥15 letters from baseline VA (cohort 1). The proportion of cohort 1 subjects who gained ≥15 letters increased with 3 loading doses of ranibizumab and was then maintained for the duration of the 12-month study. ETDRS = Early Treatment Diabetic Retinopathy Study; VA = visual acuity.

intravitreal ranibizumab in a population of subjects with CNV secondary to AMD. Ranibizumab was well tolerated, and the incidence of ocular SAEs and AEs was low and unrelated to dose. The rates of key nonocular SAEs and AEs, including APTC ATEs, MI, and vascular death, were similar across cohorts and dose groups.

The incidence of stroke in SAILOR was similar to that observed in previous ranibizumab studies.[1,2,4,5] An interim analysis of SAILOR cohort 1 safety data (October 2006) suggested a higher incidence of stroke in subjects who received 0.5 mg ranibizumab compared with those who received 0.3 mg ranibizumab and triggered a "Dear Doctor" letter in January 2007. The interim safety analysis was based on an incomplete data set, and the difference between doses was less pronounced in the final study data.

The final study data showed a difference in stroke rate between doses, with a higher rate in the 0.5 mg dose group compared with the 0.3 mg dose group. The total number of events was small, and the difference was not confirmed statistically. However, there is potentially a higher stroke rate associated with the 0.5 mg dose, which is being monitored via postmarketing surveillance and ongoing trials of ranibizumab in neovascular AMD.

A more comprehensive data set exists with regard to safety when SAILOR data are combined with data from the studies designated A Phase IIIb, Multicenter, Randomized, Double Masked, Sham Injection Controlled Study of the Efficacy and Safety of Ranibizumab in Subjects with Subfoveal Choroidal Neovascularization (CNV) with or without Classic CNV Secondary to Age-Related Macular Degeneration (PIER)[5] and RhuFab V2 Ocular Treatment Combining the Use of Visudyne to Evaluate Safety,[6] MARINA, and ANCHOR trials. Further evaluation from a meta-analysis of these studies evaluating the incidence of strokes and overall APTC ATEs will be conducted and will include additional clinical trial data as they become available.

In SAILOR there was not a difference between doses in APTC ATEs overall, which is consistent with our current understanding of ranibizumab pharmacology. As a Fab, ranibizumab has low systemic bioavailability (~1/90,0000 of intravitreal concentration) and a half-life of only several hours (Kubler P, Xu L, Jumbe N, et al. Population pharmacokinetics of ranibizumab in patients with age-related macular degeneration. Presented at: American Society of Retina Specialists Annual Meeting, December 1–5, 2007; Indian Wells, California).

Certain subgroups of subjects (e.g., those with prior cardiovascular accidents) may experience higher rates of systemic SAEs. We observed that the incidence of stroke was greater for cohort 1 subjects who had a history of stroke, congestive heart failure, or arrhythmias. However, the low incidence of stroke in SAILOR made it difficult to draw meaningful conclusions about the relationship between risk factors and stroke. Although the results of clinical trials cannot be directly compared with epidemiology studies in AMD, epidemiology stroke rates can provide a reference that aids in understanding stroke rates in SAILOR. The annual stroke rate for new-onset neovascular AMD in a large sample of Medicare subjects was 3.8%, and the annual ischemic stroke rate was 56.4% for those subjects who had experienced an ischemic stroke in the year before study entry.[7] Both of these rates are higher than those observed in SAILOR.

Ranibizumab treatment was associated with a net gain in VA in the cohort 1 0.3 mg and 0.5 mg dose groups. However, consistent with the results of MARINA and ANCHOR, 0.5 mg doses of ranibizumab tended to have a slightly greater VA benefit than 0.3 mg doses in subjects with neovascular AMD. Ranibizumab also tended to be

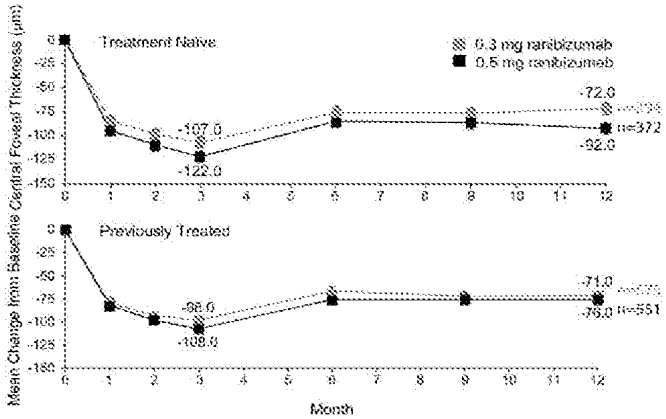

Figure 7. Change from baseline CFT (cohort 1). In cohort 1 subjects with OCT data, CFT decreased with 3 loading doses of ranibizumab. Central foveal thickness then increased from months 3 to 6 and remained stable from months 6 to 12. Error bars are ±1 standard error. CFT = central foveal thickness; ETDRS = Early Treatment Diabetic Retinopathy Study; OCT = optical coherence tomography.

more efficacious in treatment-naïve subjects than in previously treated subjects.

The VA changes observed after month 3 on the SAILOR dosing regimen were not as great as those observed with continual monthly dosing in the MARINA and ANCHOR studies, in which VA increased throughout the first study year. In the SAILOR study, VA increased with 3 loading doses of ranibizumab and then decreased from month 3 to 12. A similar trend was observed in the PIER study, in which subjects received 3 loading doses of ranibizumab followed by quarterly injections. Thus, SAILOR and PIER subjects made fewer visits and were treated less frequently than subjects in MARINA and ANCHOR, which may account for the reduced VA benefits observed with less-than-monthly dosing.

The protocol-defined retreatment criteria in SAILOR may have permitted too much disease progression before retreatment was permitted. For example, for cohort 1 subjects who were retreated according to VA and OCT criteria (81%), retreatment was not permitted until a 100 μm increase in CFT or a loss of >1 line of best-corrected VA, relative to the lowest previously recorded value, occurred. Given that the largest average decrease in CFT ranged from 98 to 122 μm, it is possible that subjects lost nearly all of their prior anatomic improvement before qualifying for retreatment.

The Prospective Optical Coherence Tomography Imaging of Patients with Neovascular AMD Treated with Intra-Ocular Ranibizumab (Lucentis) (PrONTO) study, a nonrandomized, single-institute study with more flexible retreatment criteria,[4] demonstrated that VA benefits similar to those of ANCHOR and MARINA could be obtained with less-than-monthly dosing when retreatment was based on qualitative and quantitative OCT, VA, hemorrhage, and fluid criteria. A future goal is to develop less-than-monthly treatment regimens that will prove optimal for physicians and subjects while realizing the full VA benefits of ranibizumab.

Study Limitations

Because the study did not include a control arm, safety could not be evaluated in terms of events related to ranibizumab treatment and events inherent to the elderly SAILOR subject population. Although differences in subject populations and dosing regimens prevent direct comparison across ranibizumab studies, the rates of safety events in the SAILOR study were low and similar to those of previous ranibizumab studies. Likewise, although the true benefit of ranibizumab could not be evaluated in the absence of a control group, SAILOR efficacy results were consistent with those in other controlled ranibizumab studies.

Eligibility for the SAILOR study was contingent on angiographically determined CNV. However, angiography was evaluated by individual investigators rather than a central reading center. Thus, investigator bias may have been introduced in subject selection across study sites.

Approximately 18% of cohort 1 subjects in each dose group discontinued the study before the month 12 visit, and approximately 50% of cohort 2 subjects discontinued before the end of the 12-month study. The primary reason for discontinuation from each cohort was "subject decision," and although case report forms did not provide specific reasons that subjects opted to discontinue, one can speculate reasons for doing so. For instance, subjects may have discontinued so that their fellow eye could be treated with ranibizumab. Also, subjects who did not fulfill the retreatment criteria may have discontinued the study so that they could follow a less-conservative retreatment regimen. Ranibizumab became commercially available, and bevacizumab (Avastin, Genentech, Inc.) became widely used for AMD treatment during the study period; therefore, subjects were not required to remain in the study to receive ranibizumab/anti-VEGF-A therapy. Furthermore, ranibizumab was provided to cohort 2 subjects for only 30 days after it became commercially available on June 30, 2006. Thus, many cohort 2 subjects may have discontinued the study to pursue other treatment options.

In conclusion, intravitreal ranibizumab was safe and well tolerated in a large population of subjects with neovascular AMD. Ranibizumab had a beneficial effect on VA and retina anatomy. Future investigations will seek to establish optimal dosing regimens for persons with neovascular AMD.

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References

1. Rosenfeld PJ, Brown DM, Heier JS, et al., MARINA Study Group. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–31.
2. Brown DM, Kaiser PK, Michels M, et al, ANCHOR Study Group. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N Engl J Med 2006;355: 1432–44.
3. Antiplatelet Trialists' Collaboration. Collaborative overview of randomised trials of antiplatelet therapy—I: prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. BMJ 1994; 308:81–106.
4. Fung AE, Lalwani GA, Rosenfeld PJ, et al. An optical coherence tomography-guided, variable dosing regimen with intravitreal ranibizumab (Lucentis) for neovascular age-related macular degeneration. Am J Ophthalmol 2007; 143:566–83.
5. Regillo CD, Brown DM, Abraham P, et al., PIER Study Group. Randomized, double-masked, sham-controlled trial of ranibizumab for neovascular age-related macular degeneration: PIER Study year 1. Am J Ophthalmol 2008; 145:239–48.
6. Heier JS, Boyer DS, Ciulla TA, et al., FOCUS Study Group. Ranibizumab combined with verteporfin photodynamic therapy in neovascular age-related macular degeneration: year 1 results of the FOCUS Study. Arch Ophthalmol 2006;124: 1532–42.
7. Alexander SL, Linde-Zwirble WT, Werther W, et al. Annual rates of arterial thromboembolic events in Medicare neovascular age-related macular degeneration patients. Ophthalmology 2007;114:2174–8.

Footnotes and Financial Disclosures

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[1] Retina Vitreous Associates Medical Group, Los Angeles, California.

[2] Ophthalmic Consultants of Boston, Boston, Massachusetts.

[3] Vitreoretinal Consultants, Houston, Texas.

[4] Genentech, Inc., South San Francisco, California.

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Correspondence:
David S. Boyer, MD, 1127 Wilshire Boulevard, Suite 1620, Los Angeles, CA 90017. E-mail: vitdoc@aol.com.

ORIGINAL ARTICLE

Ranibizumab versus Verteporfin for Neovascular Age-Related Macular Degeneration

David M. Brown, M.D., Peter K. Kaiser, M.D., Mark Michels, M.D., Gisele Soubrane, M.D., Jeffrey S. Heier, M.D., Robert Y. Kim, M.D., Judy P. Sy, Ph.D., and Susan Schneider, M.D., for the ANCHOR Study Group*

From Vitreoretinal Consultants, Methodist Hospital, Houston (D.M.B.); the Cole Eye Institute, Cleveland Clinic Foundation, Cleveland (P.K.K.); Retina Care Specialists, Palm Beach Gardens, FL (M.M.); the Clinique d'Ophtalmologie, University of Paris XII, Créteil, France (G.S.); Ophthalmic Consultants of Boston, Boston (J.S.H.); and Genentech, South San Francisco, CA (R.Y.K., J.P.S., S.S.). Address reprint requests to Dr. Brown at Vitreoretinal Consultants, 6560 Fannin St., Suite 750, Houston, TX 77030, or at dmbmd@houstonretina.com.

*Principal investigators in the Anti-VEGF Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization in Age-Related Macular Degeneration (ANCHOR) Study Group are listed in the Appendix.

N Engl J Med 2006;355:1432-44.

ABSTRACT

BACKGROUND

We compared ranibizumab — a recombinant, humanized, monoclonal antibody Fab that neutralizes all active forms of vascular endothelial growth factor A — with photodynamic therapy with verteporfin in the treatment of predominantly classic neovascular age-related macular degeneration.

METHODS

During the first year of this 2-year, multicenter, double-blind study, we randomly assigned patients in a 1:1:1 ratio to receive monthly intravitreal injections of ranibizumab (0.3 mg or 0.5 mg) plus sham verteporfin therapy or monthly sham injections plus active verteporfin therapy. The primary end point was the proportion of patients losing fewer than 15 letters from baseline visual acuity at 12 months.

RESULTS

Of the 423 patients enrolled, 94.3% of those given 0.3 mg of ranibizumab and 96.4% of those given 0.5 mg lost fewer than 15 letters, as compared with 64.3% of those in the verteporfin group ($P<0.001$ for each comparison). Visual acuity improved by 15 letters or more in 35.7% of the 0.3-mg group and 40.3% of the 0.5-mg group, as compared with 5.6% of the verteporfin group ($P<0.001$ for each comparison). Mean visual acuity increased by 8.5 letters in the 0.3-mg group and 11.3 letters in the 0.5-mg group, as compared with a decrease of 9.5 letters in the verteporfin group ($P<0.001$ for each comparison). Among 140 patients treated with 0.5 mg of ranibizumab, presumed endophthalmitis occurred in 2 patients (1.4%) and serious uveitis in 1 (0.7%).

CONCLUSIONS

Ranibizumab was superior to verteporfin as intravitreal treatment of predominantly classic neovascular age-related macular degeneration, with low rates of serious ocular adverse events. Treatment improved visual acuity on average at 1 year. (ClinicalTrials.gov number, NCT00061594.)

AGE-RELATED MACULAR DEGENERATION is a leading cause of severe and irreversible vision loss in the developed world among people 50 years of age or older.[1-4] The neovascular form of the disease is characterized by the growth of abnormal, choroidal blood vessels beneath the macula, which causes severe loss of vision.[5] Two main patterns of choroidal neovascularization that are associated with age-related macular degeneration, as seen on fluorescein angiography, are classic (in which intensely bright fluorescence is seen in early phases of the angiogram and leaks in late phases) and occult (in which leakage is less intense and appears in the late phases of disease).[6] Choroidal neovascular lesions that are predominantly (50% or more) classic in composition cause more severe and more rapid loss of vision than do lesions that are minimally (less than 50%) classic or occult.[7,8]

Photodynamic therapy with verteporfin[9-12] and intravitreal administration of pegaptanib sodium are approved by the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products for the treatment of neovascular age-related macular degeneration.[13] Neither treatment has resulted in clinically significant improvements in visual acuity.

Ranibizumab — a recombinant, humanized monoclonal antibody Fab that neutralizes all active forms of vascular endothelial growth factor A (VEGF-A) — was recently approved by the Food and Drug Administration for the treatment of this condition. Elsewhere in this issue of the *Journal*, Rosenfeld et al. report on a phase 3 study, called the Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular Age-Related Macular Degeneration (MARINA),[14] which demonstrated that monthly intravitreal injections of ranibizumab prevented the loss of visual acuity in approximately 95% of patients and improved visual acuity in one quarter to one third of treated patients during 24 months of treatment. In a similar manner, the addition of ranibizumab to verteporfin photodynamic therapy in patients with predominantly classic choroidal neovascularization was associated with a reduction in the loss of visual acuity, as compared with verteporfin therapy alone, and with an improvement in visual acuity over baseline in many patients.[15] We report the first-year results of a 2-year, phase 3 study, which compared the efficacy and safety of repeated intravitreal injections of ranibizumab with that of photodynamic therapy with verteporfin in patients with predominantly classic lesions associated with neovascular age-related macular degeneration.

METHODS

STUDY DESIGN

The Anti-VEGF Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization in Age-Related Macular Degeneration (ANCHOR) trial was an international, multicenter, randomized, double-blind, active-treatment–controlled study. Before the initiation of the study, we obtained approval from institutional review boards or ethics committees at all clinical centers. Patients provided written informed consent for study participation. Screening lasted as long as 28 days.

For inclusion in the study, patients had to be at least 50 years of age; have a lesion whose total size was no more than 5400 μm in greatest linear dimension in the study eye; have best-corrected visual acuity of 20/40 to 20/320 (Snellen equivalent), assessed with the use of Early Treatment Diabetic Retinopathy Study (ETDRS) charts; have no permanent structural damage to the central fovea; and have had no previous treatment (including verteporfin therapy) that might compromise an assessment of the study treatment. No patients were excluded because of preexisting cardiovascular, cerebrovascular, or peripheral vascular conditions.

STUDY TREATMENT

We randomly assigned eligible patients in a 1:1:1 ratio to receive either 0.3 or 0.5 mg of ranibizumab (Lucentis, Genentech) plus sham verteporfin therapy or sham intravitreal injections plus active verteporfin therapy. Randomization was stratified according to study center and to visual-acuity scores on day 0 (<45 letters vs. ≥45 letters, with a score of 45 letters as the approximate Snellen equivalent of 20/125 vision). In the group that received photodynamic therapy with verteporfin, intravenous administration of verteporfin (Visudyne, Novartis Pharmaceuticals) was followed by laser irradiation of the macula, according to instructions provided in the product package insert (www.visudyne.com). In the ranibizumab groups, sham verteporfin therapy was achieved by an intravenous infusion of saline rather than verteporfin, followed by laser

Table 1. **Baseline Characteristics of the Patients.***

Characteristic	Verteporfin (N=143)	0.3 mg of Ranibizumab (N=140)	0.5 mg of Ranibizumab (N=140)
Sex — no. (%)			
Male	64 (44.8)	73 (52.1)	75 (53.6)
Female	79 (55.2)	67 (47.9)	65 (46.4)
Race — no. (%)†			
White	140 (97.9)	137 (97.9)	136 (97.1)
Other	3 (2.1)	3 (2.1)	4 (2.9)
Age — yr			
Mean	77.7±7.8	77.4±7.5	76.0±8.6
Range	53–95	54–97	54–93
Age group — no. (%)			
50–64 yr	8 (5.6)	9 (6.4)	14 (10.0)
65–74 yr	35 (24.5)	28 (20.0)	41 (29.3)
75–84 yr	74 (51.7)	84 (60.0)	64 (45.7)
≥85 yr	26 (18.2)	19 (13.6)	21 (15.0)
Previous therapy — no. (%)			
Any treatment	64 (44.8)	63 (45.0)	58 (41.4)
Laser photocoagulation	19 (13.3)	23 (16.4)	20 (14.3)
Medication	1 (0.7)	1 (0.7)	1 (0.7)
Nutritional supplements	51 (35.7)	48 (34.3)	45 (32.1)
No. of letters read as a measure of visual acuity‡§			
Mean	45.5±13.1	47.0±13.1	47.1±13.2
<45 — no. (%)	66 (46.2)	63 (45.0)	60 (43.2)
≥45 — no. (%)	77 (53.8)	77 (55.0)	79 (56.8)

irradiation of the macula identical to that in the active verteporfin-therapy group.

Ranibizumab was injected into the study eye at a monthly interval (ranging from 23 to 37 days, for a total of 12 injections, excluding the injection at month 12) in the first year, beginning on day 0; sham injections were administered on the same schedule. Either verteporfin or sham verteporfin was administered on day 0 and then if needed on the basis of investigators' evaluation of angiography at months 3, 6, 9, or 12.

The study was designed and analyzed by a committee composed of Dr. Brown, representing the academic investigators, and representatives of Genentech. In analyzing the data and writing this manuscript, Dr. Brown had full and unrestricted access to the data, and all coauthors contributed to the interpretation of the data and the writing of the manuscript. The authors vouch for the accuracy and completeness of the reported data.

STATISTICAL ANALYSIS

We performed efficacy analyses on an intention-to-treat basis with the use of a last-observation-carried-forward method for missing data. Pairwise treatment comparisons were performed with the use of statistical methods adjusting for baseline scores of visual acuity (<45 letters vs. ≥45 letters) and, for lesion morphologic end points, the baseline value of the lesion characteristic. Binary end points were analyzed with the use of the Cochran chi-square test.[16] Mean changes from baseline were analyzed with the use of analysis of variance for end points with respect to visual acuity and an analysis of covariance for morphologic end points. The Hochberg–Bonferroni multiple-comparison procedure[17] was used to adjust for the two pairwise treatment comparisons of the primary end point. Safety analyses included all treated patients.

The number of patients required for statistical

Table 1. (Continued.)

Characteristic	Verteporfin (N=143)	0.3 mg of Ranibizumab (N=140)	0.5 mg of Ranibizumab (N=140)
Visual acuity (approximate Snellen equivalent) — no. (%)‡§			
20/200 or worse	46 (32.2)	35 (25.0)	32 (23.0)
Better than 20/200 but worse than 20/40	97 (67.8)	103 (73.6)	101 (72.7)
20/40 or better	0	2 (1.4)	6 (4.3)
Type of choroidal neovascularization — no. (%)			
Predominantly classic lesion	141 (98.6)	134 (95.7)	135 (96.4)
Minimally classic lesion	2 (1.4)	5 (3.6)	5 (3.6)
Occult with no classic lesion	0	1 (0.7)	0
Size of lesion — optic-disk area¶			
Mean	1.88±1.40	1.89±1.44	1.79±1.54
Range	0.07–5.75	0.12–7.20	0.05–10.00
Size of choroidal neovascularization — optic-disk area¶			
Mean	1.48±1.25	1.48±1.33	1.31±1.24
Range	0.07–5.55	0.11–6.80	0.05–7.50
Size of classic choroidal neovascularization — optic-disk area¶			
Mean	1.36±1.13	1.28±1.05	1.21±1.12
Range	0.07–5.55	0.00–6.40	0.05–5.30
Size of leakage from choroidal neovascularization plus staining of retinal pigment epithelium — optic-disk area¶			
Mean	3.06±1.81	3.00±1.92	2.92±2.08
Range	0.20–8.20	0.20–11.00	0.25–9.0

* Plus–minus values are means ±SD. Percentages may not total 100 because of rounding.
† Race was determined by the investigators.
‡ Visual acuity was measured with the use of Early Treatment Diabetic Retinopathy Study charts at a starting distance of 2 m. A score of 45 letters is the approximate Snellen equivalent of 20/125.
§ For the group that received 0.5 mg of ranibizumab, 139 patients were observed.
¶ One optic-disk area is equal to 2.54 mm^2 on the basis of one optic-disk diameter of 1.8 mm.

significance was determined on the basis of a 1:1:1 randomization ratio, the Pearson chi-square test for the two pairwise comparisons of the primary end point, and the Hochberg–Bonferroni multiple-comparison procedure at an overall type I error of 0.0497. We estimated that the enrollment of 426 patients would provide the study with a statistical power of 96% to detect a significant difference between one or both ranibizumab groups and the verteporfin group in the percentage of patients losing fewer than 15 letters at 12 months, assuming a rate of 84% in each ranibizumab group and 67% in the sham verteporfin group. (See the Supplementary Appendix, available with the full text of this article at www.nejm.org, for additional information on the study design and analysis.)

RESULTS

STUDY PATIENTS

Between June 2003 and September 2004, 423 patients were enrolled and randomly assigned to a study treatment (143 to the verteporfin group and 140 to each of the ranibizumab groups). The disposition of the patients is summarized in Table 1 of the Supplementary Appendix. Three patients in the group receiving 0.3 mg of ranibizumab did not receive any treatment: one because of the patient's decision and two because of an investigator's decision. More than 90% of patients in each group (91.5% overall) were receiving treatment at 12 months. Of a possible 12 injections of ranibizumab or sham injections, the mean number administered was 11.1 in the verteporfin group,

11.0 in the 0.3-mg group, and 11.2 in the 0.5-mg group. Including the required administration on day 0 and excluding month 12, active verteporfin therapy was administered a mean of 2.8 times in the verteporfin group, and sham verteporfin was administered a mean of 1.7 times in each of the ranibizumab groups.

Randomized treatment groups were balanced for demographic and baseline ocular and morphologic characteristics (Table 1). The independent reading center subtyped the choroidal neovascularization as predominantly classic in all patients during the expedited screening evaluation. Subsequent reevaluation confirmed the initial classification in 96.9% of patients, and 3.1% were reclassified. In each group, the mean total lesion area was slightly less than 2 optic-disk areas (1 optic-disk area equals 2.54 mm^2 on the basis of 1 optic-disk diameter of 1.8 mm).

PRIMARY AND SECONDARY END POINTS

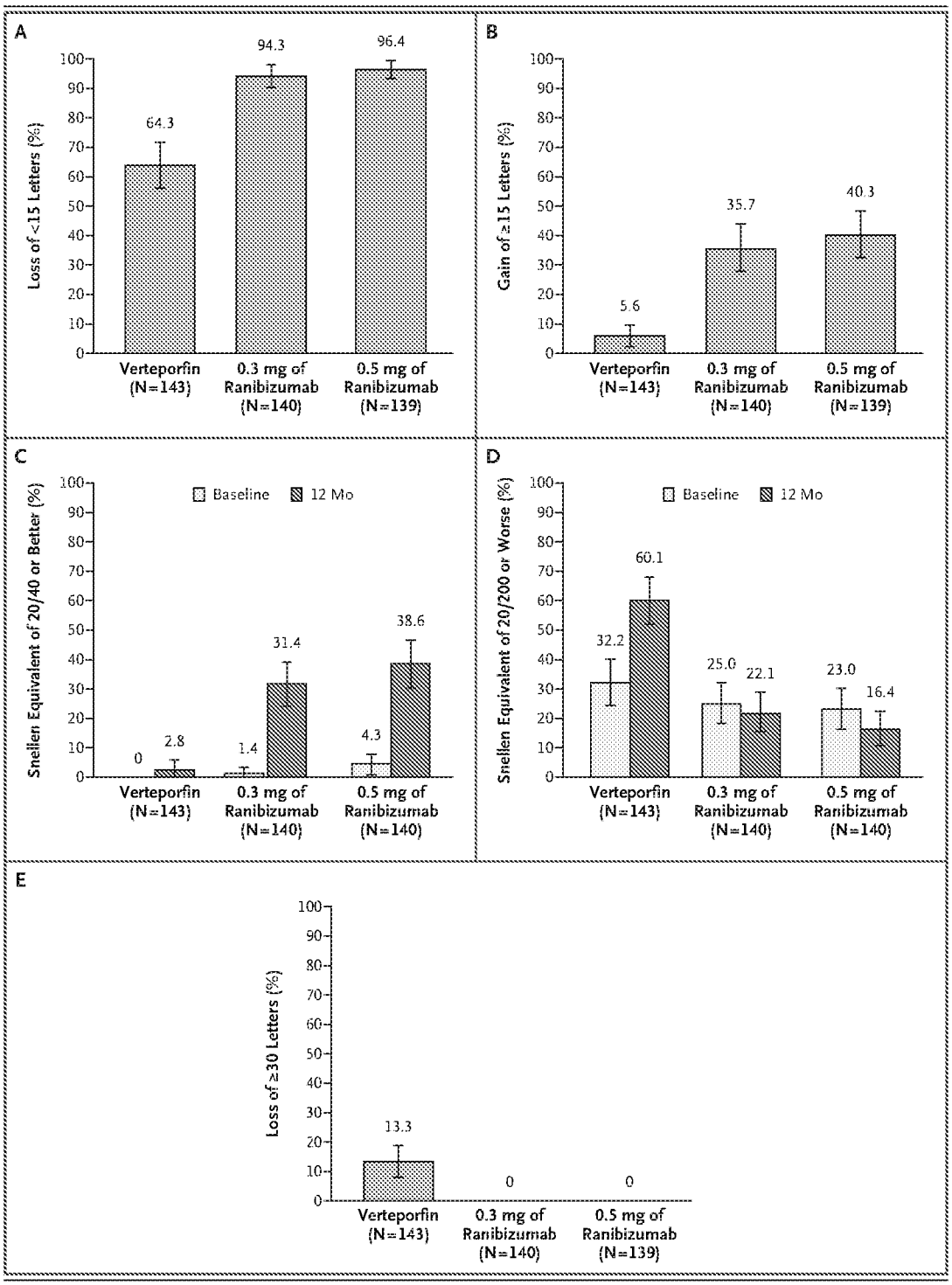
All end points with respect to visual acuity in the study eye at 12 months favored ranibizumab treatment over verteporfin therapy. With respect to the primary efficacy end point, 94.3% of patients in the 0.3-mg group and 96.4% in the 0.5-mg group lost fewer than 15 letters from baseline visual acuity, as compared with 64.3% in the verteporfin group ($P<0.001$ for each comparison) (Fig. 1A). In addition, the proportion of patients whose visual acuity improved from baseline by 15 or more letters was significantly greater among those receiving ranibizumab treatment (35.7% in the 0.3-mg group and 40.3% in the 0.5-mg group, as compared with 5.6% in the verteporfin group; $P<0.001$ for each comparison) (Fig. 1B). Significantly greater proportions of ranibizumab-treated patients than patients in the verteporfin group had visual acuity of 20/40 or better ($P<0.001$ for the comparison of each ranibizumab group with the verteporfin group) (Fig. 1C), and smaller proportions had visual acuity of 20/200 or worse ($P<0.001$ for each comparison) (Fig. 1D). A severe loss of visual acuity (defined as a decrease of 30 letters or more) did not occur in any patient in the ranibizumab groups but occurred in 13.3% of patients in the verteporfin group ($P<0.001$ for each comparison) (Fig. 1E). Although no patient had baseline visual acuity of 20/20 or better, at 12 months 7.1% of the patients in the 0.3-mg group and 6.4% in the 0.5-mg group had visual acuity of 20/20 or better, as compared with 0.7% of patients in the verteporfin group.

Figure 1 (facing page). **Visual Acuity Scores and Snellen Equivalents at 12 Months.**

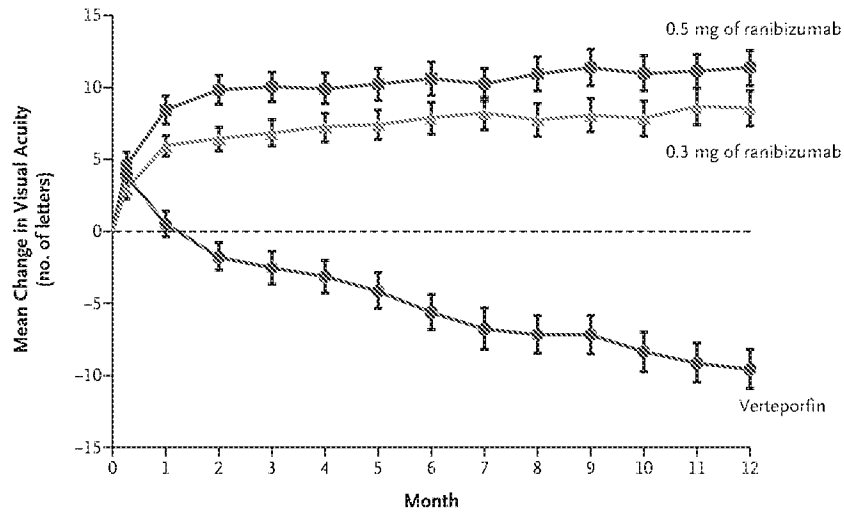
Panel A shows the percentage of patients who lost fewer than 15 letters (moderate loss) from baseline visual acuity at 12 months (the primary efficacy end point). Panel B shows the percentage of patients who gained 15 or more letters (moderate gain) from baseline at 12 months. Panels C and D show the percentage of patients with vision of the Snellen equivalent of 20/40 or better and of those with vision of 20/200 or worse, respectively, at both baseline and 12 months. (For the group that received 0.5 mg of ranibizumab, 139 patients were observed at baseline and 140 patients were observed at 12 months in Panels C and D.) Panel E shows the percentage of patients who lost 30 or more letters (severe loss) from baseline at 12 months. Treatment comparisons were based on the Cochran chi-square test stratified according to the visual-acuity score on day 0 (<45 letters vs. ≥45 letters). Confidence intervals, denoted by I bars, were based on the normal approximation and the simple (unstratified) estimates of the percentages and their standard errors. The last-observation-carried-forward method was used to impute missing data. All statistical tests were two-sided. $P<0.001$ for all comparisons of each dose of ranibizumab with verteporfin.

The tracking of mean changes in visual-acuity scores over time showed that the values in each of the ranibizumab groups were significantly superior to those in the verteporfin group at each month during the first year ($P<0.001$) (Fig. 2). On average, visual acuity of ranibizumab-treated patients increased by 5.9 letters in the 0.3-mg group and 8.4 letters in the 0.5-mg group at 1 month after the first treatment and increased further over time to a gain of 8.5 letters in the 0.3-mg group and 11.3 letters in the 0.5-mg group by 12 months. In contrast, the verteporfin group had an average loss in visual acuity at each month after the first month, with a mean loss of 9.5 letters by 12 months. Results for all end points with respect to visual acuity at 12 months were similar when the analyses used the observed data with no imputation of missing values (data not shown).

Results for prespecified secondary end points related to the morphologic characteristics of lesions are summarized in Table 2. At 12 months, the area occupied by classic choroidal neovascularization decreased by a mean of 0.52 optic-disk area in the 0.3-mg group and 0.67 optic-disk area in the 0.5-mg group, as compared with a mean increase of 0.54 optic-disk area in the verteporfin group ($P<0.001$ for each comparison). The area of leakage from choroidal neovascularization plus



intense, progressive staining of the retinal pigment epithelium at 12 months decreased by a mean of 1.80 optic-disk areas in the 0.3-mg group and 2.05 optic-disk areas in the 0.5-mg group, as compared with a mean increase of 0.32 optic-disk area in the verteporfin group ($P<0.001$ for each comparison). Figure 3 shows a representative patient with a reduction in the area of choroidal neovascularization and leakage from baseline to 12 months.



Mean Change from Baseline	(day 7)												
0.5 mg of ranibizumab	+4.6	+8.4	+9.8	+10.0	+9.9	+10.2	+10.6	+10.2	+10.9	+11.4	+10.9	+11.1	+11.3
0.3 mg of ranibizumab	+2.9	+5.9	+6.4	+6.8	+7.2	+7.4	+7.9	+8.2	+7.7	+8.1	+7.8	+8.6	+8.5
Verteporfin	+3.9	+0.5	−1.8	−2.5	−3.1	−4.1	−5.6	−6.8	−7.1	−7.1	−8.3	−9.1	−9.5

Figure 2. Mean (±SE) Changes in the Number of Letters Read as a Measure of Visual Acuity from Baseline through 12 Months.

P<0.001 for all monthly comparisons of each dose of ranibizumab with verteporfin. Pairwise analysis of variance adjusting for the visual-acuity score on day 0 (<45 letters vs. ≥45 letters) was used to analyze the mean change in visual acuity from baseline at each monthly assessment. The last-observation-carried-forward method was used to impute missing data. All statistical tests were two-sided.

The area occupied by choroidal neovascularization (classic and occult, if present) increased by a mean of 1.63 optic-disk areas in the verteporfin group, as compared with small mean increases of 0.20 optic-disk area in the 0.3-mg group and 0.22 optic-disk area in the 0.5-mg group (P<0.001 for each comparison). The mean lesion area increased in the verteporfin group to 2.56 optic-disk areas, as compared with small increases in the ranibizumab groups of 0.36 optic-disk area in the 0.3-mg group and 0.28 optic-disk area in the 0.5-mg group (P<0.001 for each comparison).

ADVERSE EVENTS

Safety results are summarized in Table 3. Serious ocular adverse events associated with treatment were uncommon. Endophthalmitis, classified as a condition treated with intravitreal or systemic antibiotics, was reported in one patient, who was in the 0.5-mg group (0.7%). An additional patient in the 0.5-mg group (0.7%) had two events of intraocular inflammation that were classified by the investigator as serious uveitis. However, since one of the events was treated with systemic antibiotics (without obtaining ocular culture specimens or treatment with intravitreal antibiotics), this patient was presumed to have had endophthalmitis, and was so classified in Table 3. Rhegmatogenous retinal detachment occurred in one patient (0.7%) in the 0.3-mg group and one in the verteporfin group, and vitreous hemorrhage occurred in one patient (0.7%) in the 0.3-mg group.

Rates of adverse events of intraocular inflammation (pooled for reported events of iritis, iridocyclitis, vitritis, uveitis, and anterior-chamber inflammation) were higher in both ranibizumab groups (10.2% in the 0.3-mg group and 15.0% in the 0.5-mg group) than in the verteporfin group (2.8%). Rates of intraocular inflammation (regardless of cause) observed during slit-lamp examination were consistent with those reported as adverse events (12.4% in the 0.3-mg group and 17.1% in the 0.5-mg group, as compared with 3.5% in the verteporfin group). Most patients in all groups had no observable inflammation during the study, and the proportion of inflammation events graded 2+ or higher among ranibizumab-treated pa-

Table 2. **Summary of Changes from Baseline in Morphologic Characteristics of Lesions at 12 Months.***

End Point	Verteporfin (N=143)	0.3 mg of Ranibizumab (N=140)	0.5 mg of Ranibizumab (N=140)	P Value†
Change in size of classic choroidal neovascularization (optic-disk area)‡				
Mean	0.54±2.37	−0.52±0.89	−0.67±1.10	<0.001
95% CI	0.15 to 0.93	−0.67 to −0.37	−0.86 to −0.49	
Change in size of leakage from choroidal neovascularization plus staining of retinal pigment epithelium (optic-disk area)‡				
Mean	0.32±3.09	−1.80±1.72	−2.05±1.98	<0.001
95% CI	−0.19 to 0.83	−2.09 to −1.51	−2.38 to −1.72	
Change in size of choroidal neovascularization (classic lesion plus occult lesion, if present) (optic-disk area)‡				
Mean	1.63±2.37	0.20±0.97	0.22±1.25	<0.001
95% CI	1.23 to 2.02	0.04 to 0.37	0.01 to 0.42	
Change in size of lesion (optic-disk area)‡				
Mean	2.56±3.09	0.36±1.06	0.28±1.29	<0.001
95% CI	2.05 to 3.07	0.18 to 0.53	0.06 to 0.49	

* Plus–minus values are means ±SD. CI denotes confidence interval.

† P values are for the comparison of each dose of ranibizumab with verteporfin therapy. Comparisons were based on pairwise analysis-of-covariance models adjusted for the stratification variable (a baseline visual-acuity score of <45 letters or ≥45 letters) and the baseline value of the end point. The last-observation-carried-forward method was used to impute missing data. All statistical tests were two-sided.

‡ One optic-disk area is equal to 2.54 mm^2 on the basis of 1 optic-disk diameter of 1.8 mm.

tients was small: three patients in each dose group (2.2% in the 0.3-mg group and 2.1% in the 0.5-mg group).

Transient changes in intraocular pressure after injection were common in the ranibizumab-treated patients. The proportion of patients with a postinjection intraocular pressure of 30 mm Hg or more was greater in both ranibizumab groups (8.8% in the 0.3-mg group and 8.6% in the 0.5-mg group) than in the verteporfin group (4.2%). However, very few patients had measurements of 40 mm Hg or more (2.9% in each ranibizumab group vs. 0.7% in the verteporfin group).

The ranibizumab groups had an increased frequency of cataract formation (10.9% in the 0.3-mg group and 12.9% in the 0.5-mg group, as compared with 7.0% in the verteporfin group). With the exception of one severe cataract in the verteporfin group, all adverse events associated with cataracts were mild or moderate. A small number of patients had changes in lens status reported during the first treatment year. Of patients whose study eye was phakic at baseline, five underwent cataract surgery during the 12 months of the study: four (5.3%) in the 0.3-mg group and one (1.2%) in the 0.5-mg group, as compared with none in the verteporfin group. Visual-acuity outcomes of these patients at 12 months were not notably different from those of the respective treatment groups overall. No traumatic lens damage was reported in the study eye of any patient during the first treatment year.

There was no overall imbalance among groups in the rates of serious nonocular adverse events: 14.6% in the 0.3-mg group and 20.0% in the 0.5-mg group, as compared with 19.6% in the verteporfin group. The numbers of deaths were similar across groups: three patients (2.2%) in the 0.3-mg ranibizumab group and two patients each (1.4%) in the 0.5-mg group and verteporfin group. With respect to specific nonocular adverse events, there were imbalances in back pain and nonocular hemorrhage (a combination of serious and nonserious events). Back pain was less common in the ranibizumab groups (3.6% in the 0.3-mg group and 1.4% in the 0.5-mg group) than in the

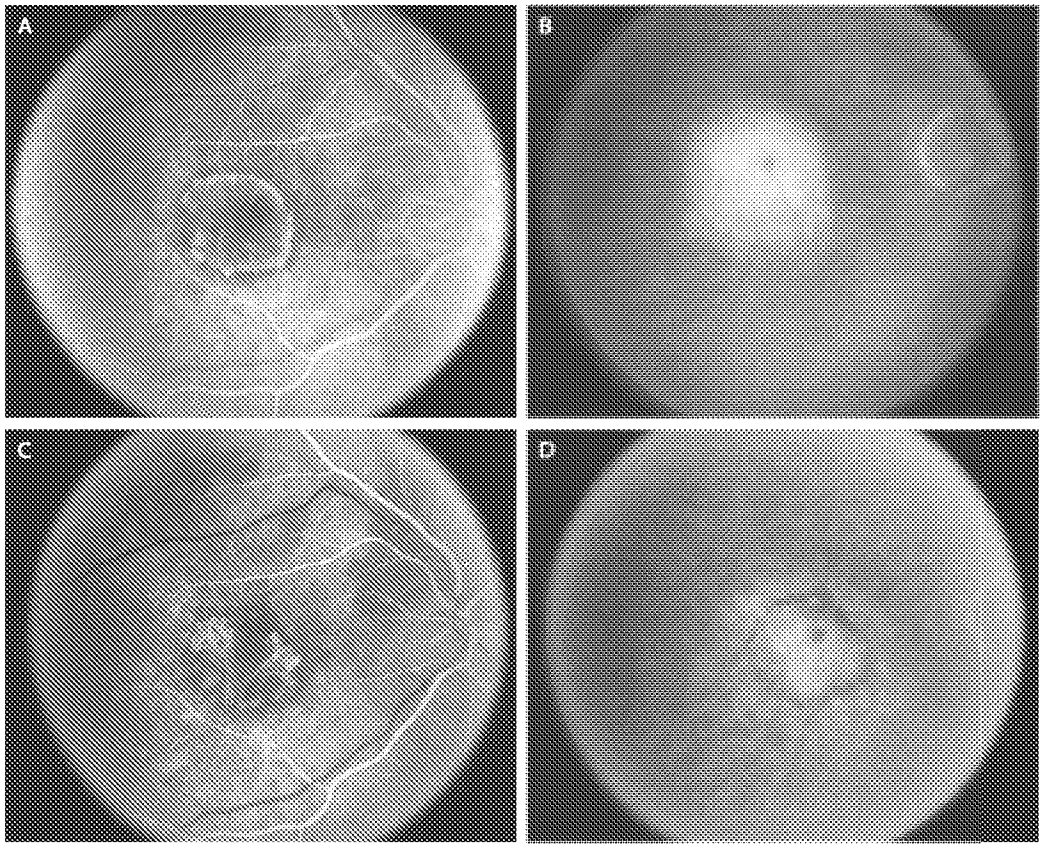


Figure 3. **Fundus Fluorescein Angiography in a 79-Year-Old Patient.**
The patient presented with a best-corrected visual acuity of 20/100 and was randomly assigned to the group receiving 0.3 mg of ranibizumab. The early-phase angiogram shows a predominantly classic lesion (Panel A); intense leakage is visible in the late phase (Panel B). At 12 months, the patient's visual acuity had improved by 3 lines to 20/50. Repeated angiography shows involution of the classic choroidal neovascularization (Panel C) with a reduction in leakage in the late phase (Panel D).

verteporfin group (9.1%) and is a well-known potential adverse reaction to verteporfin infusion.[18] The incidence of nonocular hemorrhage, an adverse event that potentially reflects systemic VEGF inhibition,[19] was higher in the ranibizumab groups (5.1% in the 0.3-mg group and 6.4% in the 0.5-mg group, as compared with 2.1% in the verteporfin group). There was no increase in the ranibizumab groups in mean systolic or diastolic blood pressure or in the rates of hypertension and proteinuria, other adverse events potentially reflecting systemic VEGF inhibition.

Serious adverse events of arterial thromboembolism were evaluated with the use of the Antiplatelet Trialists' Collaboration (APTC) criteria, in which an event is defined as a nonfatal myocardial infarction, nonfatal ischemic stroke, nonfatal hemorrhagic stroke, or death owing to vascular or unknown causes.[20] Overall, APTC-classified arterial thromboembolic events occurred in three patients (2.2%) in the 0.3-mg group, six patients (4.3%) in the 0.5-mg group, and three patients (2.1%) in the verteporfin group (Table 3). One patient (0.7%) in each group had a nonfatal cerebrovascular event. Nonfatal myocardial infarction occurred in one patient (0.7%) in the 0.3-mg group, three patients (2.1%) in the 0.5-mg group, and in one patient (0.7%) in the verteporfin group. No apparent relationship between the onset of those events and the time of study treatment was observed; the differences were not significant. One patient in the 0.3-mg group who began concomitant treatment with the systemic anti-VEGF agent bevacizumab for metastatic cancer midway through

Table 3. **Adverse Events.***

Adverse Event	Verteporfin (N=143)	0.3 mg of Ranibizumab (N=137)	0.5 mg of Ranibizumab (N=140)
Serious ocular adverse event — no. (%)			
Presumed endophthalmitis†	0	0	2 (1.4)
Culture positive	0	0	1 (0.7)‡
Culture not obtained	0	0	1 (0.7)
Uveitis	0	0	1 (0.7)§
Rhegmatogenous retinal detachment	1 (0.7)¶	1 (0.7)	0
Retinal tear‖	0	0	0
Vitreous hemorrhage	0	1 (0.7)	0
Lens damage	0	0	0
Most severe ocular inflammation — no. (%)**			
None	138 (96.5)	120 (87.6)	116 (82.9)
Trace	4 (2.8)	11 (8.0)	13 (9.3)
1+	1 (0.7)	3 (2.2)	8 (5.7)
2+	0	1 (0.7)	1 (0.7)
3+	0	2 (1.5)	1 (0.7)
4+	0	0	1 (0.7)
Nonocular adverse event			
Investigator-defined hypertension			
Treatment-emergent hypertension — no. (%)	12 (8.4)	3 (2.2)	9 (6.4)
Mean change in blood pressure from baseline — mm Hg	0.1/0.3	−2/−2	−2/1
Key arterial nonfatal thromboembolic events — no. (%)			
Myocardial infarction	1 (0.7)	1 (0.7)	3 (2.1)
Stroke	1 (0.7)	0	1 (0.7)
Cerebral infarction	0	1 (0.7)	0
Death — no. (%)	2 (1.4)††	3 (2.2)	2 (1.4)
Vascular cause (APTC criteria)	1 (0.7)‡‡	1 (0.7)‡‡	2 (1.4)§§
Nonvascular cause	1 (0.7)¶¶	2 (1.5)‖	0
Nonocular hemorrhage — no. (%)			
Reported as a serious adverse event	0	2 (1.5)	3 (2.1)
Total serious or nonserious events***	3 (2.1)	7 (5.1)	9 (6.4)

* APTC denotes Antiplatelet Trialists' Collaboration.
† Events were categorized as presumed endophthalmitis in cases in which intravitreal or systemic antibiotics were administered.
‡ Vitreous culture was positive for *Staphylococcus epidermidis*.
§ One patient had two episodes of intraocular inflammation that were reported as uveitis, but one of the episodes was classified as presumed endophthalmitis because it was treated with systemic antibiotics. In neither of these two episodes was a vitreous culture obtained, and neither was treated with intravitreal antibiotics.
¶ One patient had two episodes of rhegmatogenous retinal detachment.
‖ No serious or nonserious adverse events associated with retinal tears were reported.
** Ocular inflammation (regardless of the cause) was determined on the basis of slit-lamp examination. The grading system used to evaluate intraocular inflammation is outlined in Tables 2 and 3 of the Supplementary Appendix.
†† One patient died after withdrawing from the study because of an adverse event.
‡‡ One patient died from cardiac arrest.
§§ One patient died from cardiac failure, and one patient died from worsening of chronic heart failure.
¶¶ One patient died from chronic obstructive pulmonary disease.
‖ One patient died from respiratory arrest and one from viral syndrome.
***All nonocular hemorrhagic adverse events are listed in Table 4 of the Supplementary Appendix.

the study and continued to receive ranibizumab had an intestinal perforation, a known risk associated with systemic bevacizumab therapy.[19]

We observed immunoreactivity to ranibizumab in a percentage of patients in all treatment groups (1.5% in the verteporfin group, 3.2% in the 0.3-mg group, and 0.8% in the 0.5-mg group) before any exposure to ranibizumab. Monitoring of immunoreactivity during the first treatment year revealed no increase from baseline in the number of patients testing positive in the verteporfin group or the 0.3-mg group (1.6% in each group at 12 months), whereas the 0.5-mg group showed an increase to 3.9% of patients at 12 months. Although the small number of patients with immunoreactivity precludes drawing definitive conclusions, proportionately more ranibizumab-treated patients who were immunoreactive at any point had adverse events associated with intraocular inflammation (3 of 6 in the 0.3-mg group and 3 of 5 in the 0.5-mg group, as compared with 0 of 3 in the verteporfin group) than did patients who were never immunoreactive (11 of 127 in the 0.3-mg group and 17 of 129 in the 0.5-mg group, as compared with 3 of 129 in the verteporfin group). The presence or absence of immunoreactivity appeared to be unrelated to end points associated with visual acuity or nonocular adverse events potentially related to immunoreactivity.

DISCUSSION

Our study demonstrated that ranibizumab prevents central vision loss and improves mean visual acuity at 1 year. In this study, monthly intravitreal injections of ranibizumab were superior in efficacy to verteporfin therapy. Although our study was not designed to evaluate the superiority of one ranibizumab dose over the other, efficacy results suggest a dose–response effect.

Intravitreal injections of ranibizumab were associated with a low rate of serious ocular adverse events, including such key events as presumed endophthalmitis, severe intraocular inflammation, and retinal detachment (each of which was reported in less than 1% of the pooled ranibizumab-treated patients and in less than 0.1% of ranibizumab injections). The ocular safety profiles for the three treatment groups revealed no overall imbalance in serious and nonserious adverse events, although there were trends toward increased rates of intraocular inflammation (generally mild), cataract (consistently mild or moderate), and nonocular hemorrhage with ranibizumab. The rates of intraocular inflammation and cataract in the ranibizumab groups were similar to those for ranibizumab-treated patients in the MARINA study.[14] However, the rates of these events in the verteporfin group in our study were lower than the rates in the sham-injection group in the MARINA study.[14]

Regarding adverse events that potentially reflect systemic VEGF inhibition, no adverse events of proteinuria were reported and no imbalance in adverse events of hypertension or in blood-pressure measurements was noted in either our study or the MARINA study. In both studies, ranibizumab-treated patients had a higher percentage of nonocular hemorrhages than did patients who did not receive ranibizumab, and patients treated with a 0.5-mg dose had a higher rate of APTC-classified arterial thromboembolic events than did those who received a 0.3-mg dose or verteporfin therapy (Table 3). Since our study was not designed to distinguish small differences in rare adverse events among treatment groups, the clinical significance of these trends is unclear and requires further attention. In the MARINA study, with 2 years of study treatment, the rates of events classified as arterial thromboembolism according to APTC criteria were similar among the treatment groups.[13] Follow-up is continuing through 2 years of treatment in our study to identify these events. The clinical significance of immunoreactivity to ranibizumab observed with the assay method used in our study and in the MARINA study is also not clear.

In summary, the ANCHOR study showed that ranibizumab administered monthly by intravitreal injection was superior in efficacy to photodynamic therapy with verteporfin in patients with subfoveal, predominantly classic choroidal neovascularization associated with age-related macular degeneration. The first-year results of our study and the 2-year results of the MARINA study, considered together, demonstrate that ranibizumab was effective with an acceptable adverse-event profile in the treatment of all angiographic subtypes of choroidal neovascularization associated with age-related macular degeneration.

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APPENDIX

The following principal investigators were members of the ANCHOR Study Group: T. Aaberg, Associated Retinal Consultants, Grand Rapids, MI; P. Abraham, Black Hills Regional Eye Institute, Rapid City, SD; L. Akduman, Saint Louis University Eye Institute, St. Louis; D. Alfaro III, Retina Consultants of Charleston, Charleston, SC; A. Antoszyk, Southeast Clinical Research Associates, Charlotte, NC; J. Arnold, Marsden Eye Research, Parramatta, Australia; C. Awh, Retina Vitreous Associates, Nashville; P. Beer, New Lions Eye Institute, Slingerlands, NY; J. Belmont, Retina Diagnostic and Treatment, Philadelphia; B. Berger, Retina Research Center, Austin, TX; D. Berinstein, Retina Group of Washington, Fairfax, VA; R. Bhisitkul, University of California at San Francisco School of Medicine, San Francisco; D. Boyer, Retina–Vitreous Associates, Beverly Hills, CA; W. Bridges, Western Carolina Retinal Associates, Asheville, NC; H. Brooks, Jr., Southern Vitreoretinal Associates, Tallahassee, FL; D. Brown, Vitreoretinal Consultants, Houston; T. Ciulla, Midwest Eye Institute, Indianapolis; W. Clark, Palmetto Retina Center, Columbia, SC; T. Connor, Medical College of Wisconsin, Milwaukee; S. Cousins, Bascom Palmer Eye Institute, Palm Beach Gardens, FL; F. Devin, Clinique Monticelli, Marseilles, France; W. Dunn, Florida Retina Institute, Daytona Beach; A. Eaton, Retina Health Center, Fort Myers, FL; S. Fekrat, Duke University Eye Center, Durham, NC; C. Foja, Universitätsklinikum Leipzig, Leipzig, Germany; B. Foster, New England Retina Consultants, West Springfield, MA; A. Gaudric, Hôpital Lariboisière, Paris; M. Gilles, Save Sight Institute, Sydney; D. Glaser, Retina Associates of St. Louis, Florissant, MO; B. Godley, Retina Specialists, Desoto, TX; V. Gonzalez, Valley Retina Institute, McAllen, TX; R. Goodart, Rocky Mountain Retina Consultants, Salt Lake City; R. Guymer, University of Melbourne, Centre for Eye Research Australia, East Melbourne, Australia; L. Halperin, Retina Vitreous Consultants, Fort Lauderdale, FL; J. Heier, Ophthalmic Consultants of Boston, Boston; F. Holz, Universitätsklinikum Bonn, Bonn, Germany; B. Hubbard, Emory University, Atlanta; H. Hudson, Retina Centers, Tucson, AZ; D. Ie, Delaware Valley Retina Associates, Lawrenceville, NJ; M. Johnson, University of Michigan, Ann Arbor; A. Joussen, Universitätsklinikum Köln, Cologne, Germany; P. Kaiser, Cleveland Clinic Foundation, Cole Eye Institute, Cleveland; B. Kuppermann, University of California at Irvine, Irvine; M. Lomeo, Midwest Retina, Columbus, OH; C. MacDonald, University of Texas Health Science Center, San Antonio; D. Marcus, Southeast Retina Center, Augusta, GA; A. Martidis, Retina Diagnostic and Treatment, Philadelphia; J. Martinez, Austin Retina Associates, Austin, TX; D. Maxwell, Jr., Retinal Associates of Oklahoma, Oklahoma City; T. McMillan, Southeastern Retina Associates, Knoxville, TN; M. Michels, Palm Beach Gardens, FL; D. Miller, Retina Associates of Cleveland, Beachwood, OH; P. Michell, Westmead Hospital, Westmead, Australia; I. Orgel, Retina Vitreous Associates, Toledo, OH; R. Park, University of Arizona, Tucson; M. Paul, Danbury Eye Physicians and Surgeons, Danbury, CT; P. Pavan, University of South Florida, Tampa; M. Petersen, Cincinnati Eye Institute, Cincinnati; J. Prensky, Pennsylvania Retina Specialists, Camp Hill, PA; P. Raskauskas, Retina Consultants of Southwest Florida, Fort Myers; E. Reichel, New England Eye Center, Boston; D. Reid, Retina Associates, Annapolis, MD; P. Richmond, Central Florida Retina, Orlando; W. Rodden, Retina and Vitreous Center of Southern Oregon, Ashland, OR; P. Rosenfeld, Bascom Palmer Eye Institute, Miami; P. Runge, Ophthalmic Consultants, Sarasota, FL; S. Sadda, Doheny Eye Institute, Los Angeles; S. Sanislo, California Vitreoretinal Research Center, Menlo Park; S. Schwartz, Bascom Palmer Eye Institute, Palm Beach Gardens, FL; A. Seres, Semmelweis University, Budapest, Hungary; M. Singer, Medical Center Ophthalmology Associates, San Antonio, TX; G. Soubrane, Clinique d'Ophtalmologie, University of Paris XII, Créteil, France; P. Soucek, University Hospital Vinohrady, Prague, Czech Republic; W. Stern, Northern California Retina-Vitreous Associates, Mountain View; G. Stoller, Ophthalmic Consultants of Long Island, Rockville Centre, NY; J. Thompson, Retina Specialists, Towson, MD; D. Tom, New England Retina Associates, Hamden, CT; R. Torti, Retina Specialists, Desoto, TX; M. Trese, Associated Retinal Consultants, Royal Oak, MI; E. van Kuijk, University of Texas Medical Branch, Galveston; T. Verstaeten, Allegheny General Hospital, Pittsburgh; K. Warren, University of Kansas Medical Center, Kansas City; H. Weiss, Retina Consultants of Michigan, Southfield; J. Weiss, Retina Associates of South Florida, Margate, FL; C. Wells, Vitreoretinal Associates, Seattle; S. Wolf, Universitätsklinikum Leipzig, Leipzig, Germany; and K. Zhang, John Moran Eye Center, University of Utah, Salt Lake City.

REFERENCES

1. Bressler NM. Age-related macular degeneration is the leading cause of blindness. JAMA 2004;291:1900-1.
2. Friedman DS, O'Colmain BJ, Munoz B, et al. Prevalence of age-related macular degeneration in the United States. Arch Ophthalmol 2004;122:564-72.
3. Resnikoff S, Pascolini D, Etya'ale D, et al. Global data on visual impairment in the year 2002. Bull World Health Organ 2004;82:844-51.
4. Augood C, Fletcher A, Bentham G, et al. Methods for a population-based study of the prevalence of and risk factors for age-related maculopathy and macular degeneration in elderly European populations: the EUREYE study. Ophthalmic Epidemiol 2004;11:117-29.
5. Ferris FL III, Fine SL, Hyman L. Age-related macular degeneration and blindness due to neovascular maculopathy. Arch Ophthalmol 1984;102:1640-2.
6. Macular Photocoagulation Study Group. Subfoveal neovascular lesions in age-relat-

ed macular degeneration: guidelines for evaluation and treatment in the Macular Photocoagulation Study. Arch Ophthalmol 1991;109:1242-57.
7. Ali F, Chan WC, Stevenson MR, Muldrew KA, Chakravarthy U. Morphometric analysis of angiograms of exudative lesions in age-related macular degeneration. Arch Ophthalmol 2004;122:710-5.
8. Pauleikhoff D. Neovascular age-related macular degeneration: natural history and treatment outcomes. Retina 2005;25:1065-84.
9. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: one-year results of 2 randomized clinical trials — TAP report. Arch Ophthalmol 1999;117:1329-45. [Erratum, Arch Ophthalmol 2000;118:488.]
10. Verteporfin in Photodynamic Therapy Study Group. Verteporfin therapy of subfoveal choroidal neovascularization in age-related macular degeneration: two-year results of a randomized clinical trial including lesions with occult with no classic choroidal neovascularization — Verteporfin In Photodynamic Therapy (VIP) report 2. Am J Ophthalmol 2001;131:541-60.
11. Bressler NM. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: two-year results of 2 randomized clinical trials — TAP report 2. Arch Ophthalmol 2001;119:198-207.
12. Guidelines for using verteporfin (Visudyne) in photodynamic therapy for choroidal neovascularization due to age-related macular degeneration and other causes: update. Retina 2005;25:119-34.
13. Gragoudas ES, Adamis AP, Cunningham ET Jr, Feinsod M, Guyer DR. Pegaptanib for neovascular age-related macular degeneration. N Engl J Med 2004;351:2805-16.
14. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419-31.
15. Heier JS, Boyer DS, Ciulla TA, et al. Ranibizumab in combination with verteporfin photodynamic therapy in neovascular age-related macular degeneration (FOCUS): year 1 results. Arch Ophthalmol (in press).
16. Cochran WG. Some methods for strengthening the common chi-square tests. Biometrics 1954;10:417-51.
17. Hochberg Y. A sharper Bonferroni procedure for multiple tests of significance. Biometrika 1988;75:800-2.
18. Visudyne. East Hanover, NJ: Novartis Pharmaceuticals, 2005 (package insert).
19. Avastin. South San Francisco, CA: Genentech, 2004 (package insert).
20. Antiplatelet Trialists' Collaboration. Collaborative overview of randomised trials of antiplatelet therapy. I. Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. BMJ 1994;308:81-106. [Erratum, BMJ 1994;308:1540.]

Primary Endpoint Results of a Phase II Study of Vascular Endothelial Growth Factor Trap-Eye in Wet Age-related Macular Degeneration

David M. Brown, MD,[1] Jeffrey S. Heier, MD,[2] Thomas Ciulla, MD,[3] Matthew Benz, MD,[1] Prema Abraham, MD,[4] George Yancopoulos, MD, PhD,[5] Neil Stahl, PhD,[5] Avner Ingerman, MD,[5] Robert Vitti, MD, MBA,[5] Alyson J. Berliner, MD, PhD,[5] Ke Yang, PhD,[5] Quan Dong Nguyen, MD, MSc,[6] for the CLEAR-IT 2 Investigators

Objective: To evaluate the biologic effects and safety of vascular endothelial growth factor (VEGF) Trap-Eye during a 12-week fixed-dosing period in patients with neovascular (wet) age-related macular degeneration (AMD).

Design: Multicenter, prospective, randomized, double-masked clinical trial with initial 12-week fixed dosing period. Data were analyzed to week 16.

Participants: We included 159 patients with subfoveal choroidal neovascularization secondary to wet AMD.

Methods: Patients were randomized 1:1:1:1:1 to VEGF Trap-Eye during the fixed-dosing phase (day 1 to week 12): 0.5 or 2 mg every 4 weeks (0.5 mg q4wk, 2 mg q4wk) on day 1 and at weeks 4, 8, and 12; or 0.5, 2, or 4 mg every 12 weeks (0.5 mg q12wk, 2 mg q12wk, or 4 mg q12wk) on day 1 and at week 12.

Main Outcome Measures: The primary endpoint was change from baseline in central retinal/lesion thickness (CR/LT) at week 12; secondary outcomes included change in best-corrected visual acuity (BCVA), proportion of patients with a gain of ≥15 letters, proportion of patients with a loss of >15 letters, and safety.

Results: At week 12, treatment with VEGF Trap-Eye resulted in a significant mean decrease in CR/LT of 119 μm from baseline in all groups combined ($P<0.0001$). The reduction in CR/LT with the 2 mg q4wk and 0.5mg q4wk regimens was significantly greater than each of the quarterly dosing regimens. The BCVA increased significantly by a mean of 5.7 letters at 12 weeks in the combined group ($P<0.0001$), with the greatest mean gain of >8 letters in the monthly dosing groups. At 8 weeks, BCVA improvements were similar with 2 mg q4wk and 2 mg q12wk dosing. After the last required dose at week 12, CR/LT and visual acuity were maintained or further improved at week 16 in all treatment groups. Ocular adverse events were mild and consistent with safety profiles reported for other intraocular anti-VEGF treatments.

Conclusions: Repeated monthly intravitreal dosing of VEGF Trap-Eye over 12 weeks demonstrated significant reductions in retinal thickness and improvements in visual acuity, and was well-tolerated in patients with neovascular AMD.

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Age-related macular degeneration (AMD) is a leading cause of vision loss among older adults in Western countries.[1,2] The vast majority of patients with AMD have the dry form of the disease, but severe vision loss occurs most frequently in patients who develop choroidal neovascularization (CNV).[3] Neovascular AMD is characterized by the growth of anomalous vessels originating from the choroidal vascular network, which causes hemorrhage and leakage in the subretinal and intraretinal spaces resulting in metamorphopsia and decreased vision.

The pathophysiology of ocular neovascularization is complex, but vascular endothelial growth factor (VEGF)-A is an important stimulus for both the growth of new blood vessels and increased vascular leakage resulting in retinal edema as seen in animal models and human AMD.[4–7] The mammalian VEGF family also includes VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF), but the members predominantly involved in ocular neovascularization are VEGF-A and PlGF.[8,9] Of at least the 4 major isoforms of human VEGF-A, $VEGF_{165}$ is the most abundantly expressed, although the other isoforms are also biologically active.[8,10] The biological activities of VEGF-A are mediated through 2 receptor tyrosine kinases, VEGF receptor (VEGFR)1 and VEGFR2. Found predominantly on the surface of vascular endothelial cells, VEGFR2 plays a key role in mediating endothelial cell survival, migration, and proliferation, both during normal development as well as in a variety of pathophysiologic conditions. Initially discovered as a vascular permeability factor, VEGF–A also decreases barrier functions of the endothelium, resulting in increased extravasation of water and macromolecules.[10,11] Vascular

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endothelial growth factor-A is a potent promoter of vascular permeability (approximately 50 000 times more potent than histamine), and the onset of this effect is very rapid.

Vascular endothelial growth factor increases permeability of the pathologic choroidal vessels, leading to extravasation of fluid into and under the retina. The resulting increase in central retinal thickness is responsible in part for the decrease in central visual acuity. Although not always correlative with visual acuity, the change in central retinal thickness, as measured by optical coherence tomography, has become one of the established means of monitoring the disease and its response to treatment.

The related angiogenic factor, PlGF, binds to VEGFR1 and collaborates with VEGF-A in promoting angiogenesis and vascular permeability, particularly in pathologic conditions.[9,12,13] The mechanism of action of PlGF has not yet been fully elucidated,[11,14] but it has been shown that PlGF ligation of VEGFR1 promotes leukocyte chemotaxis,[13] and that PlGF may play a role in recruiting inflammatory cells into the diseased retina, leading to release of VEGFs and other inflammatory mediators, perpetuating the cycle of angiogenesis and inflammation.[15]

Most current anti-VEGF treatments target VEGF-A. Of the currently approved anti-VEGF agents for ocular disease, pegaptanib is specific for $VEGF_{165}$,[16] and ranibizumab targets multiple VEGF-A isoforms and their degradation products.[17] Bevacizumab, a full-length humanized monoclonal anti-VEGF antibody that is used off-label to treat AMD, is derived from the same mouse antibody as ranibizumab and is also directed against all isoforms of VEGF-A.[18,19]

Vascular endothelial growth factor Trap-Eye (VEGF Trap-Eye) is a fully human, soluble recombinant decoy VEGFR that is biologically engineered to contain key binding domains of VEGFR1 and VEGFR2 fused to the constant Fc region of IgG1.[20] Unlike currently available anti-VEGF agents, VEGF Trap-Eye inhibits PlGF in addition to all isoforms of VEGF-A.[20] Because the binding affinity of VEGF Trap-Eye for VEGF-A isoforms (K_D, 0.5–1 pmol/L) and PlGF (K_D, 39–392 pmol/L) is higher than that of native receptors (K_D of 10–30 pmol/L for VEGFR1 and 100-300 pmol/L for VEGFR2), it effectively blocks VEGF binding and activation of these receptors, even when VEGF Trap-Eye is present at low concentrations. The binding affinity of anti-VEGF monoclonal antibodies by contrast is many fold lower (K_D, 0.1–10 nmol/L).[21,22] Tight binding of VEGF Trap-Eye to all VEGF-A isoforms and PlGF could theoretically offer a differential impact on visual acuity. As shown in modeling studies, high-affinity blockade of VEGF-A and PlGF activity with VEGF Trap-Eye may increase the duration of effect, thus allowing an extended dosing interval.[23] VEGF Trap-Eye also forms a stable, inert 1:1 complex with VEGF dimers, unlike the rapidly cleared multimeric immune complexes formed with an antibody.[24]

Preclinical studies support a therapeutic role for VEGF Trap-Eye in multiple vascular eye diseases, including wet AMD. Blockade of VEGF with VEGF Trap-Eye inhibited CNV, suppressed VEGF-induced breakdown of the blood-retinal barrier, and promoted regression of newly formed and established blood vessels (Invest Ophthalmol Vis Sci 5307 [Suppl]:46,2005; Invest Ophthalmol Vis Sci 1411 [Suppl]:46,2005; and Invest Ophthalmol Vis Sci 5300 [Suppl]:46,2005).[25] Primate studies showed VEGF Trap-Eye rapidly reversed vascular leakage in retinal injury models and had a favorable ocular safety profile (Invest Ophthalmol Vis Sci 1751 [Suppl]:47,2006).

The clinical activity of VEGF Trap-Eye was initially demonstrated in a 6-week, sequential, single ascending-dose, phase 1 study (*CL*inical *E*valuation of *A*nti-angiogenesis in the *R*etina *I*ntravitreal *T*rial [CLEAR-IT 1]) in patients with neovascular AMD (Invest Ophthalmol Vis Sci 1751 [Suppl]:47,2006). After receiving single intravitreal injections of VEGF Trap-Eye (0.05–4 mg), patients showed a dose-dependent improvement in visual acuity, which correlated with anatomic improvement. At 6 weeks, an overall mean decrease in foveal thickness of 104.5 μm and mean increase in visual acuity of 4.4 letters was reported for all groups combined. In the 2 highest dose groups (2 and 4 mg) combined, best-corrected visual acuity (BCVA) increased by a mean of 13.5 letters, and by 6 weeks, vision had stabilized or improved in 95% of patients. Anatomic benefits and visual gains were maintained out to 12 weeks in 3 of 6 patients who received single administrations of higher doses. Based on these encouraging results from CLEAR-IT 1, a dose- and interval-ranging phase 2 study (*CL*inical *E*valuation of *A*nti-angiogenesis in the *R*etina *I*ntravitreal *T*rial [CLEAR-IT 2]) was designed to investigate the safety and biologic effects of VEGF Trap-Eye after repeated dosing. The study consisted of a fixed-dosing phase during which patients received 1 of 5 regimens of VEGF Trap-Eye for 12 weeks, followed by as-needed (PRN) dosing from weeks 16 through 52. The details of the PRN dosing phase are presented in the accompanying article.[26] The primary endpoint and results from the fixed-dosing period are presented herein.

Materials and Methods

Study Design

The primary objectives of the study were to assess the effect of intravitreal VEGF Trap-Eye on central retinal/lesion thickness (CR/LT) and to assess the ocular and systemic safety and tolerability of repeated doses of VEGF Trap-Eye in patients with CNV associated with wet AMD. A key secondary objective was to assess the effect of VEGF Trap-Eye on BCVA.

The CLEAR-IT 2 was a prospective, double-masked, randomized study conducted at 33 sites in the United States. Patients were enrolled between May 2006 and April 2007. Five groups of approximately 30 patients each were randomized in a balanced ratio to receive an intravitreal injection of VEGF Trap-Eye 0.5 or 2 mg every 4 weeks, (0.5 mg q4wk or 2 mg q4wk) on day 1 and at weeks 4, 8, and 12 for a total of 4 treatments or 0.5, 2, or 4 mg every 12 weeks (0.5 mg q12wk, 2 mg q12wk, or 4 mg q12wk) on day 1 and week 12 for a total of 2 treatments (Fig 1). The PRN dosing phase began at week 16 and continued through week 52.[26] The primary endpoint (change in CR/LT) and BCVA were assessed at week 12 (after 1 or 3 doses in the quarterly and monthly dosing groups, respectively) and the results of the fixed dosing phase were assessed at week 16 (after 2 or 4 doses in the quarterly and monthly dose groups, respectively). Although the primary endpoint of the study was at week 12, results at week 16 were evaluated to assess the impact of the final fixed dose from each dose group on anatomic outcomes and BCVA.

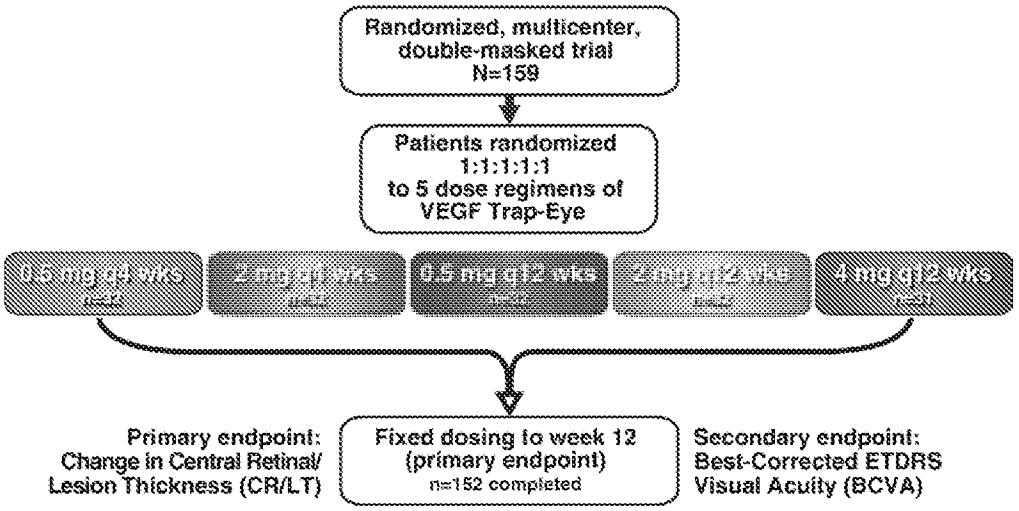

Figure 1. Study design. During the fixed-dosing phase of the CLEAR-IT 2 study, patients were randomized in equal ratios to receive 1 of 5 different regimens of VEGF Trap-Eye for 12 weeks: 0.5 or 2 mg every 4 weeks, or 0.5, 2, or 4 mg every 12 weeks. The primary endpoint, change from baseline in CR/LT, and a key secondary endpoint, BCVA, was measured at 12 weeks. BCVA = best-corrected visual acuity; CLEAR-IT = CLinical Evaluation of Anti-angiogenesis in the Retina Intravitreal Trial; CR/LT = central retinal/lesion thickness; ETDRS = Early Treatment of Diabetic Retinopathy Study; VEGF = vascular endothelial growth factor.

The study protocol was approved by the institutional review board or ethics committee at every institution and was conducted according to the recommendations of Good Clinical Practice and the Declaration of Helsinki. The study was compliant with the rules and regulations under the Health Insurance Portability and Accountability Act of 1996. All patients provided written informed consent to participate in the study. The CLEAR-IT 2 study is registered with ClinicalTrials.gov (NCT00320788).

Study Population

Patients eligible for the study were ≥50 years old, had a diagnosis of subfoveal CNV secondary to wet AMD, and met the following inclusion criteria: CR/LT ≥300 μm, Early Treatment of Diabetic Retinopathy Study (ETDRS) BCVA letter score of 73 to 34 letters (20/40–20/200), loss of ≥5 ETDRS letters in BCVA over the preceding 6 months for previously treated patients with minimally classic or occult lesions, linear diameter of lesion ≤5400 μm by fluorescein angiography, subretinal hemorrhage (if present) sparing the fovea and comprising ≤50% of total lesion, area of scar ≤25% of total lesion, and sufficient clarity of ocular media to allow retinal photography.

Exclusion criteria were vitreous hemorrhage in preceding 4 weeks; aphakia or pseudophakia with absence of a posterior capsule (unless as a result of a yttrium aluminum garnet capsulotomy); significant subfoveal atrophy or scarring; active ocular inflammation; corneal transplant; previous uveitis in either eye; or history of macular hole of grade 3 or higher. Patients who had previously received any of the following treatments in the study eye were excluded: Subfoveal thermal laser therapy, any operative intervention for AMD, extrafoveal laser coagulation treatment or photodynamic therapy in preceding 12 weeks, pegaptanib sodium in preceding 8 weeks, systemic or intravitreal treatment with VEGF Trap-Eye, ranibizumab, or bevacizumab at any time, juxtascleral steroids, anecortave acetate, or intravitreal triamcinolone acetonide or other steroids in preceding 24 weeks. Additional reasons for exclusion were other causes of CNV in either eye; active ocular infection; congenital lid anomalies that might interfere with intravitreal administration; any retinal disease other than CNV in either eye; previous trabeculectomy or pars plana vitrectomy; cup-to-disc ratio ≥0.8, intraocular pressure >25 or receipt of >2 agents for treatment of glaucoma; allergy to povidone iodine, fluorescein, or recombinant proteins; absolute neutrophil count <1000 cells/mm^3; human immunodeficiency virus positivity, active systemic infection requiring antibiotics; proteinuria >1$^+$ or urine protein:creatinine ratio ≥1 on 2 repeated determinations within 1 week; New York Heart Association class III or IV; symptomatic cardiovascular or peripheral vascular disease, malignancy other than basal cell carcinoma in preceding 2 years; and any other conditions or laboratory abnormalities that could interfere with disease assessment or patient participation in the study. The use of standard agents or other anti-VEGF agents was not permitted before week 16.

Endpoints and Assessments

The 12-week assessment measured anatomic and visual changes after administration of 3 doses of VEGF Trap-Eye in the monthly dose group and 1 dose in the quarterly dosing group. All assessments at week 12 were performed before the planned injection. Results at week 16 were evaluated to assess the impact of the final fixed dose at week 12 from each dose group on these parameters.

One eye was designated as the study eye, with all evaluations performed on that eye. Criteria, in descending order, for selection of the study eye in cases of bilateral exudative AMD were worse visual acuity, clearer ocular media, and nondominant eye. If these factors were similar in both eyes, the right eye was chosen as the study eye.

The primary efficacy endpoint was change in CR/LT from baseline at 12 weeks, as assessed by Stratus (software version 4.0 or higher) optical coherence tomography scans (Carl Zeiss Meditec, Inc., Dublin, CA) read at a masked independent central reading center (Digital Optical Coherence Tomography Reading Center [DOCTR], Cleveland, OH). The CR/LT was defined as the distance between the inner limiting membrane and the inner border of the retinal pigment epithelium/choriocapillaris complex, including any subretinal fluid and thickness of any observable choroidal neovascular membrane or scar tissue in the central 1 mm of the posterior pole scan. A posterior pole scan was obtained, consisting of a high-resolution 7-mm scan from a single scan line from the meridian of the optic disc margin, declined at a 5-degree angle

Table 1. Patient Disposition

No. of Patients	0.5 q4	2 q4	0.5 q12	2 q12	4 q12	All patients
Screened						301
Randomized	32	32	32	32	31	159
Treated	32	31	32	31	31	157
Completed week 12	31	31	31	29	30	152 (96.8%)
Withdrawn by week 12	1		1	2	1	5 (3.2%)

0.5q4 = 0.5 mg every 4 weeks; 2q4 = 2 mg every 4 weeks; 0.5q12 = 0.5 mg every 12 weeks; 2q12 = 2 mg every 12 weeks; 4q12 = 4 mg every 12 weeks.

through the presumed foveal center. The placement of the scan line was based on anatomic landmarks as visualized by a trained, certified operator to offer better registration.

Key secondary endpoints included the change in BCVA as measured by ETDRS letter score at 12 weeks and the proportions of patients with avoidance of moderate vision loss (loss of ≤15 letters), stabilization, or improvement in visual acuity (gain of ≥0 letters), and significant vision gain (gain of ≥15 letters) at 12 weeks. Certified examiners assessed BCVA using the ETDRS protocol (at 4 m). Examiners were masked to treatment assignment and performed no other study assessments. Safety was monitored with reporting of adverse events (AEs) and serious AEs, clinical laboratory tests, vital signs, and ophthalmic examination.

Statistical Analyses

Efficacy analyses were performed on the full analysis set, which included all enrolled patients who underwent baseline and ≥1 postbaseline assessment. The last observation carried-forward method was used to impute missing data. The safety analysis set included all patients who received study treatment. The primary analysis was a paired comparison *t* test of the change in CR/LT from baseline to week 12 for all groups combined. If this was significant, an analysis of covariance was done on the 5 individual groups. A similar analysis was done for BCVA measurements. Results are presented for all 5 treatment groups combined as well as for the individual groups.

Results

Disposition

Patient disposition is shown in Table 1. Among the 159 patients who were randomized, 157 received treatment. Two patients, 1 each in the 2-mg monthly and 2-mg quarterly groups, were withdrawn before receiving treatment. Of the 157 patients who received treatment, 152 (96.8%) completed the 12-week visit, and 5 patients were withdrawn. Reasons for withdrawal were death (n = 1, 4q12 group), AE (n = 1, 2q12 group), inability to attend visits (n = 1, 2q12 group), investigator decision (n = 1, 0.5q12 group), and subject request (n = 1, 0.5q4 group).

Baseline Characteristics

The study population was representative of the exudative AMD population in the United States. The mean age of patients overall was 78.2 years (range, 53–94) and a majority were women (62%). The duration of disease ranged from 0 to 67 months, with a mean of 3.9 months, and 20 patients had received previous treatment (photodynamic therapy [n =5], focal laser photocoagulation [n = 4], intravitreal pegaptanib sodium [n = 3], intravitreal triamcinolone [n = 1], and combination [n = 7]). All CNV lesion types were represented in the following distribution: Predominantly classic (38.2%), minimally classic (23.6%), and occult-no-classic (38.2%; Table 2). Of note, the baseline CR/LT was thicker (507 μm) in the 4 mg q12wk arm (Table 3).

Primary Endpoint: Change in Central Retinal Lesion Thickness

At week 12, treatment with VEGF Trap-Eye resulted in a significant decrease in mean CR/LT of 119 μm from baseline in all treatment groups combined ($P<0.0001$; Fig 2A). A significant mean improvement from baseline was observed as early as week 1 (−103 μm for all treatment groups combined; $P = 0.04$). The significant reduction in CR/LT was observed in each treatment group at week 12, with monthly dosing with 0.5 or 2 mg providing a more profound and consistent effect (Fig 2B). At 12 weeks, the mean reductions in CR/LT with the 0.5 mg q4 wk (−153.5 μm; standard deviation [SD] = 113.3) and 2 mg q4wk (−169.2 μm; SD = 138.5) regimens were significantly greater than mean reductions with each of the quarterly dosing regimens (0.5 mg q4: $P = 0.0022$, $P<0.0001$, and $P = 0.0255$; 2 mg q4: $P = 0.0010$, $P<0.0001$, and $P = 0.0129$ versus 0.5 mg q12, 2 mg q12, and 4 mg q12, respectively).

Changes in Best-corrected Visual Acuity

At week 12, BCVA, as measured by ETDRS letters score, showed a significant mean increase from baseline of 5.7 letters in all

Table 2. Baseline Demographic and Clinical Characteristics

Characteristic	All Treated Patients (n = 157)
Age, years (mean [range])	78.2 (53-94)
Gender (%M:%F)	38:62
Disease duration, mos (mean [range])	3.9 (0–67)
Previous treatment	20 (12.7%)
Lesion size (mean ± SD) in disc area	3.11 ± 2.12
Lesion type (n [%])	
Predominantly classic	60 (38.2)
Minimally classic	37 (23.6)
Occult lesions	60 (38.2)
Disease status (mean [range])	
Central retinal/lesion thickness	456 μm (186–1316 μm)
Foveal thickness	327 μm (116–1081 μm)
Best corrected visual acuity (ETDRS letters)	56 (27–83)

ETDRS = Early Treatment of Diabetic Retinopathy Study; F= Female; M = Male; SD = standard deviation.

Table 3. Baseline Disease Status by Treatment Group

	0.5q4 (n = 32)	2q4 (n = 31)	0.5q12 (n = 2)	2q12 (n = 31)	4q12 (n = 31)	All groups (n = 157)
CR/LT (μm)	434 (282–710)	453 (232–960)	442 (186–762)	447 (265–948)	507 (240–1316)	456 (186–1316)
Foveal Thickness (μm)	329 (212–509)	307 (171–524)	319 (116–559)	334 (186–762)	360 (177–1081)	327 (116–1081)
BCVA (ETDRS letters)	54 (27–76)	58 (32–83)	56 (30–72)	57 (32–72)	53 (28–80)	56 (27–83)

BCVA = best-corrected visual acuity; CR/LT = central retinal/lesion thickness; ETDRS = Early Treatment of Diabetic Retinopathy Study; 0.5q4 = 0.5 mg every 4 weeks; 2q4 = 2 mg every 4 weeks; 0.5q12 = 0.5 mg every 12 weeks; 2q12 = 2 mg every 12 weeks; 4q12 = 4 mg every 12 weeks. Values are presented as mean (range).

treatment groups combined ($P<0.0001$; Fig 3A). A significant gain in BCVA was noted as early as week 1 (mean gain of 3 letters). Each treatment group showed an improvement in visual acuity at week 12 (Fig 3B). Mean increases were similar among all treatment groups at week 8 ($P\geq0.25$ for all pairwise comparisons, analysis of covariance), after which in the monthly treatment groups of 0.5 mg q4wk and 2 mg q4wk, vision continued to improve, with a mean gain of 8.8 (SD = 9.2) and 8.3 (SD = 10.1) letters, respectively, at week 12. Of note, the mean improvement in visual acuity at 8 weeks was similar after administration of a single 2-mg dose (quarterly dose group) or 2 monthly 2-mg doses.

Frequency of Changes in Best-corrected Visual Acuity

After 12 weeks, 98% of patients in all treatment groups combined (range, 94%–100% in the individual dose groups) avoided vision

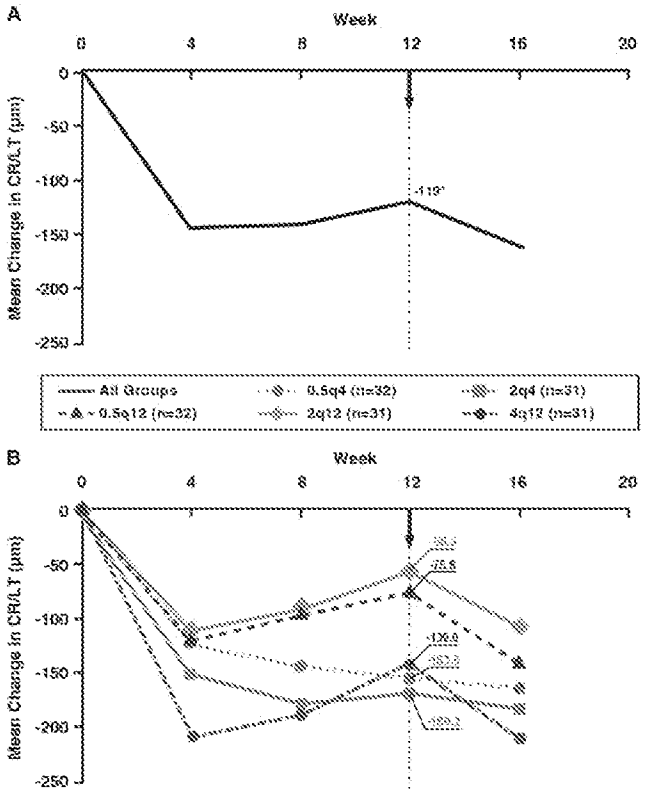

Figure 2. Mean change from baseline in central retinal/lesion thickness (CR/LT) for (A) all groups combined and (B) individual dosing groups. Change in CR/LT from baseline at 12 weeks was the primary study endpoint; in the combined treatment group, a significant decrease of 119 μm was observed at week 12. *$P<0.0001$ versus baseline. All treatment groups demonstrated a significant reduction in CR/LT from baseline at week 12, with the greatest reductions in the monthly dosing groups. The last-observation-carried-forward method was used to impute missing data. CR/LT = central retinal/lesion thickness; 0.5q4 = 0.5 mg every 4 weeks; 2q4 = 2 mg every 4 weeks; 0.5q12 = 0.5 mg every 12 weeks; 2q12 = 2 mg every 12 weeks; 4q12 = 4 mg every 12 weeks.

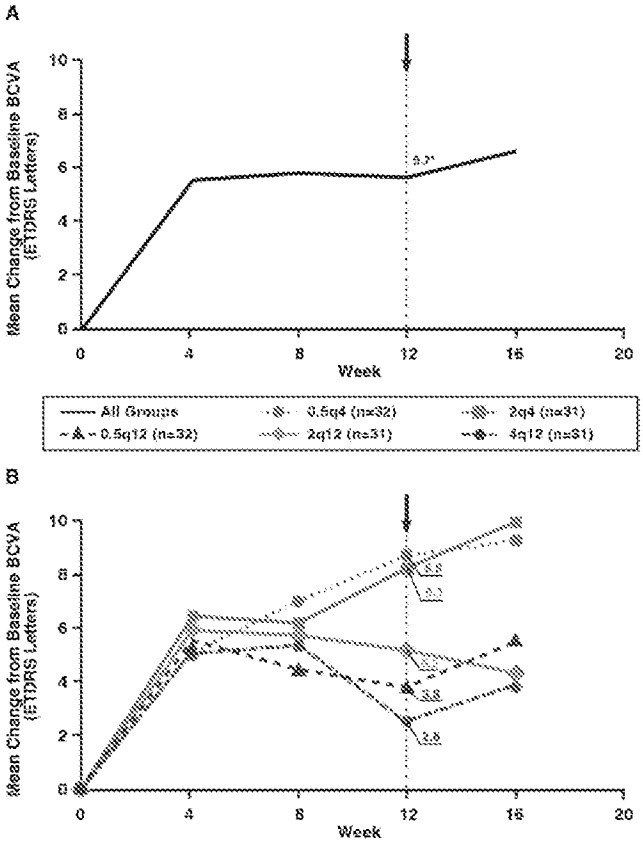

Figure 3. Mean change from baseline in best-corrected visual acuity (BCVA) for (A) all groups combined and (B) individual dosing groups. The combined treatment group showed a significant gain of 5.7 letters ($P<0.0001$ versus baseline). The BCVA was improved in all treatment groups at week 12, but the greatest improvements were observed in the monthly dosing groups. The last observation-carried-forward method was used to impute missing data. BCVA = best corrected visual acuity; ETDRS = Early Treatment of Diabetic Retinopathy Study; 0.5q4 = 0.5 mg every 4 weeks; 2q4 = 2 mg every 4 weeks; 0.5q12 = 0.5 mg every 12 weeks; 2q12 = 2 mg every 12 weeks; 4q12 = 4 mg every 12 weeks.

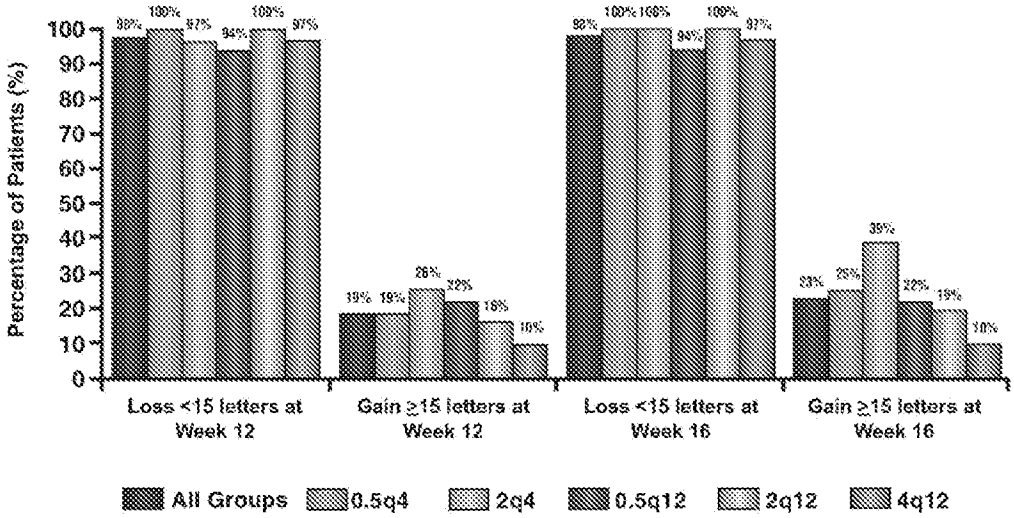

Figure 4. Visual acuity changes at weeks 12 and 16. The proportions of patients who avoided moderate vision loss (loss of ≥15 letters) or had significant vision gain (gain of ≥15 letters) in the combined treatment group and individual dosing groups are shown. At both 12 and 16 weeks, only 2% of patients in the combined treatment group experienced a loss of ≥15 letters, whereas 19% of patients showed a significant gain in vision at 12 weeks; in individual treatment groups, the proportions of patients showing a significant gain in vision remained steady or increased at week 16. 0.5q4 = 0.5 mg every 4 weeks; 2q4 = 2 mg every 4 weeks; 0.5q12 = 0.5 mg every 12 weeks; 2q12 = 2 mg every 12 weeks; 4q12 = 4 mg every 12 weeks. Decreases in visual acuity were due to retinal pigment epitheliopathy as reported by the investigators (n = 1), subretinal hemorrhage (n = 1), retinal hemorrhage (n = 1), and unexplained (n = 6).

loss of ≥15 letters (Fig 4). Overall, 4 patients (2.5%) experienced vision loss of ≥15 letters, including 2 patients in the 0.5 mg q12wk group, 1 patient in the 2 mg q4wk group, and 1 patient in the 4 mg q12wk group. In all treatment groups combined, the proportion of patients experiencing a clinically significant gain in vision (≥15 letters) was 19% at week 12. Again, the frequency of clinically significant vision gain was highest in the 2 mg q4wk group (26% at 12 weeks).

By week 12, monthly dosing reduced the proportion of patients with vision of ≤20/200, and all dose regimens of VEGF Trap-Eye (Fig 5) increased the proportion of patients with ≥20/40 vision. The proportion of patients with ≤20/200 vision was higher in the quarterly treatment groups than in the monthly treatment groups at week 12; none of the patients in the 2 mg q4wk group had ≤20/200 vision (data not shown). Conversely, a lower proportion of patients who received quarterly doses achieved ≥20/40 vision; the 2 mg q4wk dose group again had the highest proportion of patients (58%) with ≥20/40 vision.

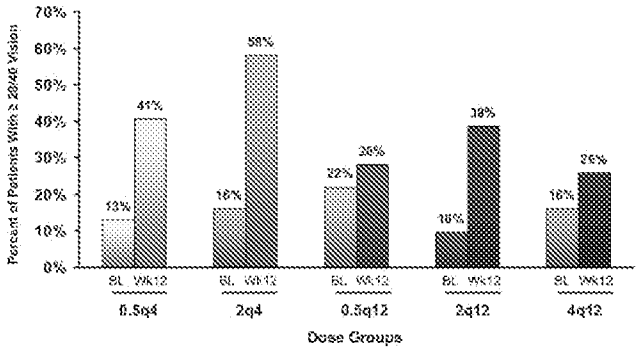

Figure 5. Snellen equivalent of ≥20/40 vision. All treatment groups showed an increase from baseline in the proportion of patients with ≥20/40 vision at week 12. The last-observation-carried-forward method was used to impute missing data. BL = baseline; 0.5q4 = 0.5 mg every 4 weeks; 2q4 = 2 mg every 4 weeks; 0.5q12 = 0.5 mg every 12 weeks; 2q12 = 2 mg every 12 weeks; 4q12 = 4 mg every 12 weeks.

Results at 16 Weeks (Fixed-Dose Phase)

Although the primary endpoint was assessed at week 12, the data collected at week 16 were indicative of the response to the last mandatory injection of the fixed-dosing phase at week 12. In the treatment groups combined, a further decrease in CR/LT was noted from a mean of −119 μm at week 12 to a mean of −160 μm at week 16 (Fig 2A). In the monthly treatment groups, CR/LT decreased continuously from baseline to week 16; in the quarterly treatment group, the reduction in CR/LT was attenuated by week 12 but was noted again at 16 weeks (after administration of the second dose at week 12).

In addition, the BCVA improved from week 12 to week 16 in the combined treatment group and in most individual treatment groups (Fig 3B). In the combined treatment group, the BCVA improved further, from a mean of 5.7 letters at week 12 to a mean of 6.6 letters at week 16. The 0.5-mg and 2-mg monthly dose groups showed a continuing and consistent improvement in BCVA to week 16. In the quarterly dose groups, the BCVA, which had declined by week 12, showed mixed results at week 16, with improved acuity in the 0.5- and 4-mg dose groups, but with worsened vision in the 2-mg dose group. The proportion of patients experiencing a gain of ≥15 letters continued to increase between weeks 12 and 16 for the overall group (from 19% to 23%) and in both monthly dose groups (from 19% to 25% in the 0.5 mg q4wk group and from 26% to 39% in the 2 mg q4wk group; Fig 4).

Safety

The mean total dose administered to each group was consistent with the anticipated amount based on the dosing schedule. The highest total exposure was in the 2 mg q4wk group, which received a mean total of 5.74 mg through week 12. All patients in the quarterly dosing groups, and 90.6% and 90.3% in the 0.5 mg q4wk

Table 4. Adverse Events in the Study Eye (Frequency ≥5% in All Groups Combined*) at Week 16

Adverse Event	Number (n)	Percent (%)
Conjunctival hemorrhage	42	26.8
Increased IOP (transient postinjection)	22	14.0
Refraction disorder	16	10.2
Retinal hemorrhage	14	8.9
Eye pain	12	7.6
Vitreous detachment	11	7.0
Detachment of retinal pigment epithelium	9	5.7
Visual acuity reduced (patient-reported)	9	5.7

IOP = intraocular pressure.
*Patients receiving treatment with vascular endothelial growth factor Trap-Eye (n = 157).

and 2 mg q4wk dose groups, respectively, received the required doses.

Most AEs were related to the injection procedure and no ocular serious AEs, clinically significant ocular inflammation, or endophthalmitis was reported in any study eyes during the first 16 weeks of the study. The ocular AEs that occurred through week 16 were mild and were similar to those reported for other intravitreally administered anti-VEGF compounds. An ocular AE was reported in 70.7% of patients in the treatment groups combined (Table 4). In general, fewer patients in the 0.5 mg q12wk and 2 mg q12wk groups (62.5% and 74.2%, respectively) reported an ocular AE compared with the 0.5 mg q4wk, 2 mg q4wk, and 4 mg q12wk groups (68.8%, 67.8%, and 80.7%, respectively).

Systemic serious AEs were observed in 12 patients. One case of angina pectoris (2 mg q4wk group), 2 cases of congestive heart failure (0.5 mg q4wk and 2 mg q4wk groups), and 2 cases of coronary artery diseases (2 mg q4wk and 4 mg q12wk groups) were reported during the treatment period. One death occurred during this part of the study from preexisting pulmonary hypertension. There did not seem to be any relationship between the VEGF Trap-Eye dose and the occurrence of any particular AE.

Discussion

During the 12-week fixed-dosing period of this phase 2 study, intravitreally administered VEGF Trap-Eye demonstrated significant anatomic and visual improvements from baseline at week 12 after repeated monthly dosing. Treatment with VEGF Trap-Eye 0.5 mg and 2 mg dosed every 4 weeks resulted in the greatest improvements in both measures at the 12-week endpoint. The CR/LT decreased by a mean of −153.5 and −169.2 μm from baseline, and BCVA mean letter score improved by 8.8 and 8.3 letters with 0.5- and 2-mg monthly dosing, respectively. In this index study, 60% of patients had occult or minimally classic lesions and 40% had predominantly classic lesions. In the pivotal trials of ranibizumab, the improvement in BCVA at 12 weeks after fixed monthly dosing was 10.0 and 6.8 letters with 0.5 and 0.3 mg ranibizumab, respectively, in patients with predominantly classic lesions[27] and 5.9 and 5.1 letters with 0.5 mg and 0.3 mg ranibizumab, respectively, in patients with minimally classic or occult lesions.[28] Although our smaller study did not compare VEGF Trap-Eye directly with ranibizumab and cross-trial comparisons must be made with caution, the improvements in BCVA with VEGF Trap-Eye are of similar magnitude to those noted at 12 weeks after fixed dosing with ranibizumab in the larger pivotal trials.[27-29]

Both monthly dose groups continued to show anatomic and vision improvements after administration of the fourth mandatory dose at week 12. Both mean visual acuity and frequency of patients with a significant gain in vision increased from weeks 12 to 16. Whether continued monthly dosing (rather than PRN dosing) beyond 12 weeks would offer further vision gains will be determined from ongoing phase 3 studies. The PRN dosing phase of the current study demonstrates that visual gains were maintained through week 52.[26]

In the phase 1 study, an extended duration of efficacy to 12 weeks was noted in 3 of 6 patients who received a single intravitreal injection of 2 or 4 mg of VEGF Trap-Eye (Invest Ophthalmol Vis Sci 1751 [Suppl]:47,2006). The phase 2 study of VEGF Trap-Eye in exudative AMD was designed to evaluate whether quarterly dosing could provide similar efficacy as could be achieved with monthly dosing. Although the fixed quarterly dosing regimens reduced retinal thickness and improved visual acuity at all time points, the effect in general was less robust than that achieved with monthly fixed dosing. The improvements in CR/LT and BCVA seen in the monthly dose groups (3 initial injections) were greater than those seen in the quarterly dose groups (1 initial injection). An initial intensive monthly loading dose period may be required to completely resolve edema and render the lesion fluid free and/or to maximize visual gain. Whether quarterly dosing could maintain efficacy after an initial, intensive anti-VEGF treatment period was not evaluated. Notably, a single 2-mg dose achieved an improvement in visual acuity that was similar to that achieved with 2 mg dosed monthly out to 8 weeks, raising the possibility that dosing with 2 mg every 8 weeks may be as effective as monthly dosing.

Based on these findings, 2 identical phase 3 pivotal studies of VEGF Trap-Eye, VIEW-1 and VIEW-2 (VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet Age-Related Macular Degeneration), were designed to test both of these hypotheses. The regimens evaluated in phase 3 were VEGF Trap-Eye at doses of 0.5 mg and 2 mg every 4 weeks and 2 mg every 8 weeks (after 3 monthly loading doses), compared with ranibizumab 0.5 mg every 4 weeks. Phase 3 data have been released (http://newsroom.regeneron.com/releasedetail.cfm?ReleaseID_532099 accessed December 21, 2010) and manuscripts are under preparation. These phase 3 results support the efficacy findings of the current study. The PRN phase of the CLEAR IT-2 study,[26] with PRN dosing from weeks 16 through 52 also provides further information on the durability of the anti-VEGF effect of VEGF Trap-Eye.

In conclusion, results from the fixed-dosing phase of the CLEAR-IT 2 study show that repeated intravitreal dosing with VEGF Trap-Eye administered monthly was associated with clinically and statistically significant improvements in CR/LT and BCVA at 12 weeks in patients with neovascular AMD. In all dosing groups, VEGF Trap-Eye was generally well-tolerated and there were no unexpected safety findings.

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References

1. Klaver CC, Wolfs RC, Vingerling JR, et al. Age-specific prevalence and causes of blindness and visual impairment in an older population: the Rotterdam Study. Arch Ophthalmol 1998;116:653–8.
2. Eye Diseases Prevalence Research Group. Prevalence of age-related macular degeneration in the United States. Arch Ophthalmol 2004;122:564–72.
3. Ferris FL III, Fine SL, Hyman L. Age-related macular degeneration and blindness due to neovascular maculopathy. Arch Ophthalmol 1984;102:1640–2.
4. Kvanta A, Algvere PV, Berglin L, Seregard S. Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. Invest Ophthalmol Vis Sci 1996;37:1929–34.
5. Tolentino MJ, Miller JW, Gragoudas ES, et al. Vascular endothelial growth factor is sufficient to produce iris neovascularization and neovascular glaucoma in a nonhuman primate. Arch Ophthalmol 1996;114:964–70.
6. Kliffen M, Sharma HS, Mooy CM, et al. Increased expression of angiogenic growth factors in age-related maculopathy. Br J Ophthalmol 1997;81:154–62.
7. Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 2002;120:338–46.
8. Bhisitkul RB. Vascular endothelial growth factor biology: clinical implications for ocular treatments. Br J Ophthalmol 2006;90:1542–7.
9. Rakic JM, Lambert V, Devy L, et al. Placental growth factor, a member of the VEGF family, contributes to the development of choroidal neovascularization. Invest Ophthalmol Vis Sci 2003;44:3186–93.
10. Kaiser PK. Vascular endothelial growth factor Trap-Eye for diabetic macular oedema. Br J Ophthalmol 2009;93:135–6.
11. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signalling—in control of vascular function. Nat Rev Mol Cell Biol 2006;7:359–71.
12. Carmeliet P, Moons L, Luttun A, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med 2001;7:575–83.
13. Ziche M, Maglione D, Ribatti D, et al. Placenta growth factor-1 is chemotactic, mitogenic, and angiogenic. Lab Invest 1997;76:517–31.
14. Cao Y. Positive and negative modulation of angiogenesis by VEGFR1 ligands. Sci Signal [serial online] 2009;2:re1. Available at: http://stke.sciencemag.org/cgi/content/abstract/sigtrans;2/59/re1. Accessed February 11, 2011.
15. Murakami M, Zheng Y, Hirashima M, et al. VEGFR1 tyrosine kinase signaling promotes lymphangiogenesis as well as angiogenesis indirectly via macrophage recruitment. Arterioscler Thromb Vasc Biol 2008;28:658–64.
16. Lee JH, Canny MD, De Erkenez A, et al. A therapeutic aptamer inhibits angiogenesis by specifically targeting the heparin binding domain of VEGF165. Proc Natl Acad Sci U S A 2005;102:18902–7.
17. Lowe J, Araujo J, Yang J, et al. Ranibizumab inhibits multiple forms of biologically active vascular endothelial growth factor in vitro and in vivo. Exp Eye Res 2007;85:425–30.
18. Kim KJ, Li B, Houck K, et al. The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. Growth Factors 1992; 7:53–64.
19. Presta LG, Chen H, O'Connor SJ, et al. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res 1997;57:4593–9.
20. Holash J, Davis S, Papadopoulos N, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci U S A 2002;99:11393–8.
21. Konner J, Dupont J. Use of soluble recombinant decoy receptor vascular endothelial growth factor trap (VEGF Trap) to inhibit vascular endothelial growth factor activity. Clin Colorectal Cancer 2004;4(suppl):S81–5.
22. Dixon JA, Oliver SC, Olson JL, Mandava N. VEGF Trap-Eye for the treatment of neovascular age-related macular degeneration. Expert Opin Investig Drugs 2009;18:1573–80.
23. Stewart MW, Rosenfeld PJ. Predicted biological activity of intravitreal VEGF Trap. Br J Ophthalmol 2008;92:667–8.
24. Rudge JS, Holash J, Hylton D, et al. VEGF Trap complex formation measures production rates of VEGF, providing a biomarker for predicting efficacious angiogenic blockade. Proc Natl Acad Sci U S A 2007;104:18363–70.
25. Saishin Y, Saishin Y, Takahashi K, et al. VEGF-TRAP(R1R2) suppresses choroidal neovascularization and VEGF-induced breakdown of the blood-retinal barrier. J Cell Physiol 2003; 195:241–8.
26. Heier JS, Boyer D, Nguyen QD, et al. The 1-year results of CLEAR-IT 2, a phase 2 study of vascular endothelial growth factor trap-eye dosed as-needed after 12-week fixed dosing. Ophthalmology 2011;118:1098–106.
27. Nguyen QD, Shah SM, Browning DJ, et al. A phase I study of intravitreal vascular endothelial growth factor Trap-Eye in patients with neovascular age-related macular degeneration. Ophthalmology 2009;116:2141–8.
28. Brown DM, Kaiser PK, Michels M, et al, ANCHOR Study Group. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N Engl J Med 2006;355:1432–44.
29. Rosenfeld PJ, Brown DM, Heier JS, et al, MARINA Study Group. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–31.

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[1] Retina Consultants of Houston, The Methodist Hospital, Houston, Texas.

[2] Ophthalmic Consultants of Boston, Boston, Massachusetts.

[3] Midwest Eye Institute, Indianapolis, Indiana.

[4] Black Hills Eye Institute, Black Hills, South Dakota.

[5] Regeneron Pharmaceuticals, Inc., Tarrytown, New York.

[6] Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland.

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Correspondence: Quan Dong Nguyen, MD, MSc, Wilmer Eye Institute, The Johns Hopkins University and Hospital, 600 North Wolfe Street, Maumenee 745, Baltimore, MD 21287. E-mail: qnguyen4@jhmi.edu

Long-term Outcomes of Ranibizumab Therapy for Diabetic Macular Edema: The 36-Month Results from Two Phase III Trials

RISE and RIDE

*David M. Brown, MD,[1,§] Quan Dong Nguyen, MD, MSc,[2,§] Dennis M. Marcus, MD,[3] David S. Boyer, MD,[4] Sunil Patel, MD, PhD,[5] Leonard Feiner, MD, PhD,[6] Patricio G. Schlottmann, MD,[7] Amy Chen Rundle, MS,[8] Jiameng Zhang, PhD,[8] Roman G. Rubio, MD,[8] Anthony P. Adamis, MD,[8] Jason S. Ehrlich, MD, PhD,[8] J. Jill Hopkins, MD,[8] on behalf of the RIDE and RISE Research Group**

Purpose: To report 36-month outcomes of RIDE (NCT00473382) and RISE (NCT00473330), trials of ranibizumab in diabetic macular edema (DME).

Design: Phase III, randomized, multicenter, double-masked, 3-year trials, sham injection–controlled for 2 years.

Participants: Adults with DME (n=759), baseline best-corrected visual acuity (BCVA) 20/40 to 20/320 Snellen equivalent, and central foveal thickness (CFT) ≥275 μm on optical coherence tomography.

Methods: Patients were randomized equally (1 eye per patient) to monthly 0.5 mg or 0.3 mg ranibizumab or sham injection. In the third year, sham patients, while still masked, were eligible to cross over to monthly 0.5 mg ranibizumab. Macular laser was available to all patients starting at month 3; panretinal laser was available as necessary.

Main Outcome Measures: The proportion of patients gaining ≥15 Early Treatment Diabetic Retinopathy Study letters in BCVA from baseline at month 24.

Results: Visual acuity (VA) outcomes seen at month 24 in ranibizumab groups were consistent through month 36; the proportions of patients who gained ≥15 letters from baseline at month 36 in the sham/0.5 mg, 0.3 mg, and 0.5 mg ranibizumab groups were 19.2%, 36.8%, and 40.2%, respectively, in RIDE and 22.0%, 51.2%, and 41.6%, respectively, in RISE. In the ranibizumab arms, reductions in CFT seen at 24 months were, on average, sustained through month 36. After crossover to 1 year of treatment with ranibizumab, average VA gains in the sham/0.5 mg group were lower compared with gains seen in the ranibizumab patients after 1 year of treatment (2.8 vs. 10.6 and 11.1 letters). Per-injection rates of endophthalmitis remained low over time (~0.06% per injection). The incidence of serious adverse events potentially related to systemic vascular endothelial growth factor inhibition was 19.7% in patients who received 0.5 mg ranibizumab compared with 16.8% in the 0.3 mg group.

Conclusions: The strong VA gains and improvement in retinal anatomy achieved with ranibizumab at month 24 were sustained through month 36. Delayed treatment in patients receiving sham treatment did not seem to result in the same extent of VA improvement observed in patients originally randomized to ranibizumab. Ocular and systemic safety was generally consistent with the results seen at month 24.

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**Group members listed online (available at http://aaojournal.org).*

In 1985, the Early Treatment Diabetic Retinopathy Study (ETDRS) established macular laser photocoagulation as the standard of care for diabetic macular edema (DME).[1] Despite widespread use of macular laser for the past quarter-century, its mechanism of action still remains largely unknown. In contrast, Folkman's pioneering work in angiogenesis led to the discovery of precise molecular mechanisms that could be specifically targeted in cancer, macular degeneration, and diabetic retinopathy (DR).[2] The subsequent cloning of vascular endothelial growth factor (VEGF) A by Ferrara and Henzel[3] and the creation of highly specific VEGF antagonists led to targeted therapy for DME with ranibizumab, a monoclonal antibody fragment (Fab, or antigen-binding fragment) that potently inhibits VEGF.[4] Randomized prospective clinical trials have demonstrated that intravitreal inhibition of VEGF with ranibizumab, given monthly for up to 24 months or less frequently using a variety of as-needed regimens, results in rapid and sustained improvements in vision and retinal anatomy in patients with DME.[5–9]

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RIDE and RISE are phase III, multicenter, randomized clinical trials that enrolled a total of 759 patients with vision loss from DME (best-corrected visual acuity [BCVA], 20/40–20/320 Snellen equivalent, and documented macular edema with central subfield thickness ≥275 μm on time-domain optical coherence tomography [OCT]), with the objective of evaluating the efficacy and safety of intravitreal ranibizumab for DME. The 24-month sham-controlled outcomes, previously published in *Ophthalmology*, demonstrated that the response to intravitreal inhibition of VEGF was rapid and substantial.[7] Compared with the control treatment of sham injections plus macular laser per protocol-specified criteria, statistically significant improvements in BCVA and reductions in retinal thickness were observed on average as early as 7 days after the first ranibizumab injection; these improvements were maintained to 24 months. Furthermore, in the first 2 years of RIDE and RISE, fewer patients treated with ranibizumab experienced significant vision loss (≥15 ETDRS letters), and fewer patients treated with ranibizumab developed proliferative DR.[10] The ocular safety of ranibizumab in patients with DME was consistent with previous phase III studies of ranibizumab in DME, age-related macular degeneration, and retinal vein occlusion.[5,10–14]

Although sham-controlled for only the first 24 months, the RIDE and RISE studies continued after the primary analysis so that additional questions could be addressed. The study design allowed for patients in the sham control group to cross over and receive monthly 0.5 mg ranibizumab injections in the third year. Patients originally randomized to ranibizumab were maintained in a masked fashion on their originally assigned regimens of monthly 0.3 or 0.5 mg. The additional data provide for evaluation of 3 important clinical questions: (1) Are the results seen after 24 months of ranibizumab treatment maintained over a third year of monthly therapy? (2) What is the effect, if any, of delayed initiation of treatment with ranibizumab in the sham crossover group? (3) Which tested dose of ranibizumab should be recommended over the long term for patients with DME, a population that differs from other populations with retinal disease treated with anti-VEGF therapy in having a higher likelihood of bilateral disease[5] and an elevated risk of cardiovascular events and mortality?[15,16] In this report, the ongoing efficacy and safety of monthly injections of 0.3 mg and 0.5 mg ranibizumab for DME through 36 months are presented, and the questions are addressed.

Materials and Methods

RIDE (registered on ClinicalTrials.gov as NCT00473382; accessed September 15, 2012) and RISE (registered on ClinicalTrials.gov as NCT00473330; accessed September 15, 2012) are methodologically identical, phase III, randomized, multicenter, double-masked, 3-year trials that were sham injection–controlled for the first 2 years. Adults with decreased vision due to center-involved DME and the presence of macular edema documented on OCT were eligible to enroll. Both trials were designed and conducted in accordance with the principles of the Declaration of Helsinki and in compliance with the Health Insurance Portability and Accountability Act. The study protocols were approved by institutional review boards, ethics committees, or as applicable. All patients provided written informed consent before enrolling as study participants.

The study methods have been reported in detail elsewhere.[7] Upon completion of the 24-month sham-controlled treatment period (time point for the primary efficacy outcome), sham patients were eligible to cross over to receive treatment with monthly 0.5 mg ranibizumab. Of note, to preserve study masking, all patients were asked if they wanted to cross over, but only patients randomized to sham injection were actually crossed over by the study management computer system. After a protocol amendment in 2010, sham patients who met predefined vision loss and OCT criteria became eligible for early (before month 25) crossover to active treatment with monthly 0.5 mg ranibizumab starting in mid-2010. Patients with study eyes originally randomized to 0.3 or 0.5 mg ranibizumab continued on the monthly schedule to which they originally had been assigned. All patients remained eligible for per-protocol macular laser beginning at month 3 and throughout the duration of the 36-month treatment period on the basis of pre-specified subjective and objective criteria.

Outcomes

The primary efficacy outcome measure was the proportion of patients who gained ≥15 ETDRS letters in BCVA score at month 24 compared with baseline. Secondary outcome measures at month 36 were analogous to the 24-month outcomes and included the proportion of patients who had gained ≥15 letters from baseline at month 36, mean change from baseline in BCVA score over time, proportion of patients who lost <15 letters in BCVA score compared with baseline, proportion of patients with BCVA Snellen equivalent of 20/40 or better, and mean change from baseline in central foveal thickness (CFT) over time, as assessed on OCT by the central reading center. Exploratory outcomes included the proportion of patients with OCT CFT ≤250 μm and the proportion of patients progressing to proliferative DR.

Analysis

The statistical methods used to analyze the data have been described in detail elsewhere.[7] Analyses for efficacy end points were based on the intent-to-treat (ITT) population, with patients grouped according to their assigned treatment. The methods used to analyze the 36-month efficacy were the same as those described for the analysis of the 24-month end points; however, because most patients in the sham group crossed over to receive 0.5 mg ranibizumab monthly in the third year of treatment, analyses of 36-month efficacy data consisted of descriptive statistics by treatment group with limited formal comparisons made post hoc. Comparisons of efficacy at month 36 were between patients actively treated for 3 years (with monthly 0.3 or 0.5 mg ranibizumab) versus patients treated with sham for 2 years followed by treatment for up to 1 year with monthly 0.5 mg ranibizumab. Missing data were imputed by last observation carried forward.

Safety analyses were based on the safety-evaluable population, defined as patients who received at least 1 dose of study drug. Patients were grouped according to the treatment received. Patients randomized to sham who inadvertently received treatment with the active study drug were classified in the active drug treatment group. For the sham group, safety outcomes were summarized during the 24-month sham-controlled period and separately for the sham/0.5 mg group during the 36-month study. The sham/0.5 mg group consists of patients who received sham only and patients who crossed over to receive treatment with monthly 0.5 mg ranibizumab in the third year of treatment.

Table 1. Patient Retention and Drug Exposure through Month 36

Category	RIDE			RISE		
	Sham/0.5 mg (N=130)	*Ranibizumab*		*Sham/0.5 mg (N=127)*	*Ranibizumab*	
		0.3 mg (N=125)	*0.5 mg (N=127)*		*0.3 mg (N=125)*	*0.5 mg (N=125)*
On study at month 24, n (%)	108 (83.1)	105 (84.0)	110 (86.6)	102 (80.3)	105 (84.0)	106 (84.8)
On study at month 36, n (%)	102 (78.5)	98 (78.4)	98 (77.2)	86 (67.7)	98 (78.4)	100 (80.0)
Drug exposure (ranibizumab or sham injections)						
Months	25–36*	1–36	1–36	25–36*	1–36	1–36
No. of patients	101†	125	124	89†	125	125
Total No. of injections	1015	3499	3765	881	3724	3562
Per patient						
Mean (SD)	10.0 (1.8)	28.0 (11.2)	30.4 (9.2)	9.9 (2.3)	29.8 (10.2)	28.5 (10.4)
Median	11	34	34	11	35	34

SD = standard deviation.
*Reflects 1 year of ranibizumab 0.5 mg exposure after crossover.
†Number of patients originally randomized to sham who crossed over to ranibizumab 0.5 mg.

Results

Patient Disposition

A total of 594 patients (78.3%) received ranibizumab treatment after month 24. At month 36, the proportion of patients remaining in the study varied from 67.7% to 80.0% across the treatment groups (Table 1). Among the 210 sham patients from both studies remaining in the study at month 24 (of 257 originally randomized to sham), a total of 190 (91%) crossed over to active treatment with monthly 0.5 mg ranibizumab. In the 2 studies, 5 sham patients (2.6%) crossed over early, at month 23. The median number of ranibizumab injections received by the patients in the sham and crossover to 0.5 mg group after crossover (between months 25 and 36) was 11, whereas patients originally randomized to ranibizumab received a median of 34 to 35 injections over their 3-year treatment period (Table 1).

Visual Acuity Outcomes

Continued treatment with ranibizumab through month 36 resulted in maintenance of the efficacy outcomes seen at earlier time points. At the 3-year time point, in RIDE, 36.8% of patients receiving 0.3 mg ranibizumab and 40.2% of patients receiving 0.5 mg ranibizumab had gained ≥15 ETDRS letters in BCVA from baseline, compared with 19.2% of patients treated with sham/0.5 mg (P=0.0026 for comparison of 0.3 mg with sham/0.5 mg, P=0.0001 for comparison of 0.5 mg with sham/0.5 mg in post hoc stratified calculations; Fig 1 and Table 2). In RISE, corresponding proportions were 51.2%, 41.6%, and 22.0%, respectively (P < 0.0001 for comparison of 0.3 mg with sham/0.5 mg, P=0.0005 for comparison of 0.5 mg with sham/0.5 mg in post hoc stratified calculations; Fig 1 and Table 2).

Consistent with the maintenance of efficacy measured in terms of ≥15-letter improvement, the average change in BCVA from baseline achieved at month 24 was sustained through month 36 in patients originally randomized to ranibizumab (Fig 2). In RIDE, the mean number of ETDRS letters change from baseline at month 24 versus change from baseline at month 36 in patients randomized to sham, 0.3 mg, and 0.5 mg ranibizumab was 2.3 versus 4.7, 10.9 versus 10.6, and 12.0 versus 11.4, respectively. In RISE, the corresponding numbers were 2.6 versus 4.3, 12.5 versus 14.2, and 11.9 versus 11.0 (Fig 2). The efficacy of the 0.3-mg and 0.5-mg doses of ranibizumab was similar over 36 months, as demonstrated in efficacy data pooled from RIDE and RISE (Fig 3).

Other measures of BCVA outcome also were consistent with the results previously observed at month 24. At month 36, fewer patients originally randomized to ranibizumab had lost ≥15 letters from baseline (0.8%–3.9%), compared with patients originally randomized to sham (7.7, 8.7%; Fig 1). Likewise, more patients treated with ranibizumab from the beginning of the study completed with a Snellen BCVA equivalent of 20/40 or better, and fewer patients originally randomized to ranibizumab completed month 36 with a Snellen BCVA of 20/200 or worse (Fig 1 and Table 2).

At baseline, the mean time from first known DME diagnosis to study enrollment was 2.3 to 2.4 years in patients randomized to sham (comparable to the baseline duration of DME in the groups originally randomized to ranibizumab).[7] Patients in the sham/0.5 mg group thus had DME for approximately 4.5 years before initiation of ranibizumab treatment. Because the sham crossover group had received only 1 year of ranibizumab treatment, a comparison was made to assess vision gains achieved after the initial 12 months of treatment (Table 3). In pooled data from the 2 studies, the mean number of letters gained after 12 months of monthly ranibizumab was 2.8 letters in the sham/0.5 mg group compared with 10.6 and 11.1 letters in the ranibizumab 0.3 mg and 0.5 mg groups, respectively. However, the conclusions that can be drawn from this observation are limited because the groups were no longer fully comparable.

In evaluating the response of the sham group to delayed ranibizumab therapy, it is notable that the average BCVA improvements in the sham group showed relatively little gain after crossover to 0.5 mg ranibizumab after month 24 (2.5 letters at month 24 and 4.5 letters at month 36 in the pooled RIDE/RISE population; Fig 3). Because the primary analysis was ITT, the mean BCVA values may have been affected by the last observation carried forward method of imputing missing data where values from patients who had discontinued from the sham group and did not receive treatment were carried forward. To better understand the potential improvements associated with ranibizumab use after 2 years of sham treatment (plus laser, when indicated, in 70%–74% of sham patients through month 24[7]), an analysis was performed in the subgroup of patients receiving ≥1 study drug injection after month 24. Sham patients who received at least 1 study drug injection after month 24 (n=190) gained on average 7.5 (RIDE)

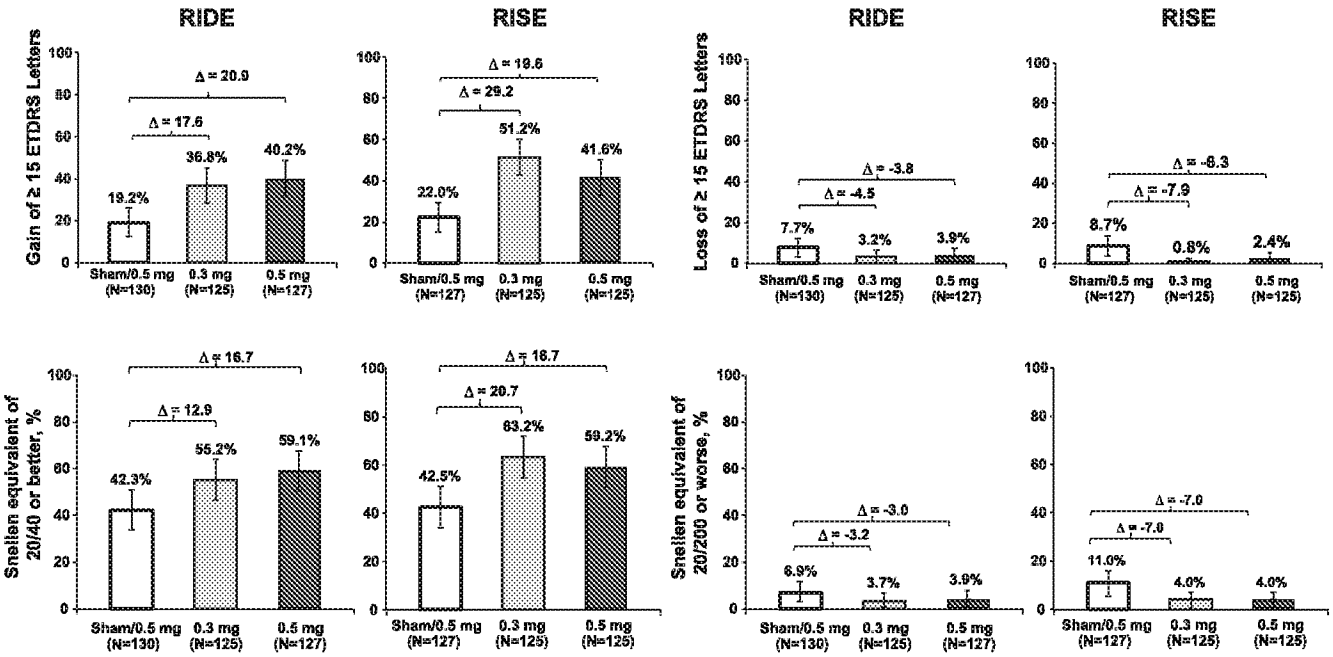

Figure 1. The visual acuity (VA) outcomes at 36 months: percentage of patients who gained ≥15 or more Early Treatment Diabetic Retinopathy Study (ETDRS) letters from baseline VA at 36 months (top left), percentage of patients who lost ≥15 ETDRS letters from baseline VA at 36 months (top right), percentage of patients with vision of the Snellen equivalent of ≥20/40 at 36 months (bottom left), and percentage of patients with vision of the Snellen equivalent of ≤20/200 at 36 months (bottom right). Vertical bars are 95% confidence intervals for the percentage. Differences shown are unadjusted for stratification variables. The last observation carried forward method was used to impute missing data.

and 7.8 (RISE) ETDRS letters from baseline (Fig 4, available at http://aaojournal.org). However, this is compared with a 12.1- to 15.6-letters average gain at month 36 in the similar subset of patients originally randomized to ranibizumab who also received at least 1 dose of study drug after month 24 (Fig 3).

Anatomic Outcomes

The mean OCT thickness in the sham group at baseline was 447.4 μm in RIDE and 467.3 μm in RISE, matching that of the originally randomized ranibizumab groups (all >450 μm). All groups at baseline also were well matched with respect to mean duration of DME (1.6–2.4 years) and prior therapy for DME (68.8%–82% in each of the sham, 0.3 mg, and 0.5 mg groups). After 12 months of monthly ranibizumab therapy, the sham/0.5 mg group experienced a reduction (SD) of −98.4 μm (142.8) compared with reductions of −237.9 μm (186.1) and −249.3 μm (194.8) in the 0.3 and 0.5 mg groups, respectively (Table 3).

The average OCT CFT at month 24, after sham treatment but before any ranibizumab exposure, was 292.5 μm in the sham group compared with 463.8 and 478.6 μm at baseline in groups originally randomized to ranibizumab. This may reflect the effect of laser or thinning associated with ongoing retinal cell loss in the diabetic retina. In patients originally randomized to ranibizumab, the significant reductions in CFT from baseline observed at month 24 also were maintained through month 36 (Fig 2). By using the ITT analysis that carried forward the last observation from sham patients who discontinued the study before month 24, OCT reductions after crossover from sham injection to 0.5 mg ranibizumab did not seem to be as great at month 36 as in patients originally randomized to ranibizumab (Fig 2). When considering only the subgroup of patients who received ≥1 study drug injection after month 24, observed OCT reductions after sham crossover to ranibizumab were greater than those seen using the ITT analysis, as shown by the steeper decline in the mean OCT CFT curve (Fig 3). Of note, the OCT thicknesses at month 36 were more similar among the groups: the sham/0.5 mg group at month 36 had an average OCT thickness of 194.1 μm, compared with 223.4 μm in the 0.3 mg group and 201.9 μm in the 0.5 mg group.

Consistent with the 24-month outcomes, patients randomized to ranibizumab were more likely to experience improvements in DR severity as measured by the ETDRS retinopathy severity scale and less likely to develop proliferative DR (Table 2). The sham crossover group also demonstrated improvements in DR severity after crossover to ranibizumab therapy (Table 2).

Use of Macular and Panretinal Laser Treatment

Compared with patients who had been randomized to receive ranibizumab, a much greater proportion of sham patients had received macular (19.7%–36% vs. 70% and 74%) or panretinal laser (0%–1.6% vs. 11% and 12.3%) at month 24.[7] These differences were maintained through 36 months, largely as a result of the difference in laser use during the 24-month sham-controlled portion of the studies. Through 36 months, the proportion of patients in the sham/0.5 mg group who received macular laser at least once over 36 months was 72.3% in RIDE and 74.0% in RISE, compared with 21.3% to 40.8% of patients originally randomized to ranibizumab (Table 2). The proportion of patients in the sham/0.5 mg group who underwent panretinal laser was 13.8% in RIDE and 12.6% in RISE over 36 months compared with 0.0% to 3.2% in patients originally randomized to ranibizumab. The proportions of patients receiving macular laser between months 24 and 36 was

Table 2. Key Efficacy Outcomes at Month 36 in the Intent-to-Treat Population

	RIDE			RISE		
		Ranibizumab			Ranibizumab	
	Sham/0.5 mg (N=130)	0.3 mg (N=125)	0.5 mg (N=127)	Sham/0.5 mg (N=127)	0.3 mg (N=125)	0.5 mg (N=125)
VA Outcomes						
Gaining ≥15 ETDRS letters, n (%)	25 (19.2%)	46 (36.8%)	51 (40.2%)	28 (22.0%)	64 (51.2%)	52 (41.6%)
95% CI for percentage	12.5–26.0	28.3–45.3	31.6–48.7	14.8–29.3	42.4–60.0	33.0–50.2
ETDRS letters change from baseline, (SD)	4.7 (13.3)	10.6 (12.9)	11.4 (16.3)	4.3 (14.9)	14.2 (12.8)	11.0 (12.9)
95% CI for mean	2.4–7.0	8.3–12.8	8.6–14.3	1.7–7.0	12.0–16.5	8.8–13.3
Gaining ≥10 ETDRS letters, n (%)	43 (33.1%)	71 (56.8%)	80 (63.0%)	49 (38.6%)	87 (69.6%)	72 (57.6%)
95% CI for percentage	25.0–41.2	48.1–65.5	54.6–71.4	30.1–47.0	61.5–77.7	48.9–66.3
Loss of <15 ETDRS letters, n (%)	120 (92.3%)	121 (96.8%)	122 (96.1%)	116 (91.3%)	124 (99.2%)	122 (97.6%)
95% CI for percentage	87.7–96.9	93.7–99.9	92.7–99.4	86.4–96.2	97.6–100	94.9–100
Snellen ≥20/40, n (%)	55 (42.3%)	69 (55.2%)	75 (59.1%)	54 (42.5%)	79 (63.2%)	74 (59.2%)
95% CI for percentage	33.8–50.8	46.5–63.9	50.5–67.6	33.9–51.1	54.7–71.7	50.6–67.8
Snellen ≤20/200, n (%)	9 (6.9%)	4 (3.2%)	5 (3.9%)	14 (11%)	5 (4.0%)	5 (4.0%)
95% CI for percentage	2.6–11.3	0.1–6.3	0.6–7.3	5.6–16.5	0.6–7.4	0.6–7.4
Anatomic Outcomes						
Mean change in CFT from baseline (SD), μm	−213.2 (193.5)	−261.8 (180.8)	−266.7 (207.8)	−200.1 (215.6)	−261.2 (196.5)	−269.1 (178.9)
95% CI for percentage	−246.8 to −179.6	−293.8 to −229.8	−303.2 to −230.2	−238.0 to −162.3	−296.0 to −226.4	−300.8 to −237.4
≥3-step progression on ETDRS scale, n (%)*	4 (3.2%)	1 (0.9%)	1 (0.8%)	5 (4.3%)	2 (1.7%)	2 (1.7%)
95% CI for percentage	0.1–6.3	0.0–2.5	0.0–2.5	0.6–8.1	0.0–4.1	0.0–4.1
≥2 step progression on ETDRS scale, n (%)*	11 (8.9%)	1 (0.9%)	2 (1.7%)	11 (9.6%)	5 (4.3%)	5 (4.3%)
95% CI for percentage	3.9–13.9	0.0–2.5	0.0–4.0	4.2–14.9	0.6–7.9	0.6–8.1
≥3-step improvement on ETDRS scale, n (%)*	5 (4.0%)	17 (14.5%)	18 (15.1%)	3 (2.6%)	18 (15.4%)	13 (11.3%)
95% CI for percentage	0.6–7.5	8.1–20.9	8.7–21.6	0.0–5.5	8.8–21.9	5.5–17.1
≥2-step improvement on ETDRS scale, n (%)*	29 (23.4%)	46 (39.3%)	45 (37.8%)	28 (24.3%)	45 (38.5%)	47 (40.9%)
95% CI for percentage	15.9–30.8	30.5–48.2	29.1–46.5	16.5–32.2	29.6–47.3	31.9–49.9
Progression to PDR by ophthalmoscopy, n (%)	18 (13.8%)	6 (4.8%)	7 (5.5%)	22 (17.3%)	3 (2.4%)	9 (7.2%)
95% CI for percentage	7.9–19.8	1.1–8.5	1.5–9.5	10.7–23.9	0.0–5.1	2.7–11.7
Laser Treatment						
Patients who received macular laser, n (%)	94 (72.3%)	46 (36.8%)	27 (21.3%)	94 (74.0%)	51 (40.8%)	47 (37.6%)
95% CI for percentage	64.6–80.0	28.3–45.3	14.1–28.4	66.4–81.6	32.2–49.4	29.1–46.1%
Patients who received PRP laser, n (%)	18 (13.8%)	4 (3.2%)	3 (2.4%)	16 (12.6%)	0	3 (2.4%)
95% CI for percentage	7.9–19.8	0.1–6.3	0.0–5.0	6.8–18.4	0.0–0.0	0.0–5.1

CFT = central foveal thickness; CI = confidence interval; ETDRS = Early Treatment Diabetic Retinopathy Study; PDR = proliferative diabetic retinopathy; PRP = panretinal photocoagulation; VA = visual acuity.
The last observation carried forward method was used to impute missing data. Stratification variables in stratified analyses: baseline VA (≤55 or >55 letters), baseline hemoglobin A1c (≤8%, >8%), and prior treatment for DME (yes, no).
*N = 124, 117, and 119 (RIDE) and 115, 117, and 115 (RISE) for sham/0.5 mg, 0.3 mg, and 0.5 mg groups, respectively.

5.5% to 9.4% across all treatment groups among patients who received at least 1 dose of study drug after month 24. Likewise, the proportions of patients who had received at least 1 dose of study drug after month 24 and underwent panretinal laser between months 25 and 36 was 0% to 2.2% among all groups.

Safety Outcomes

Safety data collected through month 36 were evaluated to assess whether the longer-term safety profile of ranibizumab was consistent with that initially observed and to further assess the relative long-term safety of ongoing monthly 0.3 mg and 0.5 mg ranibizumab doses. Because the majority of patients in the sham group crossed over to monthly 0.5 mg ranibizumab dosing after month 24 and received 12 months of exposure compared with 36 months of exposure in the originally randomized groups, comparisons between the groups need to be interpreted with caution because the populations are not directly comparable with respect to the duration of ranibizumab exposure.

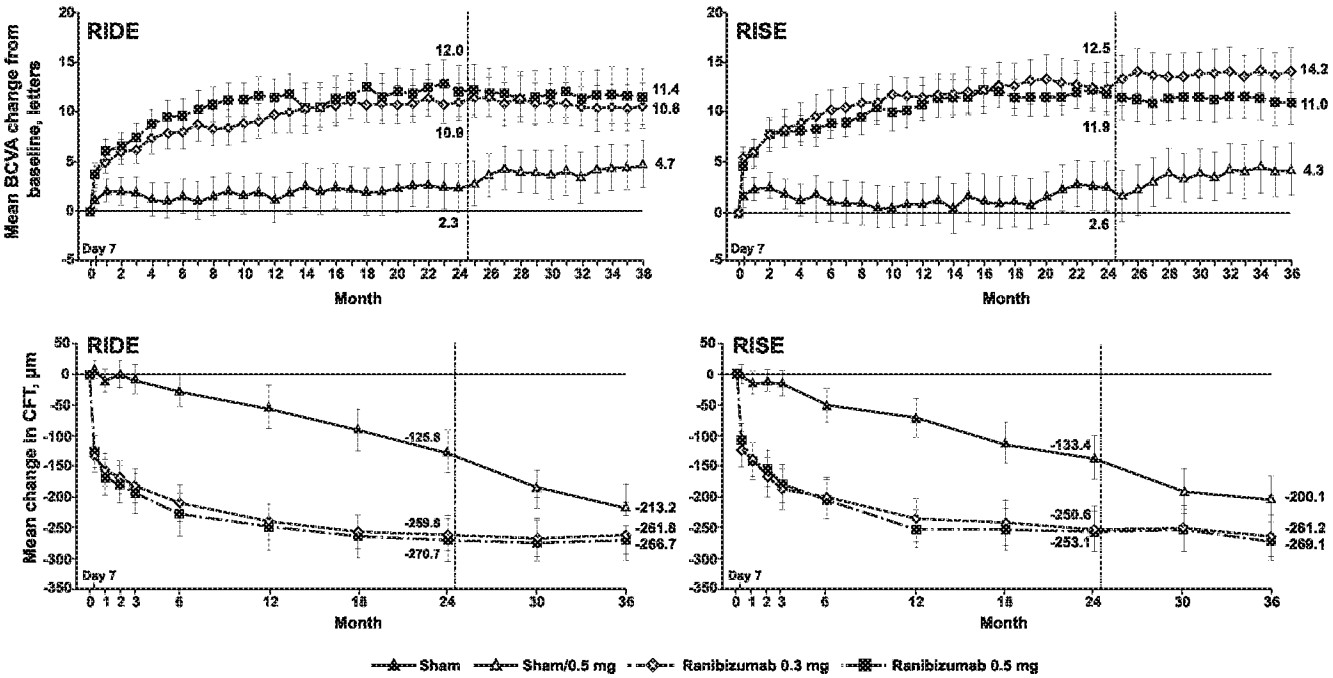

Figure 2. Mean change in best-corrected visual acuity (BCVA) and central foveal thickness (CFT) from baseline over time. Vertical bars are 95% confidence intervals. Missing data were imputed by last observation carried forward.

Ocular Safety

Key study eye ocular safety data are summarized in Table 4 (available at http://aaojournal.org). The ocular safety profile was consistent with the sham-controlled safety observations from the 24-month analysis. In particular, rates of procedure-related serious adverse events (SAEs), such as endophthalmitis and traumatic cataract, remained low. The total number of patients in the ranibizumab treatment groups experiencing endophthalmitis or traumatic cataract in the study eye over the 36-month treatment period across both studies was 6 (1.2%) and 4 (0.8%), respectively. The per-injection rate of endophthalmitis was approximately 0.06%, whereas the per-injection rate of traumatic cataract was 0.03% (Table 5, available at http://aaojournal.org). Similar proportions of the patients randomized to ranibizumab reported an adverse event (AE) of increased intraocular pressure at months 24 and 36 (Table 4, available at http://aaojournal.org). The mean pre-dose intraocular pressure in the study eye at month 36 in the sham and crossover to 0.5 mg, 0.3 mg, and 0.5 mg groups was 15.4 mmHg, 15.5 mmHg, and 14.9 mmHg, respectively.

Systemic Safety

The long-term systemic safety of ranibizumab in DME was evaluated using 2 methods. As in previous studies of intravitreal ranibizumab across several retinal vascular diseases, we first assessed rates of arterial thromboembolic events (ATEs) using the Antiplatelet Trialists' Collaboration (APTC) classification,[17] which is based on a specific and well-defined spectrum of ATE AEs: vascular deaths (including deaths of unknown cause), nonfatal myocardial infarction, and nonfatal stroke (Table 6). Overall APTC-classified AEs occurred in 7.2%, 10.8%, and 10.4% of patients in the sham/0.5 mg, 0.3 mg, and 0.5 mg groups, respectively. Among APTC-classified events occurring over 36 months, deaths of vascular and unknown causes occurred in 2%, 3.6%, and 3.6% of patients in the sham/0.5 mg, 0.3 mg, and 0.5 mg ranibizumab groups, respectively. The overall incidence of deaths through 36 months, including deaths from nonvascular causes, was 4.4% (11 patients) in the monthly 0.3 mg group, 6.4% (16 patients) in the 0.5 mg group, and 2.8% (7 patients) in the sham/0.5 mg group (Table 7, available at http://aaojournal.org). Causes of death, listed in Table 7, were mostly consistent with those typical of patients with advanced complications of diabetes.[18] Rates of stroke over 3 years were higher in the 0.5 mg group (12 [4.8%]) compared with the 0.3 mg group (5 [2.0%]) or sham/0.5 mg group (6 [2.4%]) (Table 6). The incidence of myocardial infarction through month 36 was 18 (7.2%) in the 0.3 mg group and 9 (3.6%) in the 0.5 mg group (Table 6).

Although the APTC classification system provides useful insight into the systemic safety of intraocular anti-VEGF therapy, a more thorough understanding of systemic anti-VEGF safety has developed over the last several years, primarily because of the use of intravenous agents in oncology. As clinical experience with systemic anti-VEGF agents has grown, additional types and categories of systemic AEs potentially associated with the use of systemic anti-VEGF treatment have been identified. These are considered "class" effects related to systemic VEGF inhibition.[19] Categories of these anti-VEGF class-related AEs include hypertension, proteinuria, arterial and venous thromboembolic events, bleeding/hemorrhage (central nervous system and cerebrovascular, non–central nervous system), congestive heart failure, fistulae, gastrointestinal perforation, and wound-healing complications. Categorizing SAEs using this second broader approach demonstrated that the overall incidence of SAEs potentially related to systemic VEGF inhibition was higher in patients who received 0.5 mg ranibizumab compared with 0.3 mg ranibizumab or sham/0.5 mg: 49 of 249 (19.7%) versus 42 of 250 (16.8%) and 33 of 251 (13.1%) (Table 8, available at http://aaojournal.org). The incidence of several categories (central nervous system and cerebrovascular hemorrhage, congestive heart failure, hypertension, gastrointestinal perforation, proteinuria, and wound-healing complications) appeared to increase in a dose-dependent fashion in patients with

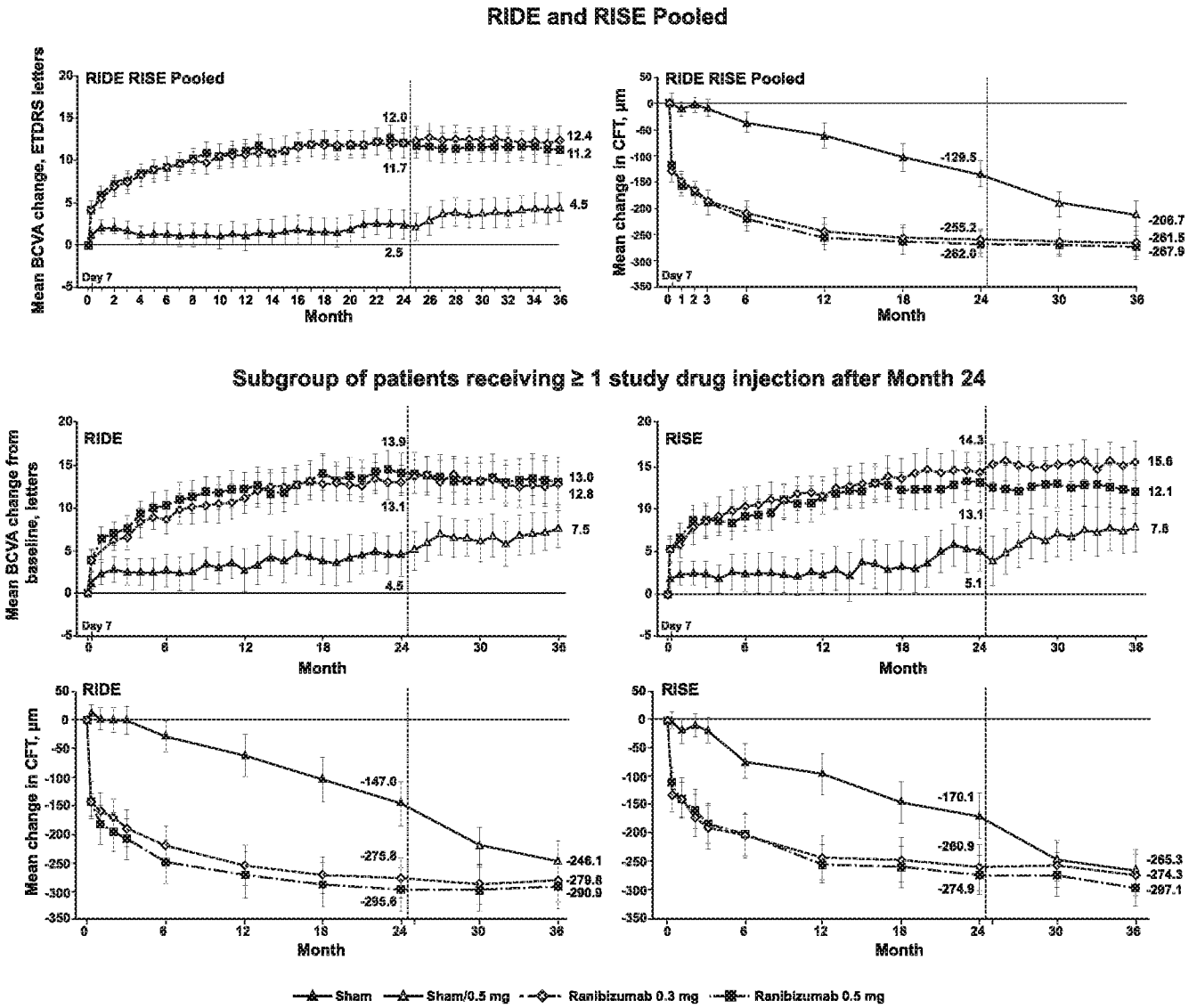

Figure 3. Mean change in best-corrected visual acuity (BCVA) and central foveal thickness (CFT) from baseline over time in the RIDE and RISE pooled population and the subgroup of patients receiving ≥1 study drug injection after month 24. Vertical bars are 95% confidence intervals. Missing data were imputed by last observation carried forward. ETDRS = Early Treatment Diabetic Retinopathy Study.

DME treated with intravitreal ranibizumab, although in each of the latter 3 categories, only 1 SAE in the 0.5 mg group was observed.

Discussion

The 36-month results from the RIDE and RISE studies demonstrate that the rapid and sustained efficacy of ranibizumab in patients with DME initially observed at 2 years is maintained over an additional third year of continued monthly treatment. A gain of ≥15 letters from baseline was experienced by 36.8% to 51.2% of ranibizumab-treated patients, and the incidence of further vision loss was significantly reduced in ranibizumab-treated eyes. Poor BCVA outcomes (such as BCVA worse than by Snellen <20/200) occurred in fewer patients initially treated with ranibizumab, confirming the long-term abilities of ranibizumab to improve vision and prevent significant vision loss in patients with DME. Reductions in retinal edema on OCT and improvements in DR severity also were maintained through 36 months.

The 36-month results provide important clinical insights into treatment outcomes after a 24-month delay in initiation of ranibizumab therapy in the sham crossover group. The relatively limited improvements in vision in this group, compared with the groups initially treated with ranibizumab, suggest that chronic retinal edema (for an average of 4.5 years before ranibizumab therapy) may result in a certain amount of potential vision gain being irreversibly lost. Retinal atrophy due to chronic edema may provide an explanation for this finding. Although OCT measurements in the sham crossover group after ranibizumab treatment showed a reduction in absolute CFT to a mean value of 190 µm (approximately 20 µm less than that observed after treatment in the

Table 3. Changes in the Study Eye at 12 Months After the First Dose of Ranibizumab for Key Efficacy Outcomes (RIDE and RISE Pooled)

	Sham and Crossover to 0.5 mg (N=191)	Ranibizumab	
		0.3 mg (N=250)	*0.5 mg (N=252)*
Total No. of ranibizumab injections by first 12-mo ranibizumab treatment, mean (SD)*	10.0 (2.0)	10.6 (2.6)	10.9 (2.2)
BCVA (ETDRS letters)			
Before ranibizumab treatment,† mean (SD)	62.0 (15.3)	56.1 (12.2)	56.9 (11.6)
12-mo after ranibizumab treatment, mean (SD)	64.8 (14.8)	67.8 (15.0)	68.9 (14.1)
Change from before treatment, mean (SD)	2.8 (9.8)	10.6 (10.6)	11.1 (10.1)
Gain of ≥15 letters from before treatment, n (%)	14 (7.3)	81 (32.4)	80 (31.7)
CFT (μm), mean (SD)			
Before ranibizumab treatment†	292.5 (167.2)	478.6 (162.3)	463.8 (160.4)
12-mo after ranibizumab treatment	194.1 (118.2)	223.4 (136.2)	201.9 (107.3)
Change from before treatment	−98.4 (142.8)	−237.9 (186.1)	−249.3 (194.8)

BCVA = best-corrected visual acuity; CFT = central foveal thickness; ETDRS = Early Treatment Diabetic Retinopathy Study; SD = standard deviation.
*Actual treatment duration for sham crossover groups is 11 months.
†Month 24 for the sham/0.5 mg group, baseline for the 0.3 mg, and 0.5 mg groups.

groups originally randomized to ranibizumab), the average improvement in BCVA was considerably smaller than that achieved in the originally treated cohorts. This outcome may represent the effect of several potential factors: neural cell loss over time in the diabetic retina, compounded by the effects of chronic edema (including neuronal retinal damage, retinal pigment epithelium pigmentation, and/or subretinal fibrosis), additional structural changes induced by repeated macular laser, and/or the natural history of DR.

In the phase III trials of ranibizumab in the treatment of age-related macular degeneration and retinal vein occlusion, there appeared to be a dose response curve favoring 0.5 mg over 0.3 mg ranibizumab for optimum efficacy. However, in the pooled data from the RIDE and RISE trials, efficacy was equivalent between the 0.3-mg and 0.5-mg doses. The comparative profile of the 2 doses of ranibizumab in DME was assessed using a structured, systematic approach based on the Benefit Risk Action Team framework (Fig 4A–C, available at http://aaojournal.org).[20,21] These figures help demonstrate that, although concentrations of ranibizumab in the systemic circulation are lower than vitreous concentrations, the use of 0.3 mg may reduce risks potentially related to systemic VEGF suppression while still maintaining optimal efficacy. This may be particularly appropriate in the management of DME because not only do 40% to 50% of patients with DME have bilateral disease requiring contemporaneous treatment,[22] but also diabetic patients have an underlying increased risk of mortality and cardiovascular disease, including stroke and silent myocardial ischemia.[23] In light of these considerations, Genentech recommended the 0.3-mg dose; the US Food and Drug Administration ultimately approved use of 0.3 mg ranibizumab for DME on August 10, 2012.

Table 6. Antiplatelet Trialists' Collaboration (APTC) Events through Month 36 (Safety-Evaluable Population)

Category/Event	Sham Months 0–24 (N=250)	Sham/0.5 mg* Months 0–36 (N=251†)	Ranibizumab	
			0.3 mg Months 0–36 (N=250)	*0.5 mg Months 0–36 (N=249)*
Total APTC-classified events	13 (5.2%)	18 (7.2%)	27 (10.8%)	26 (10.4%)
Deaths	3 (1.2%)	7 (2.8%)	11 (4.4%)	16 (6.4%)
Vascular	3 (1.2%)	5 (2.0%)	8 (3.2%)	8 (3.2%)
Nonvascular	0	2 (0.8%)	2 (0.8%)	7 (2.8%)
Unknown cause	0	0	1 (0.4%)	1 (0.4%)
Myocardial infarction	9 (3.6%)	13 (5.2%)	18 (7.2%)	9 (3.6%)
Fatal	2 (0.8%)	4 (1.6%)	3 (1.2%)	1 (0.4%)
Nonfatal	7 (2.8%)	9 (3.6%)	15 (6.0%)	8 (3.2%)
Stroke (CVA)	4 (1.6%)	6 (2.4%)	5 (2.0%)	12 (4.8%)
Fatal	1 (0.4%)	2 (0.8%)	1 (0.4%)	3 (1.2%)
Nonfatal	3 (1.2%)	4 (1.6%)	4 (1.6%)	9 (3.6%)

CVA = cerebrovascular accident.
APTC events include vascular deaths, deaths of unknown cause, nonfatal myocardial infarctions, and nonfatal strokes.
*Patients initially randomized to sham including those who crossed over to ranibizumab 0.5 mg during year 3. There is no pure sham control group at month 36, so it is not valid to compare the sham groups with the ranibizumab treatment arms.
†One sham patient received 0.5 mg ranibizumab starting at month 23. This patient was classified in the ranibizumab 0.5 mg group for the 24-month analyses per the prespecified definition of treatment groups for safety analyses. For the 36-month analyses, it was determined that this patient crossed over early and thus was classified in the sham/0.5 mg crossover group.

Study Limitations

As with all clinical trials, certain limitations exist in extrapolating the RIDE and RISE study observations to routine clinical practice. One potential limitation is that some patients discontinued their participation, with 67.7% to 80.0% of patients completing month 36 across the various treatment groups. However, interpretation of the study results did not change when performing sensitivity analyses using a variety of methods for missing data imputation (data not shown). A more important potential limitation is that ranibizumab was administered on a continuous monthly dosing schedule, which may optimize efficacy but not be practical for many patients with DME. However, additional large phase III and phase III-scope studies using 0.5 mg with less than monthly dosing have provided important additional data on the efficacy and safety of ranibizumab in DME.[5,6,8,24] For example, in the DRCRnet study of ranibizumab, macular laser, or triamcinolone for DME, significant visual acuity benefits were observed with a median of 8 to 9 ranibizumab injections in the first year, 2 to 3 injections in the second year, and 1 to 2 injections in the third year.[5,9,14] In the RESTORE phase III study of DME, which compared 0.5 mg ranibizumab (with individualized pro re nata dosing) with or without macular laser with laser alone, significant improvements in BCVA and OCT outcomes were observed with ranibizumab with an average of 6.8 to 7.0 injections over 12 months.[6] Mean BCVA gain was maintained or improved through 36 months.[25] These studies also provide insights into the systemic safety of ranibizumab in separate DME populations enrolled and studied contemporaneously with RIDE and RISE. No systemic safety imbalances emerged with 0.5 mg ranibizumab dosed on a less than monthly basis compared with control in the DRCRnet Protocol I or RESTORE studies. In DRCRnet Protocol I, patients in the sham group experienced higher rates of APTC-classified systemic events than patients receiving ranibizumab.[14] In RESTORE, no meaningful differences in the number of ATEs or other systemic events potentially related to VEGF inhibition were observed between the ranibizumab and laser control groups, although patients with a history of stroke or transient ischemic attack were excluded from this study (Abstract PO532. Annual Meeting of the American Academy of Ophthalmology, November 10–13, 2012, Chicago, IL).[6] Forthcoming data from the open-label extension phase of RIDE and RISE, in which ranibizumab is administered less frequently, will also contribute information on this question.

Another limitation is that it is unknown whether the results with ranibizumab for the management of DME as demonstrated in RIDE and RISE are applicable to other anti-VEGF agents. The various commonly used intravitreal anti-VEGFs have different molecular characteristics, leading to differences in potency, systemic clearance, and systemic VEGF inhibition.[25] To help address these questions, a comparative study of 3 anti-VEGF agents for DME is now being recruited by the Diabetic Retinopathy Clinical Research Network (NCT01627249).[26]

In conclusion, the 36-month results of RIDE and RISE confirm the long-term efficacy and safety of ranibizumab in DME. These data further highlight the importance of expanding DR screening programs and greater awareness of and adherence to already-recommended national screening guidelines. Recent reports suggest that 93% of patients with DR and 63% of patients with vision-threatening DR were unaware they had DR; 83% with vision-threatening DR had no scheduled follow-up eye examination (Abstract 1287/A37. Association for Research in Vision and Ophthalmology Annual Meeting, May 1–5, 2011, Fort Lauderdale, FL). Prompt treatment with anti-VEGF therapy at the time of initial diagnosis may avoid the considerable visual morbidity associated with chronic DME. Ophthalmologists now have considerable evidence from multiple clinical studies demonstrating that intraocular anti-VEGF therapy with ranibizumab offers a new and substantially better approach to the treatment of DME, one of the leading causes of vision loss in working-aged adults, and thus has set a new standard of care for DME.

References

1. Early Treatment Diabetic Retinopathy Study Research Group. Photocoagulation for diabetic macular edema. Early Treatment Diabetic Retinopathy Study report number 1. Arch Ophthalmol 1985;103:1796–806.
2. Cooke R. Dr. Folkman's War: Angiogenesis and the Struggle to Defeat Cancer. New York: Random House; 2001.
3. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989;161:851–8.
4. Ferrara N, Damico L, Shams N, et al. Development of ranibizumab, an anti-vascular endothelial growth factor antigen binding fragment, as therapy for neovascular age-related macular degeneration. Retina 2006;26:859–70.
5. Diabetic Retinopathy Clinical Research NetworkElman MJ, Aiello LP, Beck RW, et al. Randomized trial evaluating ranibizumab plus prompt or deferred laser or triamcinolone plus prompt laser for diabetic macular edema. Ophthalmology 2010;117:1064–77.
6. Mitchell P, Bandello F, Schmidt-Erfurth U, et al, RESTORE Study Group. The RESTORE Study: ranibizumab monotherapy or combined with laser versus laser monotherapy for diabetic macular edema. Ophthalmology 2011;118:615–25.
7. Nguyen QD, Brown DM, Marcus DM, et al, RISE and RIDE Research Group. Ranibizumab for diabetic macular edema: results from 2 phase III randomized trials: RISE and RIDE. Ophthalmology 2012;119:789–801.
8. Nguyen QD, Shah SM, Heier JS, et al, READ-2 Study Group. Primary end point (six months) results of the Ranibizumab for Edema of the mAcula in Diabetes (READ-2) study. Ophthalmology 2009;116:2175–81.
9. Diabetic Retinopathy Clinical Research Network, Elman MJ, Qin H, Aiello LP, et al. Intravitreal ranibizumab for diabetic macular edema with prompt versus deferred laser treatment: three-year randomized trial results. Ophthalmology 2012;119:2312–8.
10. Ip MS, Domalpally A, Hopkins JJ, et al. Long-term effects of ranibizumab on diabetic retinopathy severity and progression. Arch Ophthalmol 2012;130:1145–52.
11. Brown DM, Campochiaro PA, Singh RP, et al, CRUISE Investigators. Ranibizumab for macular edema following central retinal vein occlusion: six-month primary end point results of a phase III study. Ophthalmology 2010;117:1124–33.

12. Brown DM, Kaiser PK, Michels M, et al, ANCHOR Study Group. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N Engl J Med 2006;355: 1432–44.
13. Rosenfeld PJ, Brown DM, Heier JS, et al, MARINA Study Group. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–31.
14. Diabetic Retinopathy Clinical Research Network, Elman MJ, Bressler NM, Qin H, et al. Expanded 2-year follow-up of ranibizumab plus prompt or deferred laser or triamcinolone plus prompt laser for diabetic macular edema. Ophthalmology 2011;118:609–14.
15. Gregg EW, Cheng YJ, Saydah S, et al. Trends in death rates among U.S. adults with and without diabetes between 1997 and 2006: findings from the National Health Interview Survey. Diabetes Care 2012;35:1252–7.
16. Nguyen-Khoa BA, Goehring EL Jr, Werther W, et al. Hospitalized cardiovascular events in patients with diabetic macular edema. BMC Ophthalmol[serial online] 2012;12:11. Available at: http://www.biomedcentral.com/1471-2415/12/11. Accessed January 5, 2013.
17. Antiplatelet Trialists' Collaboration. Collaborative overview of randomised trials of antiplatelet therapy—I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. BMJ 1994;308:81–106.
18. American Diabetes Association. Standards of medical care in diabetes—2012. Diabetes Care 2012;35(Suppl):S11–63.
19. Chen HX, Cleck JN. Adverse effects of anticancer agents that target the VEGF pathway. Nat Rev Clin Oncol 2009;6: 465–77.
20. Levitan BS, Andrews EB, Gilsenan A, et al. Application of the BRAT framework to case studies: observations and insights. Clin Pharmacol Ther 2011;89:217–24.
21. Coplan PM, Noel RA, Levitan BS, et al. Development of a framework for enhancing the transparency, reproducibility and communication of the benefit-risk balance of medicines. Clin Pharmacol Ther 2011;89:312–5.
22. Zhang X, Saaddine JB, Chou CF, et al. Prevalence of diabetic retinopathy in the United States, 2005—2008. JAMA 2010;304:649–56.
23. Hernandez C, Candell-Riera J, Ciudin A, et al. Prevalence and risk factors accounting for true silent myocardial ischemia: a pilot case-control study comparing type 2 diabetic with non-diabetic control subjects. Cardiovasc Diabetol [serial online] 2011;10:9. Available at: http://www.cardiab.com/content/10/1/9. Accessed January 5, 2013.
24. Nguyen QD, Shah SM, Khwaja AA, et al, READ-2 Study Group. Two-year outcomes of the Ranibizumab for Edema of the mAcula in Diabetes (READ-2). Study. Ophthalmology 2010;117:2146–51.
25. IVAN Study Investigators, Chakravarthy U, Harding SP, Rogers CA, et al. Ranibizumab versus bevacizumab to treat neovascular age-related macular degeneration: one-year findings from the IVAN randomized trial. Ophthalmology 2012;119:1399–411.
26. Diabetic Retinopathy Clinical Research Network. Comparative effectiveness study of intravitreal aflibercept, bevacizumab, and ranibizumab for DME (Protocol T). ClinicalTrials.gov Identifier: NCT01627249. Available at: http://clinicaltrials.gov/ct2/show/NCT01627249. Accessed September 15, 2012.

Footnotes and Financial Disclosures

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[1] Retina Consultants of Houston, The Methodist Hospital, Houston, Texas.

[2] Stanley M. Truhlsen Eye Institute, University of Nebraska Medical Center, Omaha, Nebraska.

[3] Southeast Retina Center, Augusta, Georgia.

[4] Retina Vitreous Associates Medical Group, Los Angeles, California.

[5] West Texas Retina, Abilene, Texas.

[6] Retina Associates of New Jersey, Teaneck, New Jersey.

[7] Organizacion Medica de Investigacion, Buenos Aires, Argentina.

[8] Genentech, Inc., South San Francisco, California.

*A full listing of the RIDE and RISE Research Group is available as an online supplement to Nguyen et al. Ophthalmology 2012;119:789—801.

[§]Dr. David M. Brown and Dr. Quan Dong Nguyen contributed equally to this work.

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Correspondence:
Quan Dong Nguyen, MD, MSc, Stanley M. Truhlsen Eye Institute, University of Nebraska Medical Center, 985540 Nebraska Medical Center Omaha, NE 68198-5540 E-mail: quan.nguyen@unmc.edu.

Ranibizumab for Macular Edema following Branch Retinal Vein Occlusion

Six-Month Primary End Point Results of a Phase III Study

*Peter A. Campochiaro, MD,[1] Jeffrey S. Heier, MD,[2] Leonard Feiner, MD,[3] Sarah Gray, PhD,[4] Namrata Saroj, OD,[4] Amy Chen Rundle, MS,[4] Wendy Yee Murahashi, MD,[4] Roman G. Rubio, MD,[4] for the BRAVO Investigators**

Purpose: To assess efficacy and safety of intraocular injections of 0.3 mg or 0.5 mg ranibizumab in patients with macular edema following branch retinal vein occlusion (BRVO).

Design: Prospective, randomized, sham injection-controlled, double-masked, multicenter clinical trial.

Participants: A total of 397 patients with macular edema following BRVO.

Methods: Eligible patients were randomized 1:1:1 to receive monthly intraocular injections of 0.3 mg or 0.5 mg of ranibizumab or sham injections.

Main Outcome Measures: The primary efficacy outcome measure was mean change from baseline best-corrected visual acuity (BCVA) letter score at month 6. Secondary outcomes included other parameters of visual function and central foveal thickness (CFT).

Results: Mean (95% confidence interval [CI]) change from baseline BCVA letter score at month 6 was 16.6 (14.7–18.5) and 18.3 (16.0–20.6) in the 0.3 mg and 0.5 mg ranibizumab groups and 7.3 (5.1–9.5) in the sham group ($P<0.0001$ for each ranibizumab group vs sham). The percentage of patients who gained ≥15 letters in BCVA at month 6 was 55.2% (0.3 mg) and 61.1% (0.5 mg) in the ranibizumab groups and 28.8% in the sham group ($P<0.0001$ for each ranibizumab group vs sham). At month 6, significantly more ranibizumab-treated patients (0.3 mg, 67.9%; 0.5 mg, 64.9%) had BCVA of ≥20/40 compared with sham patients (41.7%; $P<0.0001$ for each ranibizumab group vs sham); and CFT had decreased by a mean of 337 μm (0.3 mg) and 345 μm (0.5 mg) in the ranibizumab groups and 158 μm in the sham group ($P<0.0001$ for each ranibizumab group vs sham). The median percent reduction in excess foveal thickness at month 6 was 97.0% and 97.6% in 0.3 mg and 0.5 mg groups and 27.9% in the sham group. More patients in the sham group (54.5%) received rescue grid laser compared with the 0.3 mg (18.7%) and 0.5 mg (19.8%) ranibizumab groups. The safety profile was consistent with previous phase III ranibizumab trials, and no new safety events were identified in patients with BRVO.

Conclusions: Intraocular injections of 0.3 mg or 0.5 mg ranibizumab provided rapid, effective treatment for macular edema following BRVO with low rates of ocular and nonocular safety events.

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**Group members listed online in Appendix 1 (available at http://aaojournal.org).*

The blood supply to the retina is unique in several respects. It involves 2 vascular beds: the retinal vessels supply the inner two thirds of the retina, whereas both the retinal and choroidal vessels supply the outer one third of the retina, via diffusion. The retinal vessels emanate from the central retinal artery, which enters the eye at the optic disc and sends branches along the surface of the retina to the far periphery. Blood flow extends from larger to smaller branches along the retinal surface and through penetrating branches to the inner plexiform layer to form the superficial, intermediate, and deep capillary beds. The capillaries drain into a network of veins that reverse the process, sending blood into progressively larger branches to the central retinal vein, which exits through the optic nerve. Major branch arteries and veins on the surface of the retina often run in close approximation to each other and share an adventitial sheath.

The retinal vascular bed is highly organized with little or no overlap in vessel distribution. When retinal vessels are obstructed, there are few functional collaterals to compensate, and the retina becomes ischemic. Diseases that damage retinal vessels and lead to vessel closure are referred to as ischemic retinopathies and include diabetic retinopathy, retinal vein occlusions (RVOs), hypertensive retinopathy, sickle cell retinopathy, and several others. Diabetic retinopathy is the most prevalent retinal vascular disease and the most common cause of moderate and severe vision loss in working-aged Americans.[1] The RVOs are the second most common type of retinal vascular disease and include branch RVOs (BRVOs), hemiretinal vein occlusions, and central

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RVOs.[2] The incidence of RVOs is estimated at 180 000 eyes per year in the United States, and BRVOs account for nearly 80% of those.[3,4] Hypertension and atherosclerosis are risk factors for BRVO, and both cause thickening of arteriole walls. Most BRVOs occur at sites where arterioles cross over veins, and pathologic findings support the hypothesis that, because of a common adventitial sheath, thickening of the arteriole wall compresses the lumen of the retinal vein, altering flow and promoting thrombosis.[5] Increased vascular permeability leads to hemorrhage and edema throughout the area of retina that is drained by the vein. Because most BRVOs occur at proximal arteriole–venous crossings on the temporal side of the optic nerve, the macula is included in the distribution of the occluded vein, resulting in hemorrhage and fluid in the macula (macular edema) and reduced vision. The severity of BRVO varies depending upon the location of the occlusion; in general, the more proximal the occlusion, the more severe the edema.

The amount of hemorrhage that occurs acutely in BRVO varies, but it is usually sufficient to impede visualization of retinal vessels by fluorescein angiography (FA). Once hemorrhages clear, which may take several months, FA generally shows areas of capillary nonperfusion in the region of the retina drained by the obstructed vein. Severe nonperfusion of perifoveal capillaries is an additional source of reduced vision, but in most patients macular edema is the predominant cause of vision loss.

The Branch Vein Occlusion Study (BVOS) Group investigated the effects of grid laser treatment in 139 eyes of patients with macular edema following BRVO occurring within 3–18 months of study entry, with best-corrected visual acuity (BCVA) ≤20/40 and sufficient clearing of retinal hemorrhage to allow safe laser photocoagulation.[6] At the 3-year primary end point, patients treated with laser photocoagulation showed a significant mean improvement of 1.33 lines of vision compared with 0.23 lines in the control group. In the control group, 34% of patients had a visual acuity of ≥20/40 at the third-year visit. Since publication of the BVOS results, grid laser therapy has become the standard of care for BRVO. However, because many patients with BRVO present with BCVA of ≤20/80, an average improvement of 1.33 lines may leave affected patients with substantial visual disability in the affected eye. Because visual improvement occurs very slowly after laser treatment, there is a need for more effective treatments that provide rapid and complete restoration of vision.

Elevated intraocular levels of vascular endothelial growth factor (VEGF) have been demonstrated in patients with RVOs.[7–9] Sustained release of VEGF in primate eyes causes vascular leakage and macular edema.[10] Thus, there is strong rationale for testing VEGF antagonists in patients with macular edema following RVO. Ranibizumab (Lucentis, Genentech, Inc., South San Francisco, CA) a humanized, affinity-matured VEGF antibody fragment that neutralizes all isoforms of VEGF-A and their biologically active degradation products, provides benefit to patients with neovascular age-related macular degeneration and has been approved by the Food and Drug Administration for that indication.[11,12]

In a pilot trial,[8] 20 patients with BRVO and 20 patients with central RVO were randomized to receive 3 monthly intraocular injections of 0.3 mg or 0.5 mg of ranibizumab. At the month 3 primary end point, approximately 90% of excess foveal thickness (EFT) was eliminated across all treatment groups, and mean improvement in BCVA ranged from 10 to 18 Early Treatment Diabetic Retinopathy Study[13] (ETDRS) letters. Here we report the month 6 primary and key secondary end points of the Ranibizuma*B* for the treatment of macular edema following B*RA*nch Retinal *V*ein *O*cclusion: Evaluation of Efficacy and Safety (BRAVO) study, a phase III multicenter trial in which patients with macular edema following BRVO were randomized to receive monthly intraocular injections of 0.3 mg or 0.5 mg of ranibizumab or sham injections.

Materials and Methods

The BRAVO 6-month, phase III, multicenter, randomized, injection-controlled study, with an additional 6-months of follow up (total 12 months), was designed to evaluate efficacy and safety of intraocular injections of ranibizumab in patients with macular edema following BRVO. The study included a 28-day screening period (days −28 to −1); a 6-month treatment period (day 0 to month 6), during which patients received monthly intraocular injections of 0.3 mg or 0.5 mg ranibizumab or sham injections; and a 6-month observation period (month 6 to month 12), during which all patients could receive monthly intraocular ranibizumab if they met prespecified functional and anatomic criteria (i.e., Snellen equivalent study eye BCVA ≤20/40 according to the ETDRS chart or mean central subfield thickness ≥250 μm on optical coherence tomography [OCT]; Fig 1). The BRAVO trial is registered at www.clinicaltrials.gov (NCT00486018; accessed December 18, 2009). The protocol was approved by the institutional review board at each study site, and the study was conducted according to the International Conference on Harmonisation E6 Guideline for Good Clinical Practice and any national requirements. All patients provided informed consent before participation in the study. The primary efficacy outcome was the mean change from baseline BCVA in the study eye at month 6.

Screening and Eligibility

Eligibility was determined by the investigating physician at individual studies sites using the criteria listed in Table 1. During the screening visit, patients who provided informed consent provided a medical history and underwent a physical examination, a complete eye examination (including measurement of BCVA), OCT, FA, and laboratory tests. The BCVA was measured by the procedure described in the ETDRS. If the investigating physician judged a patient to be eligible for participation in the study, the patient's OCT using the Zeiss Stratus and the FastMac protocol (Carl Zeiss Meditec, Inc., Dublin, CA) was evaluated by certified personnel at the University of Wisconsin Fundus Photograph Reading Center (UWFPRC, Madison, WI). If that evaluation and all laboratory tests supported inclusion, the patient was scheduled for the day 0 study visit.

Randomization

Eligible patients were randomized 1:1:1 to receive monthly injections of 0.3 mg or 0.5 mg ranibizumab or sham injections, using a dynamic randomization method.[14] Randomization was stratified by baseline BCVA letter score (≤34 [approximate Snellen equivalent <20/200], 35–54 [approximate Snellen equivalent 20/200 to <20/80], or ≥55 [approximate Snellen equivalent ≥20/80]) and

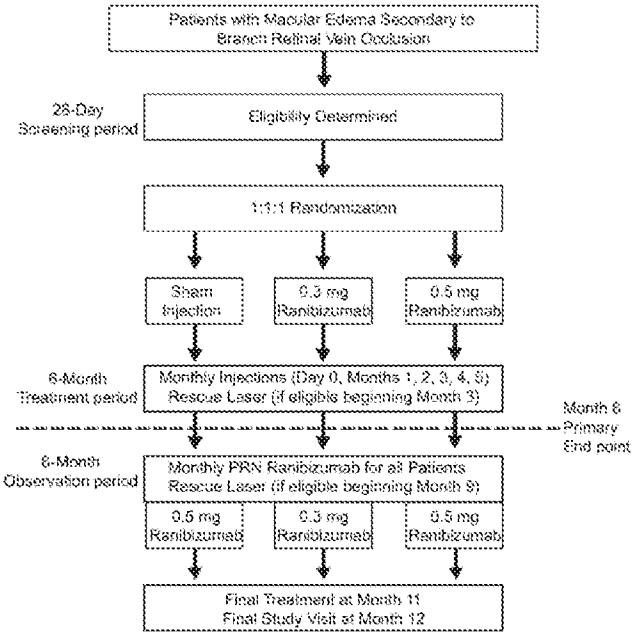

Figure 1. Study design. Eligible patients were randomized 1:1:1 to receive monthly injections of 0.3 mg or 0.5 mg ranibizumab or sham injections during the 6-month treatment period (day 0, months 1–5). During the 6-month observation period, subjects were eligible to receive monthly intraocular ranibizumab if they had Snellen equivalent study eye best-corrected visual acuity (BCVA) ≤20/40 according to the Early Treatment Diabetic Retinopathy Study chart or mean central subfield thickness ≥250 μm according to optical coherence tomography. Patients were eligible for laser treatment once during the treatment period and once during the observation period, beginning at months 3 and 9, respectively, if hemorrhages had cleared sufficiently to allow safe application of laser and the following criteria were met: Snellen equivalent BCVA ≤20/40 or mean central subfield thickness ≥250 μm, and compared with the visit 3 months before the current visit, patient had a gain of <5 letters in BCVA or a decrease of <50 μm in mean central subfield thickness. PRN = pro re nata.

study center. One eye was chosen as the study eye for each patient. If both eyes were eligible, the eye with the worse BCVA at screening was selected. Patients, certified BCVA examiners, and evaluating physicians were masked to treatment and dose. Injecting physicians, who did not perform examinations or outcome assessments, were masked to dose but not treatment.

Study Visits and Assessments

During the 6-month treatment period, study visits occurred on days 0 and 7 and months 1–6. At each visit, patients were given a complete eye examination with OCT assessment of central foveal thickness (CFT). Patients provided a medical history, vital signs were measured (except for day 7), concomitant medication was reviewed, and safety was assessed. Any new sign, symptom, illness, or worsening of any preexisting medical condition was recorded as an adverse event (AE). An AE was classified as a serious AE if it led to death, was life threatening, required prolonged hospitalization, resulted in persistent or significant disability, resulted in a congenital anomaly/birth defect, or was considered a significant medical event by the investigator. Patients who discontinued the study before the month 12 visit were encouraged to return for an early termination visit 30 days after their last injection or study visit to record AEs and serious AEs that had occurred since the patient's last visit and complete other study assessments. Patient-reported visual function was assessed with the National Eye Institute Visual Functioning Questionnaire-25 (NEI VFQ-25) at day 0 and months 1, 3, and 6.

Intraocular Injections

Patients received their assigned treatment at day 0 and months 1–5 for a maximum of 6 injections. Injection procedures were identical to those previously described.[11,12] Briefly, topical anesthetic drops were given, a lid speculum was inserted, and after subconjunctival injection of 2% lidocaine and cleaning of the injection site with 5% povidone iodine, a 30-gauge needle was inserted through the pars plana, and 0.05 mL of ranibizumab was injected. Patients who were randomized to the sham group were treated similarly to those in the ranibizumab groups, except that a needleless hub of a syringe was placed against the injection site; and the plunger of the syringe was depressed to mimic an injection. The ability to count fingers with the study eye was assessed 15 minutes after injection, and intraocular pressure was measured within 50–70 minutes of an injection.

Rescue Laser Photocoagulation

Rescue grid laser treatment was allowed based upon the precedent of the BVOS.[6] As was the case in the BVOS, patients were observed for 3 months after study entry before laser treatment was considered. Starting at month 3, patients were eligible for laser treatment if hemorrhages had cleared sufficiently to allow safe application of laser and the following criteria were met: Snellen equivalent BCVA ≤20/40 or mean central subfield thickness ≥250 μm, and compared with the visit 3 months before the current visit, patient had a gain of <5 letters in BCVA or a decrease of <50 μm in mean central subfield thickness. If rescue laser was not given at month 3, the same criteria were applied at month 4, and if rescue laser was not given at month 4, the criteria were applied at month 5. Fluorescein angiography obtained within 30 days before laser grid application was used to guide treatment.

Outcome Measures

The primary efficacy outcome measure was mean change from baseline BCVA at month 6. Secondary efficacy outcome measures included mean change from baseline BCVA letter score over time to month 6, percentage of patients who gained ≥15 letters from baseline BCVA at month 6, percentage of patients who lost <15 letters from baseline BCVA at month 6, percentage of patients with CFT ≤250 μm at month 6, and mean change from baseline CFT over time to month 6. Exploratory efficacy outcomes included percentage of patients with Snellen equivalent BCVA ≥20/40 at month 6, percentage of patients with Snellen equivalent BCVA ≤20/200 at month 6, mean change from baseline EFT over time to month 6, and mean change from baseline NEI VFQ-25 composite score over time to month 6. The upper limit of normal for central subfield thickness is 212 μm, based on measurements of a population of normal patients.[15] Thus, EFT was estimated by subtracting 212 μm from the central subfield thickness. Safety outcomes included the incidence and severity of ocular and nonocular AEs and serious AEs.

Optical coherence tomography scans obtained at day 0 and months 1, 2, 3, and 6 during the 6-month treatment period were evaluated by masked graders at the UWFPRC; the CFT was recorded as the center point thickness provided by Stratus 3 software (Carl Zeiss Meditec, Inc.), unless there was an error in computer recognition of the outer or inner boundaries of the retina or the center point. If that occurred, the grader determined the CFT

Table 1. Key Inclusion/Exclusion Criteria

Key Inclusion Criteria*	Key Exclusion Criteria*
Age ≥18 years of age with foveal center-involved ME secondary to BRVO† diagnosed within 12 months before study initiation.	Prior episode of RVO.
BCVA 20/40 to 20/400 Snellen equivalent using the ETDRS charts.	Brisk afferent pupillary defect (i.e., obvious and unequivocal).
Mean central subfield thickness ≥250 μm from 2 OCT measurements (central 1 mm diameter circle with a Stratus OCT3) on 2 measurements, one at screening confirmed by University of Wisconsin Fundus Photograph Reading Center, the other on day 0 confirmed by the investigating physician.	>10-letter improvement in BCVA between screening and day 0.
	History of radial optic neurotomy or sheathotomy.
	Intraocular corticosteroid use in study eye within 3 months before day 0.
	History or presence of wet or dry AMD.
	Panretinal scatter photocoagulation or sector laser photocoagulation within 3 months before day 0 or anticipated within 4 months after day 0.
	Laser photocoagulation for ME within 4 months before day 0 (for patients who had previously received grid laser photocoagulation, the area of leakage at day 0 must have extended into the fovea [i.e., prior laser treatment was inadequate], and there could be no evidence of laser damage to the fovea).
	Evidence upon examination of any diabetic retinopathy.
	CVA or MI within 3 months before day 0.
	Prior anti-VEGF treatment in study or fellow eye within 3 months before day 0 or systemic anti-VEGF or pro-VEGF treatment within 6 months before day 0.

AMD = age-related macular degeneration; BCVA = best-corrected visual acuity; BRVO = branch retinal vein occlusion; CVA = cerebrovascular accident; ETDRS = Early Treatment Diabetic Retinopathy Study; ME = macular edema; MI = myocardial infarction; RVO = retinal vein occlusion; VEGF = vascular endothelial growth factor.
*Pertains to study eye, except where noted otherwise.
†BRVO was defined as an eye that had retinal hemorrhage or other biomicroscopic evidence of RVO (e.g., telangiectatic capillary bed) and a dilated (or previously dilated) venous system in one quadrant or less of the retina drained by the affected vein. Hemiretinal vein occlusion (HRVO) is an RVO that involves 2 altitudinal quadrants. In this study, eyes with HRVO were treated the same as eyes with BRVO.

with a caliper. Software-generated central subfield thickness was recorded at UWFPRC and was used to calculate EFT. Fluorescein angiographs were evaluated by masked graders at the UWFPRC.

Statistical Analysis

Unless otherwise noted, the intent-to-treat approach was used for efficacy analyses and included all patients as randomized. Missing values for efficacy outcomes were imputed using the last-observation-carried-forward method. For each efficacy outcome, 2 pairwise comparisons were made: 0.3 mg ranibizumab versus sham and 0.5 mg ranibizumab versus sham. Unless otherwise noted, efficacy outcome analyses were stratified by baseline BVCA letter score (≤34 vs 35–54 vs ≥55). For the primary outcome, the mean change from baseline BCVA at month 6 was compared between each ranibizumab group and the sham injection group, using an analysis of variance model stratified by baseline BCVA, with no additional adjustments for covariates, and using the Hochberg-Bonferroni multiple comparison procedure to maintain an overall type I error rate of 0.05. Cochran-Mantel-Haenszel chi-square tests, stratified by baseline BCVA, were used for secondary and exploratory binary end point group comparisons (except for percentage of patients who had lost <15 letters from baseline BCVA at month 6 and percentage of patients who had Snellen equivalent ≤20/200 at month 6, for which the Fisher exact test was used because the percentage of patients meeting that end point was high [for the former] and low [for the latter] in all treatment groups). Analysis of variance or analysis of covariance models were used to analyze continuous outcome measures. To manage type I error across secondary end points, a type I error rate of 0.05 was allocated for each dose, and a staged hierarchical testing procedure was used with a Hochberg-Bonferroni procedure at each stage. To determine the earliest time point at which statistically significant between-group differences were obtained for mean change from baseline BCVA, CFT, EFT, and the NEI VFQ-25 composite score, a hierarchical testing procedure for significance at each time point was performed sequentially for each end point, beginning with month 6 and working backward to the time point at which the test for between-group differences resulted in $P>0.05$. Additional analyses were performed to assess sensitivity of the results to the statistical methods used. National Eye Institute VFQ-25 scores were calculated according to published guidelines. The mean of all of the NEI VFQ-25 subscales was used to calculate the overall composite score (available from: http://www.rand.org/health/surveys_tools.html; accessed December 15, 2009). The incidence of ocular and nonocular AEs and serious AEs was summarized by treatment group.

Results

Baseline Characteristics and Patient Disposition

Between July 2007 and November 2008, 397 patients were randomized to receive intraocular injections of 0.3 mg (n = 134) or 0.5 mg (n = 131) ranibizumab or sham injections (n = 132) at 93 centers in the United States. Patient demographics and baseline ocular characteristics were similar across treatment groups (Table 2). The average age of patients was 66 years, and 53% were male. The mean time from diagnosis of BRVO to screening was 3.5 months (median, 2 months for each treatment group), with duration ≤3 months in 65% of patients. Mean study eye baseline BCVA letter score was

Table 2. Patient Demographics and Baseline Ocular Characteristics

Parameter	Sham (n = 132)	Ranibizumab 0.3 mg (n = 134)	Ranibizumab 0.5 mg (n = 131)
Age (yrs)			
Mean (SD)	65.2 (12.7)	66.6 (11.2)	67.5 (11.8)
Median	64.0	66.5	67.0
Range	26–89	43–90	41–91
Gender, n (%)			
Male	74 (56.1)	67 (50.0)	71 (54.2)
Female	58 (43.9)	67 (50.0)	60 (45.8)
Race,* n (%)			
White	108 (81.8)	112 (83.6)	107 (81.7)
Black/African American	13 (9.8)	11 (8.2)	13 (9.9)
Other	8 (6.0)	3 (2.2)	5 (3.8)
Unavailable	4 (3.0)	9 (6.7)	6 (4.6)
Study eye characteristics			
Months from RVO diagnosis to screening			
Mean (SD)	3.7 (3.7)	3.6 (4.1)	3.3 (3.1)
Median	2	2	2
Range	0–16	0–35	0–13
Distribution, n (%)			
≤3	85 (64.4)	85 (63.4)	88 (67.2)
>3 to ≤6	17 (12.9)	29 (21.6)	20 (15.3)
>6 to ≤9	12 (9.1)	9 (6.7)	14 (10.7)
>9 to ≤12	16 (12.1)	8 (6.0)	7 (5.3)
>12	2 (1.5)	3 (2.2)	2 (1.5)
HRVO classification,† n (%)	17 (13.1)	16 (12.0)	17 (13.2)
BCVA			
ETDRS letter score			
Mean (SD)	54.7 (12.2)	56.0 (12.1)	53.0 (12.5)
Range	16–73	25–73	22–79
Distribution, n (%)			
<34	9 (6.8)	9 (6.7)	13 (9.9)
35–54	50 (37.9)	48 (35.8)	49 (37.4)
≥55	73 (55.3)	77 (57.5)	69 (52.7)
Approximate Snellen equivalent, median	20/80	20/63–20/80	20/80
IOP (mmHg),¶ mean (SD)	14.8 (3.0)	15.0 (3.3)	14.9 (3.3)
Taking IOP-lowering medication, n (%)	10 (7.6)	20 (14.9)	16 (12.2)
Phakic eye,** n (%)	93 (78.8)	103 (85.1)	94 (80.3)
Imaging data			
CFT(μm), mean (SD)	488.0 (192.2)	522.1 (201.9)	551.7 (223.5)
Total macular volume (mm^3),‡ mean (SD)	9.641 (1.831)	9.640 (1.833)	9.839 (2.151)
Total area of retinal hemorrhage, central subfield (DA), calculated,†† mean (SD)	0.121 (0.137)	0.103 (0.129)	0.117 (0.131)
Area of fluorescein leakage within grid (DA),¶¶ median	7	6	7
>10 DA of capillary nonperfusion (%)	0	0	0
Fellow eye characteristics			
Fellow eye BCVA (ETDRS letters), mean (SD)	79.8 (17.4)	79.4 (13.7)	81.4 (13.8)
Fellow eye vision compared with study eye, n (%)			
Better	121 (91.7)	118 (88.1)	125 (95.4)
Worse	8 (6.1)	9 (6.7)	4 (3.1)
Same	3 (2.3)	7 (5.2)	2 (1.5)

BCVA = best-corrected visual acuity; CFT = central foveal thickness; DA = disc area; ETDRS = Early Treatment Diabetic Retinopathy Study; HRVO = hemiretinal vein occlusion; IOP = intraocular pressure; RVO = retinal vein occlusion; SD = standard deviation.
*Multiracial patients were counted in each race category that they indicated. Number of patients in Other category may be overestimated. Number assessed in sham, 0.3 mg, and 0.5 mg groups was †130, 133, and 129; ¶131, 134, 130; **118, 121, and 117; ‡81, 96, and 85; ††129, 132, and 131; ¶¶131, 133, 130.

54.6 letters (approximate Snellen equivalent 20/80), and mean baseline CFT was 520.5 μm. Approximately 13% of patients had a diagnosis of hemiretinal vein occlusion.

Of patients in the 0.3 mg, 0.5 mg, and sham groups, 95.5%, 95.4%, and 93.2%, respectively, completed the study through month 6. The most common reason for study discontinuation was a decision made by the patient to do so. All but 2 of the 397 patients received study drug; for those who did, the mean number of ranibizumab or sham injections received during the 6-month treatment period was 5.7 and was similar across treatment groups.

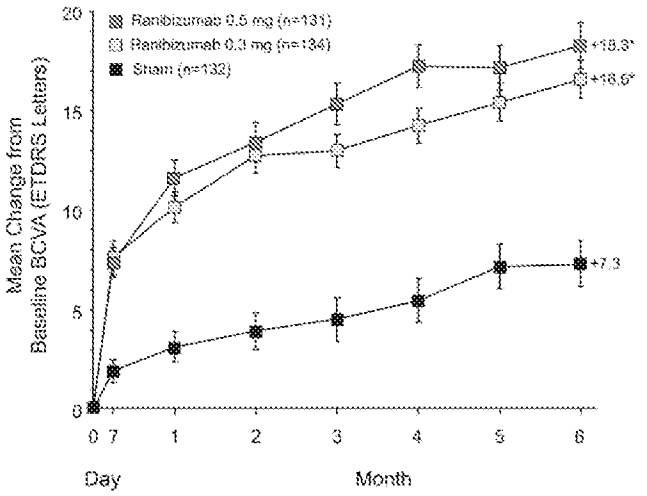

Figure 2. Mean change from study eye baseline BCVA over time to month 6. *$P<0.0001$ versus sham. Earliest statistically significant group difference ($P<0.0001$ vs sham) was at day 7. Vertical bars are ±1 standard error of the mean. The last-observation-carried-forward method was used to impute missing data. BCVA = best-corrected visual acuity; ETDRS = Early Treatment Diabetic Retinopathy Study.

Five (3.7%) patients in the 0.3 mg group, 5 (3.8%) in the 0.5 mg group, and 9 (6.8%) in the sham group discontinued treatment at or before month 5. More patients in the sham group (54.5%) received rescue grid laser therapy compared with the 0.3 mg (18.7%) and 0.5 mg (19.8%) ranibizumab groups.

Functional Outcomes at Month 6

Change from Baseline BCVA. The primary efficacy outcome was mean change from baseline BCVA at month 6. At month 6, patients in the 0.3 mg and 0.5 mg ranibizumab treatment groups had gained a mean (95% confidence interval [CI]) of 16.6 (14.7–18.5) and 18.3 (16.0–20.6) letters compared with 7.3 (5.1–9.5) letters in the sham group ($P<0.0001$ for each ranibizumab group vs sham; Fig 2, Table 3). The improvement in BCVA after injection of ranibizumab was rapid and dramatic, with patients having gained an average of 7.5 letters 7 days after the first injection, and significantly greater than that of the sham group at day 7 and all subsequent monthly assessments. The group differences in BCVA were maintained when analyzed by subgroup (Table 4). In all treatment groups, the mean improvement in BCVA letter score was greater for patients who were diagnosed with BRVO <3 months before study screening (sham = 8.2; 0.3 mg = 17.0; 0.5 mg = 19.9 letters) compared with those diagnosed ≥3 months before screening (sham = 6.3; 0.3 mg = 16.1; 0.5 mg = 16.1 letters). Although some of the subgroups were small, the mean change in BCVA at month 6 was greater for patients with worse BCVA and CFT ≥450 μm at baseline.

Percentage of Patients Who Gained ≥15 Early Treatment Diabetic Retinopathy Study Letters. At month 6, 55.2% and 61.1% of patients in the 0.3 mg and 0.5 mg ranibizumab groups had gained ≥15 letters from baseline BCVA letter score compared with 28.8% of patients in the sham group ($P<0.0001$ for each ranibizumab group vs sham). The percentage of patients who gained ≥15 letters increased rapidly after injection of ranibizumab and was 20.1% in the 0.3 mg group and 14.5% in the 0.5 mg group compared with 3.8% in the sham group at day 7. This difference was significant, as were the differences at all subsequent assessments ($P<0.005$ ranibizumab vs sham at day 7 and months 1–5).

Table 3. Change from Study Eye Baseline Best-Corrected Visual Acuity at Month 6

Parameter	Sham (n = 132)	Ranibizumab 0.3 mg (n = 134)	Ranibizumab 0.5 mg (n = 131)
ETDRS letter score			
Mean (SD)	7.3 (13.0)	16.6 (11.0)	18.3 (13.2)
95% CI for mean	5.1, 9.5	14.7, 18.5	16.0, 20.6
Difference in means (vs sham)		9.3	11.0
95% CI for difference		6.4–12.2	7.8–14.2
P (ranibizumab vs sham)*		<0.0001	<0.0001
Distribution of change at month 6, n (%)			
Gain (letters)			
≥15	38 (28.8)	74 (55.2)	80 (61.1)
10–14	15 (11.4)	25 (18.7)	23 (17.6)
5–9	27 (20.5)	18 (13.4)	8 (6.1)
No change, ±4.0	31 (23.5)	13 (9.7)	17 (13.0)
Loss (letters)			
5–9	11 (8.3)	3 (2.2)	1 (0.8)
10–14	4 (3.0)	1 (0.7)	0
≥15	6 (4.5)	0	2 (1.5)
≥15-letter gain, %			
Day 7	3.8	20.1†	14.5†
Month 1	8.3	29.9†	32.8†
Month 2	16.7	39.6†	39.7†
Month 3	17.4	38.1†	50.4†
Month 6	28.8	55.2‡	61.1‡

CI = confidence interval; ETDRS = Early Treatment Diabetic Retinopathy Study; SD = standard deviation.
*Based on pairwise analysis of variance models adjusting for baseline ETDRS letter score (≤34 vs 35–54 vs ≥55). The last-observation-carried-forward method was used to impute missing data.
†$P<0.005$ versus sham (post hoc analyses).
‡$P<0.0001$ versus sham (prespecified secondary end point).

Table 4. Change from Study Eye Baseline Best-Corrected Visual Acuity by Subgroup

Subgroup	No. of Patients Sham/0.3 mg/ 0.5 mg Ranibizumab	Visual Acuity Outcomes at Month 6 Compared with Baseline					
		Mean Change (95% CI)			Gained ≥15 ETDRS Letters, %		
		Sham	0.3 mg	0.5 mg	Sham	0.3 mg	0.5 mg
Baseline BCVA, ETDRS letter score							
≤34	9/9/13	13.6 (2.3–24.9)	28.8 (19.2–38.4)	30.7 (25.9–35.5)	33.3	77.8	100
35–54	50/48/49	8.9 (5.0–12.9)	19.6 (16.1–23.1)	21.8 (17.8–25.8)	36.0	66.7	63.3
≥55	73/77/69	5.4 (2.6–8.2)	13.3 (11.3–15.2)	13.4 (10.8–16.1)	23.3	45.5	52.2
Baseline CFT, μm							
<450	61/53/48	8.0 (5.4–10.5)	14.7 (12.0–17.5)	13.8 (10.2–17.5)	24.6	49.1	47.9
≥450	71/81/83	6.8 (3.2–10.4)	17.8 (15.2–20.4)	20.9 (18.0–23.7)	32.4	59.3	68.7
Time from BRVO diagnosis to screening (mos)							
<3	71/69/75	8.2 (5.0–11.4)	17.0 (14.1–20.0)	19.9 (16.9–23.0)	32.4	55.1	69.3
≥3	61/65/56	6.3 (3.1–9.4)	16.1 (13.7.18.5)	16.1 (12.6–19.5)	24.6	55.4	50.0

BCVA = best-corrected visual acuity; BRVO = branch retinal vein occlusion; CFT = central foveal thickness; CI = confidence interval; ETDRS = Early Treatment Diabetic Retinopathy Study.
The last-observation-carried-forward method was used to impute missing data.

Percentage of Patients Who Lost <15 Early Treatment Diabetic Retinopathy Study Letters. A large percentage of patients in each treatment group had lost <15 letters from BCVA letter score at month 6, with 100%, 98.5%, and 95.5% the 0.3 mg, 0.5 mg, and sham groups, respectively. The percentage of ranibizumab-treated patients who lost <15 letters compared with the sham group was significant only for the 0.3 mg group ($P<0.05$).

Percentage of Patients with Snellen Equivalent BCVA ≥20/40. A Snellen equivalent of ≥20/40 is generally sufficient to support reading and driving and is considered an excellent outcome. The percentage of patients that obtained this outcome at month 6 was 67.9% in the 0.3 mg group and 64.9% in the 0.5 mg group compared with 41.7% in the sham group ($P<0.0001$ for each ranibizumab group vs sham; Table 5).

Percentage of Patients with Snellen Equivalent BCVA of ≤20/200. Snellen equivalent BCVA ≤ 20/200 is considered a poor visual outcome. This outcome occurred in the study eye at month 6 in 1.5% (0.3 mg) and 0.8% (0.5 mg) of patients treated with ranibizumab compared with 9.1% of patients in the sham group ($P<0.01$ for each ranibizumab group vs sham; Table 5).

Impact on Patient-Reported Outcomes Because of Visual Function. An improvement from baseline in the mean NEI VFQ-25 composite score was observed as early as month 1 in ranibizumab-treated patients. At month 6 the mean (95% CI) change from baseline score was 9.3 (7.2–11.4), 10.4 (8.3–12.4), and 5.4 (3.6–7.3 points in the 0.3 mg [n = 133], 0.5 mg [n = 130], and sham [n = 129] groups, respectively [$P<0.005$ for each ranibizumab group vs sham]; Fig 3).

Anatomic Outcomes at Month 6

Change from Baseline Central Foveal Thickness. Concomitant with the improvement in BCVA, there was a rapid and dramatic reduction in CFT after treatment with ranibizumab. At day 7, the mean reduction from baseline CFT was >250 μm in both ranibizumab groups compared with no reduction in the sham group (Fig 4). The difference at day 7 was significant, as were differences at all subsequent graded assessments ($P<0.0001$ for each ranibizumab group vs sham at each time point). At month 6, the mean (95% CI) change in CFT was −337.3 (−375.6 to −298.9) μm and −345.2 (−386.4 to −304.0) μm in the 0.3 mg and 0.5 mg ranibizumab groups compared with −157.7 (−196.3 to −119.1) μm in the sham group.

Residual Edema. In addition to assessing the absolute reduction in CFT, it is important to determine how much macular edema is eliminated by treatment. The upper limit of normal central subfield thickness is 212 μm; thus foveal thickness >212 μm is considered excess. At baseline, the mean EFT was 276.0, 279.3, and 271.2 μm for the 0.3 mg, 0.5 mg, and sham groups, respectively. At month 6, the mean (95% CI) EFT had decreased to 57.2

Table 5. Snellen Equivalent Study Eye Best-Corrected Visual Acuity at Baseline and Month 6

Study Eye BCVA (Approximate Snellen Equivalent), n (%)	Baseline			Month 6*		
		Ranibizumab			Ranibizumab	
	Sham (n = 132)	0.3 mg (n = 134)	0.5 mg (n = 131)	Sham (n = 132)	0.3 mg (n = 134)	0.5 mg (n = 131)
≥20/20	0	0	0	9 (6.8)	27 (20.1)	26 (19.8)
20/25–20/40	19 (14.4)	21 (15.7)	15 (11.5)	46 (34.8)	64 (47.8)	59 (45.0)
20/50–20/63	44 (33.3)	46 (34.3)	36 (27.5)	27 (20.5)	25 (18.7)	25 (19.1)
20/80–20/160	55 (41.7)	53 (39.6)	59 (45.0)	38 (28.8)	16 (11.9)	20 (15.3)
20/200–20/500	14 (10.6)	14 (10.4)	21 (16.0)	12 (9.1)	2 (1.5)	1 (0.8)
<20/500	0	0	0	0	0	0

BCVA = best-corrected visual acuity.
*Last-observation-carried forward method was used to impute missing data.

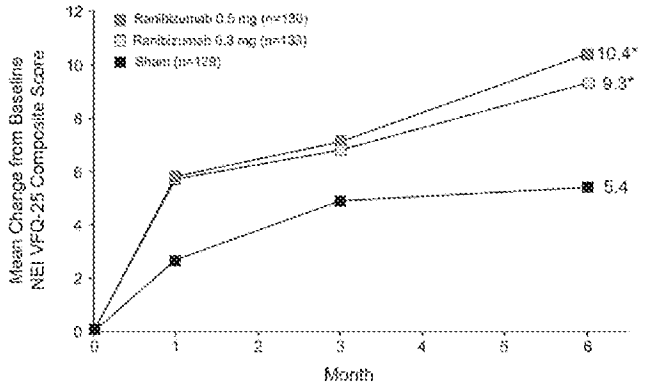

Figure 3. Mean change from baseline National Eye Institute Visual Functioning Questionnaire-25 (NEI VFQ-25) composite score over time to month 6. *$P<0.005$ versus sham. The last-observation-carried-forward method was used to impute missing data.

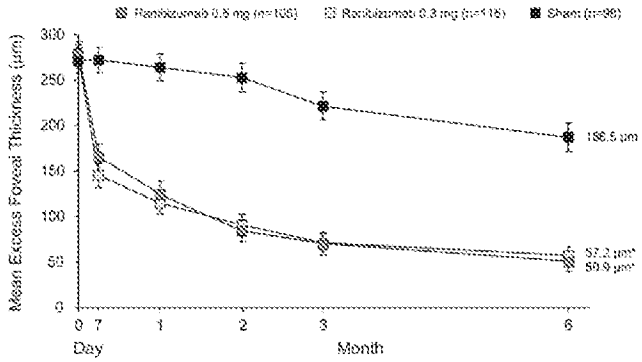

Figure 5. Mean study eye excess foveal thickness over time to month 6. *$P<0.0001$ versus sham (prespecified exploratory end point). $P<0.0001$ ranibizumab versus sham at day 7 and months 1–3 (post hoc analyses). Vertical bars are ±1 standard error of the mean.

(38.7–75.7) µm (0.3 mg, n = 115) and 50.9 (29.5–72.3) µm (0.5 mg, n = 105) in the ranibizumab groups, and 186.5 (155.4–217.7) µm in the sham group (n = 98; Fig 5). The median percent reduction from baseline EFT was 97.0% and 97.6% in 0.3 mg and 0.5 mg groups and 27.9% in the sham group at month 6. Another method of assessing residual edema is to determine the percentage of patients with CFT ≤ 250 µm at month 6, which was 91.0% (0.3 mg) and 84.7% (0.5 mg) in ranibizumab-treated patients compared with 45.5% in the sham group ($P<0.0001$ for each ranibizumab group vs sham).

Safety Outcomes through Month 6

All patients who received ≥1 injection of ranibizumab or sham injection were evaluated for safety (sham = 131; 0.3 mg = 134; 0.5 mg = 130; Table 6). A retinal detachment and retinal tear occurred in the same patient in the 0.3 mg ranibizumab group. One patient in the 0.5 mg group developed endophthalmitis, a recognized complication of intraocular injections, which led to study discontinuation. Four patients in the sham injection group, 1 patient in the 0.3 mg ranibizumab group, and 4 patients in the 0.5 mg ranibizumab group were reported to have an AE of cataract.

Some nonocular serious AEs are potentially associated with systemic VEGF inhibition and warrant close scrutiny (Table 7). One patient in the sham group had a hemorrhagic stroke. In the 0.3 mg ranibizumab group, 2 patients had hypertension, and 2 patients had nonocular hemorrhages: 1 intra-abdominal hematoma and 1 rectal hemorrhage. In the 0.5 mg ranibizumab group, there was 1 fatal cerebral hemorrhage, 1 nonfatal myocardial infarction, 1 unstable angina, 1 hemorrhage after colonoscopy, and 1 intestinal perforation in a patient with intestinal obstruction from adhesions. Three of these serious AEs qualified as thromboembolic events based on Antiplatelet Trialists' Collaboration criteria[16]—1 in the sham group (nonfatal hemorrhagic stroke) and 2 in the 0.5 mg group (fatal hemorrhagic stroke and nonfatal myocardial infarction).

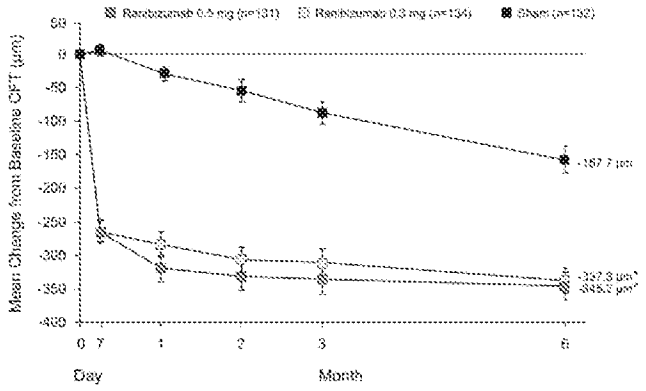

Figure 4. Mean change from study eye baseline central foveal thickness over time to month 6. *$P<0.0001$ versus sham. Earliest statistically significant difference at day 7. Vertical bars are ±1 standard error of the mean. The last-observation-carried-forward method was used to impute missing data. Independent review of optical coherence tomography was performed at the University of Wisconsin Fundus Photograph Reading Center. CFT = central foveal thickness.

Discussion

Although a small pilot study suggested that VEGF plays an important role in macular edema following BRVO,[8] this is

Table 6. Key Study Eye Adverse Events through Month 6

Adverse Events, n (%)	Sham (n = 131)	Ranibizumab 0.3 mg (n = 134)	Ranibizumab 0.5 mg (n = 130)
Any intraocular inflammation event	4 (3.1)	2 (1.5)	0
Iridocyclitis	0	1 (0.7)	0
Iritis	4 (3.1)	1 (0.7)	0
Vitritis	0	0	0
Endophthalmitis	0	0	1 (0.8)*
Lens damage	0	0	0
Cataract	4 (3.1)	1 (0.7)	4 (3.1)
Iris neovascularization	3 (2.3)	0	0
Neovascular glaucoma	0	0	0
Rhegmatogenous retinal detachment	0	1 (0.7)*†	0
Retinal tear	0	1 (0.7)*†	0
Vitreous hemorrhage	6 (4.6)	6 (4.5)	2 (1.5)

*Reported as serious.
†Same patient had rhegmatogenous retinal detachment and retinal tear.

Table 7. Key Nonocular Serious Adverse Events through Month 6

Serious Adverse Events, n (%)	Sham (n = 131)	Ranibizumab 0.3 mg (n = 134)	Ranibizumab 0.5 mg (n = 130)
Potentially related to VEGF inhibition			
Hemorrhagic stroke	1 (0.8)	0	1 (0.8)*
Ischemic stroke	0	0	0
Acute myocardial infarction	0	0	1 (0.8)
Unstable angina	0	0	1 (0.8)
Hypertension	0	2 (1.5)	0
Nonocular hemorrhage, other	0	2 (1.5)†	1 (0.8)‡
Intestinal perforation	0	0	1 (0.8)
Proteinuria	0	0	0
Antiplatelet Trialists' Collaboration arterial thromboembolic events			
Vascular death	0	0	1 (0.8)§
Nonfatal myocardial infarction	0	0	1 (0.8)
Nonfatal hemorrhagic stroke	1 (0.8)	0	0
Nonfatal ischemic stroke	0	0	0

VEGF = vascular endothelial growth factor.
*Fatal event.
†One intra-abdominal hematoma and 1 rectal hemorrhage.
‡Postprocedural (colonoscopy) hemorrhage.
§Also reported as hemorrhagic stroke potentially related to VEGF inhibition.

the first study to definitively prove that this is the case. Blocking VEGF with intraocular injections of ranibizumab has a rapid beneficial effect on visual function. There was a mean improvement of approximately 7.5 letters 1 week after the first treatment with either dose of ranibizumab. The mean improvement of between 3 and 4 lines of vision after 6 months of treatment with either dose of ranibizumab compared with 1.5 lines in the sham group is large and clinically meaningful. Differences in other parameters of visual function were equally impressive, with more than half of patients in the 2 ranibizumab treatment groups improving by ≥3 lines of BCVA compared with roughly 29% in the sham group. Probably the most notable finding was that >65% of patients treated with ranibizumab were ≥20/40 in the study eye at month 6, compared with only 42% in the sham group. Whereas <10% of patients were affected in their better-seeing eye, the impact on a patient's reported outcome based on visual function, measured by the NEI VFQ changes from baseline, indicated that the visual acuity results in the study eye translated into meaningful visual function results for the patient. With ranibizumab, roughly twice as much improvement had occurred at month 6 on the NEI VFQ-25, a validated test that measures the impact of visual function on activities of daily life.

The effect of ranibizumab on macular edema assessed by OCT was also rapid, with a reduction in mean CFT >250 μm at day 7 in the 2 ranibizumab groups compared with no reduction in the sham group. More important than the absolute reduction in CFT is an indication of the amount of residual edema. To determine this precisely would require knowing the normal premorbid CFT for each patient, which was not available. A reasonable alternative was to use population-based normative data as an estimate. Thus, we calculated EFT by subtracting the upper limit of normal of the central subfield thickness for each patient, which provided a reasonable estimate of residual edema. Of the patients with available data at month 6, the median percent reduction in EFT was 97%–98% in the 2 ranibizumab groups compared with 28% in the sham group. Thus, treatment with ranibizumab for 6 months essentially eliminated macular edema in most patients with BRVO; this is the ultimate anatomic goal and helps to explain the impressive impact of ranibizumab on visual function.

No new risks of treatment with ranibizumab were identified in patients with RVO compared with patients with neovascular age-related macular degeneration. One patient developed endophthalmitis, and it is clear that this is a very small, but definite, risk of any treatment that involves intraocular injections. One patient developed a retinal tear and a retinal detachment. Because it is possible that repeated intraocular injections can cause or exacerbate vitreous traction, it is possible that these events were also related to the study procedure. There is evidence of increased thromboembolic events in patients receiving systemic treatment with VEGF antagonists,[17] but it remains unclear whether intraocular injections of ranibizumab are associated with increased risk of such events. Using Antiplatelet Trialists' Collaboration criteria, thromboembolic events were identified in 1 patient in the sham group (a hemorrhagic stroke) and 2 patients in the 0.5 mg ranibizumab group (a hemorrhagic stroke and a myocardial infarction). Thus, the incidence of these events was small and does not provide any evidence to suggest particular concerns in patients with RVO.

The BRAVO trial did not directly compare efficacy of ranibizumab injections and grid laser treatment for macular edema following BRVO, in part because they are very different types of treatment. Laser treatment cannot be given initially to most patients owing to retinal hemorrhages in the macula. Hemorrhages on the surface of the retina increase toxicity and reduce the effectiveness of laser photocoagulation by changing light absorption from the retinal pigment epithelium below the retina to blood on the surface of the retina. This may cause damage to ganglion cell bodies and axons, which is more likely to cause visual field defects and reduce vision. It often takes several months for hemorrhages to clear sufficiently to make laser treatment less dangerous; during that time, patients may experience severe edema. It is likely that severe edema compromises retina cells and leads to permanent vision loss over time, but the extent and timing of permanent vision loss from edema are unknown.

In the BVOS,[6] patients randomized to grid laser photocoagulation were observed for 3 months after study entry and were then given grid laser photocoagulation. We followed the same protocol, and 3 months after study entry, all patients were eligible for grid laser photocoagulation if there had been sufficient clearing of retinal hemorrhages and they had not shown substantial visual and anatomic improvement from baseline. If laser was deferred, it could be given at month 4 or 5, according to the same criteria. Compared with the sham group, in which 54.5% of patients received rescue grid laser therapy, only 18.7% (0.3 mg) and 19.8% (0.5 mg) in the ranibizumab groups received laser

treatment. Between baseline and month 3 there was an 87.2 μm reduction in mean CFT in the sham group; between months 3 and 6, there was an additional reduction of 70.5 μm. This suggests that the modest improvement in the sham group may be attributable in part to laser treatment, but in some instances may represent spontaneous improvement.

Because injections were safe and well-tolerated, it would be reasonable to consider treating with ranibizumab soon following BRVO is diagnosed if the baseline criteria of this study are met. However, it should be noted that this approach would result in unnecessary treatment for the small percentage of patients who undergo complete, spontaneous resolution. Treating physicians would have to decide if the potential benefits of rapid elimination of macular edema by immediate treatment with ranibizumab outweigh the risks and the added cost and inconvenience of treatment for the small percentage of patients who would resolve spontaneously.

Although it is clear from this study that 6 monthly injections of ranibizumab provided tremendous benefit to patients with macular edema following BRVO, many important questions still exist, some of which will be unable to be addressed given the lack of a comparator group during the 6-month observation period. For instance, what percentage of patients remains edema-free following ranibizumab treatment is discontinued? For patients with recurrent edema, can ranibizumab-induced visual gains be maintained when therapy is administered if retreatment criteria are met, and if so, what is the average number of injections required to do so? Will treatment with ranibizumab after a 6-month delay allow the sham group to achieve similar visual outcomes to those seen in the ranibizumab groups at 12 months? Are there any clinical, FA, or OCT features that help to predict outcome of ranibizumab treatment? What are the effects of long-term edema on visual acuity? Continued follow-up of patients in the BRAVO trial will help to answer some of these questions. If the functional gains observed in the 6-month treatment period are maintained with longer term follow-up of the BRAVO cohort, it is likely that this therapy will be considered a "standard of care" for the treatment of macular edema following BRVO.

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References

1. Klein R. Retinopathy in a population-based study. Trans Am Ophthalmol Soc 1992;90:561–94.
2. Gewaily D, Greenberg PB. Intravitreal steroids versus observation for macular edema secondary to central retinal vein occlusion. Cochrane Database Syst Rev 2009;1:CD007324.
3. Klein R, Moss SE, Meuer SM, Klein BE. The 15-year cumulative incidence of retinal vein occlusion: the Beaver Dam Eye Study. Arch Ophthalmol 2008;126:513–8.
4. U.S. Census Bureau. Annual Estimates of the population by sex and five-year age groups for the United States: April 1, 2000 to July 1, 2007. NC-EST2007-01. Release Date: May 1, 2008. Available at: http://www.census.gov/popest/national/asrh/NC-EST2007-sa.html. Accessed February 16, 2010.
5. Frangieh GT, Green WR, Barraquer-Somers E, Finkelstein D. Histopathologic study of nine branch retinal vein occlusions. Arch Ophthalmol 1982;100:1132–40.
6. Branch Vein Occlusion Study Group. Argon laser photocoagulation for macular edema in branch vein occlusion. Am J Ophthalmol 1984;98:271–82.
7. Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 1994;331:1480–7.
8. Campochiaro PA, Hafiz G, Shah SM, et al. Ranibizumab for macular edema due to retinal vein occlusions: implication of VEGF as a critical stimulator. Mol Ther 2008;16:791–9.
9. Noma H, Funatsu H, Yamasaki M, et al. Pathogenesis of macular edema with branch retinal vein occlusion and intraocular levels of vascular endothelial growth factor and interleukin-6. Am J Ophthalmol 2005;140:256–61.
10. Ozaki H, Hayashi H, Vinores SA, et al. Intravitreal sustained release of VEGF causes retinal neovascularization in rabbits and breakdown of the blood-retinal barrier in rabbits and primates. Exp Eye Res 1997;64:505–17.
11. Brown DM, Kaiser PK, Michels M, et al, ANCHOR Study Group. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N Engl J Med 2006;355:1432–44.
12. Rosenfeld PJ, Brown DM, Heier JS, et al, MARINA Study Group. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–31.
13. Early Treatment Diabetic Retinopathy Study Research Group. Photocoagulation for diabetic macular edema: Early Treatment Diabetic Retinopathy Study report number 1. Arch Ophthalmol 1985;103:1796–806.
14. Signorini DF, Leung O, Simes RJ, et al. Dynamic balanced randomization for clinical trials. Stat Med 1993;12:2343–50.
15. Chan A, Duker JS, Ko TH, et al. Normal macular thickness measurements in healthy eyes using Stratus optical coherence tomography. Arch Ophthalmol 2006;124:193–8.
16. Antiplatelet Trialists' Collaboration. Collaborative overview of randomized trials of antiplatelet therapy—I: prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. BMJ 1994; 308:81–106.
17. Lucentis [package insert]. San Francisco: Genentech; 2007. Available at: http://www.gene.com/gene/products/information/pdf/lucentis-prescribing.pdf. Accessed February 15, 2010.

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[1] Departments of Ophthalmology and Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland.

[2] Ophthalmic Consultants of Boston, Boston, Massachusetts.

[3] Retina Associates of New Jersey, Kenilworth, New Jersey.

[4] Genentech, Inc., South San Francisco, California.

A list of study investigators is available at http://aaojournal.org

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Peter A. Campochiaro – Consultant, Genentech, GlaxoSmithKline, LPath, Regeneron, Potentia

Jeffrey Heier – Consultant – Genentech, Regeneron, Allergan; Support – Alimera

Leonard Feiner – Consultant – Allergan; Consultant, Lecturer – Genentech, Novartis

Sarah Gray – Employee – Genentech

Namrata Saroj – Employee – Genentech

Amy Chen Rundle – Employee – Genentech

Wendy Yee Murahasi – Employee – Genentech; Equity Owner – Roche

Roman G. Rubio – Employee – Genentech; Equity Owner – Roche

Correspondence:
Peter A. Campochiaro, MD, 719 Maumenee, The Wilmer Eye Institute, The Johns Hopkins School of Medicine, 600 N. Wolfe Street, Baltimore, MD 21287-9277. E-mail: pcampo@jhmi.edu.

Appendix 1. BRAVO Investigators

P. Abraham, Black Hills Regional Eye Institute, Rapid City, SD; D. V. Alfaro III, Charleston Neuroscience Institute, Charleston, SC; C. Awh, Tennessee Retina, PC, Nashville, TN; C. Baker, Paducah Retinal Center, Paducah, KY; S. Bakri, Mayo Clinic, Rochester, MN; G. Barile, Columbia University, New York, NY; M. Bennett, Retina Center of Hawaii, LLC, Honolulu, HI; B. Berger, Retina Research Center, Austin, TX; R. Bhisitkul, UCSF, San Francisco, CA; G. Blaha, Lahey Clinic, Peabody, MA; D. Boyer, Retina Vitreous Associates Medical Group, Beverly Hills, CA; H. L. Brooks, Jr., Southern Vitreoretinal Associates, Tallahassee, FL; D. Brown, Retina Consultants of Houston, Houston, TX; A. Burrows, Retina Vitreous Consultants of NJ, LLC, Englewood, NJ; C. Campbell, South Texas Retinal Consultants, Corpus Christi, TX; P. Campochiaro, Johns Hopkins School of Medicine, Baltimore, MD; K. Carnevale, Ophthalmic Consultants of Long Island, Lynbrook, NY; M. Cassell, Truman Medical Center and Sabates Eye Center, Kansas City, MO; C. Chan, Southern California Desert Retina Consultants, Palm Springs, CA; R. Chandran, Sylvan Eye Center, Modesto, CA; T. Chang, Retina Institute of California, Pasadena, CA; N. Chaudhry, New England Retina Associates, New London, CT; T. A. Ciulla, MD, PP, Indianapolis, IN; W. L. Clark, Palmetto Retina Center, LLC, West Columbia, SC; T. Cleland, Retina Associates of South Texas, PA, San Antonio, TX; G. Cowan, Retina Consultants, PA, Fort Worth, TX; A. Dessouki, Retinal Diagnostic Center, Campbell, CA; R. Dreyer, Retina Northwest, Portland, OR; P. Dugel, Retina Consultants of Arizona, Ltd., Phoenix, AZ; A. Eaton, Retina Health Center, Fort Myers, FL; N. Engelbrecht, Barnes Retina Institute, Saint Louis, MO; D. W. Faber, Rocky Mountain Retina Consultants, Salt Lake City, UT; L. Feiner, Retina Associates of New Jersey, Teaneck, NJ; R. Feldman, Florida Eye Clinic, Altamonte Springs, FL; P. Ferrone, Vitreoretinal Consultants of Long Island, Great Neck, NY; G. Fox, Retina Associates, Shawnee Mission, KS; R. Frenkel, East Florida Eye Institute, Stuart, FL; R. Gallemore, Retina Macula Institute, Torrance, CA; A. Gordon, Associated Retina Consultants, Phoenix, AZ; E. Guillet, Retina Associates of Western New York, Rochester, NY; S. Gupta, MD, PA, Pensacola, FL; S. Hariprasad, University of Chicago, Chicago, IL; Y. He, University of Texas Southwestern Medical Center at Dallas, Dallas, TX; J. Heier, Ophthalmic Consultants of Boston; Boston, MA; A. Ho, Mid-Atlantic Retina, Philadelphia, PA; D. Hoffert, Maine Vitreoretinal Consultants, Bangor, ME; J. Hoskins, Southeastern Retina Associates, PC, Knoxville, TN; B. Hubbard, Emory Eye Center, Atlanta, GA; D. Ie, Delaware Valley Retina Associates, Lawrenceville, NJ; C. Javid, Retina Associates Southwest, PC, Tucson, AZ; R. Johnson, West Coast Retina Medical Group, Inc., San Francisco, CA; R. Katz, Florida Eye Microsurgical Institute, Boynton Beach, FL; A. Kimura, Porter Adventist Hospital Centura, Denver, CO; S. Y. Lee, Retina Research Institute of Texas, Abilene, TX; N. Leonardy, Retina Vitreous Associates, Toledo, OH; E. Lit, East Bay Retina Consultants, Oakland, CA; L. Lobes, Retina Vitreous Consultants, Pittsburgh, PA; E. Madson, Midwest Eye Care, Omaha, NE; N. Mandava, Rocky Mountain Lions Eye Institute, Aurora, CO; D. Marcus, Southeast Retina Center, Augusta, GA; J. Martinez, Austin Retina Associates, Austin, TX; M. Michels, Retina Care Specialists, Palm Beach, FL; R. Mittra, VitreoRetinal Surgery, Edina, MN; G. Novalis, Retina Centers, PC, Tucson, AZ; A. Patel, Retinal Consultants Medical Group, Sacramento, CA; M. Paul, Danbury Eye Physicians and Surgeons, Danbury, CT; P. Pavan, University of South Florida Eye Institute, Tampa, FL; D. Pieramici, California Retina Consultants, Santa Barbara, CA; J. Prensky, Pennsylvania Retina Specialists, PC, Camp Hill, PA; R. Rosa, Robert Scott and White Hospital, Temple, TX; K. Rosenberg, Retina Group of Florida, Fort Lauderdale, FL; D. Roth, Retina Vitreous Center, New Brunswick, NJ; P. Runge, Ophthalmic Consultants, Sarasota, FL; D. Saperstein, Vitreoretinal Associates, Seattle, WA; T. Schneiderman, Retina Center Northwest, Silverdale, WA; L. Schocket, Union Memorial Hospital, Baltimore, MD; J. Sebag, VMR Institute, Huntington Beach, CA; M. Singer, Medical Center Ophthalmology Associates, San Antonio, TX; B. Sippy, Rocky Mountain Eye Center, Missoula, MT; A. Thach, Retina Consultants of Nevada, Las Vegas, NV; M. Tolentino, Center for Retina and Macular Disease, Winter Haven, FL; D. Tom, New England Retina Associates, Hamden, CT; R. Torti, Retina Specialists, Desoto, TX; E. Tu, Kaiser Permanente Southern California, Baldwin Park, CA; A. Verne, Bay Area Retina Associates, Walnut Creek, CA; T. Verstraeten, Allegheny Ophthalmic and Orbital, Pittsburgh, PA; J. Walker, National Ophthalmic Research, Fort Myers, FL; P. Weishaar, VitreoRetinal Consultants, Wichita, KS; M. Wieland, Northern California Retina Vitreous Associates, Mountain View, CA; M. Wood, Eye Surgical Associates, Lincoln, NE; W. Wood, Retina Associates of Kentucky, Lexington, KY; J. Wroblewski, Cumberland Valley Retina, PC, Hagerstown, MD; L. Young, Mass Eye and Ear Infirmary, Boston, MA.

Sustained Benefits from Ranibizumab for Macular Edema following Central Retinal Vein Occlusion: Twelve-Month Outcomes of a Phase III Study

Peter A. Campochiaro, MD,[1] David M. Brown, MD, FACS,[2] Carl C. Awh, MD,[3] S. Young Lee, MD,[4] Sarah Gray, PhD,[5] Namrata Saroj, OD,[5] Wendy Yee Murahashi, MD,[5] Roman G. Rubio, MD[5]

Purpose: Assess the 12-month efficacy and safety of intraocular injections of 0.3 mg or 0.5 mg ranibizumab in patients with macular edema after central retinal vein occlusion (CRVO).

Design: Prospective, randomized, sham injection-controlled, double-masked, multicenter clinical trial.

Participants: We included 392 patients with macular edema after CRVO.

Methods: Eligible patients were randomized 1:1:1 to receive 6 monthly intraocular injections of 0.3 mg or 0.5 mg of ranibizumab or sham injections. After 6 months, all patients with BCVA ≤20/40 or central subfield thickness ≥250 μm could receive ranibizumab.

Main Outcome Measures: Mean change from baseline best-corrected visual acuity (BCVA) letter score at month 12, additional parameters of visual function, central foveal thickness (CFT), and other anatomic changes were assessed.

Results: Mean (95% confidence interval) change from baseline BCVA letter score at month 12 was 13.9 (11.2–16.5) and 13.9 (11.5–16.4) in the 0.3 mg and 0.5 mg groups, respectively, and 7.3 (4.5–10.0) in the sham/0.5 mg group ($P<0.001$ for each ranibizumab group vs. sham/0.5 mg). The percentage of patients who gained ≥15 letters from baseline BCVA at month 12 was 47.0% and 50.8% in the 0.3 mg and 0.5 mg groups, respectively, and 33.1% in the sham/0.5 mg group. On average, there was a marked reduction in CFT after the first as-needed injection of 0.5 mg ranibizumab in the sham/0.5 mg group to the level of the ranibizumab groups, which was sustained through month 12. No new ocular or nonocular safety events were identified.

Conclusions: On average, treatment with ranibizumab as needed during months 6 through 11 maintained the visual and anatomic benefits achieved by 6 monthly ranibizumab injections in patients with macular edema after CRVO, with low rates of ocular and nonocular safety events. After sham injections for 6 months, treatment with ranibizumab as needed for 6 months resulted in rapid reduction in CFT in the sham/0.5 mg group to a level similar to that in the 2 ranibizumab treatment groups and an improvement in BCVA, but not to the same level as that in the 2 ranibizumab groups. Intraocular injections of ranibizumab provide an effective treatment for macular edema after CRVO.

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Central retinal vein occlusion (CRVO) is an important cause of vision loss and is estimated to have a 15-year cumulative incidence of 0.5% in a population study based in Wisconsin[1] and to affect 0.4% of the population in Australia.[2] As the name indicates, the inciting event is thought to be thrombosis within the central retinal vein, and there is pathologic evidence to support that contention.[3] Occlusion of the major outflow channel of the retinal circulation markedly increases intraluminal venous pressure, resulting in hemorrhages and edema. Massive swelling within the retina also causes variable amounts of capillary closure in some, but not all, patients. Vision is reduced if there are hemorrhages and/or edema in the macula or if there is closure of a substantial proportion of the perifoveal capillaries, resulting in macular ischemia. Hemorrhages are gradually resorbed, leaving edema and/or ischemia in the macula as the major causes of reduced vision, with the former predominant in most patients.

Recent studies have demonstrated that, while increased venous pressure may be the precipitating event for hemorrhages and edema, increased production of vascular endothelial growth factor (VEGF) occurs early in the disease process and is a major contributor to macular edema.[4–6] Those studies were made possible by the development of ranibizumab (Lucentis, Genentech, Inc., South San Francisco, CA), a humanized, affinity-matured anti-VEGF anti-

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body fragment that binds to and neutralizes all isoforms of VEGF-A and their biologically active degradation products. A small, interventional pilot study in patients with CRVO or branch retinal vein occlusion demonstrated that monthly injections of 0.3 mg or 0.5 mg ranibizumab for 3 months caused a marked reduction in macular edema and a mean improvement in best-corrected visual acuity (BCVA) of approximately 15 letters in all ranibizumab treatment groups.[4] Other pilot trials had similar results.[5,6] This provided the rationale for 2 large, multicenter trials—Ranibizumab for the Treatment of Macular Edema after *C*entral *R*etinal Vein Occl*UsI*on *S*tudy: *E*valuation of Efficacy and Safety (CRUISE) and the Ranibizuma*B* for the Treatment of Macular Edema after B*RA*nch Retinal *V*ein *O*cclusion: Evaluation of Efficacy and Safety (BRAVO) study—which were designed to determine the efficacy and safety of ranibizumab in patients with macular edema following retinal vein occlusion.[7,8] After 6 monthly intraocular injections of 0.3 mg or 0.5 mg ranibizumab in patients with CRVO, mean improvement in BCVA letter score was 12.7 and 14.9 letters compared with 0.8 letters in the sham injection group. Starting at month 6, all patients were eligible to receive ranibizumab treatment as needed based on prespecified criteria. Herein, we report the 12-month outcomes of CRUISE.

Materials and Methods

Study Design

The CRUISE Study was a 12-month, phase III, multicenter, randomized trial that included a 6-month, injection-controlled treatment period followed by a 6-month observation period, designed to evaluate efficacy and safety of intraocular injections of ranibizumab in patients with macular edema following CRVO. Details of the CRUISE methodology were previously reported[7] and are briefly summarized here. During the treatment period (day 0–month 5) patients received monthly intraocular injections of 0.3 mg or 0.5 mg ranibizumab or sham injections. During the observation period (months 6–11) all patients could receive monthly intraocular ranibizumab if study eye Snellen equivalent BCVA was ≤20/40 or mean central subfield thickness assessed by the investigator was ≥250 μm as measured by Zeiss Stratus 3 (Carl Zeiss Meditec, Inc. Dublin, CA) optical coherence tomography. The CRUISE trial is registered at www.clinicaltrials.gov (NCT00485836; accessed October 20, 2010). The protocol was approved by the institutional review board at each study site, and the study was conducted according to the International Conference on Harmonisation E6 Guideline for Good Clinical Practice and any national requirements. All patients provided informed consent before participation in the study.

Patients

Eligible patients were ≥18 years of age with foveal center-involved macular edema following CRVO diagnosed within 12 months of screening, study eye Snellen equivalent BCVA of 20/40 to 20/320, and mean central subfield thickness ≥250 μm (assessments at both screening and day 0). Patients were randomized 1:1:1 to receive monthly injections of 0.3 mg or 0.5 mg ranibizumab or sham injections for 6 months.[7] Randomization was stratified by study center and baseline BCVA letter score ≤34 (approximate Snellen equivalent <20/200), 35 to 54 (approximate Snellen equivalent 20/200 to <20/80), and ≥55 (approximate Snellen equivalent ≥20/80).

During months 6 through 12, patients continued to be evaluated monthly with a complete eye examination, optical coherence tomography, measurement of vital signs, review of medical history, including concomitant medications and concurrent ocular procedures, and safety assessments. Fluorescein angiography was performed at months 6, 9, and 12. At months 6 and 12, the National Eye Institute Visual Functioning Questionnaire-25 (NEI VFQ-25) was administered. At each visit from months 6 to 11, all patients with BCVA ≤20/40 or mean central subfield thickness ≥250 μm in the study eye were to receive intraocular ranibizumab. Patients in the 0.3 mg and 0.5 mg groups received their assigned dose; and patients in the sham group, hereafter referred to as the sham/0.5 mg group, received 0.5 mg ranibizumab.

Patients who discontinued the study before the month 12 visit were encouraged to return for an early termination visit 30 days after their last injection and/or study visit to record adverse events (AEs) and serious AEs (SAEs) that had occurred since the patient's last visit and to complete other study assessments.

Outcome Measures

The primary endpoint of CRUISE was mean change from baseline BCVA letter score at month 6. Secondary outcome measures included mean change from baseline BCVA letter score over time to month 12, proportion of patients who gained ≥15 letters from baseline BCVA letter score at month 12, proportion of patients who lost ≥15 letters from baseline BCVA letter score at month 12, mean change from baseline CFT over time to month 12, and proportion of patients with CFT ≤250 μm at month 12. Exploratory and post hoc outcomes included mean change from the baseline NEI VFQ-25 composite score over time to month 12, proportion of patients with study eye Snellen equivalent ≥20/40 at month 12, proportion of patients with study eye Snellen equivalent ≤20/200 at month 12, proportion of patients with >10 retinal hemorrhages over time to month 12, and proportion of patients with zero retinal hemorrhages over time to month 12. Safety outcomes included the incidence and severity of ocular and nonocular AEs and SAEs.

Optical coherence tomography scans, fundus photographs, and fluorescein angiography were evaluated by masked graders at the University of Wisconsin Fundus Photograph Reading Center (Madison, WI); CFT was recorded as the center point thickness provided by Stratus 3 software, unless there was an error in computer recognition of the outer or inner boundaries of the retina or the center point. If the latter occurred, the grader determined CFT with a caliper.

Statistical Analysis

Analyses of efficacy endpoints for the observation period were based on the intent-to-treat population, with subjects grouped according to their assigned treatment. Missing values were imputed using the last-observation-carried-forward method, unless otherwise noted. The study was not powered to compare efficacy outcomes between the treatment groups during the 6-month observation period (i.e., at months 7–12). Thus, efficacy analyses during that time were based on descriptive statistics, and presented statistical comparisons of efficacy outcomes between the sham/0.5 mg and ranibizumab treatment groups were performed post hoc. For visual acuity and CFT outcomes, post hoc subgroup analyses based on month 6 treatment status were performed using observed data (i.e., without imputation for missing values). The incidence of key study eye ocular AEs, SAEs potentially related to VEGF inhibition, and Antiplatelet Trialists' Collaboration[9] arterial throm-

Table 1. Patient Disposition and Treatment

	Sham/0.5 mg (n = 130)	Ranibizumab 0.3 mg (n = 132)	Ranibizumab 0.5 mg (n = 130)
Completed study, n (%)			
Through month 6	115 (88.5)	129 (97.7)	119 (91.5)
Through month 12	109 (83.8)	126 (95.5)	114 (87.7)
Mean number of injections/patient*			
Treatment period	5.4	5.8	5.5
Observation period	3.7	3.8	3.3
Patients receiving first as-needed injection at month 6, n (%)	100 (76.9)	74 (56.1)	64 (49.2)

*During the 6-month treatment period (day 0–month 5), sham patients received sham injections; during the 6-month observation period (months 6–11), sham patients received 0.5 mg ranibizumab if they met prespecified criteria.

boembolic events (ATE) were summarized by treatment group. Safety outcomes for the 0.3 mg and 0.5 mg groups were summarized for the cumulative 12-month study period. Safety outcomes for the sham/0.5 mg group were summarized separately for the treatment and observation periods.

Results

Patient Characteristics and Disposition

We randomized 392 patients to receive intraocular injections of 0.3 mg ranibizumab (n = 132) or 0.5 mg ranibizumab (n = 130) or sham injections (n = 130) at 95 centers in the United States. Patient demographics and baseline ocular characteristics were similar across treatment groups. The mean time from diagnosis of CRVO to screening was 3.3 months (median 2 months for each treatment group), with a duration of ≤3 months in 69% of patients. Mean baseline BCVA letter score was 48.3 letters (approximate Snellen equivalent 20/100) and the mean baseline CFT was 685 μm. Approximately 93% of enrolled patients completed the study through month 6, and 89% completed through month 12 (Table 1). The most common reason for study discontinuation was physician's decision. During the 6-month observation period, the percentage of patients treated with ranibizumab when the protocol-specified treatment criteria were met ranged from 79% to 94% across treatment groups and time points. Between months 6 and 12, the mean number of as-needed ranibizumab injections among all randomized patients was 3.8, 3.3, and 3.7 in the 0.3 mg, 0.5 mg, and sham/0.5 mg groups; and the percentage of patients who did not receive any injections during the observation period was 9.1%, 14.6%, and 15.4%, respectively. Twenty-nine of the 392 patients discontinued from the study before month 6. Excluding those patients, the mean number of as-needed ranibizumab injections received during the observation period was 3.9, 3.6, and 4.2 in the 0.3 mg, 0.5 mg, and sham/0.5 mg groups; and the percentage of patients who did not receive any injections during the observation period was 7.0, 6.7, and 4.3, respectively.

Functional Outcomes at Month 12

Change from Baseline BCVA. At month 6, the primary endpoint, the mean change from baseline BCVA letter score was 12.7 and 14.9 in the 0.3 mg and 0.5 mg ranibizumab groups compared with 0.8 in the sham group. In the 0.3 mg and 0.5 mg treatment groups, these improvements were maintained with as-needed ranibizumab during the observation period, with a mean (95% confidence interval) change from baseline BCVA letter score of 13.9 (11.2–16.5) and 13.9 (11.5–16.4), respectively, at month 12.

The sham/0.5 mg group experienced an overall improvement in BCVA letter score during the observation period, with a mean (95% confidence interval) change from baseline of 7.3 (4.5–10.0) at month 12. The mean improvement from baseline BCVA at month 12 in the sham/0.5 mg group was significantly less than that of the 0.3 mg and 0.5 mg treatment groups ($P<0.001$ for each ranibizumab group vs sham/0.5 mg; Fig 1).

From months 6 to 7, the mean BCVA letter score decreased in the 0.3 mg and 0.5 mg groups and increased in the sham/0.5 mg group. Across treatment groups, 43.9% (0.3 mg), 50.8% (0.5 mg), and 23.1% (sham/0.5 mg) of patients did not receive ranibizumab treatment at month 6. Most patients who did not receive an injection showed worsening of BCVA from month 6 to 7, with mean decreases in BCVA letter score of 4.6 (0.3 mg), 7.2 (0.5 mg), and 2.4 (sham/0.5 mg), whereas most of those who received an injection showed improvement in BCVA, with mean increases of 1.7 (0.3 mg and 0.5 mg) and 4.9 (sham/0.5 mg; Fig 2, available online at http://aaojournal.org).

Percentage of Patients Who Had a BCVA Letter Score Gain or Loss ≥15. The percentage of patients who had an improvement from baseline BCVA letter score of ≥15 at the month 6 time point was 46.2% (0.3 mg) and 47.7% (0.5 mg) in the ranibizumab groups and 16.9% in the sham group. This was maintained in the ranibizumab groups during the observation period when ranibizumab was given as needed, and at month 12 the percentage of patients who had an improvement from baseline BCVA letter score ≥15 was 47.0% (0.3 mg) and 50.8% (0.5 mg; Table 2). The sham/0.5 mg group showed improvement from ranibizumab injections given as needed throughout the observation period; however, the 33.1% of patients who gained ≥15 in BCVA letter score at month 12 was less than that observed in the ranibizumab groups ($P<0.05$ for each ranibizumab group vs sham/0.5 mg). The percentage of patients who lost ≥15 from baseline BCVA letter score was 3.8% (0.3 mg), 1.5% (0.5 mg), and 15.4% (sham) at month 6 compared with 3.8% (0.3 mg), 2.3% (0.5 mg), and 10.0% (sham/0.5 mg) at month 12.

Percentage of Patients with Snellen Equivalent BCVA ≥20/40. A Snellen BCVA of ≥20/40 is generally sufficient to support reading and driving and is considered an excellent outcome. The percentage of patients with Snellen equivalent BCVA ≥20/40 was 43.9% (0.3 mg), 46.9% (0.5 mg), and 20.8% (sham) at month 6, compared with 43.2% (0.3 mg), 43.1% (0.5 mg), and 34.6% (sham/0.5 mg) at month 12. Snellen equivalent BCVA outcomes are broken down into several categories in Table 3.

Percentage of Patients with Snellen Equivalent BCVA ≤20/200. Snellen equivalent BCVA ≤20/200 is a poor visual outcome and is defined as legal blindness. This outcome occurred in the study eye in 15.2% (0.3 mg), 11.5% (0.5 mg), and 27.7% (sham) of patients at month 6, compared with 12.1% (0.3 mg), 12.3% (0.5 mg), and 20.0% (sham/0.5 mg) at month 12.

Impact of Visual Outcome on Daily Life Activities. At month 6, the mean increase from baseline NEI VFQ-25 composite score was 7.1 points (0.3 mg) and 6.2 points (0.5 mg) in the ranibizumab-treatment groups compared with 2.8 points in the sham group. Treatment with ranibizumab as needed from months 6–11 maintained, on average, the increases in the 2 ranibizumab groups (7.1 points in the 0.3 mg group and 6.6 points in the 0.5 mg group) and resulted in an increase (from baseline) of 5.0 points in the sham/0.5 mg group (Fig 3).

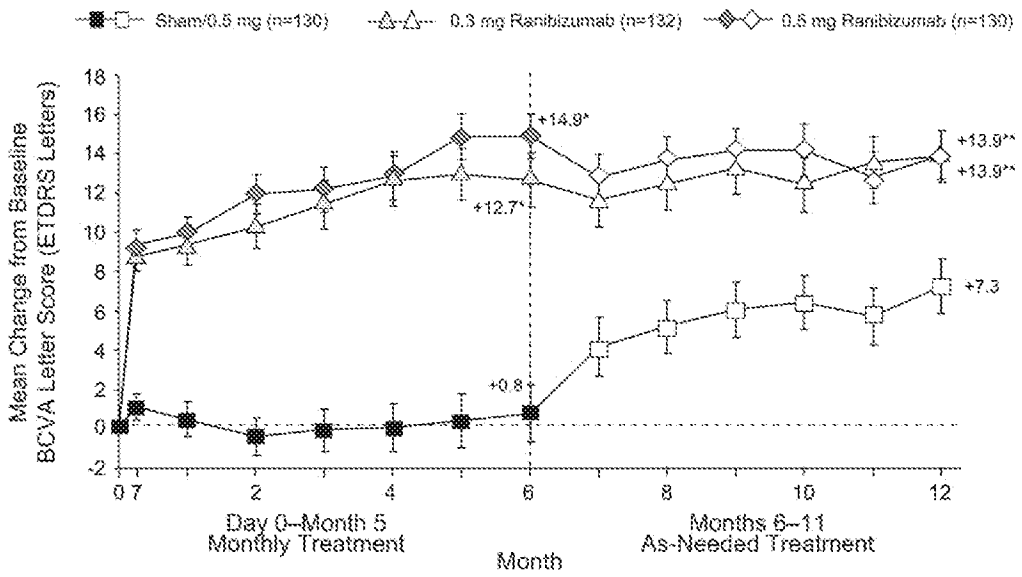

Figure 1. Mean change from study eye baseline best-corrected visual acuity letter score over time to month 12. *$P<0.0001$ versus sham, **$P<0.001$ versus sham/0.5 mg. Earliest statistically significant group difference was at day 7. The last-observation-carried-forward method was used to impute missing values. Vertical bars are ±1 standard error of the mean. On average, visual gains during the treatment period were maintained in the ranibizumab treatment groups during the observation period. There was substantial improvement in visual acuity in the sham/0.5 mg group during the observation period; however, the mean change from baseline best-corrected visual acuity score of the sham/0.5 mg patients remained significantly different from that of the 0.3 mg and 0.5 mg groups at month 12. BCVA = best-corrected visual acuity; ETDRS = Early Treatment Diabetic Retinopathy Study.

Table 2. Change from Baseline Study Eye Best-Corrected Visual Acuity at Month 12

	Sham/0.5 mg (n = 130)	Ranibizumab 0.3 mg (n = 132)	Ranibizumab 0.5 mg (n = 130)
Change from baseline BCVA (ETDRS letter score) at month 12			
Mean (SD)	7.3 (15.9)	13.9 (15.2)	13.9 (14.2)
95% CI for mean	4.5-10.0	11.2-16.5	11.5-16.4
Difference in means (vs. sham/0.5 mg)		6.6	6.7
95% CI for difference		2.8-10.4	3.0-10.4
P-value (ranibizumab vs. sham/0.5 mg)		0.0007	0.0006
Distribution of change at month 12, n (%)			
Gain (letters)			
≥15	43 (33.1)	62 (47.0)	66 (50.8)
10–14	22 (16.9)	23 (17.4)	20 (15.4)
5–9	13 (10.0)	21 (15.9)	14 (10.8)
No change, ±4.0	29 (22.3)	13 (9.8)	23 (17.7)
Loss (letters)			
5–9	7 (5.4)	6 (4.5)	2 (1.5)
10–14	3 (2.3)	2 (1.5)	2 (1.5)
≥15	13 (10.0)	5 (3.8)	3 (2.3)
≥15-letter gain, %			
Month 7	25.4	42.4	43.1
Month 8	26.2	43.9	53.8
Month 9	31.5	43.2	48.5
Month 10	31.5	45.5	51.5
Month 11	30.8	45.5	46.2

BCVA = best-corrected visual acuity; CI = confidence interval; ETDRS = Early Treatment Diabetic Retinopathy Study; SD = standard deviation. Last-observation-carried forward method was used to impute missing data.

Anatomic Outcomes at Month 12

Change from Baseline CFT. At the month 6 time point, the mean change from baseline CFT was a reduction of 433.7 and 452.3 μm in the 0.3 mg and 0.5 mg ranibizumab groups compared with a reduction of 167.7 μm in the sham group. In the 0.3 mg and 0.5 mg treatment groups, these reductions were maintained with as-needed ranibizumab during the observation period, with a mean reduction from baseline CFT of 452.8 and 462.1 μm, respectively, at month 12 (Fig 4). The sham/0.5 mg group experienced an overall improvement in CFT during the observation period, with a mean reduction from baseline of 427.2 μm at month 12. The mean improvement from baseline CFT at month 12 in the sham/0.5 mg group was not significantly less than that of the 0.3 mg or 0.5 mg treatment groups ($P>0.40$ for each ranibizumab group vs sham/0.5 mg).

Most patients in the 0.3 mg, 0.5 mg, and sham/0.5 mg groups who did not receive an injection of as-needed ranibizumab at month 6 showed worsening of CFT from months 6 to 7, with mean increases of 176, 200, and 21 μm, respectively, from months 6 to 7, whereas most who received an injection showed improvement or no change in CFT from months 6 to 7, with mean reductions of 11, 19, and 295 μm, respectively, from months 6 to 7 (Fig 5, available online at http://aaojournal.org).

Residual Edema. In addition to assessing the absolute reduction in CFT, it is important to determine how much macular edema a treatment eliminates. One way to assess this is to determine the percentage of patients with CFT ≤250 μm. At the month 6 time point, 75.0% (0.3 mg) and 76.9% (0.5 mg) of ranibizumab-treated patients had CFT ≤250 μm compared with 23.1% of the sham group patients. At month 12, the percentages in the ranibizumab groups were similar to those at month 6—75.8% (0.3 mg) and 77.7% (0.5 mg)—and had increased markedly to 70.8% in the sham/0.5 mg group (Table 4).

Retinal Hemorrhages. Indirect ophthalmoscopy and/or biomicroscopy by investigators indicated that 0.8% (0.3 mg), 1.5 % (0.5

Table 3. Snellen Equivalent Study Eye Best-Corrected Visual Acuity (BCVA)

Study Eye BCVA (Approximate Snellen Equivalent), n (%)	Baseline			Month 6*			Month 12*		
		Ranibizumab			Ranibizumab			Ranibizumab	
	Sham (n = 130)	0.3 mg (n = 132)	0.5 mg (n = 130)	Sham† (n = 130)	0.3 mg (n = 132)	0.5 mg (n = 130)	Sham/0.5 mg† (n = 130)	0.3 mg (n = 132)	0.5 mg (n = 130)
≥20/20	0	0	0	2 (1.5)	8 (6.1)	17 (13.1)	9 (6.9)	9 (6.8)	11 (8.5)
20/25–20/40	12 (9.2)	9 (6.8)	7 (5.4)	25 (19.2)	50 (37.9)	44 (33.8)	36 (27.7)	48 (36.4)	45 (34.6)
20/50–20/63	36 (27.7)	28 (21.2)	38 (29.2)	26 (20.0)	17 (12.9)	21 (16.2)	23 (17.7)	27 (20.5)	30 (23.1)
20/80–20/160	47 (36.2)	54 (40.9)	46 (35.4)	41 (31.5)	37 (28.0)	33 (25.4)	36 (27.7)	32 (24.2)	28 (21.5)
20/200–20/500	35 (26.9)	40 (30.3)	39 (30.0)	31 (23.8)	18 (13.6)	15 (11.5)	25 (19.2)	16 (12.1)	15 (11.5)
<20/500	0	1 (0.8)	0	5 (3.8)	2 (1.5)	0	1 (0.8)	0	1 (0.8)

Baseline and month 6 data are based on month 6 database.
*Last-observation-carried forward method was used to impute missing data.
†During the 6-month treatment period (day 0–month 5), sham patients received sham injections; during the 6-month observation period (month 6–11), sham patients received 0.5 mg ranibizumab if they met prespecified criteria.

mg), and 1.5% (sham) of patients had no intraretinal hemorrhages at baseline (Fig 6, available online at http://aaojournal.org), whereas 82.6%, 87.7%, and 86.9%, respectively, had >10 hemorrhages. A greater increase was observed in the percentage of patients with no intraretinal hemorrhage in the 0.3 mg and 0.5 mg groups compared with the sham group at month 6 and the sham/0.5 mg group at month 12; and the percentage of patients who had >10 intraretinal hemorrhages decreased more rapidly in the ranibizumab treatment groups compared with the sham/0.5 mg group.

Safety Outcomes at Month 12

Key study eye AEs were infrequent, and the only one with a greater incidence in the ranibizumab groups during the 12-month study period compared with the sham group during the first 6 months and the sham/0.5 mg group during the second 6 months was cataract (Table 5). If this small increase in the incidence of cataract in the ranibizumab groups—3.8% (0.3 mg; 12-month rate) and 7.0% (0.5 mg; 12-month rate) compared with 0% (sham; 6-month rate)—was not due to chance, it could have been related to the procedure (intraocular injections) or to ranibizumab.

There were few nonocular SAEs potentially related to VEGF inhibition (Table 6). Throughout the 12-month study, there were 2 such SAEs in the 0.3-mg group and 4 in the 0.5 mg group, compared with 2 SAEs in the sham group during the first 6 months. This included 1 Antiplatelet Trialists' Collaboration ATE in the 0.3-mg group, 3 in the 0.5 mg group, and 1 in the sham group. There were no nonocular SAEs potentially related to VEGF inhibition in the sham/0.5 mg group between months 6 and 12, when patients were received ranibizumab injections as needed.

Discussion

Monthly intraocular injections of 0.3 mg or 0.5 mg of ranibizumab for 6 months provided substantial benefit in

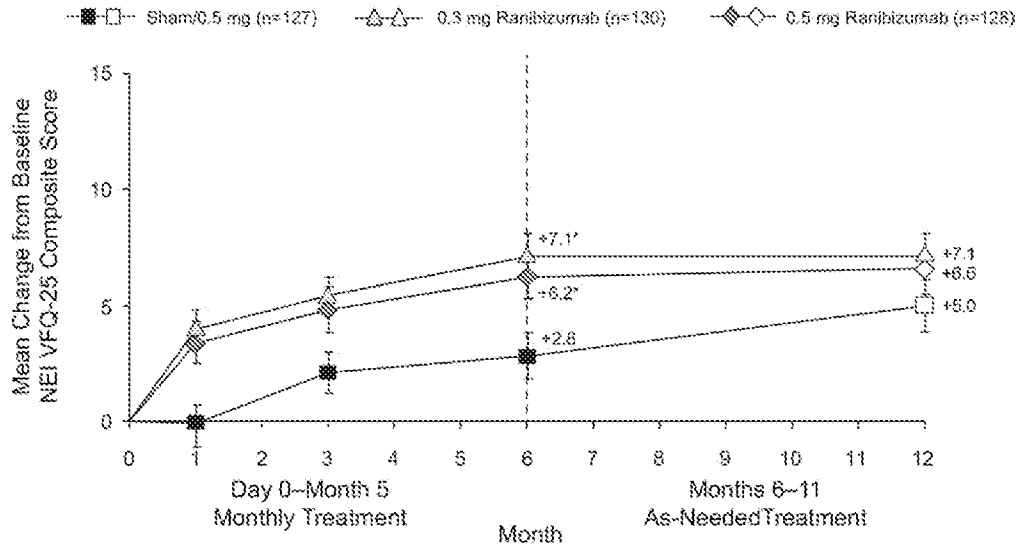

Figure 3. Mean change from baseline National Eye Institute Visual Function Questionnaire-25 composite score over time to month 12. *$P<0.01$ versus sham. The last-observation-carried-forward method was used to impute missing data. Vertical bars are ±1 standard error of the mean. The composite score increased rapidly and was significantly greater in the ranibizumab treatment groups compared with the sham group at month 6. During the observation period, on average, the composite score remained stable in the ranibizumab groups and increased substantially in the sham/0.5 mg group, which was no longer significantly different than the ranibizumab groups at month 12. NEI VFQ-25 = National Eye Institute Visual Functioning Questionnaire-25.

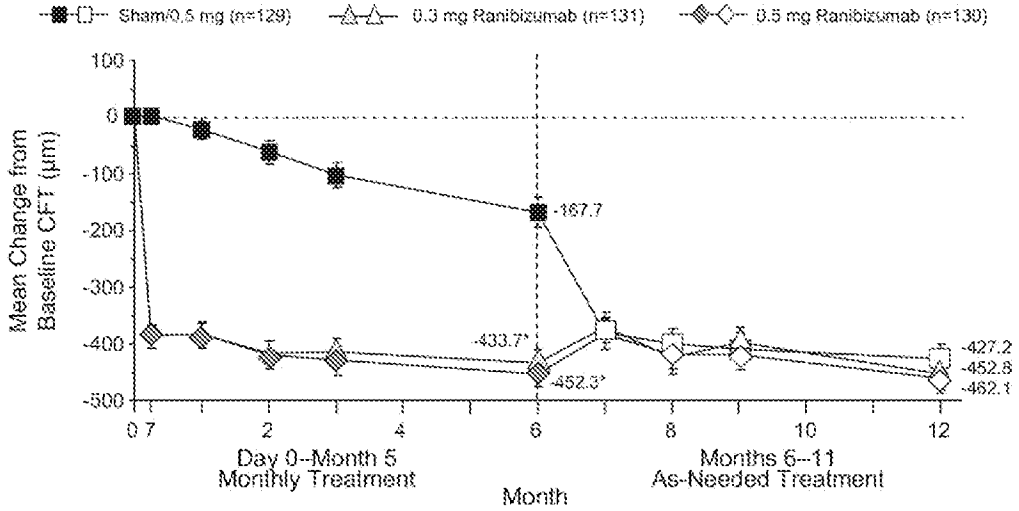

Figure 4. Mean change from baseline central foveal thickness over time to month 12. Month 6 values are based on month 6 database. *$P<0.0001$ versus sham. The last-observation-carried-forward method was used to impute missing values. The earliest significant group difference was at day 7. Vertical bars are ±1 standard error of the mean. On average, improvements in central foveal thickness during the treatment period were maintained in the ranibizumab groups during the observation period. There was substantial improvement in the sham/0.5 mg group during the observation period, and the mean change from baseline central foveal thickness of sham/0.5 mg patients was similar to that of the 0.3 mg and 0.5 mg groups at month 12. CFT = central foveal thickness.

patients with CRVO, resulting in mean improvements from baseline BCVA letter score of 12.7 and 14.9. This benefit was maintained during the subsequent 6 months in which injections were given only if retreatment criteria were met, so that at 12 months, the mean improvement in BCVA letter score was 13.9 in each ranibizumab treatment group. This indicates that after a period of aggressive treatment with ranibizumab, visual benefits can be maintained by close follow-up and treatment if there is evidence of persistent or recurrent disease. What is not answered by this trial is whether even better visual outcomes would have resulted by continuing monthly injections during the second 6 months of the study. In fact, the trial was designed to ensure that during the observation period, patients who might benefit from ranibizumab treatment would receive it. It was thought that even if CFT was ≤250 µm, patients should continue to receive treatment, unless their BCVA had improved to the point that one could potentially question whether the risk/benefit ratio favored another injection. It was our judgment that this level was ≥20/40. However, some investigators questioned whether a patient with CFT ≤250 µm should receive an injection, regardless of BCVA, and deferred treatment. This could be a source of undertreatment during the observation period. It is clear that although monthly injections of ranibizumab suppressed the effects of VEGF in the majority of patients, they did not eliminate VEGF production, because the majority of patients who did not receive an injection of ranibizumab at month 6 had an increase in CFT and reduced vision and required an injection at month 7. During the observation period, recurrent/persistent edema or BCVA ≤20/40 was common, necessitating an injection of ranibizumab approximately two thirds of the time in each of the groups.

Table 4. Study Eye Central Foveal Thickness

	Sham/0.5 mg (n = 130)	Ranibizumab 0.3 mg (n = 132)	Ranibizumab 0.5 mg (n = 130)
Baseline, n (%)			
≤250 µm	2 (1.6)	4 (3.1)	6 (4.6)
>250–400 µm	14 (10.9)	8 (6.1)	8 (6.2)
>400 µm	113 (87.6)	119 (90.8)	116 (89.2)
Month 6*, n (%)			
≤250 µm	30 (23.1)	99 (75.0)	100 (76.9)
>250–400 µm	17 (13.1)	14 (10.6)	12 (9.2)
>400 µm	83 (63.8)	19 (14.4)	18 (13.8)
Month 12*, n (%)			
≤250 µm	92 (70.8)	100 (75.8)	101 (77.7)
>250–400 µm	14 (10.8)	11 (8.3)	13 (10.0)
>400 µm	24 (18.5)	21 (15.9)	16 (12.3)

Baseline and month 6 data are based on month 6 database. One sham/0.5 mg patient and one 0.3 mg patient did not have an assessment at baseline.
*Last-observation-carried-forward method was used to impute missing data at post baseline time points.

On average, there was substantial improvement in the sham/0.5 mg group during the observation period when patients received injections of 0.5 mg of ranibizumab if they met retreatment criteria. In fact, after 77% of sham/0.5 mg patients received an injection of 0.5 mg ranibizumab at month 6, there was a dramatic reduction in macular edema at month 7, and mean CFT was similar to that in the 2 ranibizumab treatment groups and remained so through month 12. There was also substantial improvement in BCVA in the sham/0.5 mg group during the observation period; however, unlike the mean CFT, which no longer differed from that of the 0.3 mg and 0.5 mg ranibizumab groups at month 7 and beyond, there remained a significant difference in mean improvement from baseline BCVA at month 12 between the sham/0.5 mg group and the 0.3 mg

Table 5. Key Study Eye Adverse Events through Month 12[†]

Adverse Events, n (%)	Sham* Day 0–Month 6 (n = 129)	Sham/0.5 mg[†] Months 6–12 (n = 110)	Ranibizumab 0.3 mg Day 0–Month 12 (n = 132)	Ranibizumab 0.5 mg Day 0–Month 12 (n = 129)
Any intraocular inflammation event (iridocyclitis, iritis, vitritis)	5 (3.9)	2 (1.8)	3 (2.3)	2 (1.6)
Endophthalmitis	0	0	0	0
Lens damage	0	0	0	0
Cataract	0	2 (1.8)[‡]	5 (3.8)	9 (7.0)
Iris neovascularization	9 (7.0)	2 (1.8)	2 (1.5)	5 (3.9)
Neovascular glaucoma	2 (1.6)	0	0	1 (0.8)
Rhegmatogenous retinal detachment	0	0	0	0
Retinal tear	0	2 (1.8)[‡]	0	2 (1.6)
Vitreous hemorrhage	9 (7.0)[‡]	2 (1.8)[‡]	7 (5.3)	7 (5.4)

*Outcomes during 6-month treatment period for safety-evaluable sham-group patients (received ≥1 sham injection).
[†]Outcomes during 6-month observation period for safety-evaluable sham/0.5 mg group patients (i.e., received at least one 0.5 mg ranibizumab injection).
[‡]One event reported as serious.

and 0.5 mg groups. Just as it is unknown whether the 0.3 mg and 0.5 mg groups may have had even better outcomes at month 12 if they had continued to receive monthly injections of ranibizumab during the second 6 months of the study, it is unknown whether the sham/0.5 mg group would have had even greater improvement if monthly injections were mandated; however, what is clear is that a 6-month period of monthly treatments followed by treatment as needed for 6 months is superior to observation for 6 months followed by treatment as needed for 6 months. This suggests that there may be a visual penalty incurred by delaying ranibizumab injections in patients with macular edema following CRVO.

In addition to providing a major impact on macular edema, monthly injections of ranibizumab accelerated the resolution of retinal hemorrhages. The mechanism by which hemorrhages are cleared from the retina is not completely understood, but it is felt that macrophages and microglia

Table 6. Key Nonocular Serious Adverse Events through Month 12

	Sham* Day 0–Month 6 (n = 129)	Sham/0.5 mg[†] Months 6–12 (n = 110)	Ranibizumab 0.3 mg Day 0–Month 12 (n = 132)	Ranibizumab 0.5 mg Day 0–Month 12 (n = 129)
Serious Adverse Events Potentially Related to VEGF Inhibition, n (%)				
Hemorrhagic stroke	0	0	0	0
Ischemic stroke	0	0	0	1 (0.8)
Transient ischemic attack	0	0	1 (0.8)	1 (0.8)[‡]
Myocardial infarction	1 (0.8)	0	1 (0.8)	1 (0.8)
Angina pectoris	0	0	0	1 (0.8)[‡]
Hypertension	1 (0.8)	0	0	0
Nonocular hemorrhage, other	0	0	0	0
Proteinuria	0	0	0	0
APTC ATEs, n (%)	1 (0.8)	0	1 (0.8)	3 (2.3)
Vascular death	0	0	0	0
Death from unknown cause	0	0	0	1 (0.8)
Nonfatal myocardial infarction	1 (0.8)	0	1 (0.8)	1 (0.8)
Nonfatal hemorrhagic stroke	0	0	0	0
Nonfatal ischemic stroke	0	0	0	1 (0.8)

APTC ATEs = Antiplatelet Trialists' Collaboration arterial thromboembolic events; VEGF = vascular endothelial growth factor.
*Outcomes during 6-month treatment period for safety-evaluable sham-group patients (i.e., received at least one sham injection).
[†]Outcomes during 6-month observation period for safety-evaluable sham/0.5 mg group patients (i.e., received at least one 0.5 mg ranibizumab injection).
[‡]Both events occurred in the same patient.

play a role. Because VEGF promotes influx of macrophages in the retina, and this is suppressed by VEGF blockade, it is unlikely that ranibizumab accelerates the removal of hemorrhages from the retina. Another possibility is that hemorrhages do not occur all at once at the onset of retinal vein occlusion, but rather are ongoing. Perhaps the large and sustained increases in VEGF that occur in retinal vein occlusions compromise the blood–retinal barrier to such an extent that influx of red blood cells accompanies influx of plasma. Thus, the rate of clearance of hemorrhage may result from 2 opposing processes: Ongoing hemorrhage that is gradually reduced under normal circumstances, and removal of hemorrhages by macrophages and microglia. Ranibizumab may help to reduce the influx of RBCs, just as it reduces influx of plasma, and thus tip the balance toward hemorrhage removal, resulting in more rapid clearance of hemorrhages.

These data suggest that RVOs are not simply acute events followed by gradual recovery that is accelerated by blockade of VEGF. Instead, it seems that vascular occlusion is an inciting event that causes reduced perfusion, retinal ischemia, and increased production of VEGF. The level of upregulation of VEGF is likely to be influenced by several factors, including the amount of compromise of perfusion from the occlusion itself (possibly related to the location or extent of occlusion); the amount of preexistent arterial insufficiency; and the amount of retinal infarction, which can reduce the total area of retinal ischemia. If the upregulation of VEGF is sufficiently high, it can become a major exacerbating factor. This may explain why the level of VEGF at baseline has an inverse correlation with visual outcome.[4]

In conclusion, 6 monthly intraocular injections of ranibizumab in patients with CRVO resulted in large gains in BCVA and improved quality of life that were maintained over a subsequent 6 months during which ranibizumab was given as needed. Patients met retreatment criteria and received injections roughly two thirds of the time during the observation period, and it is likely that treatment for longer than a year will be needed for many patients, as demonstrated in a previous uncontrolled trial.[10] Additional studies are needed to provide longer follow-up of patients with CRVO treated with ranibizumab to determine whether dependence on injections is reduced over time and whether strategies such as scatter photocoagulation to areas of retinal nonperfusion provide added benefit.

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References

1. Klein R, Moss SE, Meuer SM, Klein BE. The 15-year cumulative incidence of retinal vein occlusion: the Beaver Dam Eye Study. Arch Ophthalmol 2008;126:513–8.
2. Mitchell P, Smith W, Chang A. Prevalence and associations of retinal vein occlusion in Australia: the Blue Mountains Eye Study. Arch Ophthalmol 1996;114:1243–7.
3. Green WR, Chan CC, Hutchins GM, Terry JM. Central vein occlusion: a prospective histological study of 29 eyes in 28 cases. Trans Am Ophthalmol Soc 1981;79:371–422.
4. Campochiaro PA, Hafiz G, Shah SM, et al. Ranibizumab for macular edema due to retinal vein occlusions: implication of VEGF as a critical stimulator. Mol Ther 2008;16:791–9.
5. Spaide RF, Chang LK, Klancnik JM, et al. Prospective study of intravitreal ranibizumab as a treatment for decreased visual acuity secondary to central retinal vein occlusion. Am J Ophthalmol 2009;147:298–306.
6. Pieramici DJ, Rabena M, Castellarin AA, et al. Ranibizumab for the treatment of macular edema associated with perfused central retinal vein occlusions [report online]. Ophthalmology 2008;115:e47–54. Available at: http://www.ophthalmologyjournaloftheaao.com/article/S0161-6420%2808%2900634-9/abstract. Accessed February 21, 2011.
7. Brown DM, Campochiaro PA, Singh RP, et al, CRUISE Investigators. Ranibizumab for macular edema following central retinal vein occlusion: six-month primary end point results of a phase III study. Ophthalmology 2010;117:1124–33.
8. Campochiaro PA, Heier JS, Feiner L, et al, BRAVO Investigators. Ranibizumab for macular edema following branch retinal vein occlusion: six-month primary endpoint results of a phase III study. Ophthalmology 2010;117:1102–12.
9. Antiplatelet Trialists' Collaboration. Collaborative overview of randomised trials of antiplatelet therapy—I: Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. BMJ 1994;308:81–106.
10. Campochiaro PA, Hafiz G, Channa R, et al. Antagonism of vascular endothelial growth factor for macular edema caused by retinal vein occlusion: two-year outcomes. Ophthalmology 2010;117:2387–94.

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[1] Departments of Ophthalmology and Neuroscience, The Johns Hopkins School of Medicine, Baltimore, Maryland.

[2] Retinal Consultants of Houston, Houston, Texas.

[3] Tennessee Retina, Nashville, Tennessee.

[4] Retina Research Institute of Texas, Abilene, Texas.

[5] Genentech, Inc., South San Francisco, California.

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Roman G. Rubio – Genentech – Employee; Roche – Equity Ownership/Stock Options.

Correspondence:

Peter A. Campochiaro, MD, 719 Maumenee, The Wilmer Eye Institute, The Johns Hopkins School of Medicine, 600 N. Wolfe Street, Baltimore, MD 21287-9277. E-mail: pcampo@jhmi.edu

Safety Implications of Vascular Endothelial Growth Factor Blockade for Subjects Receiving Intravitreal Anti–Vascular Endothelial Growth Factor Therapies

KARL CSAKY AND DIANA V. DO

• PURPOSE: To evaluate potential safety risks associated with nonspecific inhibition of vascular endothelial growth factor (VEGF).

• DESIGN: A perspective, reviewing the current literature.

• METHODS: Herein, we discuss the systemic safety of VEGF-targeted therapies, address safety issues for VEGF-targeted therapies in neovascular age-related macular degeneration, and propose the consideration of methods for identifying low rate systemic safety signals from patients treated with these agents.

• RESULTS: Several prospective, randomized clinical trials have demonstrated that intravitreal anti-VEGF therapies generally are well tolerated. However, within these trials, there is some circumstantial evidence that links systemic VEGF inhibition to systemic adverse events, particularly systemic thromboembolic events. Because all of the intravitreal anti-VEGF agents have been associated with detectable levels in the systemic circulation, there is a scientific rationale for the occurrence of potential systemic adverse events. However, if safety issues are present, they occur at very low rates and may go undetected in controlled clinical trials of premarketed drugs.

• CONCLUSIONS: We propose that highly sensitive methodologies be put into place for identifying low rate safety signals, including postmarketing clinical trials, chart reviews, electronic medical records, and various national and international registries and databases, to evaluate the systemic safety of antiangiogenic agents in ocular diseases such as neovascular age-related macular degeneration. (Am J Ophthalmol 2009;148:647–656. © 2009 by Elsevier Inc. All rights reserved.)

AGE-RELATED MACULAR DEGENERATION (AMD) IS the leading cause of irreversible blindness in developed countries, predominantly affecting adults 50 years of age or older.[1] The vast majority of vision loss resulting from the disease occurs in patients with the exudative, or neovascular, form of AMD. Neovascular AMD often is characterized by the development of choroidal neovascularization (CNV) penetrating the Bruch membrane, disrupting the retinal pigment epithelium, and resulting in scarring and vision loss.[2]

Vascular endothelial growth factor A (VEGF-A) is a proangiogenic growth factor that has been implicated in the pathogenesis of neovascular AMD. Based on the observation that VEGF-A may play a role in CNV secondary to AMD, therapies for neovascular AMD that target VEGF-A and proangiogenic pathways have been or continue to be investigated. These therapies vary greatly in the specificity of their targets (Figure).

Currently, there are two therapies approved by the United States Food and Drug Administration (FDA) that target VEGF-A for the treatment of neovascular AMD: pegaptanib sodium (Macugen; OSI Pharmaceuticals, Melville, New York, USA), which binds $VEGF_{165}$, and ranibizumab (Lucentis; Genentech Inc, South San Francisco, California, USA), which binds all VEGF-A isoforms and their biologically active degradation products. In addition, bevacizumab (Avastin; Genentech Inc), which also binds all VEGF-A isoforms and is FDA approved for the treatment of breast, colorectal, and lung cancers, has been used off-label to treat CNV secondary to neovascular AMD. Bevacizumab currently is being evaluated in a phase 3 clinical trial for this indication. A decoy VEGF receptor known as VEGF Trap also currently is under investigation in phase 3 clinical trials for the treatment of CNV secondary to neovascular AMD. Furthermore, several other agents that target VEGF receptors and downstream signaling pathways are in preclinical and clinical development (Table 1).

Vascular endothelial growth factor ligands and their receptors are essential for development and for a wide variety of physiologic functions through adulthood, including visual function.[4] Indeed, Rosenfeld and associates demonstrated that the most common cause of three-line vision loss among ranibizumab-treated eyes was the development of geographic atrophy (Rosenfeld PJ, et al. Comparison of lesion characteristics between ranibizumab treated patients who lost or gained visual acuity [VA] in the MARINA and ANCHOR trials. Paper presented at the Annual Meeting of the Macula Society, March 26 to 29, 2008). These data suggest that VEGF-A may play a role in the protection of the retinal pigment epithelium; however, geographic atrophy as part of the normal disease

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From the Ophthalmic Unit, Duke Clinical Research Institute, Duke University Medical Center, Durham, North Carolina (K.C.); and The Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland (D.V.D.).

Inquiries to Karl Csaky, Ophthalmic Unit, DCRI, Duke University Medical Center, Durham, NC 27710; e-mail: karl.csaky@duke.edu

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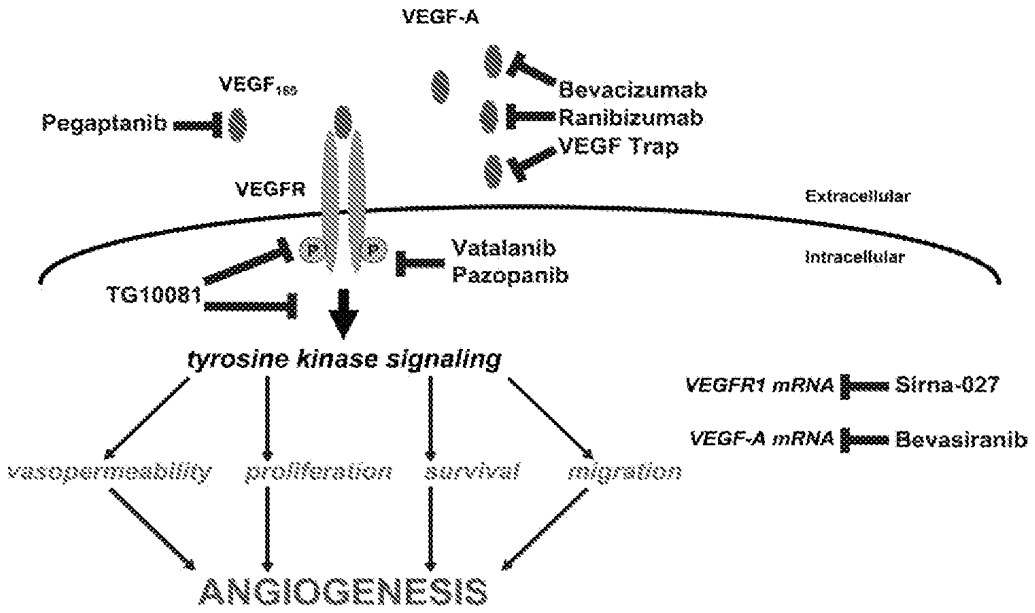

FIGURE. Diagram showing vascular endothelial growth factor (VEGF) signaling pathways, indicating sites of inhibition by current and emerging anti-VEGF agents. Current and pipeline anti-VEGF agents block VEGF signaling at different points in the pathway and have varying degrees of specificity. In general, anti-VEGF antibodies, antibody fragments, and decoy receptors inhibit extracellular VEGF. Pegaptanib specifically blocks the VEGF-A isoform $VEGF_{165}$, whereas bevacizumab, ranibizumab, and VEGF trap block all VEGF-A isoforms. In contrast, tyrosine kinase inhibitors and small interfering ribonucleic acids (RNA) inhibit VEGF-stimulated intracellular signaling. Vatalanib targets VEGF receptor (VEGFR)-1, VEGFR-2, and VEGFR-3 tyrosine kinase activity, and pazopanib targets the activity of multiple tyrosine receptor kinases, including VEGFR-1 and VEGFR-2. TG100801 nonspecifically targets tyrosine kinases, including VEGFRs. Sirna-027 and bevasiranib target VEGFR-1 and VEGF-A messenger RNA.

process after neovascularization is arrested cannot be ruled out.

Given the ubiquitous physiologic role of VEGF, evaluation of potential safety risks associated with nonspecific inhibition of VEGF-A and other VEGF-related targets is imperative. Herein, we discuss potential safety issues with current VEGF-targeted drug therapies in neovascular AMD and consider methods for identifying low rate systemic safety signals from neovascular AMD patients treated with these agents.

ROUTE OF ADMINISTRATION OF ANTI–VASCULAR ENDOTHELIAL GROWTH FACTOR AGENTS AND SYSTEMIC SAFETY

THE ROUTE OF ADMINISTRATION MUST BE CONSIDERED TO understand potential systemic effects of anti-VEGF agents used to treat neovascular AMD. Interestingly, intraocular drugs also have been found in the systemic circulation, despite the presence of the blood-ocular barrier (ie, blood-retinal barrier), which shields the retina from circulating blood.[5] Ocular drugs that are injected into the eye may enter the systemic circulation after absorption through uveal vessels (iris or ciliary body) or by aqueous humor outflow (through the trabecular meshwork into the episcleral vessels).[6]

Systemic circulation of topically applied ocular drugs (eg, eye drops, gels, or ointments) also is possible through several other routes. Penetration of topical ocular treatments into ocular tissues is relatively poor, and thus the active ingredients in ophthalmic drugs often are highly concentrated.[7] Approximately 40% of a standard 50-μl eye drop directly enters the highly vascular tear drainage apparatus,[6] which results in drug absorption through mucous vessels in the nasal cavity. The conjunctiva represents another route of drug entry into the systemic circulation. It has been estimated that up to 80% of topical ocular drugs may reach the systemic circulation after their ocular administration.[8] For example, timolol maleate (Timoptic; Merck & Co Inc, Whitehouse Station, New Jersey, USA), a β-adrenergic receptor blocker used as a topical ocular therapy for glaucoma, can enter the systemic circulation and cause significant adverse events (AEs). Although the amount of timolol that reaches the systemic circulation is suspected to be low, these levels nevertheless have produced significant AEs (eg, cardiovascular and bronchopulmonary AEs) in predisposed patients; however, these AEs were not discovered until after timolol was marketed.[9,10] Timolol

TABLE 1. Anti-Vascular Endothelial Growth Factor Agents Categorized Based on Specificity of Target in the Angiogenesis Pathways

Agent	Administration	Mechanism of Action	Clinical Development Status
VEGF-A targeted			
Pegaptanib sodium (EyeTech Pharmaceuticals)	Intravitreal	Anti–VEGF-A aptamer that targets $VEGF_{165}$ and larger isoforms	FDA approved in 2004
Ranibizumab (Genentech Inc)	Intravitreal	Humanized anti–VEGF-A antibody-binding fragment that binds all isoforms and biologically active degradation products	FDA approved in 2006
Bevacizumab (Genentech Inc)	Intravitreal	Humanized anti–VEGF-A antibody that binds all isoforms and biologically active degradation products	Off-label use; ongoing phase 3 CATT trial to compare ranibizumab with bevacizumab in neovascular AMD
VEGF Trap[a] (Regeneron Pharmaceuticals)	Intravitreal	Soluble fusion protein of VEGFR-1 and VEGFR-2 Ig domains fused to IgG Fc domain; binds VEGF-A	Ongoing phase 3 study (VIEW 1) comparing VEGF Trap with ranibizumab for neovascular AMD
Bevasiranib[b] (Acuity Pharmaceuticals)	Intravitreal	siRNA that targets VEGF-A	Ongoing phase 3 clinical trial in combination with ranibizumab for neovascular AMD
VEGFR targeted			
Sirna-027[b] (Sirna Therapeutics)	Intravitreal	siRNA directed against VEGFR-1	Ongoing phase 2 clinical trial in neovascular AMD
TG100801 (TargeGen Pharmaceuticals)	Topical	Multitarget tyrosine kinase (including VEGFR) antagonist that blocks neovascularization in preclinical trials	Ongoing phase 2 trial in neovascular AMD
Vatalanib[a] (Novartis Pharma AG/Schering AG)	Oral	Receptor kinase inhibitor that targets VEGFR-1, VEGFR-2, and VEGFR-3	Ongoing phase 1/2 trial in combination with PDT for treatment of neovascular AMD
Pazopanib[a] (GlaxoSmithKline)	Topical	Small-molecule tyrosine kinase inhibitor of VEGFR-1, VEGFR-2, and VEGFR-3	A phase 1 trial in neovascular AMD was recently completed (clinicaltrials.gov ID no. NCT00463320)

AMD = age-related macular degeneration; CATT = Comparison of AMD Treatment Trials; FDA = Food and Drug Administration; Ig = immunoglobulin; PDT = photodynamic therapy; siRNA = small interfering ribonucleic acid; VEGF = vascular endothelial growth factor; VEGFR = vascular endothelial growth factor receptor.

[a]Also being evaluated for treatment of solid tumor cancers.

[b]Despite encouraging findings from gene-targeted therapies, the effects of bevasiranib and Sirna-027 recently were attributed in part to a class effect, with both agents nonspecifically targeting toll-like receptor 3 (TLR3), potentially through another method of administration.[3]

was suspected of contributing to 32 deaths within the first 7 postmarketing years.[11]

SYSTEMIC SAFETY OF SYSTEMICALLY ADMINISTERED ANTI–VASCULAR ENDOTHELIAL GROWTH FACTOR AGENTS

SYSTEMIC AES WITH VEGF ISOFORM INHIBITION MUST BE considered because of the ubiquitous distribution of the molecule and because VEGF isoform levels can change in response to disease conditions. It is possible that patients with lower systemic levels of VEGF-A have lower thresholds of tolerance to anti-VEGF agents and are at higher risk for systemic AEs that result directly from VEGF inhibition. To gain an understanding of potential systemic AEs that may emerge with intravitreal administration of anti-VEGF agents, we review the effects of such agents when administered systemically.

In some patients treated with systemic anti–VEGF-A agents, inhibition of essential VEGF-A functions has been detrimental to systemic health. VEGF-A is believed to function as a homeostatic factor for blood pressure (BP), and inhibition of this function is thought to increase vascular tension.[12] For example, systemic inhibition of VEGF-A has been associated with an increased risk of arterial thromboembolic events (ATEs) in colorectal cancer patients treated with intravenous bevacizumab. In a phase 3 trial of bevacizumab in combination with chemotherapy, grade 3 hypertension was reported in 4.0% of patients receiving bevacizumab compared with 1.0% receiving placebo; ATEs occurred in 2.0% of patients receiving bevacizumab compared with 1.0% receiving placebo.[13] In another phase 3 trial of bevacizumab in combination with chemotherapy, grade 3 hypertension was reported in 16% and 3% of patients receiving bevacizumab plus chemotherapy vs chemotherapy, respectively.[13]

An analysis of 5 randomized controlled trials that evaluated safety of bevacizumab in 1,745 patients with colorectal,

TABLE 2. Ocular and Systemic Safety Rates[a] for Ranibizumab from Randomized Clinical Trials

	FOCUS – 2 Year		MARINA – 2 Years			ANCHOR – 2 Years			PIER – 2 Years			SAILOR[b] – 1 Year	
			Ranibizumab			Ranibizumab			Ranibizumab			Ranibizumab	
	0.5 mg Ranibizumab + PDT (n = 105)	PDT (n = 56)	0.3 mg (n = 238)	0.5 mg (n = 239)	Sham (n = 236)	0.3 mg (n = 137)	0.5 mg (n = 140)	PDT (n = 143)	0.3 mg (n = 59)	0.5 mg (n = 61)	Sham[c] (n = 62)	0.3 mg (n = 1,169)	0.5 mg (n = 1,209)
Ocular AE, n (%)													
Presumed endophthalmitis	3 (2.9)	0	2 (0.8)	3 (1.3)	0	0	3 (2.1)	0	0	0	—	2 (0.2)	5 (0.4)
Uveitis	4 (3.8)	0	3 (1.3)	3 (1.3)	0	0	1 (0.7)	0	0	0	—	0	1 (0.1)
Traumatic cataract	0	0	0	1 (0.4)	0	0	0	0	0	0	—	0	0
Rhegmatogenous retinal detachment	0	0	0	0	1 (0.4)	2 (1.5)	0	1 (0.7)	0	0	—	1 (0.1)	0
Retinal tear	1 (1.0)	0	1 (0.4)	1 (0.4)	0	0	1 (0.7)	0	0	0	—	0	1 (0.1)
Vitreous hemorrhage	2 (1.9)	2 (3.6)	1 (0.4)	1 (0.4)	2 (0.8)	2 (1.5)	0	0	0	0	—	4 (0.3)	1 (0.1)
Systemic AE, n (%)													
APTC ATE	5 (4.8)	4 (7.1)	11 (4.6)	11 (4.6)	9 (3.8)	6 (4.4)	7 (5.0)	6 (4.2)	1 (1.7)	0	—	30 (2.6)	34 (2.8)
Nonfatal MI	0	3 (5.4)	8 (3.4)	3 (1.3)	4 (1.7)	1 (0.7)	5 (3.6)	2 (1.4)	0	0	—	14 (1.2)	15 (1.2)
Nonfatal stroke	5 (4.8)	0	3 (1.3)	8 (3.3)	3 (1.3)	3 (2.2)	0	2 (1.4)	1 (1.7)	0	—	8 (0.7)	15 (0.2)
Vascular death	0	1 (1.8)	3 (1.3)	3 (1.3)	4 (1.7)	2 (1.5)	2 (1.4)	3 (2.1)	1 (1.7)	0	—	12 (1.0)	11 (0.9)

AE = adverse event; APTC = Antiplatelet Trialists' Collaboration; ATE = arterial thromboembolic event; MI = myocardial infarction; PDT = photodynamic therapy.

[a]Values shown for safety-evaluable patients.

[b]Cohort 1.

[c]Two-year data for sham cohort not shown because most patients switched to ranibizumab after year 1.

breast, and lung cancers demonstrated that the addition of bevacizumab to chemotherapy increased the risk for an ATE (hazard ratio, 2.0; 95% confidence interval, 1.05 to 3.75; $P = .031$) compared with chemotherapy alone.[14] Risk factor analysis revealed that development of an ATE was associated significantly with prior occurrence of an ATE ($P < .001$) and age older than 65 years ($P = .01$). The average age of patients in this study was not disclosed, but the majority of patients were younger than 65 years. Although the precise role of anti–VEGF-A agents in the development of ATEs is not understood fully, it has been suggested that inhibition of VEGF-A may disrupt the regulated expression of proinflammatory genes that promote arteriovascular disease leading to thrombosis.[14]

In the management of cancer, where VEGF-A plays a critical role in tumor vascularization, currently there is a lack of agreement about the clinical relevance of circulating vs tumor VEGF-A levels. Furthermore, the absence of a predefined cut-off value for determining clinical usefulness of VEGF-A measurement for therapy selection, as well as the absence of a standard VEGF-A detection assay, preclude the use of such measurements in treating solid tumors.[15]

ANTIANGIOGENIC AGENTS FOR NEOVASCULAR AGE-RELATED MACULAR DEGENERATION: OCULAR AND SYSTEMIC SAFETY

• **PEGAPTANIB SODIUM:** Pegaptanib sodium is a 28-base ribonucleic aptamer that binds with high affinity to $VEGF_{165}$ and larger isoforms. In the phase 3 VEGF Inhibition Study in Ocular Neovascularization (VISION) clinical trial, intravitreal pegaptanib sodium was well tolerated. Serious ocular AEs associated with pegaptanib sodium in the first year of the VISION were endophthalmitis, traumatic cataract, and retinal detachment, which were attributed to the injection preparation or procedure rather than to the drug itself.[16] No AEs related to systemic VEGF-A inhibition were identified; however, patients with a history or evidence of severe cardiac disease or myocardial infarction (MI) within 6 months and stroke within 1 year before the study were excluded from the VISION trial.[16]

• **RANIBIZUMAB:** Ranibizumab is a humanized antigen-binding fragment (48 kD) with a broader molecular target profile than pegaptanib sodium, binding to all isoforms of VEGF-A and their biologically active degradation products. Ranibizumab is the first and only FDA-approved treatment for neovascular AMD that improves vision in patients. In the phase 3 Anti–VEGF Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization in AMD (ANCHOR) and Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular AMD (MARINA) trials, intravitreal ranibizumab injections were associated with a low rate of ocular AEs, including endophthalmitis, uveitis, and transient increases in intraocular pressure. In these trials, a slightly increased but still low rate of systemic AEs, such as Antiplatelet Trialists' Collaboration (APTC) ATEs and nonocular hemorrhages, were reported (Boyer DS, et al. A safety overview of ranibizumab in patients with wet AMD: ANCHOR, MARINA, PIER, and SAILOR. Presented at the Annual Meeting of the American Academy of Ophthalmology, November 8 to 11, 2008, Atlanta, Georgia).[17,18] Two-year data from the phase 3 MARINA trial showed that the rate of APTC ATEs was 4.6% in both ranibizumab dosage groups compared with 3.8% in the sham injection group (Table 2), and serious nonocular hemorrhages were reported in 2.1% and 1.3% of patients in the 0.5-mg and 0.3-mg ranibizumab groups, respectively, compared with 0.8% of patients in the sham group. At 2-year follow-up of the phase 3 ANCHOR trial, APTC ATE rates were 5.0% of patients in the 0.5-mg group compared with 4.4% in the 0.3-mg group and 4.2% in the verteporfin photodynamic therapy (PDT) group (Table 2); serious nonocular hemorrhages were reported in 2.1% and 2.9% of patients in the 0.5-mg and 0.3-mg ranibizumab groups, respectively, compared with 0.7% of patients in the verteporfin group.

Low rates of MI and stroke were reported in MARINA and ANCHOR. Although MARINA and ANCHOR did not exclude patients with a recent MI or stroke, investigators could exclude patients with any condition that might have contraindicated the use of an investigational drug or put the subject at high-risk for treatment complication. The rates of MI and stroke for all patients enrolled in MARINA were 1.8% and 1.5%, respectively, and in ANCHOR these were 1.5% and 1.2%, respectively (at the 24-month follow-up).[17,18] The annual rates of MI and stroke in a general inpatient population are 2.2% and 4.1%, respectively.[19]

In the 2-year, Phase IIIb, Multicenter, Randomized, Double-Masked Sham Injection-Controlled Study of the Efficacy and Safety of Ranibizumab in Subjects with Subfoveal Choroidal Neovascularization with or without Classic CNV Secondary to AMD (PIER) clinical trial, in which ranibizumab (0.3 mg and 0.5 mg) was administered as 3 monthly injections followed by quarterly injections, few ocular and systemic AEs were reported. One stroke (1.7%) was reported in a patient receiving 0.3 mg ranibizumab; no MIs were reported among patients receiving either dose of ranibizumab (Table 2; Boyer DS, et al. A safety overview of ranibizumab in patients with wet AMD: ANCHOR, MARINA, PIER, and SAILOR. Presented at the Annual Meeting of the American Academy of Ophthalmology, November 8 to 11, 2008, Atlanta, Georgia). In the 2-year, phase 1/2 rHu-Fab V2 Ocular Treatment Combining the Use of Visudyne to Evaluate Safety (FOCUS) trial, in which patients received 0.5 mg ranibizumab plus PDT or PDT alone, APTC ATE rates were

4.8% and 7.1%, respectively (Table 2). No MIs were reported in the ranibizumab plus PDT treatment arm; however, 4.8% of patients experienced a stroke (Table 2). In the 1-year, phase 3b Safety Assessment of Intravitreal Lucentis for AMD (SAILOR) trial (cohort 1), in which patients received 0.3 mg or 0.5 mg intravitreal ranibizumab followed by treatment as needed, APTC ATE rates were 2.6% and 2.8%, respectively (Table 2). The rate of MI was 1.2% for each ranibizumab dose group. The rate of stroke was higher among patients receiving 0.5 mg ranibizumab (1.2%) than among patients receiving 0.3 mg ranibizumab (0.6%), although this difference was not statistically significant (Boyer DS, et al. A safety overview of ranibizumab in patients with wet AMD: ANCHOR, MARINA, PIER, and SAILOR. Presented at the Annual Meeting of the American Academy of Ophthalmology, November 8 to 11, 2008, Atlanta, Georgia).

A meta-analysis of the systemic safety of intravitreal ranibizumab, based on a pooling of first-year data from MARINA, ANCHOR, PIER, FOCUS, and SAILOR and on second-year data from MARINA, ANCHOR, PIER, and FOCUS showed that overall APTC ATE rates for the total patient population are similar across treatment groups (Boyer DS, et al. A safety overview of ranibizumab in patients with wet AMD: ANCHOR, MARINA, PIER, and SAILOR. Presented at the Annual Meeting of the American Academy of Ophthalmology, November 8 to 11, 2008, Atlanta, Georgia). Further analysis of APTC ATE rates for the aforementioned clinical trials is ongoing.

• **BEVACIZUMAB:** Bevacizumab is a full-length monoclonal antibody (148 kD) that binds to all isoforms of VEGF-A and its bioactive degradation forms. Bevacizumab targets the same VEGF-A isoforms as ranibizumab and, when administered intravitreally, is detected longer in systemic circulation than intravitreal ranibizumab.[20–22]

In a small, nonrandomized clinical study, intravenous bevacizumab for the treatment of neovascular AMD resulted in a significant elevation of systolic and diastolic BP, evident at 3 weeks (compared with baseline; $P < .001$).[23] Although the safety of intravitreal bevacizumab for neovascular AMD has not been investigated in large, randomized, masked clinical trials, retrospective case series evaluating intravitreal bevacizumab have shown that bevacizumab is well tolerated in most patients with neovascular AMD and other retinal and choroidal vascular diseases.[24–26] A prospective, nonrandomized clinical study also demonstrated the ocular and systemic safety of intravitreal bevacizumab for the treatment of neovascular AMD.[27] Furthermore, the International Intravitreal Bevacizumab Safety Survey, which collected self-reported safety data from retinal physicians through the Internet between November 2005 and April 2006 from patients from 12 countries, showed that bevacizumab generally was safe: for 5,228 patients, a total of 7,113 injections were reported. The most common potential drug-related ocular AE was inflammation or uveitis (0.14%), and the most common potential drug-related systemic AE was BP elevation (0.21%).[28] The rates of AEs reported in this survey were lower than those reported for ranibizumab in the MARINA and ANCHOR trials, which raises the possibility that self-reporting methods may not be reliable to determine true safety rates. The safety of intravitreal bevacizumab, as well as its efficacy, compared with ranibizumab currently are being evaluated in the National Eye Institute-sponsored randomized, phase 3 Comparison of AMD Treatment Trials (CATT) Study.

• **VASCULAR ENDOTHELIAL GROWTH FACTOR TRAP:** VEGF Trap is a soluble fusion protein (110 kD) containing the extracellular sequences for VEGF receptors 1 and 2 that consequently acts as a VEGF decoy receptor.[29] Based on its molecular structure, VEGF Trap has very broad targeting, binding to all VEGF family members. VEGF Trap binds VEGF-A with higher affinity than antibodies (it has an approximately 140-fold higher affinity than that of ranibizumab),[30] which may make it active at lower concentrations and may reduce the frequency of dosing relative to other anti-VEGF agents.[31] Based on the size and binding affinity of VEGF Trap, modeling of its predicted biologic activity demonstrated that on days 73, 83, and 87 after a 0.5-mg, 2-mg, and 4-mg injection, respectively, its biologic activity is comparable with that of ranibizumab dosed at 0.5 mg at 30 days.[30] It is unclear whether the higher affinity of VEGF Trap for its targets, its relatively broad molecular targeting, and predicted intravitreal half-life will be associated with greater systemic safety risks.

In a randomized, double-masked, escalating-dose, placebo-controlled phase 1 trial of 25 patients with neovascular AMD treated with intravenous VEGF Trap, a dose-dependent increase in hypertension was observed, and the clinical trial and clinical development of systemic VEGF Trap for neovascular AMD were halted. Systemic VEGF Trap no longer is being evaluated for ocular disease.[32]

Consequently, the Clinical Evaluation of Antiangiogenesis in the Retina (CLEAR-IT) AMD 1 phase 1 study evaluated intravitreal injection of VEGF Trap (0.15 or 4 mg), which was well tolerated, with no ocular inflammation.[33] In the CLEAR-IT AMD two randomized, controlled phase 2 study of the safety and efficacy of VEGF Trap at different doses and dosing regimens, VEGF Trap also generally was well tolerated, with no drug-related systemic AEs. The most common AEs were associated with the intravitreal injections (Do DV, et al. Results of a phase I study of intravitreal VEGF Trap in subjects with diabetic macular edema: the CLEAR-IT DME Study. IOVS 2007;48:ARVO E-Abstract 1430/B486). The VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet AMD (VIEW) 1 and VIEW 2 are ongoing phase 3 trials designed to investigate different dosing intervals of VEGF Trap and should help to elucidate the ocular and systemic safety profiles of VEGF Trap in neovascular AMD patients.

TABLE 3. Methods for Identifying Safety Signals in Investigational and Approved Drugs

	Description	Pros	Cons
Clinical trials (including postmarketing trials)	Studies that evaluate safety and tolerability of new drugs under development	Postmarketing trials may help to identify AEs not detected during clinical development	Often lack statistical power to detect rare AEs or an increased rate of AEs in certain patient population; ethical concerns with conducting these trials
Meta-analyses	Studies that pool clinical trial data of new or approved drugs	Increase the number of patients evaluated to detect rare adverse events	Often combine trials with heterogeneous study designs and patient populations; may not be statistically powered to detect very rare AEs
Chart reviews	Physician-recorded patient history	Useful for verification of information obtained through other methods	Impractical as first-line approach
EMRs	Health information technology systems of patient records	Computerized search programs; large data sets; allows combination of variables to identify new diagnoses or AEs	Inconsistencies in reporting and oversight of potentially important variables; tend to be small and expensive
Registries and databases	Disease- or product-based databases	Large and relatively inexpensive; sampling of patients with few or no exclusion criteria	Nonrandomized treatment may result in inaccurate assessments based on registry data; require close attention to patient enrollment plans, data collection methods, and study endpoints
	Spontaneous reporting databases	May represent the best method for detecting low-frequency AEs	Underreporting of AEs; delays in reporting; inability to assess incidence rates; reporting bias; lack of control group
Healthcare utilization databases	CMS population-based databases	Large data sets; relatively inexpensive; able to capture data on routine clinical care	Lack of detail; may include misdiagnoses or incomplete diagnoses; difficulty in distinguishing incidence vs prevalence; applicable to elderly or low-income populations only

AE = adverse events; CMS = Center for Medicare and Medicaid Systems; EMR = electronic medical record.

MECHANISMS FOR IDENTIFYING LOW RATE SYSTEMIC SAFETY SIGNALS

IT IS USEFUL TO INTERPRET TRIAL-GENERATED SAFETY PROfiles to help predict AE risk for real-life patients. Notably, however, safety profiles of marketed drugs used in the real-life setting may differ from those anticipated based on clinical trial data because extremely rare AEs may be undetected during clinical trials.

Even large-scale clinical trials, including the MARINA (n = 716), ANCHOR (n = 423), SAILOR (n = 4,300), and the ongoing CATT (n = 1,200) and VIEW 1 (n = 1,200) and VIEW 2 (n = 1200) trials of anti-VEGF agents for the treatment of neovascular AMD may not have sufficient statistical power to detect low rates of AEs, including the differences between rates across treatment arms. For example, a clinical trial involving 50,000 patients would be needed to demonstrate statistical significance in the increase of an AE rate from 0.1% to 0.2%.[34] Even more difficult to detect is an increase in the rate of already established AEs (eg, an increased stroke rate in a group of diabetic patients already prone to stroke).

The clinical significance of identifying low rate systemic safety signals is exemplified by the recent experience with rofecoxib. The failure to recognize the increased risk of rofecoxib-induced MI was a consequence of poor AE reporting and the interpretation of findings from clinical trials insufficiently powered to determine such risks.[35]

It is estimated that adverse drug reactions account for 3% to 6% of hospital admissions and up to 100,000 deaths annually in the United States.[36] It therefore is essential to have highly sensitive methodologies in place for identifying low rate systemic safety signals. Several common approaches are described below and are summarized in Table 3.

- **CLINICAL TRIALS (INCLUDING POSTMARKETING TRIALS):** Clinical trials are designed to evaluate the safety and tolerability of new drugs under development. However, as mentioned previously, most trials do not have sufficient statistical power to detect very rare AEs or an increase in the rate of established AEs in certain patient populations. Furthermore, clinical trials may exclude patients with certain preexisting medical conditions who could have adverse outcomes with comorbidities or

when the trial drugs and other medications are combined. The systematic assessment of potential safety issues in larger postmarketing clinical trials may help to identify AEs not detected during the clinical development stage. However, there are ethical concerns with conducting these trials unless there is evidence that patients may derive benefit from them.

- META-ANALYSES: Meta-analyses may be performed to evaluate larger pools of patient safety data. The strongest meta-analyses are those based on a specific research question on which a hypothesis can be formulated and tested and those that include studies that are relatively homogeneous with respect to patient populations and design. Well-conducted meta-analyses offer an integration of a larger data pool that can provide much stronger support for a drug's safety profile. Nevertheless, meta-analyses often are limited by the inclusion of heterogeneous patient populations and varying doses and treatment regimens. Similar to individual clinical trials, meta-analyses also may lack sufficient statistical power.

- CHART REVIEWS: Although impractical as a first-line approach, chart review remains the gold standard for identifying AEs and may be useful for the verification of information obtained through other methods, such as Centers for Medicare and Medicaid Systems (CMS) databases, etc.

- ELECTRONIC MEDICAL RECORDS: Electronic medical records are health information technology systems that maintain patient records, including several detailed data fields, and that allow a combination of variables to identify new diagnoses or AEs.[37] Computerized search programs, including free-text searches, can be used to detect drug-induced AEs with moderate sensitivity. Such methods for detecting AEs are limited by inconsistencies in reporting and omissions in reporting potentially important variables, such as the use of over-the-counter medications.[38] Although electronic medical records typically provide extensive data sets, they also tend to be small and expensive.[37]

- REGISTRIES AND DATABASES: Registries are large disease-based or product-based databases that include sampling of patients with few or no exclusion criteria; however, because treatment is not randomized, accurate assessment of safety based on registry data requires close attention to patient enrollment plans, data collection methods, and study endpoints. Other databases include spontaneous reporting databases, population-based databases, and healthcare utilization databases.

National and international spontaneous reporting databases, such as those supported by the World Health Organization (WHO), have the advantages of large size (with 200,000 patients included per year)[39] and relatively low cost. However, the disadvantages of these spontaneous reporting databases include underreporting of AEs[40] (eg, it has been estimated that less than 1% of serious AEs are reported to the FDA),[41,42] delay in reporting AEs (which may delay detection of safety signals), inability to assess incidence rates, reporting bias, and lack of a control group.[37] Nonetheless, it has been suggested that spontaneous reporting to these databases is the best method for detecting low-frequency AEs because of the minimal number of case reports (range, 3 to 9) needed for a signal.[39,43]

The FDA is responsible for monitoring safety of marketed drugs by means of its AE Reporting System Database. The database is updated with mandatory reports from pharmaceutical companies of physician- and pharmacist-communicated AEs and with AE reports submitted directly to the FDA's MedWatch program by healthcare professionals (physicians, pharmacists, nurses, and dentists) and patients.[40] Other countries have similar mechanisms to capture AEs reported by healthcare professionals. In the United Kingdom, the Medicines and Healthcare Products Regulatory Agency and the Commission on Human Medicines have collected more than 500,000 reports of AEs through their electronic database.[36] In Canada, the Canadian Adverse Drug Reaction Information System Database is used to monitor the safety of marketed drugs and to corroborate safety trends in other countries (http://www.hc-sc.gc.ca/dhp-mps/alt_formats/hpfb-dgpsa/pdf/medeff/cadris-2-eng.pdf; Accessed: April 20, 2009).

The WHO Collaborating Centre for International Drug Monitoring in Uppsala, Sweden, compiles worldwide data from various national spontaneous reporting systems, including the FDA, thus allowing comparisons of AEs that occur in different countries.[39] With coordination among international organizations, this methodology may amplify the signal for the detection of extremely rare AEs.

International Internet-based databases (or surveys) also may prove useful in estimating the rates of rare drug-related AEs. Self-reported AEs can be solicited from physicians to provide a real-world risk assessment of heterogeneous populations, similar to the aforementioned intravitreal bevacizumab survey.[26] However, the AE rates calculated from such surveys should be interpreted with caution because of the voluntary nature of reporting safety events and the lack of standard and systematic methods of measuring parameters such as BP, intraocular pressure, and inflammation.[44]

Health administration databases such as the CMS database contain data from millions of patients and can be used to assess drug-related AEs. The advantages of these large, linked, administrative databases include the collection of large data sets, relatively low cost, and ability to capture data related to routine clinical care.[37] Limitations include lack of detail (eg, with respect to diagnoses), the potential to include misdiagnoses or incomplete diagnoses, and the difficulty in distinguishing between incidence and prevalence (because patients may have had preexisting medical conditions before their inclusion in the CMS database), as

well as the applicability to elderly or low-income populations only.[37] The FDA currently is coordinating collaborative efforts with CMS to integrate CMS safety data into the AE Reporting System safety analysis (http://www.fda.gov/oc/oms/ofm/budget/2007/HTML/5DrugSafetyPOM.htm; Accessed: April 20, 2009). In addition, pharmacoepidemiologic studies using these large databases can be useful in identifying rare events, as well as drug interactions.

In summary, the specificity of molecular targeting of a therapeutic agent can impact its efficacy and safety profiles: in general, the more broadly acting an agent, the greater is its potential for efficacy, but so, too, is its potential for causing AEs. Two main potential determinants of systemic AEs with intravitreal anti-VEGF therapies include degree of systemic exposure (blood levels) and the degree of systemic anti-VEGF blockade. Data from animal studies suggest that systemic levels after intravitreal injection of 0.5 mg ranibizumab[45] are similar to those after an intravitreal injection of 0.3 mg pegaptanib.[46] However, the exact isoforms that may play a role in maintaining systemic tissue health are unknown. Therefore, it is reasonable to hypothesize that a more selective VEGF blocker (such as pegaptanib)[47] may elicit fewer systemic side effects than a pan-VEGF blocker (such as ranibizumab).[45]

The systemic safety of VEGF-targeted therapies currently is under investigation, with predominantly circumstantial evidence from clinical trials linking VEGF inhibition to systemic AEs. Additional data are needed to ascertain whether intravitreal administration of more broadly targeted antiangiogenic agents is associated more frequently with systemic AEs than the intravitreal administration of more VEGF isoform-selective agents. We propose implementing methodologies, including postmarketing clinical trials, chart reviews, electronic medical records, various national and international registries and databases, and meta-analyses for the detection of low rate safety signals to evaluate systemic safety of antiangiogenic drugs in ocular diseases such as neovascular AMD. Stricter FDA guidelines may be required to enforce policies of premarketing and postmarketing drug safety surveillance.

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REFERENCES

1. Friedman DS, O'Colmain BJ, Munoz B, et al. Prevalence of age-related macular degeneration in the United States. Arch Ophthalmol 2004;122:564–572.
2. Bressler S, Rosberger D. Non-neovascular (non-exudative) age-related macular degeneration. In Guyer D, Yannuzzi L, Chang S, Shields J, Green WR, editors. Retina-Vitreous-Macula. Philadelphia, Pennsylvania: W. B. Saunders Co., 1999: 79–93.
3. Kleinman ME, Yamada K, Takeda A, et al. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature 2008;452:591–597.
4. Saint-Geniez M, Maharaj AS, Walshe TE, et al. Endogenous VEGF is required for visual function: evidence for a survival role on Muller cells and photoreceptors. PLoS ONE 2008;3:e3554.
5. Cunha-Vaz JG. The blood-ocular barriers: past, present, and future. Doc Ophthalmol 1997;93:149–157.
6. Shell JW. Pharmacokinetics of topically applied ophthalmic drugs. Surv Ophthalmol 1982;26:207–218.
7. Labetoulle M, Frau E, Le Jeunne C. Systemic adverse effects of topical ocular treatments. Presse Med 2005;34:589–595.
8. Le Jeunne C, Hugues FC, Munera Y, Haas C. [Systemic effects of eye drops]. Ann Med Interne (Paris) 1990;141:674–677.
9. Botet C, Grau J, Benito P, Coll J, Vivancos J. Timolol ophthalmic solution and respiratory arrest. Ann Intern Med 1986;105:306–307.
10. Van Buskirk EM. Adverse reactions from timolol administration. Ophthalmology 1980;87:447–450.
11. Stewart WC, Garrison PM. Beta-blocker-induced complications and the patient with glaucoma. Newer treatments to help reduce systemic adverse events. Arch Intern Med 1998;158: 221–226.
12. Los M, Roodhart JM, Voest EE. Target practice: lessons from phase III trials with bevacizumab and vatalanib in the treatment of advanced colorectal cancer. Oncologist 2007;12:443–450.
13. Kabbinavar FF, Hambleton J, Mass RD, Hurwitz HI, Bergsland E, Sarkar S. Combined analysis of efficacy: the addition of bevacizumab to fluorouracil/leucovorin improves survival for patients with metastatic colorectal cancer. J Clin Oncol 2005;23:3706–3712.
14. Scappaticci FA, Skillings JR, Holden SN, et al. Arterial thromboembolic events in patients with metastatic carcinoma treated with chemotherapy and bevacizumab. J Natl Cancer Inst 2007;99:1232–1239.
15. Longo R, Gasparini G. Challenges for patient selection with VEGF inhibitors. Cancer Chemother Pharmacol 2007;60: 151–170.
16. Chakravarthy U, Adamis AP, Cunningham ET Jr, et al. Year 2 efficacy results of 2 randomized controlled clinical trials of pegaptanib for neovascular age-related macular degeneration. Ophthalmology 2006;113:1508–1525.
17. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–1431.
18. Brown DM, Michels M, Kaiser PK, Heier JS, Sy JP, Ianchulev T. Ranibizumab versus verteporfin photodynamic therapy for neovascular age-related macular degen-

eration: two-year results of the ANCHOR Study. Ophthalmology 2009;116:57–65.
19. Alexander SL, Linde-Zwirble W, Werther W, et al. Annual rates of arterial thromboembolic events in Medicare neovascular age-related macular degeneration patients. Ophthalmology 2007;114:2174–2178.
20. Bakri SJ, Snyder MR, Reid JM, Pulido JS, Ezzat MK, Singh RJ. Pharmacokinetics of intravitreal ranibizumab (Lucentis). Ophthalmology 2007;114:2179–2182.
21. Gaudreault J, Fei D, Beyer JC, et al. Pharmacokinetics and retinal distribution of ranibizumab, a humanized antibody fragment directed against VEGF-A, following intravitreal administration in rabbits. Retina 2007;27:1260–1266.
22. Bakri SJ, Snyder MR, Reid JM, Pulido JS, Singh RJ. Pharmacokinetics of intravitreal bevacizumab (Avastin). Ophthalmology 2007;114:855–859.
23. Moshfeghi AA, Rosenfeld PJ, Puliafito CA, et al. Systemic bevacizumab (Avastin) therapy for neovascular age-related macular degeneration: 24-week results of an uncontrolled open-label clinical study. Ophthalmology 2006;113:e1–e12.
24. Avery RL, Pieramici DJ, Rabena MD, Castellarin AA, Nasir MA, Giust MJ. Intravitreal bevacizumab (Avastin) for neovascular age-related macular degeneration. Ophthalmology 2006;113:363–372.e5.
25. Spaide RF, Laud K, Fine HF, et al. Intravitreal bevacizumab treatment of choroidal neovascularization secondary to age-related macular degeneration. Retina 2006;26:383–390.
26. Wong LJ, Desai RU, Jain A, et al. Surveillance for potential adverse events associated with the use of intravitreal bevacizumab for retinal and choroidal vascular disease. Retina 2008;28:1151–1158.
27. Bashshur ZF, Haddad ZA, Schakal A, Jaafar RF, Saab M, Noureddin BN. Intravitreal bevacizumab for treatment of neovascular age-related macular degeneration: a one-year prospective study. Am J Ophthalmol 2008;145:249–256.
28. Fung AE, Rosenfeld PJ, Reichel E. The International Intravitreal Bevacizumab Safety Survey: using the internet to assess drug safety worldwide. Br J Ophthalmol 2006;90:1344–1349.
29. Konner J, Dupont J. Use of soluble recombinant decoy receptor vascular endothelial growth factor trap (VEGF trap) to inhibit vascular endothelial growth factor activity. Clin Colorectal Cancer 2004;4:S81–S85.
30. Stewart MW, Rosenfeld PJ. Predicted biological activity of intravitreal VEGF trap. Br J Ophthalmol 2008;92:667–668.
31. Economides AN, Carpenter LR, Rudge JS, et al. Cytokine traps: multi-component, high-affinity blockers of cytokine action. Nat Med 2003;9:47–52.
32. Kaiser PK. Anti-vascular endothelial growth factor agents and their development: therapeutic implications in ocular diseases. Am J Ophthalmol 2006;142:660–668.
33. Nguyen QD, Shah SM, Hafiz G, et al. A phase I trial of an IV-administered vascular endothelial growth factor trap for treatment in patients with choroidal neovascularization due to age-related macular degeneration. Ophthalmology 2006;113:1522e1–1522e14.
34. Berlin JA, Glasser SC, Ellenberg SS. Adverse event detection in drug development: recommendations and obligations beyond phase 3. Am J Public Health 2008;98:1366–1371.
35. Mukherjee D, Nissen SE, Topol EJ. Risk of cardiovascular events associated with selective COX-2 inhibitors. JAMA 2001;286:954–959.
36. Jones D. Keeping vigilant about drug safety. Nat Rev Drug Discov 2007;6:855–856.
37. Wilson AM, Thabane L, Holbrook A. Application of data mining techniques in pharmacovigilance. Br J Clin Pharmacol 2004;57:127–134.
38. Honigman B, Lee J, Rothschild J, et al. Using computerized data to identify adverse drug events in outpatients. J Am Med Inform Assoc 2001;8:254–266.
39. Meyboom RH, Egberts AC, Edwards IR, Hekster YA, de Koning FH, Gribnau FW. Principles of signal detection in pharmacovigilance. Drug Saf 1997;16:355–365.
40. Ahmad SR. Adverse drug event monitoring at the Food and Drug Administration. J Gen Intern Med 2003;18:57–60.
41. Scott HD, Rosenbaum SE, Waters WJ, et al. Rhode Island physicians' recognition and reporting of adverse drug reactions. R I Med J 1987;70:311–316.
42. Rogers AS, Israel E, Smith CR, et al. Physician knowledge, attitudes, and behavior related to reporting adverse drug events. Arch Intern Med 1988;148:1596–1600.
43. Edwards IR, Lindquist M, Wiholm BE, Napke E. Quality criteria for early signals of possible adverse drug reactions. Lancet 1990;336:156–158.
44. Ziemssen F, Grisanti S, Bartz-Schmidt KU. The international intravitreal bevacizumab safety survey. Br J Ophthalmol 2006;90:1440–1441.
45. Gaudreault J, Fei D, Rusit J, Suboc P, Shiu V. Preclinical pharmacokinetics of ranibizumab (rhuFabV2) after a single intravitreal administration. Invest Ophthalmol Vis Sci 2005;46:726–733.
46. Drolet DW, Nelson J, Tucker CE, et al. Pharmacokinetics and safety of an anti-vascular endothelial growth factor aptamer (NX1838) following injection into the vitreous humor of rhesus monkeys. Pharm Res 2000;17:1503–1510.
47. Moshfeghi AA, Puliafito CA. Pegaptanib sodium for the treatment of neovascular age-related macular degeneration. Expert Opin Investig Drugs 2005;14:671–682.

One-Year Outcomes of the DA VINCI Study of VEGF Trap-Eye in Eyes with Diabetic Macular Edema

*Diana V. Do, MD,[1] Quan Dong Nguyen, MD, MSc,[1] David Boyer, MD,[2] Ursula Schmidt-Erfurth, MD,[3] David M. Brown, MD,[4] Robert Vitti, MD,[5] Alyson J. Berliner, MD,[5] Bo Gao, PhD,[5] Oliver Zeitz, MD,[6,7] Rene Ruckert, MD,[6] Thomas Schmelter, PhD,[6] Rupert Sandbrink, MD, PhD,[6,8] Jeff S. Heier, MD,[9] for the DAVINCI Study Group**

Purpose: To compare different doses and dosing regimens of Vascular Endothelial Growth Factor (VEGF) Trap-Eye with laser photocoagulation in eyes with diabetic macular edema (DME).

Design: Randomized, double-masked, multicenter, phase 2 clinical trial.

Participants: Diabetic patients (n = 221) with center-involved DME.

Methods: Participants were assigned randomly to 1 of 5 treatment regimens: VEGF Trap-Eye 0.5 mg every 4 weeks (0.5q4); 2 mg every 4 weeks (2q4); 2 mg every 8 weeks after 3 initial monthly doses (2q8); or 2 mg dosing as needed after 3 initial monthly doses (2PRN), or macular laser photocoagulation.

Main Outcome Measures: The change in best-corrected visual acuity (BCVA) at 24 weeks (the primary end point) and at 52 weeks, proportion of eyes that gained 15 letters or more in Early Treatment of Diabetic Retinopathy Study (ETDRS) BCVA, and mean changes in central retinal thickness (CRT) from baseline.

Results: As previously reported, mean improvements in BCVA in the VEGF Trap-Eye groups at week 24 were 8.6, 11.4, 8.5, and 10.3 letters for 0.5q4, 2q4, 2q8, and 2PRN regimens, respectively, versus 2.5 letters for the laser group ($P \leq 0.0085$ versus laser). Mean improvements in BCVA in the VEGF Trap-Eye groups at week 52 were 11.0, 13.1, 9.7, and 12.0 letters for 0.5q4, 2q4, 2q8, and 2PRN regimens, respectively, versus −1.3 letters for the laser group ($P \leq 0.0001$ versus laser). Proportions of eyes with gains in BCVA of 15 or more ETDRS letters at week 52 in the VEGF Trap-Eye groups were 40.9%, 45.5%, 23.8%, and 42.2% versus 11.4% for laser ($P = 0.0031$, $P = 0.0007$, $P = 0.1608$, and $P = 0.0016$, respectively, versus laser). Mean reductions in CRT in the VEGF Trap-Eye groups at week 52 were −165.4 μm, −227.4 μm, −187.8 μm, and −180.3 μm versus −58.4 μm for laser ($P < 0.0001$ versus laser). Vascular Endothelial Growth Factor Trap-Eye generally was well tolerated. The most frequent ocular adverse events with VEGF Trap-Eye were conjunctival hemorrhage, eye pain, ocular hyperemia, and increased intraocular pressure, whereas common systemic adverse events included hypertension, nausea, and congestive heart failure.

Conclusions: Significant gains in BCVA from baseline achieved at week 24 were maintained or improved at week 52 in all VEGF Trap-Eye groups. Vascular Endothelial Growth Factor Trap-Eye warrants further investigation for the treatment of DME.

Financial Disclosure(s): Proprietary or commercial disclosure may be found after the references.
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Diabetic macular edema (DME) is the most common cause of vision loss for patients with diabetes mellitus.[1] The Wisconsin Epidemiologic Study found that the prevalence of macular edema was associated with an increasing duration of diabetes.[2,3] Worldwide, the prevalence of adult diabetes is anticipated to rise from 4.0% in 1995 to 5.4% by 2025.[4] Given this rising prevalence, it is expected that diabetic retinopathy and DME will continue to be common and will be important causes of vision impairment.

The complex pathophysiology of DME has been under investigation in recent years. In individuals with diabetic retinopathy, fluid can accumulate within the retina as a result of a breakdown in the blood–retinal barrier. Hyperglycemia associated with diabetes stimulates an inflammatory response, which causes detrimental effects on the retinal vasculature.[5] Vascular occlusion and ischemia results, and can lead to local hypoxia.[6] Vascular endothelial growth factor (VEGF) and a host of other growth factors are upregulated during hypoxic conditions, and an inflammatory cascade of events can ensue.

Vascular endothelial growth factor is thought to be a key factor in the pathogenesis of DME[5,7] and is a vasoactive cytokine that both induces vascular permeability and stimulates angiogenesis. It is approximately 50 000-fold more potent in inducing permeability than histamine[8–10] and affects endothelial tight junction proteins. Vascular endothelial growth factor is known to cause a breakdown of the blood–retinal barrier, followed by extracellular fluid accumulation and retinal edema.[11]

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Vascular endothelial growth factor concentrations are elevated in both the vitreous fluid and aqueous humor of patients with active proliferative diabetic retinopathy.[12,13] One study reported that VEGF concentrations in aqueous humor were elevated nearly 5-fold in DME eyes compared with that of age-matched controls.[14] Another study showed that the VEGF concentrations in the aqueous humor of eyes with DME were 3-fold higher than in the plasma.[12] Moreover, these elevated VEGF levels were correlated significantly with the severity of DME.[12] Elevated VEGF concentrations are associated with extensive macular leakage in diabetic eyes, and numerous studies have shown that VEGF inhibitors are effective for reducing retinal thickness and improving visual acuity.[15–22]

Vascular Endothelial Growth Factor Trap-Eye is a 115-kDA recombinant fusion protein comprising the key VEGF binding domains of human VEGF receptors 1 and 2 fused to the Fc domain of human immunoglobulin G1.[23] Vascular Endothelial Growth Factor Trap-Eye is a panisoform VEGF-A inhibitor whose binding affinity to VEGF is substantially greater than that of either bevacizumab or ranibizumab,[23] leading to a mathematical model predicting it could have substantially longer duration of action in the eye.[24] In addition, VEGF Trap-Eye binds placental growth factors 1 and 2, which have been shown to contribute to excessive vascular permeability and retinal neovascularization.[25]

The phase 2 clinical trial DME And VEGF Trap-Eye: INvestigation of Clinical Impact (DA VINCI) was designed to compare intravitreal VEGF Trap-Eye with macular laser photocoagulation. Results at week 24 (primary end point data) from the current study have been published previously,[26] and all VEGF Trap-Eye arms showed significant gains in visual acuity compared with laser treatment ($P \leq 0.0085$) at week 24. Patients in this study continued with their assigned dosing regimen and continued follow-up to determine if these visual acuity gains were maintained through week 52. The 1-year results are reported here.

Patients and Methods

The DA VINCI study was a randomized, double-masked, active-controlled multicenter phase 2 clinical trial. Thirty-nine sites in the United States, Canada, and Austria participated in the trial, and patients were enrolled between December 2008 and June 2009. The primary objective was to assess the efficacy of various doses and dose intervals of intravitreal VEGF Trap-Eye (aflibercept injection) on BCVA. The primary end point was the change in BCVA from baseline to week 24. Secondary objectives were to assess the effects of intravitreal VEGF Trap-Eye on retinal thickness assessed by optical coherence tomography (OCT) and to assess safety and tolerability of intravitreal VEGF Trap-Eye in eyes with DME. Secondary outcomes were the change in BCVA from baseline at week 52, the proportion of eyes that gained at least 15 ETDRS letters in BCVA compared with baseline at weeks 24 and 52, the change in central retinal thickness (CRT; central subfield on OCT) from baseline to weeks 24 and 52, and the number of focal laser treatments given.

The study protocol was approved by the institutional review board or ethics committee at every institution and was conducted according to the recommendations of Good Clinical Practice and the tenets of the Declaration of Helsinki. The study was compliant with the rules and regulations under the Health Insurance Portability and Accountability Act of 1996. All patients provided written informed consent to participate in the study. The DA VINCI study is registered with ClinicalTrials.gov (NCT00789477).

Participants

The study enrolled adult patients 18 years of age or older with type 1 or 2 diabetes mellitus with clinically significant DME with center involvement of the fovea, defined as a central subfield measurement of 250 μm or more on time-domain OCT (Stratus OCT; Carl Zeiss Meditec, Jena, Germany). In addition, patients had an ETDRS BCVA letter score at 4 m of 73 to 24 (20/40 to 20/320) in the study eye.[27,28] Patients were excluded if any of the following were present in the study eye: history of vitreoretinal surgery, panretinal or macular laser photocoagulation within 3 months of screening, previous use of intraocular or periocular corticosteroids within 3 months of screening, or other ocular disorders that could contribute to vision loss and could confound the study results. In addition, previous treatment with antiangiogenic drugs for either eye (pegaptanib sodium, anecortave acetate, bevacizumab, ranibizumab, etc.) was not allowed within 3 months of screening. Patients with uncontrolled diabetes mellitus or hypertension (systolic blood pressure >180 mmHg or >160 mmHg on 2 consecutive measurements or diastolic blood pressure >100 mmHg on optimal medical regimen) also were excluded from the study.

Treatments

Eyes were assigned randomly using a 1:1:1:1:1 ratio to one of the following treatment regimens (Fig 1): (1) 0.5 mg VEGF Trap-Eye every 4 weeks (0.5q4); (2) 2 mg VEGF Trap-Eye every 4 weeks (2q4); (3) 2 mg VEGF Trap-Eye every 8 weeks after 3 initial monthly doses (2q8); (4) 2 mg VEGF Trap-Eye, with dosing as needed after 3 initial monthly doses (2PRN); (5) laser photocoagulation using a modified ETDRS protocol[27] at baseline and then as needed (but no more frequently than every 16 weeks). Eyes in the laser group also received a sham injection every 4 weeks.

Vascular Endothelial Growth Factor Trap-Eye, provided by Regeneron Pharmaceuticals, Inc (Tarrytown, New York), was administered by intravitreal injection with a 30-gauge needle using standard ophthalmic techniques. Vascular Endothelial Growth Factor Trap-Eye was formulated as a sterile liquid to a final concentration of either 10 mg/ml or 40 mg/ml VEGF Trap-Eye. The injection volume was 50 μl (0.05 ml), which provided the delivery of 0.5 mg or 2 mg of VEGF-Trap-Eye. Sham injections were performed following the identical treatment protocol used for the active injections, but only gentle application of the hub of the syringe (without the needle) to the sclera was used to mimic an injection.

Laser photocoagulation was performed using the modified ETDRS protocol (baseline treatment at week 1).[3,28] After topical anesthesia and placement of a contact lens, grid therapy was applied to the thickened areas of the retina with diffuse leakage, focal therapy, or both being applied to leaking microaneurysms within the areas of retinal thickening. Sham laser treatments consisted of placing a contact lens on the study eye and positioning the patient in front of the laser machine for the approximate duration of a laser treatment, while the laser remained in the off position.

Retreatment Criteria

After the 3 initial monthly doses, eyes assigned to the 2PRN arm received an injection of study drug if any one of the following criteria were present: a more than 50-μm increase in CRT com-

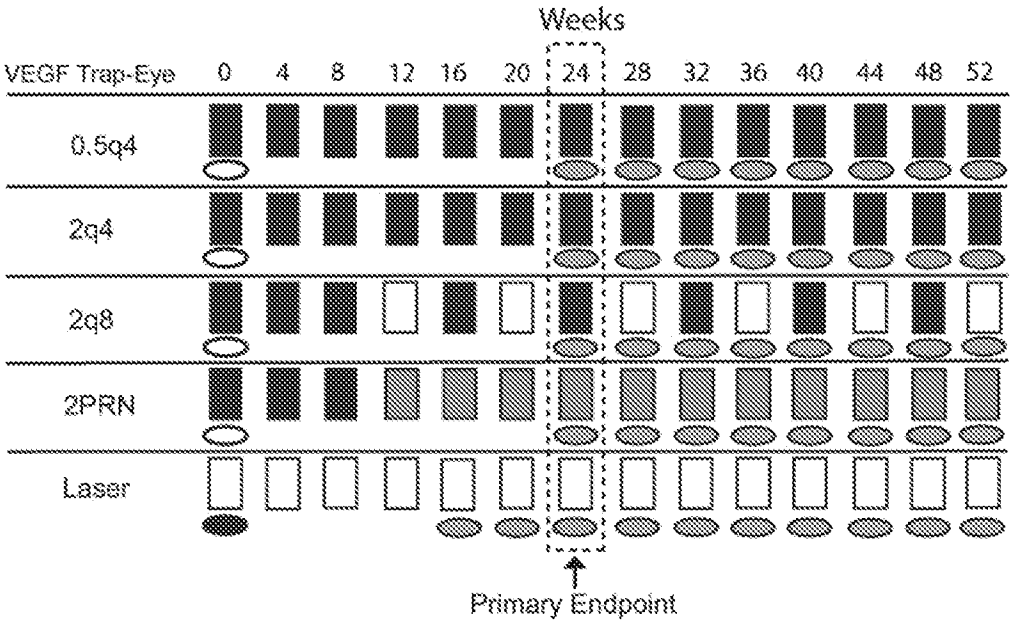

Figure 1. Diagram showing study design with interventions and schedule of visits throughout the course of the 12-month study. 0.5q4 = 0.5 mg every 4 weeks; 2q4 = 2 mg every 4 weeks; 2q8 = 2 mg for 3 initial monthly doses then every 8 weeks; 2PRN = 2 mg for 3 initial monthly doses then as needed; box = injection; grey = as needed; oval = laser; outline = sham; solid = active; VEGF = vascular endothelial growth factor.

pared with the lowest previous measurement; new or persistent cystic retinal changes, subretinal fluid, or persistent diffuse edema of 250 μm or more on OCT; a loss of 5 or more letters of BCVA from the best previous measurement in conjunction with any increase in CRT; and an increase in BCVA between the current and most recent visit of 5 letters or more. Eyes assigned to the 2PRN arm received sham injections if none of the retreatment criteria above were met.

Eyes in the laser photocoagulation arm of the study received their initial laser at week 1 (Fig 1). Starting at week 16, eyes were assessed for retreatment according to the following ETDRS criteria and were retreated if any one of the criteria were met: an increase in retinal thickness at or within 500 μm of the center of the macula; hard exudates at or within 500 μm of the center of the macula, if associated with thickening of adjacent retina; zone(s) of retinal thickening 1 disc area or larger (any part of which was within 1 disc diameter of the center of the macula).

Starting at week 24 (month 6), these same three criteria were used to assess eyes in the VEGF Trap-Eye arms for laser rescue. Eyes in the VEGF Trap-Eye arms that met the criteria for laser rescue received laser 1 week after the scheduled visit, which they qualified for laser rescue. Subsequent laser rescue treatments could be performed at 16-week intervals.

Masking

Treatments (study drug injection, sham injection, laser or sham laser photocoagulation) were performed by an unmasked physician. A separate masked physician was assigned to assess adverse events (AEs) and retreatment and rescue criteria and to supervise the masked assessment of efficacy. Every effort was made to ensure that all other study site personnel remained masked to treatment assignment to facilitate an unbiased assessment of efficacy and safety.

Measurements

Visual acuity was measured using the ETDRS protocol.[28] Retinal and lesion characteristics of the study eye were evaluated using time-domain OCT (Zeiss Stratus OCT equipped with software version 3.0 or greater; Carl Zeiss Meditec, Jena, Germany). The study eye was evaluated by dilated funduscopic examination, fundus photography, and fluorescein angiography. The severity of each patient's diabetic retinopathy was assessed using the Diabetic Retinopathy Severity Score.[29] Intraocular pressure of the study eye was measured using Goldmann applanation tonometry (Haag-Streit AG, KÖniz, Switzerland) or the Tono-Pen (Reichert Technologies, Depew, New York) before dosing and again approximately 5 to 10 minutes after dosing. Safety assessments included ophthalmic examinations, clinical AEs, laboratory measures, and serum samples for potential development of anti-VEGF Trap-Eye antibodies.

Concomitant Medications

Patients were not allowed to receive any treatment for their DME in the study eye other than the assigned study treatment with VEGF Trap-Eye or laser until week 52 or until the early termination visit assessments were completed.

Statistical Analyses

The full analysis set, which was used for the efficacy analysis, included all randomized patients who received any study medication and had at least 1 assessment after baseline. The safety analysis set, used for all safety and tolerability assessments, included all participants who received any study medication. The last observation carried forward approach was used to account for missing data. A sample size of 200 patients (40 per group) provided 84% power to detect an 8-letter difference between each of the 4 VEGF Trap-Eye arms and the laser arm (assuming a standard

Table 3. Treatment and Exposure Summary for Vascular Endothelial Growth Factor Trap-Eye and Laser Treatments over the Course of the First 48 Weeks

Study Arm	Mean No. of Vascular Endothelial Growth Factor Trap-Eye Injections (SD)	Mean No. of Laser Treatments (SD)
0.5q4 (n = 44)	11.7 (2.49)	0.8 (0.83)
2q4 (n = 44)	10.8 (2.87)	0.5 (0.66)
2q8 (n = 42)	7.2 (1.74)	0.8 (0.86)
2PRN (n = 45)	7.4 (3.19)	0.7 (0.77)
Laser (n = 44)	N/A	2.5 (0.87)

0.5q4 = 0.5 mg every 4 weeks; 2q4 = 2 mg every 4 weeks; 2q8 = 2 mg for 3 initial monthly doses then every 8 weeks; 2PRN = 2 mg for 3 initial monthly doses then as needed; N/A = not applicable; SD = standard deviation.

deviation of 10 letters per group, with a 2-sided t test at an α level of 5%/4 = 0.0125). Change from baseline in BCVA and OCT were analyzed using analysis of covariance, models with the baseline value as covariate and the treatment as fixed factor. Hochberg's procedure was used for the primary analysis to control for the multiple comparisons. No adjustments for multiplicity were made for the secondary variables. The proportions of patients in the VEGF Trap-Eye arms gaining 10 letters or more (15 letters or more) were compared with the laser arm using the Fisher exact test. Other secondary end points, as well as demographic, baseline, and safety data, were evaluated using summary statistics.

Results

Patient Disposition and Demographics

A total of 221 eyes were randomized, 219 were treated, and 176 completed the 52-week study (Table 1, available at http://aaojournal.org). Forty-three patients discontinued the study after receiving at least 1 treatment for the following reasons: lost to follow-up (n = 11), withdrew consent (n = 11), death (n = 6), treatment failures (n = 2), AE (n = 7), protocol deviation (n = 2), other (n = 4). Discontinuations were distributed evenly among all the treatment groups. Demographic information and baseline characteristics are provided in Table 2 (available at http://aaojournal.org). The groups generally were similar, although the VEGF Trap-Eye 2q8 group had a higher prevalence of proliferative diabetic retinopathy (regressed at baseline) compared with the other treatment groups. In addition, a history of cardiac disease was more common in the VEGF Trap-Eye groups compared with the laser group.

Treatment and Exposure Summary

Over the 52 weeks of the study, the mean number of VEGF Trap-Eye injections administered was similar to the number of required injections for the group (Table 3). The VEGF Trap-Eye groups received an average of less than 1 laser treatment between month 6 and month 12 (up to 2 laser treatments were allowed from week 24 to week 48). For the laser treatment group, the mean number of laser treatments was 2.5 (up to 4 laser treatments were allowed from baseline to week 48).

Efficacy

Treatment with VEGF Trap-Eye produced statistically significant improvements in BCVA in all treatment groups compared with laser at both week 24 (the primary outcome) and week 52 (week 52, $P<0.001$; Fig 2).[27] The ranges of improvement were +8.5 to +11.4 letters at week 24 and +9.7 to +13.1 letters at week 52. No significant differences were observed among the VEGF Trap-Eye treatment groups. Waterfall plots displaying BCVA changes for individual eyes indicate that few patients in the VEGF Trap-Eye groups experienced any loss of vision (Fig 3). At week 52, the proportion of eyes that gained 15 letters or more was statistically greater ($P\leq0.001$) than that in the laser treatment group in all VEGF Trap-Eye groups except 2q8 (Fig 4). The percentages of eyes that gained 10 letters or more were 57%, 71%, 45%, 62%, and 30%, for the 0.5q4, 2q4, 2q8, 2PRN, and the laser groups, respectively.

Eyes treated with each VEGF Trap-Eye dosing regimen experienced statistically significant reductions in CRT compared with eyes undergoing laser treatment (week 52, $P < 0.0001$; Fig 5). For eyes on the VEGF Trap-Eye treatment regimens, CRT continued to decrease through week 52.

For each study eye, baseline diabetic retinopathy severity was recorded using the Diabetic Retinopathy Severity Score (Table 2, available at http://aaojournal.org). At week 52, 40%, 31%, 64%, and 32% of the 0.5q4, 2q4, 2q8, and 2PRN VEGF Trap-Eye groups, respectively, had an improvement in their Diabetic Retinopathy Severity Score compared with 12% in the laser group. In addition, eyes treated with VEGF Trap-Eye were less likely to have worsening of their Diabetic Retinopathy Severity Score compared with laser-treated eyes (0%, 13%, 0%, and 14% in the 0.5q4, 2q4, 2q8, and 2PRN VEGF Trap-Eye groups and 24% in the laser group).

Safety

Vascular Endothelial Growth Factor Trap-Eye was well tolerated, and the most common ocular AEs that occurred were typical of those associated with intravitreal injections (Table 4, available at http://aaojournal.org). The most frequent were conjunctival hemorrhage, eye pain, increased intraocular pressure, ocular hyperemia, cataract, and vitreous floaters. Approximately 11% of patients treated with VEGF Trap-Eye experienced an AE of increased intraocular pressure immediately after the intravitreal injection; however, only 2 of these patients had an increase of more than 10 mmHg. Two patients who were randomized to

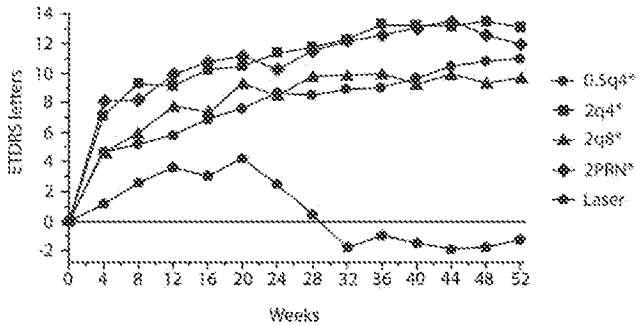

Figure 2. Graph showing mean changes in best-corrected visual acuity letter score by treatment groups (laser and Vascular Endothelial Growth Factor [VEGF] Trap-Eye) using last observation carried forward analysis: n = 44 (laser; VEGF Trap-Eye 0.5 mg every 4 weeks [0.5 q4] and 2 mg every 4 weeks [2q4]); n = 42 (VEGF Trap-Eye 2 mg for 3 initial monthly doses then every 8 weeks [2q8]); n = 45 (VEGF Trap-Eye 2 mg for 3 initial monthly doses then as needed [2PRN]). Difference between each treatment versus laser at week 52 was assessed using an analysis of covariance. *$P < 0.0001$. ETDRS = Early Treatment Diabetic Retinopathy Study.

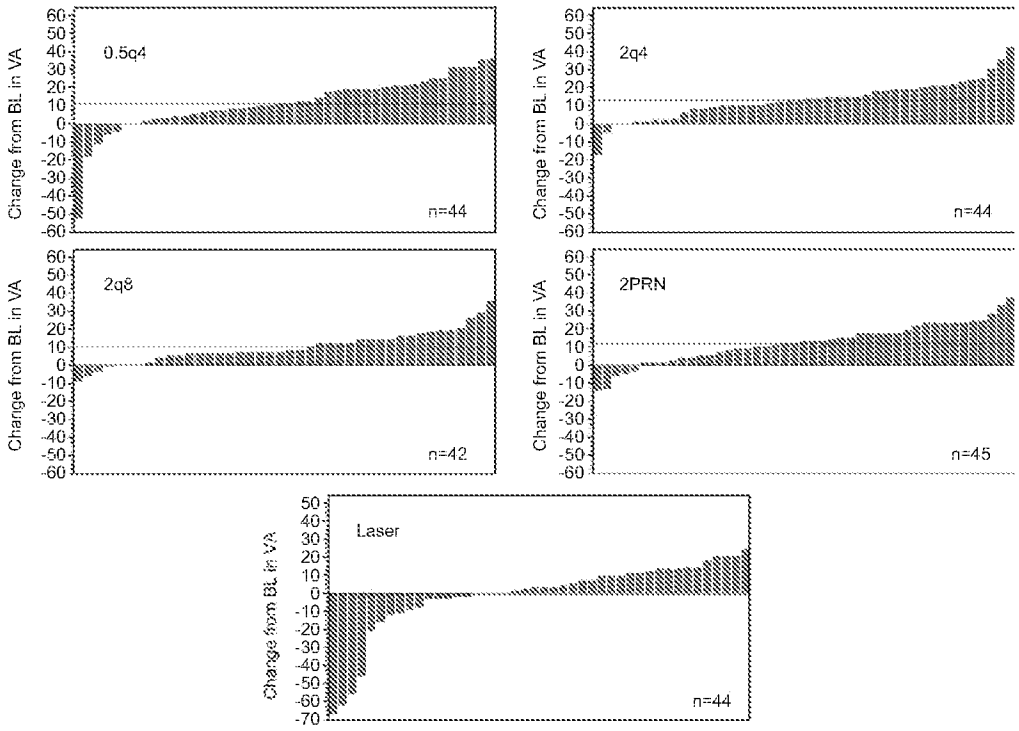

Figure 3. Graphs showing individual changes in best-corrected visual acuity (BCVA) letter score by treatment groups (laser and Vascular Endothelial Growth Factor Trap-Eye). Each bar corresponds to an individual patient. Dotted line represents median BCVA. 0.5q4 = 0.5 mg every 4 weeks; 2q4 = 2 mg every 4 weeks; 2q8 = 2 mg for 3 initial monthly doses then every 8 weeks; 2PRN = 2 mg for 3 initial monthly doses then as needed; BL = baseline; PRN = as needed; VA = visual acuity.

VEGF Trap-Eye experienced injection-related endophthalmitis, and uveitis developed in 1 patient. Serious nonocular AEs were infrequent in all treatment groups (Table 5). The most common systemic AEs were hypertension, nausea, and congestive heart failure. Because of its limited sample size, this phase 2 study was not powered adequately to assess the significance of differences in AEs among the treatment arms.

Seven deaths occurred during the study. One patient in the laser group died of cardiac arrest. One patient in the 0.5q4 group died of multiorgan failure. Three patients in the 2q4 group died: one of cerebral infarction, another from non–small-cell lung cancer, and the third from sudden death. Two patients in the 2q8 group died: one of renal failure and the other of acute coronary syndrome. None of the events that led to death in these patients was judged by

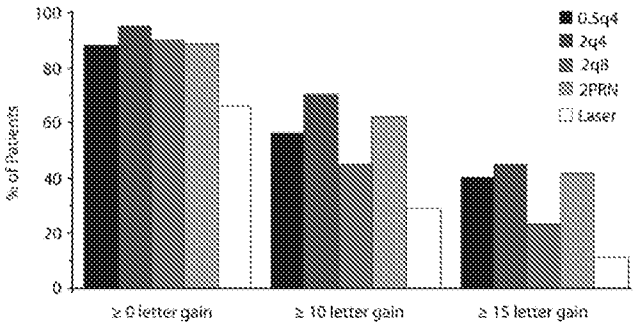

Figure 4. Bar graph showing percentage of patients with changes in changes in best-corrected visual acuity at 12 months by treatment groups (laser and Vascular Endothelial Growth Factor [VEGF] Trap-Eye) using last observation carried forward analysis: n = 44 (laser; VEGF Trap-Eye 0.5 mg every 4 weeks [0.5q4], 2 mg every 4 weeks [2q4]); n = 42 (VEGF Trap-Eye 2 mg for 3 initial monthly doses then every 8 weeks [2q8]); n = 45 (VEGF Trap-Eye 2 mg for 3 initial monthly doses then as needed [2PRN]). $P = 0.0031$, 0.5q4; $P = 0.0007$, 2q4; $P = 0.1608$, 2q8; $P = 0.0016$, 2PRN; all are compared with laser (analysis of covariance).

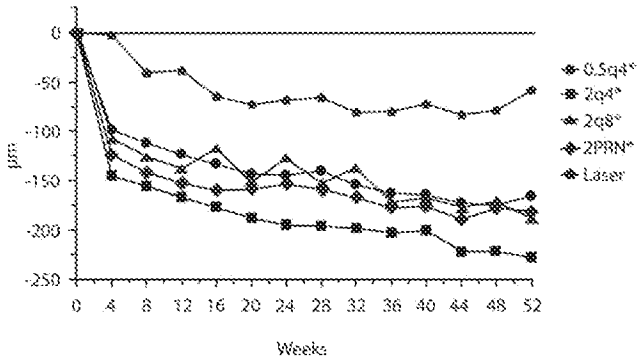

Figure 5. Graph showing mean change in central retinal thickness (in micrometers) by treatment groups (laser and Vascular Endothelial Growth Factor [VEGF] Trap-Eye) over the course of 12 months using last observation carried forward analysis: n = 44 (laser; VEGF Trap-Eye 0.5 mg every 4 weeks [0.5q4], 2 mg every 4 weeks [2q4]); n = 42 (VEGF Trap-Eye 2 mg for 3 initial monthly doses then every 8 weeks [2q8]); n = 45 (VEGF Trap-Eye 2 mg for 3 initial monthly doses then as needed [2PRN]). *$P < 0.0001$, difference between each treatment versus laser analysis of covariance.

Table 5. Serious Systemic Adverse Events and Deaths by Treatment Group of 4% or More in Any Treatment Arm

		Vascular Endothelial Growth Factor Trap-Eye Treatment Groups				
	Macular Laser Photocoagulation	0.5 mg Every 4 Weeks	2 mg Every 4 Weeks	2 mg for 3 Initial Monthly Doses Then Every 8 Weeks	2 mg for 3 Initial Monthly Doses Then as Needed	All Vascular Endothelial Growth Factor Trap-Eye
No. (safety analysis set)	44	44	44	42	45	175
No. of subjects with at least 1 AE, n (%)	10 (22.7%)	14 (31.8%)	13 (29.5%)	12 (28.6%)	6 (13.3%)	45 (25.7%)
Cardiac failure, congestive	0	0	3 (6.8%)	1 (2.4%)	2 (4.4%)	6 (3.4%)
Cellulitis	0	3 (6.8%)	2 (4.5%)	0	1 (2.2%)	6 (3.4%)
Chest pain	0	0	2 (4.5%)	0	3 (6.7%)	5 (2.9%)
Cerebrovascular accident	1 (2.3%)	1 (2.3%)	2 (4.5%)	0	0	3 (1.7%)
Hypertension	0	0	2 (4.5%)	1 (2.4%)	0	3 (1.7%)
Anemia	0	1 (2.3%)	2 (4.5%)	0	0	3 (1.7%)
Dehydration	0	0	0	2 (4.8%)	0	2 (1.1%)
Hyperglycemia	0	0	2 (4.5%)	0	0	2 (1.1%)
Myocardial infarction and acute myocardial infarction	0	3 (6.8%)	0	0	0	3 (1.7%)
Deaths*	1 (2.3%)	1 (2.3%)	3 (6.8%)	2 (4.5%)	0	7 (4.0%)

AE = adverse event.
*One death occurred after a patient in the 2 mg every 4 weeks group discontinued because of an AE.

the investigators to be related to the study drug or to the study procedure.

Discussion

In this phase 2 clinical trial, all VEGF Trap-Eye doses and dosing regimens were found to be superior to macular laser photocoagulation for the treatment of DME over the course of 52 weeks and produced similar results in terms of preserving and improving visual acuity. Patients who received VEGF Trap-Eye benefited from significantly greater increases in mean visual acuity at 1 year (+9.7 to +13.1 letters of improvement) compared with laser treatment alone (−1.3 letters change; $P<0.0001$). However, it should be noted that this study was not powered adequately to be able to discern differences with regard to efficacy among the VEGF Trap-Eye treatment groups. In addition, a study of longer duration may be able to detect further improvements in visual acuity for the laser treatment arm.

The administration of VEGF Trap-Eye over the course of this study generally was consistent with the number of treatments that had been planned, indicating good compliance with the protocol. There were a similar number of injections in the 2PRN (7.2) and 2q8 (7.4) groups. These numbers are consistent with the number of injections in the RESTORE (Efficacy and Safety of Ranibizumab [Intravitreal Injections] in Patients with Visual Impairment Due to Diabetic Macular Edema) trial for patients treated over 12 months with ranibizumab or ranibizumab plus laser (7.0 and 6.8 injections, respectively).[22] Longer intervals between dosing may provide advantages compared with monthly dosing in terms of less frequent monitoring visits and a decreased number of injections. Benefits of an extended dosing interval may include not only improved safety with fewer injection-related complications such as endophthalmitis, but also a decreased burden to the patient and their caregivers with fewer office visits. This benefit holds particularly true for the 2q8 treatment schedule, which could reduce the number of visits by half (after the loading phase), whereas monthly visits would be needed for determining the need for treatment in a PRN schedule.

The average number of laser treatments administered to eyes randomized to VEGF Trap-Eye was fewer than 1 (of a maximum of 2 possible lasers), with most patients not requiring laser photocoagulation, indicating that the visual acuity and anatomic benefits achieved were the result of VEGF Trap-Eye and not laser treatment. Eyes that were randomized to the laser group received an average of 2.5 laser treatments (of a maximum of 4 possible lasers), indicating that nearly the maximum amount of laser was applied during the 52-week study period. For comparison, during the first year of Protocol I from the Diabetic Retinopathy Clinical Research Network (DRCR) study, eyes that were randomized to macular laser photocoagulation received a median of 3 laser treatments, with 40% of eyes requiring 2, 1, or 0 additional treatments after the initial laser.[30]

A larger proportion of eyes in all the VEGF Trap-Eye treatment groups experienced 15-letter or more gains in visual acuity at week 52 compared with eyes in the laser arm, and these differences were statistically significant for 0.5q4, 2q4, and PRN treatments. The 2q4 treatment group had the highest percentage of eyes with visual acuity improvements at every level (≥0, ≥10, and ≥15 letters gained). The 2q8 group seemed to have less improvement in BCVA than in the other 2-mg groups. However, this difference was observed during the first 3 months of the study, despite the identical 2-mg loading dose, and persisted through the end of the study; therefore, this difference in visual acuity gains likely is attributable to baseline differ-

ences among treatment groups, rather than to the dosing interval.

In this clinical trial, combination treatment of VEGF Trap-Eye with laser photocoagulation was not investigated formally. Although eyes randomized to VEGF Trap-Eye could receive macular laser photocoagulation starting at week 24, most study eyes achieved gains in visual acuity with VEGF Trap-Eye monotherapy and did not require the addition of laser. Similarly, other studies[20,21] demonstrated that the combination of VEGF inhibitor with laser does not seem to provide any additional benefit in visual acuity gains or reductions in retinal thickness compared with VEGF inhibition alone.

Significantly greater mean reductions in retinal thickness were observed at week 52 for eyes undergoing the VEGF Trap-Eye regimens than for those treated with laser alone. Retinal thickness continued to decrease for eyes in the VEGF Trap-Eye arms after the week 24 primary end point.

Eyes randomized to VEGF Trap-Eye also were more likely to have an improvement in their diabetic retinopathy severity scale compared with laser-treated eyes. The biologic activity of VEGF Trap-Eye not only may treat DME, but also it can reduce the severity of diabetic retinopathy. This positive effect can be beneficial to patients who are at risk for severe vision loss associated with the development of proliferative diabetic retinopathy.

Vascular Endothelial Growth Factor Trap-Eye was well tolerated, and the incidence of ocular AEs was low. The rate of endophthalmitis was consistent with that observed for ranibizumab in the RESOLVE (Safety and Efficacy of Ranibizumab in Diabetic Macular Edema With Center Involvement) study (2%).[31] Most of the systemic AEs observed were attributed to the underlying medical conditions and cardiovascular comorbidities of these diabetic patients. Studies have shown that individuals with diabetes seem to have an approximately 2- to 4-fold greater risk for both heart disease and stroke.[32,33,34] Most of the deaths that occurred in this study were associated with pre-existing heart disease. The DA VINCI study was not powered sufficiently to assess the relationship between VEGF inhibition and systemic AEs or mortality. The results from this study suggest that intravitreal VEGF blockade with VEGF Trap-Eye may be a safe treatment that confers an acceptable benefit-to-risk ratio for eyes with DME over a 1-year period.

Because there is considerable individual variation in the progression of DME, patients could benefit from an individualized, as-needed treatment regimen.[31] At the same time, such individualized regimens may require close follow-up and monthly monitoring, which can be burdensome to patients and their caregivers. This intensive monitoring schedule may be mitigated by a dosing interval extended to 2 months. The results of this study support additional phase 3 clinical studies with every 2-month dosing of VEGF Trap-Eye after an initial loading dose.

Two phase 3 clinical studies of VEGF Trap-Eye, both with a primary end point of the change from baseline of BCVA in ETDRS letter score, have been initiated. The VIVID (DME and VEGF Trap-Eye: Investigation of Clinical Impact) DME study will evaluate 2 different dosing regimens of VEGF Trap-Eye compared with laser over the course of 1 year. The VISTA (Study of Intravitreal Administration of VEGF Trap-Eye [Bayer86-5321] in Patients with Diabetic Macular Edema) study will assess the efficacy of 2 different dosing regimens of VEGF Trap-Eye compared with laser over a 2-year period.

In conclusion, eyes receiving VEGF Trap-Eye experienced statistically significant improvements in BCVA compared with laser treatment at 6 months (primary end point), and these results were maintained or improved through 12 months. The long duration of efficacy (at least 8 weeks) is consistent with the tight binding characteristics and enhanced pharmacokinetic profile of VEGF Trap-Eye. Vascular Endothelial Growth Factor Trap-Eye generally was well tolerated. The ocular AEs were typical of those associated with intravitreal injections. Vascular Endothelial Growth Factor Trap-Eye represents a promising therapeutic agent for the management of diabetic macular edema.

References

1. Singh A, Stewart JM. Pathophysiology of diabetic macular edema. Int Ophthalmol Clin 2009;49:1–11.
2. Klein R, Klein BE, Moss SE, et al. The Wisconsin Epidemiologic Study of Diabetic Retinopathy. IV. Diabetic macular edema. Ophthalmology 1984;91:1464–74.
3. Early Treatment Diabetic Retinopathy Study Research Group. Treatment techniques and clinical guidelines for photocoagulation of diabetic macular edema: Early Treatment Diabetic Retinopathy Study report number 2. Ophthalmology 1987;94:761–74.
4. King H, Aubert RE, Herman WH. Global burden of diabetes, 1995–2025: prevalence, numerical estimates, and projections. Diabetes Care 1998;21:1414–31.
5. Khurana RN, Do DV, Nguyen QD. Anti-VEGF therapeutic approaches for diabetic macular edema. Int Ophthalmol Clin 2009;49:109–19.
6. Nguyen QD, Shah SM, Van Anden E, et al. Supplemental oxygen improves diabetic macular edema: a pilot study. Invest Ophthalmol Vis Sci 2004;45:617–24.
7. Grant MB, Afzal A, Spoerri P, et al. The role of growth factors in the pathogenesis of diabetic retinopathy. Expert Opin Investig Drugs 2004;13:1275–93.
8. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol 1995;146:1029–39.
9. Dvorak HF, Orenstein NS, Carvalho AC, et al. Induction of a fibrin-gel investment: an early event in line 10 hepatocarcinoma growth mediated by tumor-secreted products. J Immunol 1979;122:166–74.
10. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med 2003;9:669–76.
11. Gardner TW, Antonetti DA, Barber AJ, et al. Diabetic retinopathy: more than meets the eye. Surv Ophthalmol 2002; 47(suppl):S253–62.
12. Funatsu H, Yamashita H, Noma H, et al. Increased levels of vascular endothelial growth factor and interleukin-6 in the aqueous humor of diabetics with macular edema. Am J Ophthalmol 2002;133:70–7.
13. Funatsu H, Yamashita H, Nakanishi Y, Hori S. Angiotensin II and vascular endothelial growth factor in the vitreous fluid of patients with proliferative diabetic retinopathy. Br J Ophthalmol 2002;86:311–5.
14. Funk M, Schmidinger G, Maar N, et al. Angiogenic and inflammatory markers in the intraocular fluid of eyes with

diabetic macular edema and influence of therapy with bevacizumab. Retina 2010;30:1412–9.
15. Nguyen QD, Tatlipinar S, Shah SM, et al. Vascular endothelial growth factor is a critical stimulus for diabetic macular edema. Am J Ophthalmol 2006;142:961–9.
16. Chun DW, Heier JS, Topping TM, et al. A pilot study of multiple intravitreal injections of ranibizumab in patients with center-involving clinically significant diabetic macular edema. Ophthalmology 2006;113:1706–12.
17. Fraser-Bell S, Kaines A, Hykin PG. Update on treatments for diabetic macular edema. Curr Opin Ophthalmol 2008;19:185–9.
18. Macugen Diabetic Retinopathy Study Group. A phase II randomized double-masked trial of pegaptanib, an anti-vascular endothelial growth factor aptamer, for diabetic macular edema. Ophthalmology 2005;112:1747–57.
19. Ng EW, Adamis AP. Anti-VEGF aptamer (pegaptanib) therapy for ocular vascular diseases. Ann N Y Acad Sci 2006; 1082:151–71.
20. Diabetic Retinopathy Clinical Research Network. A phase II randomized clinical trial of intravitreal bevacizumab for diabetic macular edema. Ophthalmology 2007;114:1860–7.
21. Nguyen QD, Shah SM, Khwaja AA, et al, READ-2 Study Group. Two-year outcomes of the Ranibizumab for Edema of the mAcula in Diabetes (READ-2) study. Ophthalmology 2010;117:2146–51.
22. Mitchell P, Bandello F, Schmidt-Erfurth U, et al, RESTORE Study Group. The RESTORE Study: ranibizumab monotherapy or combined with laser versus laser monotherapy for diabetic macular edema. Ophthalmology 2011;118:615–25.
23. Holash J, Davis S, Papadopoulos N, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci U S A 2002;99:11393–8.
24. Stewart MW, Rosenfeld PJ. Predicted biological activity of intravitreal VEGF Trap. Br J Ophthalmol 2008;92:667–8.
25. Rakic JM, Lambert V, Devy L, et al. Placental growth factor, a member of the VEGF family, contributes to the development of choroidal neovascularization. Invest Ophthalmol Vis Sci 2003;44:3186–93.
26. Do DV, Schmidt-Erfurth U, Gonzalez VH, et al. The DA VINCI Study: phase 2 primary results of VEGF Trap-Eye in patients with diabetic macular edema. Ophthalmology 2011; 118:1819–26.
27. Writing Committee for the Diabetic Retinopathy Clinical Research Network. Comparison of the modified Early Treatment Diabetic Retinopathy Study and mild macular grid laser photocoagulation strategies for diabetic macular edema. Arch Ophthalmol 2007;125:469–80.
28. Early Treatment Diabetic Retinopathy Study Research Group. Photocoagulation for diabetic macular edema: Early Treatment Diabetic Retinopathy Study report number 1. Arch Ophthalmol 1985;103:1796–806.
29. Wilkinson CP, Ferris FL III, Klein RE, et al, Global Diabetic Retinopathy Project Group. Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. Ophthalmology 2003;110:1677–82.
30. Treatment techniques and clinical guidelines for photocoagulation of diabetic macular edema. Early Treatment Diabetic Retinopathy Study Report Number 2. Early Treatment Diabetic Retinopathy Study Research Group. Ophthalmology 1987;94:761-74.
31. Diabetic Retinopathy Clinical Research Network, Elman MJ, Aiello LP, Beck RW, et al. Randomized trial evaluating ranibizumab plus prompt or deferred laser or triamcinolone or deferred laser or triamcinolone plus prompt laser for diabetic macular edema. Ophthalmology 2010;117:1064–77.
32. Massin P, Bandello F, Garweg JG, et al. Safety and efficacy of ranibizumab in diabetic macular edema (RESOLVE Study): a 12-month, randomized, controlled, double-masked, multicenter phase II study. Diabetes Care 2010;33:2399–405.
33. Gregg EW, Gu Q, Cheng YJ, et al. Mortality trends in men and women with diabetes, 1971 to 2000. Ann Intern Med 2007;147:149–55.
34. American Diabetes Association. Diabetes Statistics. Available at: http://www.diabetes.org/diabetes-basics/diabetes-statistics/. Accessed February 3, 2011.

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[1] Wilmer Eye Institute, Department of Ophthalmology, Johns Hopkins University School of Medicine, Baltimore, Maryland.

[2] Retina Vitreous Associates Medical Group, Los Angeles, California.

[3] Department of Ophthalmology, Medical University of Vienna, Vienna, Austria.

[4] Retina Consultants of Houston, The Methodist Hospital, Houston, Texas.

[5] Regeneron Pharmaceuticals, Inc, Tarrytown, New York.

[6] Bayer Healthcare, Berlin, Germany.

[7] Universitätsklinikum Hamburg-Eppendorf, Klinik und Poliklinik für Augenheilkunde, Hamburg, Germany.

[8] Department of Neurology, Heinrich-Heine-Universität, Düsseldorf, Germany.

[9] Ophthalmic Consultants of Boston, Boston, Massachusetts.

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Correspondence:
Diana V. Do, MD, The Wilmer Eye Institute, 600 North Wolfe Street, Maumenee No. 745, Baltimore, MD 21287. E-mail: ddo@jhmi.edu.

"TREAT AND EXTEND" DOSING OF INTRAVITREAL ANTIVASCULAR ENDOTHELIAL GROWTH FACTOR THERAPY FOR TYPE 3 NEOVASCULARIZATION/RETINAL ANGIOMATOUS PROLIFERATION

MICHAEL ENGELBERT, MD, PHD,*† SANDRINE A. ZWEIFEL, MD,*†‡
K. BAILEY FREUND, MD*†

Purpose: The purpose of this study was to analyze long-term outcomes for the treatment of type 3 neovascularization/retinal angiomatous proliferation using a "Treat and Extend" dosing regimen for antivascular endothelial growth factor therapy.

Methods: This was a retrospective analysis of visual acuity and optical coherence tomography data of 11 eyes of 10 consecutive patients with newly diagnosed type 3 neovascularization/retinal angiomatous proliferation treated with intravitreal bevacizumab and/or ranibizumab with at least a 12-month follow-up. Three monthly injections were followed by continued treatment at intervals increasing by 2 weeks per visit, to a maximum of 10 weeks, unless clinical or optical coherence tomography evidence of persistent or recurrent fluid was present, in which case, the interval was shortened.

Results: Mean baseline Snellen visual acuity was 20/80, improved to 20/40 at 1 month, and was maintained throughout the 36-month period (n = 11 at 12 months, n = 10 at 24 months, and n = 8 at 36 months) ($P < 0.04$, paired t-test). The mean center point optical coherence tomography thickness decreased from 320 μm to 180–230 μm, and was maintained during the study period ($P < 0.02$). The mean number of injections was seven in the first year, six in the second year, and seven in the third year.

Conclusion: "Treat and Extend" antivascular endothelial growth factor dosing in type 3 neovascularization/retinal angiomatous proliferation delivers promising outcomes at a reduced burden for the patient and health care system compared with monthly and optical coherence tomography-guided dosing regimens.

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Type 3 neovascularization (otherwise known as retinal angiomatous proliferation [RAP])[1,2] is a subtype of neovascular age-related macular degeneration (AMD) with distinct angiographic and optical coherence tomography (OCT) features related to intraretinal proliferation of the abnormal vessels with associated retinal–retinal and retinal–choroidal anastomosis. Its natural course is typically worse than other more frequent lesion types such as subretinal pigment epithelium neovascularization (type 1)/occult choroidal neovascularization or subneurosensory neovascularization (type 2)/well-defined (classic) choroidal neovascularization.[3–5] Many different treatment strategies[6] such as photocoagulation,[3,4,7] transpupillary thermotherapy,[3,8] photodynamic therapy (PDT),[9–13] intravitreal antivascular endothelial growth factor (anti-VEGF) agents,[6,14,15] intravitreal triamcinolone acetonide, surgical excision,[16,17] and many combinations of the above[18–21] have been tried in small case series with limited follow-up.

Monthly injections of antiangiogenic agents have become the standard of care for the treatment of neovas-

From the *LuEsther T. Mertz Retinal Research Center, Manhattan Eye, Ear and Throat Hospital; †Vitreous-Retina-Macula Consultants of New York, New York, New York; and ‡University Hospital Zurich, Department of Ophthalmology, Frauenklinikstrasse 24, 8032 Zurich, Switzerland.

Supported by The Macula Foundation Inc.

Reprint requests: K. Bailey Freund, MD, 460 Park Avenue, 5th Floor, NY 10022; e-mail: kbfny@aol.com

cular AMD[22,23] but are expensive and difficult to sustain in this elderly patient population. However, the less frequent dosing in the PIER trial,[24] in which patients received quarterly injections after an initial series of three monthly injections, could not reproduce the excellent results obtained in trials using monthly dosing. The PrONTO Study[25,26] attempted to tailor the dosing to the individual needs of the patient based on acuity decline, clinical findings, or OCT evidence of disease activity and was able to demonstrate good visual results after a >24-month period.

Although PrONTO-style dosing has become widely adopted in the retinal community and seems to yield favorable results, this strategy does require monthly visits, clinical examinations, and OCTs with patients uncertain if or when they will need treatment. Because eyes with type 3 neovascularization/RAP typically manifest retinal–choroidal anastomosis, recurrent exudation may occur earlier and more frequently than with other neovascular lesion types. In our experience, some patients managed with this strategy will return for assessments having already developed macular hemorrhages in the injection-free interval with irreversible vision loss.[27–29] In theory, a dosing regimen that does not maintain the macula in a "dry" state could deny some patients the opportunity for further visual recovery.

The "Treat and Extend" dosing regimen is a strategy intended to resolve macular exudation and maintain the macula in this "dry" state indefinitely with, when possible, fewer patient visits and treatments than monthly dosing.[30,31] The strategy consists of an initial induction or "loading" sequence of at least three monthly injections. If stable visual acuity, an absence of macular hemorrhage, and a dry OCT have been achieved at this point, patients continue to receive regular maintenance injections at increasing intervals. At 6 weeks after the last of the three monthly injections, visual acuity, clinical findings, and OCT changes are recorded again, and patients receive an injection regardless of the presence or absence of disease activity. However, the interval to the next visit (and scheduled injection) is based on an observed change in the above parameters. If there are no changes, the next visit is scheduled for 8 weeks later. If there is a change, the patient comes for another scheduled injection and examination after 4 weeks. The observation and scheduled treatment interval is extended (hence the term "Treat and Extend") to a maximum of 10 weeks. We report on 11 eyes of 10 patients with type 3 neovascularization/RAP managed with the "Treat and Extend" dosing regimen and with follow-up of between 12 and 36 months.

Materials and Methods

A waiver of authorization for use of protected health information for the above-referenced research and a waiver of consent for this retrospective chart review were obtained from the Institutional Review Board committee of the Manhattan Eye Ear and Throat Hospital, New York, NY.

The diagnosis of type 3 neovascularization/RAP was made by the treating physician (K.B.F.) based on the characteristic clinical, OCT, and angiographic features including intraretinal hemorrhage, cystoid macular edema, intraretinal vascular anastomosis, retinal–choroidal anastomosis, and in some cases, the presence of pigment epithelial detachment (PED) on OCT. Patients treated previously with thermal laser, PDT, or intravitreal pegaptanib (Macugen, Pfizer Inc., New York, NY), or who presented with subfoveal fibrosis or atrophy, a history of vitrectomy, aphakia or absence of posterior capsule, history of idiopathic or autoimmune associated uveitis in either eye, or diabetic retinopathy more severe than mild nonproliferative stage, were excluded from this study. Patients with preexisting cardiac or cerebrovascular conditions were not excluded from the study.

The treatment consisted of intravitreal injection of 1.25 mg of bevacizumab (Avastin, Genentech Inc., South San Francisco, CA) or 0.5 mg ranibizumab (Lucentis, Genentech Inc.) suspended in 0.05 mL. For the purpose of this analysis, no distinction between either antiangiogenic drug was made. Before intravitreal injection, topical anesthesia and surface disinfection with 5% povidone–iodine was performed. Intravitreal injections were administered at the time of diagnosis and subsequently followed a protocol we termed "Treat and Extend." Patients all received at least 3 monthly injections followed by continued treatment at intervals increasing by 2 weeks per visit once visual acuity was stable, OCT showed an absence of intra- and subretinal fluid, and all hemorrhage had resolved. Resolution of PED was not required before treatment intervals were lengthened. The treatment interval was extended to a maximum of 10-week "maintenance" unless clinical examination or OCTdetected new hemorrhage or persistent/recurrent fluid. In those cases, the interval was shortened by 2 weeks and maintained at that duration, provided this resolved the fluid.

The main outcome measure in this study was visual acuity after treatment. Decrease in retinal thickness, number of injections needed, and change in funduscopic or tomographic appearance were assessed as well.

Snellen visual acuity was measured by a certified ophthalmic technician. Snellen acuity was converted into logarithm of the minimum angle of resolution (logMAR) for statistical analysis at baseline and at 1, 3, 12, 24, and 36 months after injection of an antiangiogenic agent. Changes in logMAR-converted acuities were tested with a paired Student's t-test and accepted as significant if $P < 0.05$. Also, the proportions of patients

approximately halving (≥0.3 logMAR, but <0.6 logMAR-converted Snellen visual acuity improvement) or approximately quartering their visual angle (≥0.6 logMAR-converted Snellen visual acuity improvement), as well as those that remained stable (<0.3 logMAR-converted visual acuity improvement) or lost lines on the Snellen chart compared with baseline, were reported.

The quantitative assessments of center point retinal thickness were made using Stratus OCT (Carl Zeiss Meditec, Dublin, CA) and Topcon OCT (Topcon 3D OCT-1000, Topcon Medical Systems, Paramus, NJ). The center point retinal thickness was defined as the distance between the internal limiting membrane and the retinal pigment epithelium under the fovea and did not include any fluid under the retinal pigment epithelium. For Topcon OCT images, the calipers provided by the Topcon image analysis software were used. The Stratus OCT measurements were made manually on the IMAGEnet software on a single horizontal line scan through the fovea (Topcon Medical Systems), and the calculated data in pixels were multiplied with a conversion factor of 8 μm/pixel. This conversion factor had been derived from previous comparisons of controls on the different imaging platforms (based on 20 normal eyes measured on the 2 platforms, Howard F. Fine, personal communication).

The qualitative assessment included identification of intraretinal cysts, neovascular complex within the retinal layers, and PED. Additional funduscopic and tomographic changes and their development over time were recorded as well. Specifically, the presence of intraretinal hemorrhage or development of a pigment epithelial rip on funduscopy and the presence of intra- or subretinal fluid or PED on high-resolution B-scans were determined. Because staging of type 3 neovascularization/RAP is difficult and of controversial significance, this was not performed.

Results

Eleven eyes of 10 patients were included in this study. Eleven eyes completed the 12-month follow-up, 10 eyes completed the 24-month follow-up, and 8 eyes completed the 36-month follow-up.

Patient demographics, baseline, and follow-up visual acuity, center point retinal thickness data, and number of injections in the first, second, and third year are presented in Table 1. Median patient age was 85 years (range, 71–92 years). Seven of 10 patients were women. Two contralateral eyes had evidence of antecedent type 3 neovascularization/RAP lesions, and 2 patients developed disease in the contralateral eye during the study period. Only one of these two latter eyes was treated with a "Treat and Extend" protocol and included

Table 1. Summary of Patient Data

				Baseline							Second			Third	
Patient	Gender	Age (Years)	Baseline VA	OCT (μm)	Month 1 VA	Month 1 OCT (μm)	Month 12 VA	Month 12 OCT (μm)	Month 12 No Inj	Month 24 VA	Month 24 OCT (μm)	Year No Inj	Month 36 VA	Month 36 OCT (μm)	Year No Inj	RAP in Fellow Eye
1 OD	F	89	20/400	257	20/160	299	20/100	174	5	20/200	174	5	20/80	181	6	P
2 OS	F	85	20/50	241	20/50	183	20/60	150	6	20/40	177	5	20/40	163	7	P
3 OD	F	84	20/25	246	20/25	192	20/25	186	9	20/25	117	10	---	---	---	P
3 OS	—	—	20/200	433	20/40	289	20/25	118	10	20/30	108	8	20/40	136	10	L
4 OS	F	83	20/80	282	20/30	199	20/20	166	7	20/25	174	5	---	---	---	---
5 OS	M	92	20/200	410	20/70	174	20/100	232	7	---	---	---	---	---	---	L
6 OS	M	71	20/20	182	20/25	133	20/25	121	8	20/20	174	8	20/20	134	6	---
7 OS	F	76	20/50	548	20/30	374	20/50	254	6	20/30	196	5	20/30	199	7	---
8 OD	F	92	20/30	200	20/40	224	20/40	216	6	20/25	224	6	20/30	225	6	---
9 OS	M	82	20/200	388	20/70	206	20/40	315	6	20/40	224	6	20/40	218	5	---
10 OS	F	91	20/70	336	20/40	246	20/50	166	8	20/60	175	7	20/50	197	7	—

Inj, injections; P, at presentation; L, later.

Table 2. Optical Coherence Tomography Center Point Retinal Thickness of Eyes With Type 3 Neovascularization/RAP Treated With the "Treat and Extend" Dosing Regimen (n at baseline and 1, 3, and 12 Months = 11, n at 24 Months = 10, and n at 36 Months = 8)

	Baseline	Month 1	Month 3	Month 12	Month 24	Month 36
Mean	320	229	183	191	181	182
Median	282	206	174	174	175	189

This difference was statistically significant at all time points (paired 2-tailed Student's t-test, $P < 0.02$).

in the study. The mean number of injections was seven in the first year, six in the second year, and seven in the third year.

Mean Snellen visual acuity at presentation was 20/80 at baseline (n = 11), improved to 20/40 at 1 month and 20/30 at 3 months, and was maintained at a level of 20/40 during the rest of the 36-month study period (n = 11 at 1, 3, and 12 months, n = 10 at 24 months, and n = 8 at 36 months; Tables 1 and 2; Figures 1 and 2). The difference in logMAR-converted visual acuity was statistically significant at all time points (paired 2-tailed t-test, $P < 0.04$).

The center point retinal thickness measurements improved in all patients (Table 2) and more rapidly

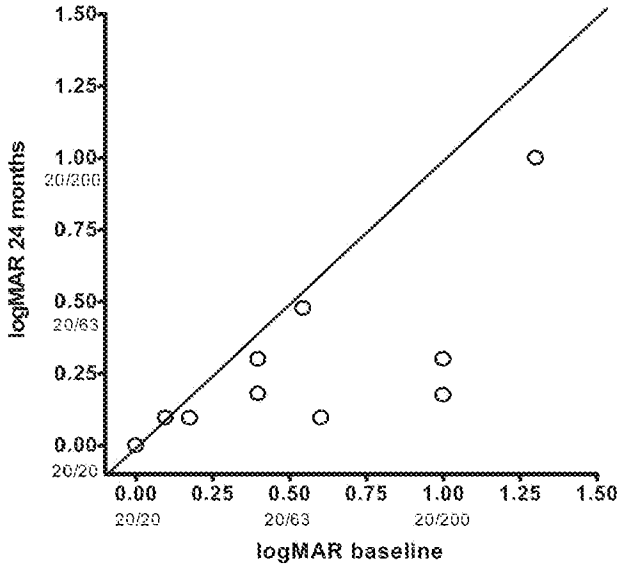

Fig. 1. Scatter plot of logMAR-converted visual acuity change of eyes with type 3 neovascularization/RAP treated with the "Treat and Extend" dosing regimen (n at 24 months = 10). Mean Snellen visual acuity at presentation was 20/80 at baseline (n = 11), improved to 20/40 at 1 month and 20/30 at 3 months, and was maintained thereafter at a level of 20/40. The difference in logMAR-converted visual acuity was statistically significant at all time points (paired 2-tailed t-test, $P < 0.04$).

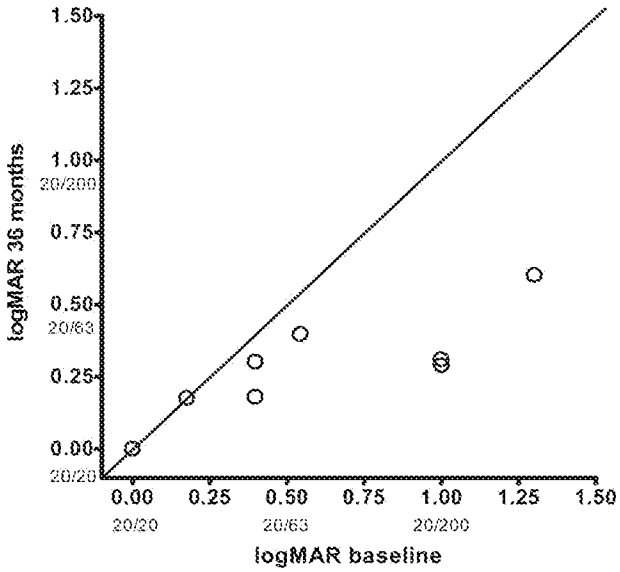

Fig. 2. Scatter plot of logMAR-converted visual acuity change of eyes with type 3 neovascularization/RAP treated with the "Treat and Extend" dosing regimen (n at 36 months = 8). The difference in logMAR-converted visual acuity was statistically significant at all time points (paired 2-tailed t-test, $P < 0.04$).

than visual acuity, even in those patients who experienced initial worsening in visual acuity. Mean center point retinal thickness at the time of diagnosis was ~320 μm and rapidly decreased to ~230 μm 1 month after the first injection. After the first 3 monthly injections, center point retinal thickness had decreased to ~180 μm and remained stable at that level during the 36-month observation period. This difference was statistically significant (paired 2-tailed Student's t-test, $P < 0.02$ at all time points).

The majority of eyes (9 of 11) had PEDs in the area of type 3 neovascularization/RAP. During the treatment period of 36 months, the size of the PED diminished in seven of eight eyes and resolved completely in four out of eight eyes.

In the 10 patients we followed for at least 24 months, 16 recurrences of fluid occurred, 12 during the first year. After establishment of a defined treatment interval, 6 recurrences occurred in 10 patients (Figure 3) during the first 24 months of observation. During the cumulative observation period of 336 months, a total of 21 recurrences of fluid occurred. Because fluid would not always quickly regress within 1 month, a "wet" macula after the initial 3 monthly injections was encountered for a total of 35 of 240 cumulative months of observation in the group of 10 patients we followed for 24 months.

Despite the presence of a presumably vascularized PED at presentation in most patients and frequent injections (mean of 20 injections after 36 months), we did not observe any tears of the pigment epithelium during the

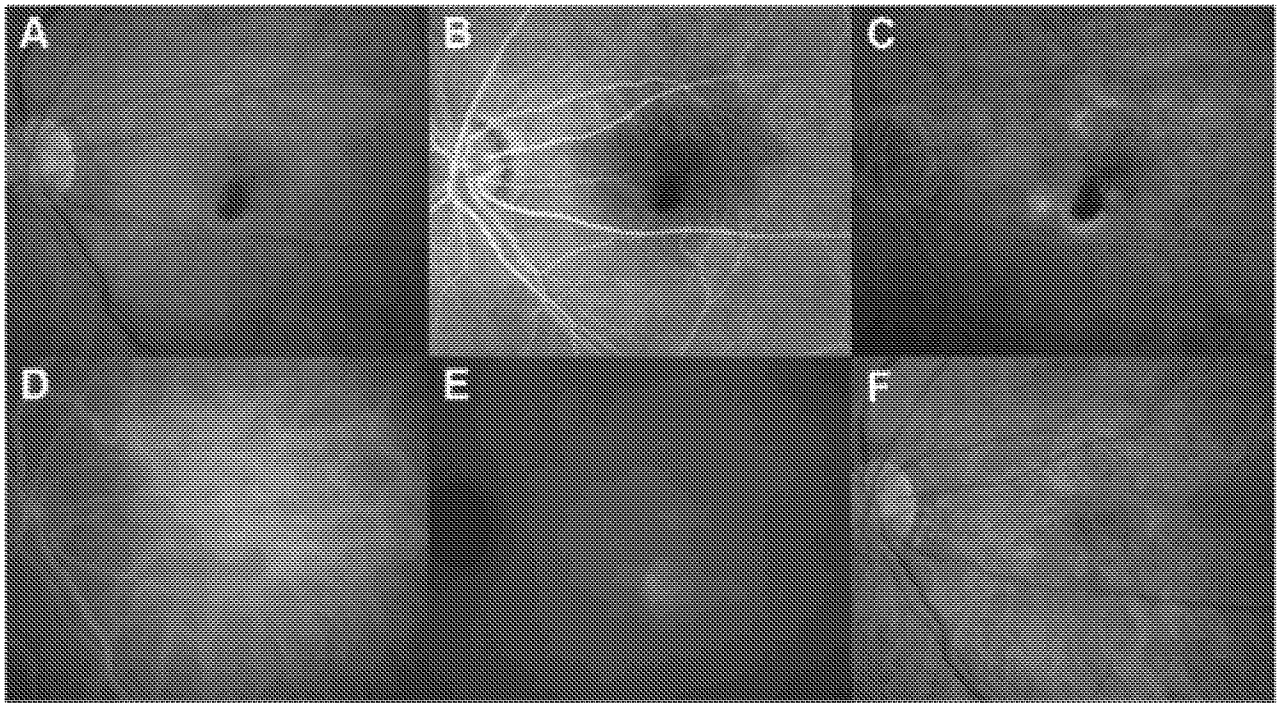

Fig. 3. Color fundus photograph (**A**) at baseline of an 85-year-old woman showing intraretinal hemorrhages and retinal edema at the inferior edge of the fovea characteristic of type 3 neovascularization/RAP. Early- (**B**) and late-phase (**C**) fluorescein angiograms at baseline show poorly defined intraretinal leakage. Early- (**D**) and late-phase (**E**) indocyanine angiograms show a focal area of increasing hyperfluorescence ("hot spot") consistent with type 3 neovascularization/RAP. Color fundus photograph (**F**) at month 39 shows increased pigment hyperplasia and no evidence of exudative changes.

study period. There were no injection-related complications such as endophthalmitis or retinal detachment.

Figure 3 illustrates the case of an 85-year-old woman with type 3 neovascularization/RAP treated with the "Treat and Extend" dosing strategy. Color fundus photograph (A) at baseline demonstrates intraretinal hemorrhages and retinal edema at the inferior edge of the fovea, characteristic of type 3 neovascularization/RAP. Early- (B) and late-phase (C) fluorescein angiograms at baseline show poorly defined intraretinal late leakage. Early- (D) and late-phase (E) indocyanine angiograms show a focal area of increasing hyperfluorescence ("hot spot"), consistent with type 3 neovascularization/RAP. A color fundus photograph (F) at month 39 shows increased pigment hyperplasia and no evidence of exudative changes. Optical coherence tomography images of the same patient as shown in Figure 3 at baseline are shown in Figure 4A, and the response to treatment after 1, 12, 14, 15, 36, and 44 months is shown in Figure 4, B–G. Intra- and subretinal fluid present at baseline decreased after the first injection (B), with a corresponding visual acuity improvement from 20/200 at baseline to 20/40. After 3 monthly injections, the patient received injections every 6 weeks to 7 weeks until, at month 14, a mild recurrence of intraretinal fluid was observed (C), with a decline in visual acuity from 20/40 to 20/50. The injection interval was reduced to 5 weeks. At month 15, the fluid was resolved, and visual acuity had returned to 20/40 and remained stable until the most recent follow-up visit at 44 months (G). The patient continues to receive injections at 5-week intervals.

Discussion

Although intravitreal anti-VEGF therapy has revolutionized the treatment of neovascular AMD, the optimal dosing regimen for these agents remains uncertain. Whether different neovascular lesion types warrant different dosing regimens is also unclear. Type 3 neovascularization/RAP is a subtype of neovascular AMD which has been difficult to treat[21,32] and usually involves the second eye within 3 years of onset in the first involved eye.[33]

Preliminary short-term data on visual acuity outcomes for the treatment of type 3 neovascularization/RAP with bevacizumab have been promising but have not yet led to an established consistent dosing regimen.[6,15,34–36]

Although monthly dosing of anti-VEGF agents for neovascular AMD have produced results far superior to previous treatments such as thermal laser and PDT,[22,23] cost, convenience, and safety concerns have prompted studies of less frequent dosing regimens. The PIER study[24] explored a regimen consisting of

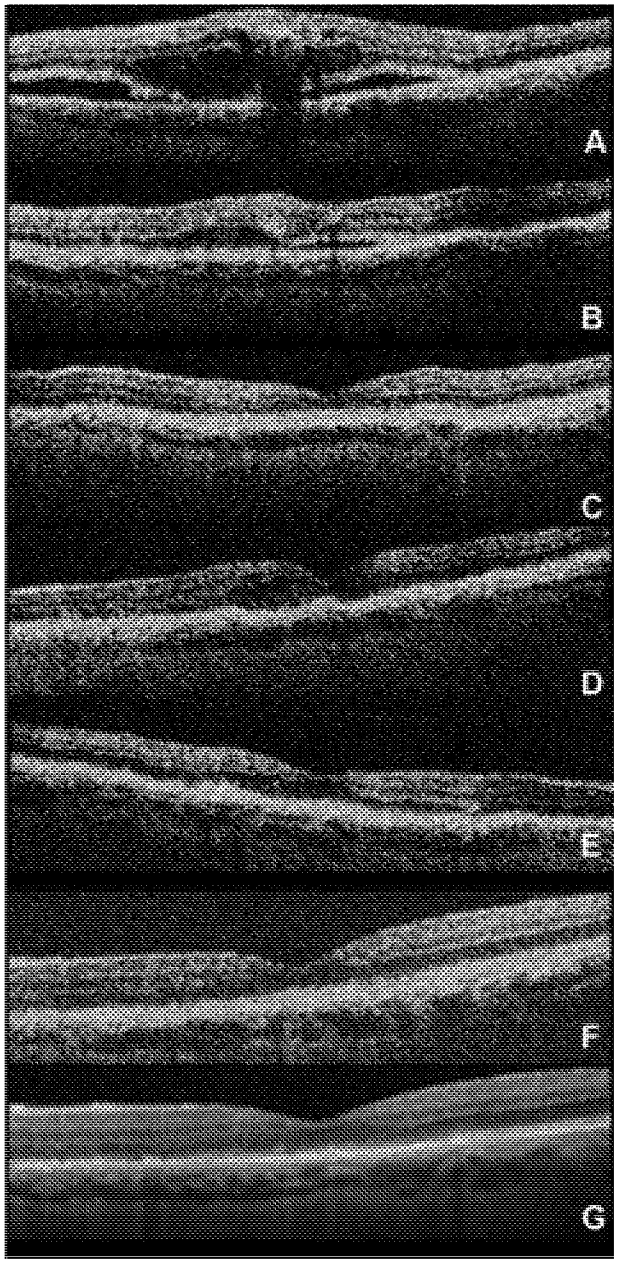

Fig. 4. Optical coherence tomography images of the same patient as shown in Fig. 3 at baseline (**A**) and after 1, 12, 14, 15, 36, and 44 months (**B**–**G**). Intra- and subretinal fluid present at baseline decreased after the first injection (**B**), with a corresponding visual acuity improvement from 20/200 at baseline to 20/40. After 3 monthly injections, the patient received injections every 6 weeks to 7 weeks until, at month 14, a mild recurrence of intraretinal fluid was observed (**C**), with loss of 1 line of visual acuity to 20/50. The injection interval was reduced to 5 weeks. At month 15, the fluid was resolved, and visual acuity had returned to 20/40 and remained stable until the most recent follow-up visit at 44 months (**G**). The patient continues to receive injections at 5-week intervals.

three monthly injections followed by mandated quarterly injections. However, this dosing regimen gave disappointing results, and this particular fixed-dosing strategy has largely been abandoned. The PrONTO study explored three monthly injections followed by dosing on an as-needed or PRN basis guided by changes in visual acuity, clinical findings, and OCT evaluation.[25,26] This open-label, prospective, nonrandomized study yielded results similar to those of the ANCHOR and MARINA studies[22,23] with fewer injections but a similar number of patient visits.

Although the PrONTO dosing regimen has gained popularity, it might not be ideal for patients with disease that follows a more relentless course, such as type 3 neovascularization/RAP. Furthermore, elderly patients with comorbidities often find it difficult or impossible to adhere to the monthly visits required when following a PrONTO-style dosing regimen.[25,26] Recurrent fluid or possibly a sudden macular hemorrhage may put patients at risk for irreversible vision loss in this "wait and watch, treat if necessary" strategy. Also, noncompliant patients or patients forced to miss follow-up visits as a result of illness and/or hospitalization may not be suitable candidates for a PrONTO-style regimen. Finally, although the number of injections and cost are reduced with PrONTO-style dosing, patients still require monthly OCT evaluations.

Type 3 neovascularization/RAP tends to follow a more aggressive course and has a higher risk of bilaterality than other lesion types. These patients tend to be older than the average patient with neovascular AMD (median age = 85 years in this series). With the intention of reducing the risk of recurrent exudation or vision loss and the burden of monthly visits and the overall cost of treatment for these patients, we investigated a dosing regimen that we call "Treat and Extend." The "Treat and Extend" regimen consists of a minimum of three monthly injections followed by examination and treatment intervals which are gradually extended provided there is stable visual acuity, no hemorrhage on clinical examination, and neither intra- nor subretinal fluid on OCT. The interval between examinations and treatment is extended in 2-week increments until a maintenance interval of ≤10 weeks is reached. If new hemorrhage or fluid is detected on any visit, the interval between evaluations and treatment is reduced until an interval is found that maintains the macula in a "dry" state.

The "Treat and Extend" dosing regimen is a tailored maintenance regimen which typically achieves reductions in patient visits, decreased imaging studies, and fewer injections compared with other dosing regimens, in particular continuous monthly dosing. Although patients treated with a PrONTO-style regimen typically receive fewer injections than those receiving monthly dosing, these patients continue to undergo monthly examinations and OCT evaluations. In our series, patients following the "Treat and Extend" reg-

imen were seen on average 13.6 ± 2.8 times (range, 9–18 times) during the first 24 months and 20.3 ± 4.1 times during 36 months. Although patients on a "Treat and Extend" regimen receive a mandated injection at each visit, the number of injections our eyes received (average of 7.1 ± 1.5; range, 5–10) was similar to that in the PrONTO study (average of 7.1 ± 2.2 for 10 patients with type 3 neovascularization/RAP, 5.6 for all choroidal neovascularization) during the first 12 months.[25] The reduction in patient visits without an increase in number of treatments could potentially decrease the burden on practitioners, patients, and the health care system as a whole. During the course of 2 years, the mean number of treatments was higher with the "Treat and Extend" regimen compared with the PrONTO study[26] (13.6 ± 2.8 vs. 11.6 ± 5.9 in 10 PrONTO patients with type 3 neovascularization/RAP), but we cannot calculate whether this is statistically significant.

In our attempt to evaluate long-term results of the "Treat and Extend" dosing regimen, we investigated whether visual acuity and center point retinal thickness could be improved and maintained with this anti-VEGF treatment regimen and how any such an effect observed would compare with historical outcomes reported for the large randomized trials ANCHOR and MARINA, PrONTO, and smaller case series focused on the treatment of type 3 neovascularization/RAP. We evaluated 11 eyes managed with the "Treat and Extend" regimen and followed for at least 1 year, the majority having 3 years of follow-up. Both visual acuity and central point retinal thickness improved significantly during the follow-up period.

To the best of our knowledge, this is the first study demonstrating a long-term benefit with anti-VEGF therapy in the treatment of type 3 neovascularization/RAP. Median Snellen visual acuity at presentation was 20/80 at baseline, improved to 20/40 at 1 month, and was maintained throughout the 36-month study period (n = 11 at 12 months, n = 10 at 24 months, and n = 8 at 36 months). Of the 11 eyes we treated and followed, only 2 did not regain or maintain reading vision, and not 1 eye worsened from baseline at the last follow-up visit. At 12 months, 4 of 11 (36%) eyes had more than one half their initial visual angle, and the rest remained essentially stable. This improvement was maintained during the 36-month study period. The improvement in logMAR-converted visual acuity at 12, 24, and 36 months was statistically significant (paired t-test, $P < 0.04$). Accordingly, there were statistically significant decreases in mean OCT from 320 μm to 170 and 190 μm at 12, 24, and 36 months ($P < 0.02$). The mean number of injections was seven in the first year, six in the second year, and seven in the third year.

These positive long-term outcomes contrast sharply with other treatment strategies tried before the availability of effective anti-VEGF therapy. Results with other treatment modalities, including our own "sequenced combined" regimen with intravitreal triamcinalone acetonide followed by PDT, have been universally inferior to anti-VEGF maintenance, although comparison with and between these studies are difficult because of generally short follow-up and differences in patient population.[6,10–15,18] Outcomes after laser therapy alone are generally poor, even if the localization of type 3 neovascularization/RAP lesion is juxta- or extravofeal with results typically in the 20/100 to 20/200 range.[3,32] The same is true for PDT,[11] where, in 1 study, visual acuity dropped from an average of 20/73 to 20/174 after an average of 13.7-month observation with 1.7 PDT sessions. Bottoni et al[3] experimented with focal- or grid-laser, PDT, and transpupillary therapy and reviewed their experience with 104 eyes treated for type 3 neovascularization/RAP, which they categorized by stages. In the majority of eyes treated in this large series, visual loss could not be prevented, and only in the subgroup with early type 3 neovascularization/RAP lesions could visual acuity be improved from an average of 20/200 to 20/100, which may not be much different than the natural history of these early lesions.[5] Our group eventually abandoned the "sequenced combined" strategy in favor of intravitreal anti-VEGF therapy because of progressive atrophic macular changes and associated visual decline occurring with recurrent exudation and retreatment seen with longer-term follow-up in our cohort of patients (unpublished data). Combined intravitreal triamcinolone and focal laser has also been studied by Krieglstein et al.[37] Although outcomes seemed superior to laser or PDT alone, this approach appears to have visual results inferior to the anti-VEGF therapy in our study. The population in the combined laser/triamcinolone study was probably similar to this study, with similar baseline acuities and lesion types, but vision improved only to a mean of 20/60 at 4 months compared with 20/30 after 3 monthly injections in our study.

In summary, "Treat and Extend" dosing of intravitreal anti-VEGF agents for type 3 neovascularization/RAP seems to yield improvements in visual acuity comparable with the gold standard monthly injection regimen in this aggressive subtype of neovascular AMD and seems capable of maintaining these results long term.

Key words: retinal angiomatous proliferation, type 3 neovascularization, RAP, bevacizumab, ranibizumab, lucentis, avastin, Treat and Extend.

References

1. Yannuzzi LA, Negrao S, Iida T, et al. Retinal angiomatous proliferation in age-related macular degeneration. Retina 2001; 21:416–434.
2. Freund KB, Ho IV, Barbazetto IA, et al. Type 3 neovascularization: the expanded spectrum of retinal angiomatous proliferation. Retina 2008;28:201–211.
3. Bottoni F, Massacesi A, Cigada M, Viola F, Musicco I, Staurenghi G. Treatment of retinal angiomatous proliferation in age-related macular degeneration: a series of 104 cases of retinal angiomatous proliferation. Arch Ophthalmol 2005;123:1644–1650.
4. Bressler NM. Retinal anastomosis to choroidal neovascularization: a bum rap for a difficult disease. Arch Ophthalmol 2005;123:1741–1743.
5. Viola F, Massacesi A, Levi G, Bottoni F, Staurenghi G, Orzalesi N. Retinal angiomatous proliferation: natural history. Invest Ophthalmol Vis Sci 2004;45:E-abstract 5254.
6. Joeres S, Heussen FM, Treziak T, Bopp S, Joussen AM. Bevacizumab (Avastin) treatment in patients with retinal angiomatous proliferation. Graefes Arch Clin Exp Ophthalmol 2007;245:1597–1602.
7. Slakter JS, Yannuzzi LA, Schneider U, et al. Retinal choroidal anastomoses and occult choroidal neovascularization in age-related macular degeneration. Ophthalmology 2000;107:742–753; discussion 753–744.
8. Kuroiwa S, Arai J, Gaun S, Iida T, Yoshimura N. Rapidly progressive scar formation after transpupillary thermotherapy in retinal angiomatous proliferation. Retina 2003;23:417–420.
9. Boscia F, Furino C, Sborgia L, Reibaldi M, Sborgia C. Photodynamic therapy for retinal angiomatous proliferations and pigment epithelium detachment. Am J Ophthalmol 2004;138:1077–1079.
10. Silva RM, Faria de Abreu JR, Travassos A, Cunha-Vaz JG. Stabilization of visual acuity with photodynamic therapy in eyes with chorioretinal anastomoses. Graefes Arch Clin Exp Ophthalmol 2004;242:368–376.
11. Boscia F, Parodi MB, Furino C, Reibaldi M, Sborgia C. Photodynamic therapy with verteporfin for retinal angiomatous proliferation. Graefes Arch Clin Exp Ophthalmol 2006;244:1224–1232.
12. Panagiotidis D, Karagiannis DA, Baltatzis S. Photodynamic therapy in retinal angiomatous proliferation stage I. Eur J Ophthalmol 2006;16:326–329.
13. Silva RM, Cachulo ML, Figueira J, de Abreu JR, Cunha-Vaz JG. Chorioretinal anastomosis and photodynamic therapy: a two-year follow-up study. Graefes Arch Clin Exp Ophthalmol 2007;245:1131–1139.
14. Costagliola C, Romano MR, dell'Omo R, Cipollone U, Polisena P. Intravitreal bevacizumab for the treatment of retinal angiomatous proliferation. Am J Ophthalmol 2007;144:449–451.
15. Meyerle CB, Freund KB, Iturralde D, et al. Intravitreal bevacizumab (Avastin) for retinal angiomatous proliferation. Retina 2007;27:451–457.
16. Sakimoto S, Gomi F, Sakaguchi H, Tano Y. Recurrent retinal angiomatous proliferation after surgical ablation. Am J Ophthalmol 2005;139:917–918.
17. Shiragami C, Iida T, Nagayama D, Baba T, Shiraga F. Recurrence after surgical ablation for retinal angiomatous proliferation. Retina 2007;27:198–203.
18. Freund KB, Klais CM, Eandi CM, et al. Sequenced combined intravitreal triamcinolone and indocyanine green angiography-guided photodynamic therapy for retinal angiomatous proliferation. Arch Ophthalmol 2006;124:487–492.
19. van de Moere A, Kak R, Sandhu SS, Talks SJ. Anatomical and visual outcome of retinal angiomatous proliferation treated with photodynamic therapy and intravitreal triamcinolone. Am J Ophthalmol 2007;143:701–704.
20. Reche-Frutos J, Calvo-Gonzalez C, Donate-Lopez J, et al. Retinal angiomatous proliferation reactivation 6 months after high-dose intravitreal acetonide triamcinolone and photodynamic therapy. Eur J Ophthalmol 2007;17:979–982.
21. Krebs I, Krepler K, Stolba U, Goll A, Binder S. Retinal angiomatous proliferation: combined therapy of intravitreal triamcinolone acetonide and PDT versus PDT alone. Graefes Arch Clin Exp Ophthalmol 2008;246:237–243.
22. Brown DM, Kaiser PK, Michels M, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. N Engl J Med 2006;355:1432–1444.
23. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–1431.
24. Regillo CD, Brown DM, Abraham P, et al. Randomized, double-masked, sham-controlled trial of ranibizumab for neovascular age-related macular degeneration: PIER Study year 1. Am J Ophthalmol 2008;145:239–248.
25. Fung AE, Lalwani GA, Rosenfeld PJ, et al. An optical coherence tomography-guided, variable dosing regimen with intravitreal ranibizumab (Lucentis) for neovascular age-related macular degeneration. Am J Ophthalmol 2007;143:566–583.
26. Lalwani GA, Rosenfeld PJ, Fung AE, et al. A variable-dosing regimen with intravitreal ranibizumab for neovascular age-related macular degeneration: year 2 of the PrONTO Study. Am J Ophthalmol 2009;148:43–58.
27. Barbazetto I, Saroj N, Freund KB. Dosing regimen and the frequency of macular hemorrhages in neovascular age-related macular degeneration treated with ranibizumab. Presented at the Annual Meeting of the Retina Society, Scottsdale, AZ, September 2008.
28. Margolis R, Freund KB. Hemorrhagic recurrence of neovascular age-related macular degeneration not predicted by spectral domain optical coherence tomography. Retin Cases Brief Rep. In press.
29. Levine JP, Marcus I, Sorenson JA, Spaide RF, Cooney MJ, Freund KB. Macular hemorrhage in neovascular age-related degeneration after stabilization with anti-angiogenic therapy. Retina 2009;29:1074–1079.
30. Yannuzzi LA. Neovascular AMD: out of the forest and into the trees. Retina 2007;27:655–661.
31. Spaide R. Ranibizumab according to need: a treatment for age-related macular degeneration. Am J Ophthalmol 2007;143:679–680.
32. Kuhn D, Meunier I, Soubrane G, Coscas G. Imaging of chorioretinal anastomoses in vascularized retinal pigment epithelium detachments. Arch Ophthalmol 1995;113:1392–1398.
33. Gross NE, Aizman A, Brucker A, Klancnik JM Jr, Yannuzzi LA. Nature and risk of neovascularization in the fellow eye of patients with unilateral retinal angiomatous proliferation. Retina 2005;25:713–718.
34. Abi-Ayad N, Mauget-Faysse M, Amara-Allieu S, Ardisson P. Systemic bevacizumab for retinal angiomatous proliferation associated with retinal pigment epithelial detachment. Eur J Ophthalmol 2007;17:987–991.
35. Kang JH, Park KA, Chung SE, Kang SW. Retinal angiomatous proliferation and intravitreal bevacizumab injection. Korean J Ophthalmol 2007;21:213–215.
36. Gharbiya M, Allievi F, Recupero V, Martini D, Mazzeo L, Gabrieli CB. Intravitreal bevacizumab as primary treatment for retinal angiomatous proliferation: twelve-month results. Retina 2009;29:740–749.
37. Krieglstein TR, Kampik A, Ulbig M. Intravitreal triamcinolone and laser photocoagulation for retinal angiomatous proliferation. Br J Ophthalmol 2006;90:1357–1360.

LONG-TERM FOLLOW-UP FOR TYPE 1 (SUBRETINAL PIGMENT EPITHELIUM) NEOVASCULARIZATION USING A MODIFIED "TREAT AND EXTEND" DOSING REGIMEN OF INTRAVITREAL ANTIVASCULAR ENDOTHELIAL GROWTH FACTOR THERAPY

MICHAEL ENGELBERT, MD, PHD,*† SANDRINE A. ZWEIFEL, MD,†‡ K. BAILEY FREUND, MD*†§

Purpose: The purpose of the study was to analyze long-term outcomes for the treatment of type 1 (subretinal pigment epithelium) neovascularization using a modified "treat and extend" antivascular endothelial growth factor dosing regimen.

Methods: We performed a retrospective, noncomparative analysis of visual acuity, funduscopic, and optical coherence tomography data for 18 eyes of 16 consecutive patients with newly diagnosed type 1 neovascularization treated with intravitreal bevacizumab and/or ranibizumab with at least 24-month follow-up. Three monthly injections were followed by continued treatment at intervals increasing by 2 weeks per visit to a maximum of 10 weeks. The interval was shortened if clinical or optical coherence tomography evidence of recurrent fluid at the foveola or increased extrafoveolar fluid was detected.

Results: Median baseline logarithm of the minimum angle of resolution visual acuity was 0.53 (20/69 Snellen equivalent) and remained stable at 24 months (logarithm of the minimum angle of resolution 0.52, $P = 0.84$) after an average of 12 injections (range, 8–19 injections) and at 36 months (logarithm of the minimum angle of resolution 0.52, $P = 0.68$) after an average of 20 injections (range, 18–25 injections). Although most eyes (15 of 18 [83%]) continued to manifest extrafoveolar subretinal fluid throughout the course of treatment, only 1 eye developed geographic atrophy overlying the areas of choroidal neovascularization. During a cumulative observation period of 540 months, no eyes developed a sight-threatening submacular hemorrhage.

Conclusion: A modified "treat and extend" dosing regimen of intravitreal antivascular endothelial growth factor therapy reduces the need for monthly visits and imaging and allows for stable long-term visual acuity in eyes with type 1 neovascularization.

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Type 1 neovascularization occurs as a subtype of neovascular age-related macular degeneration (AMD) in which the abnormal vessels are located between Bruch membrane and the basal surface of the retinal pigment epithelium (RPE). Type 1 neovascularization typically exhibits an occult pattern with fluorescein angiography and always manifests some degree of RPE elevation with optical coherence tomography (OCT). The type 1 pattern has a different natural course and treatment response from the type 2 (classic) and type 3 (retinal angiomatous proliferation) neovascular patterns. For example, eyes with type 1 (occult) neovascularization presented with better visual acuity in the MARINA study[1] than eyes with type 2 (classic) neovascularization in the ANCHOR study.[2] In addition, in these trials, patients with type 1 neovascularization did not gain as many letters[1] as patients with type 2 neovascularization,[2] although they were treated with the same dosing regimen of continuous monthly intravitreal injections of ranibizumab during 24 months.

It has been hypothesized that the type 1 neovascular pattern may be a compensatory form of neovascular

growth occurring in response to an ischemic outer retina.[3] Type 1 vessels may represent a more mature form of neovascularization confined to the sub-RPE space and may be less responsive to antivascular endothelial growth factor (anti-VEGF therapy) than other neovascular patterns. In addition, by recapitulating the choriocapillaris, these vessels may provide nutritional support to the outer retina and could theoretically protect against the advent of geographic atrophy (GA).

Although a monthly dosing regimen of intravitreal ranibizumab has the greatest scientific support for efficacy, it may be difficult to sustain in the elderly population with AMD. In addition, by inhibiting a potentially compensatory neovascular response, GA could be accelerated in eyes treated with more aggressive anti-VEGF dosing regimens. However, the relatively infrequent dosing in the PIER trial,[4] in which patients received quarterly injections after an initial series of three monthly injections, resulted in inferior visual results when compared with the trials that were using monthly dosing.

The PrONTO Study[5] attempted to customize the dosing to the individual needs of the patient based on acuity decline, clinical findings, or OCT evidence of disease activity. The results from the 37 patients who completed this trial seemed to be favorable at both during 12- and 24-month period.[5,6] As a result, PrONTO-style dosing has become popular in the retina community. Nonetheless, this strategy does require monthly visits, clinical examinations, and OCTs, and patients are uncertain if or when they will need treatment. In addition, there have been more recent concerns that patients who are no longer receiving regular maintenance intravitreal anti-VEGF injections can occasionally experience sudden sight-threatening macular hemorrhages within days or weeks after a stable clinical examination and an OCT showing no apparent sub- or intraretinal fluid.[7–9]

The "treat and extend" dosing regimen is a strategy intended to resolve macular exudation and then maintain the macula in this "dry" state indefinitely with, when possible, fewer patient visits and treatments than monthly dosing.[10] We recently reported encouraging long-term results using this regimen in eyes with type 3 neovascularization.[11] The dosing strategy consists of an initial induction or "loading" sequence of at least three initial monthly injections. If stable visual acuity, an absence of macular hemorrhage, and a dry OCT have been achieved at this point, patients continue to receive regular maintenance injections at increasing intervals. At 6 weeks after the last of the 3 initial monthly injections, visual acuity, clinical findings, and OCT changes are recorded again, and patients receive an injection regardless of the presence or absence of disease activity. However, the interval to the next visit (and scheduled injection) is based on an observed change in these parameters. If there are no changes, the next visit is scheduled for 8 weeks. If there is a change, the patient returns for another scheduled injection and examination after 4 weeks. The observation and scheduled treatment interval is extended (hence the phrase "treat and extend"). In our clinical experience, the risk of recurrent sight-threatening hemorrhages seems to increase because the interval between injections of anti-VEGF agents is extended. Because of this concern, 10 weeks was chosen as the longest interval between office visits and treatments in this and in our previous study of eyes with type 3 neovascularization.[7–9,11]

We now report on 18 eyes of 16 consecutive patients with newly diagnosed type 1 neovascularization treated with intravitreal bevacizumab and/or ranibizumab using a modified "treat and extend" dosing regimen and followed for at least 24 months.

Materials and Methods

Waiver of authorization for use of protected health information for the referenced research and a waiver of consent for this retrospective chart review were obtained from the Institutional Review Board Committee of the Manhattan Eye Ear and Throat Hospital, New York, NY.

The diagnosis of type 1 neovascularization was performed by the treating physician (K.B.F.) based on the clinical, fluorescein angiographic, OCT, and, in some cases, indocyanine green angiographic findings. To be considered as having the type 1 neovascular pattern, eyes had to have clinical and OCT evidence of subretinal fluid and/or hemorrhage with an associated elevation of the RPE. Fluorescein angiography of these eyes exhibited late leakage and staining in an indistinct or "occult" pattern. When available, a well-delineated "plaque" found on indocyanine green angiography was correlated with the OCT finding of a vascularized pigment epithelial detachment (PED). Only patients with recent symptoms, hemorrhage, or evidence of recent disease progression were included in this

From the *Edward S. Harkness Eye Institute, Columbia University College of Physicians and Surgeons, New York, New York; the †LuEsther T. Mertz Retinal Research Center, New York, New York; the ‡Department of Ophthalmology, University Hospital Zurich, Zurich, Switzerland; and the §Vitreous-Retina-Macula Consultants of New York, New York.

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Reprint requests: K. Bailey Freund, MD, Vitreous Retina Macula Consultants of New York, 460 Park Avenue, 5th Floor, New York, NY 10022; e-mail: kbfnyf@aol.com

analysis. Patients treated previously with thermal laser, photodynamic therapy, or intravitreal pegaptanib (Macugen, Eyetech Pharmaceuticals Inc., Palm Beach Gardens, FL) or who presented with subfoveal fibrosis or atrophy, a history of vitrectomy, aphakia, a history of idiopathic or autoimmune-associated uveitis in either eye, or diabetic retinopathy more severe than mild nonproliferative stage were excluded from this study. Patients with preexisting cardiac or cerebrovascular conditions were not excluded from the study.

Patient characteristics, including age, sex, and presence or absence of disease in the contralateral eye, were recorded. Treatment consisted of intravitreal injection of 1.25 mg/0.05 mL bevacizumab or 0.5 mg/0.05 mL ranibizumab. For the purpose of this analysis, no distinction was made between the antiangiogenic drugs. Before intravitreal injection, topical anesthesia and surface disinfection with 5% povidone-iodine were performed. Intravitreal injections were administered at the time of diagnosis and subsequently following a protocol we have termed "treat and extend." In contrast to a PrONTO-style regimen, patients did not have to return for monthly examinations. Instead, all the patients received at least 3 initial monthly injections followed by continued examination and treatment at intervals increasing by 2 weeks per visit once visual acuity was stable and clinical examination and OCT showed an absence of intra- and subretinal fluid at the foveola, resolution of all macular hemorrhage, and no further reduction in extrafoveolar subretinal fluid. Because most eyes with the type 1 neovascular pattern continued to manifest PED and/or extrafoveolar subretinal fluid after the initial 3 monthly injections, resolution of PEDs and/or extrafoveolar subretinal fluid that was judged not to affect visual acuity was not required before the treatment intervals were lengthened. The treatment interval was extended to a maximum of 10 weeks "maintenance" unless clinical examination or OCT detected new hemorrhage, persistent/recurrent intra- or subretinal fluid at the foveola, or an increase in PED size and/or extrafoveolar subretinal fluid. In those cases, the interval was shortened by 2 weeks and maintained at that duration provided this restored the clinical and OCT findings back to their previous level.

In this study, the main outcome measure was visual acuity after treatment. The number of injections needed and change in funduscopic or tomographic appearance were also assessed. Specifically, presence of a PED, subretinal fluid, sight-threatening submacular hemorrhage, defined as a subretinal hemorrhage of any size within 200 μm of the foveal center or a subretinal hemorrhage of at least 2 disk areas within the temporal vascular arcades as well as presence and progression of GA were recorded.

Snellen visual acuity was measured by a certified ophthalmic technician. Snellen acuity was converted into logarithm of the minimum angle of resolution (logMAR) for statistical analysis at baseline and subsequently at 1, 2, 3, 24, and 36 months after injection of an antiangiogenic agent. Changes in logMAR-converted acuities were tested with a paired Student's t-test and accepted as significant if the P value was < 0.05.

Qualitative assessments of retinal thickness were initially made using Stratus OCT (Carl Zeiss Meditec, Dublin, CA). Later in the study, including at last follow-up visits, the Topcon OCT (Topcon 3D OCT-1000, Topcon Medical Systems, Paramus, NJ) or Spectralis HRA + OCT (Heidelberg Engineering, Inc., Heidelberg, Germany) was used.

Fundus photography, fluorescein angiograms, enface OCT scan images, and, where available, autofluorescence photography were examined for the presence and progression of GA overlying the areas of type 1 neovascularization.

Results

Eighteen eyes of 16 consecutive patients with newly diagnosed type 1 neovascularization treated with intravitreal injections of bevacizumab and/or ranibizumab with at least 24-month follow-up were included in this study. Nine eyes completed 36-month follow-up.

Median patient age was 79 years (range, 67–90 years). Twelve of 16 patients were women. Four contralateral eyes had evidence of neovascular AMD and 2 of these eyes were treated with a "treat and extend" regimen and included in the study.

Median logMAR visual acuity at presentation was 0.53 (Snellen equivalent 20/69) and transiently improved to 0.41 (Snellen equivalent 20/51) at 1 month and maintained at this level during the next 2 months (Figure 1). The difference in logMAR-converted visual acuity was statistically significant at all early time points (paired 2-tailed t-test, $P < 0.05$). At 24 months and after an average of 12 injections (range, 8–19), median logMAR visual acuity was 0.52, which was not statistically significantly different from baseline ($P = 0.84$). For 9 eyes, 36-month follow-up data after an average of 20 injections (range, 18–25 injections) were available. Visual acuity remained stable compared with the 24-month time point with a logMAR of 0.52, which also was no different from the baseline visual acuity of 0.54 for these 9 eyes ($P = 0.68$).

As mandated by our inclusion criteria, all eyes had serous or vascularized PEDs present at the initiation of anti-VEGF treatment. Ten of the 18 eyes had OCT evidence of intraretinal fluid on presentation, and 17 of

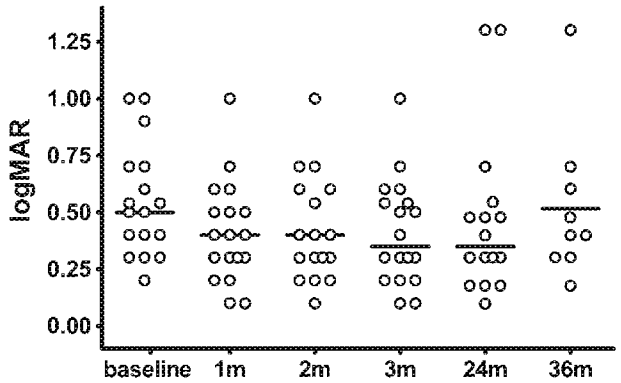

Fig. 1. Mean Snellen visual acuity of patients with type 1 choroidal neovascularization on a "treat and extend" regimen was assessed at baseline and 1, 2, 3, 24, and 36 months and plotted after conversion to logMAR. Horizontal lines represent the median.

18 eyes had subretinal fluid. In all these latter cases, the subretinal fluid involved the foveola. At the last follow-up examination, PEDs had resolved in only 3 of 18 eyes (17%). Most eyes (15 of 18 [83%]) continued to manifest extrafoveolar subretinal fluid throughout the course of treatment. Of the 10 eyes with intraretinal fluid on presentation, complete resolution of this fluid occurred in only 3 eyes. In the remaining seven eyes, the intraretinal fluid resolved at the foveola but persisted elsewhere within the macula. Three eyes that initially presented without intraretinal fluid developed extrafoveolar intraretinal fluid during the follow-up period. One eye developed a new PED while on the anti-VEGF treatment regimen.

Only 1 of 18 eyes developed GA overlying the areas of type 1 neovascularization. Accordingly, visual acuity decreased from 20/200 to 20/400 in this patient. During a cumulative observation period of 540 months, no eyes developed a sight-threatening submacular hemorrhage.

Figure 2 shows a representative case of a 75-year-old woman treated with the modified "treat and extend" dosing regimen (31 ranibizumab injections) during 36 months. The patient's visual acuity remained stable despite persistent extrafoveolar subretinal and intraretinal fluid.

Discussion

Although intravitreal anti-VEGF therapy for neovascular AMD has produced visual outcomes superior to previous therapies, the optimal dosing regimen for these agents remains uncertain. Similarly, whether different neovascular patterns respond differently or require different dosing regimens remains unclear. Although monthly dosing of anti-VEGF agents gives visual outcomes superior to previous treatments,[1,2] it can place a tremendous burden on patients, retinal practices, and the healthcare system as a whole. In addition, safety concerns about long-term monthly injections argue in favor of exploring alternative dosing regimens.

The only randomized, double-blind, sham-controlled trial investigating an alternative dosing scheme is the PIER study,[4] which showed that a regimen consisting of three initial monthly injections followed by mandated quarterly dosing gives inferior visual results compared with a monthly dosing regimen. Presumably, persistent and/or recurrent exudation occurring during the extended intervals between treatments was related to these inferior visual outcomes. The PrONTO study investigated a strategy intended to limit macular exudation in which three initial monthly injections were followed by dosing on an as-needed basis based on changes in visual acuity, clinical findings, and evaluation of OCT.[5,6] Although this open-label, nonrandomized study seemed to show that visual results similar to monthly dosing could be achieved with fewer injections, patients still required monthly visits, examinations, and OCTs. Furthermore, after the initial mandated series of three injections, fluid was allowed to reaccumulate at the foveola before the treatment was repeated, raising concerns regarding incremental long-term vision loss and the possibility of new hemorrhages occurring during long periods without VEGF inhibition.

Type 1 (occult) neovascularization tends to have a variable but often less aggressive natural course compared with type 2 (well-defined [classic]) neovascularization and type 3 (retinal angiomatous proliferation) neovascularization based on the presenting acuities and long-term natural history data. Patients with type 1 neovascularization who were enrolled in the MARINA study presented with a mean Early Treatment Diabetic Retinopathy Study letter score of 53 (Snellen equivalent 20/80^{-}),[1] whereas patients with type 2 neovascularization enrolled in the ANCHOR study presented with only 45 letters (Snellen equivalent of 20/125).[2] It is well known that some patients who have evidence of type 1 (occult) neovascularization may never experience vision loss (often despite continued growth of the lesion),[12] or they may experience only a mild gradual visual decline. This benign natural course may relate to the theory that, in some eyes, the type 1 neovascular growth pattern may develop as a compensatory form of neovascularization providing nutritional support to an ischemic outer retina by recapitulating the normal choriocapillaris.[3] Our finding that only 1 of 18 eyes (6%) developed GA overlying the areas of type 1 neovascularization may support this hypothesis. However, in some eyes, the type 1 neovascular pattern

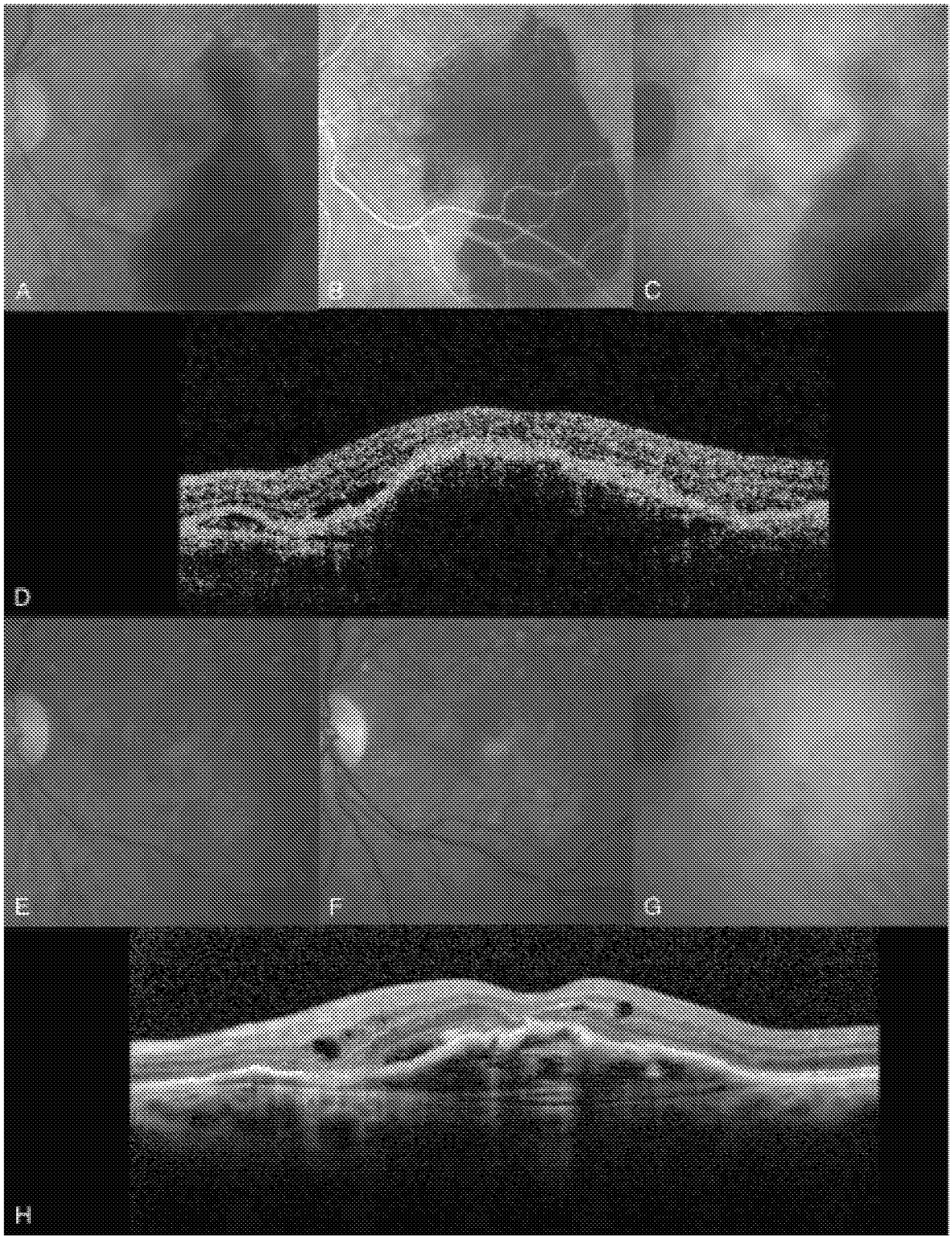

Fig. 2. Representative case. **A–D**. Left eye of a 75-year-old woman with 20/200 visual acuity at baseline. **A**. Color fundus photograph showing a large area of subretinal hemorrhage temporal to a vascularized PED. **B**. Fluorescein angiogram shows type 1 neovascularization nasal to blocked fluorescence resulting from sub-RPE and subretinal hemorrhage. **C**. Late indocyanine green angiogram shows a plaque of choroidal neovascularization representing type 1 neovascularization. **D**. Spectral domain OCT scan (Topcon 3D) shows a vascularized PED and subretinal fluid. **E–H**. Images at 36-month follow-up dosed according to the "treat and extend" protocol. Visual acuity is stable. **E**. Color fundus photograph. **F**. Red-free photograph shows resolution of the hemorrhage. **G**. Late indocyanine green angiogram shows a persistent plaque representing type 1 neovascularization. **H**. Spectral domain OCT scan (Heidelberg) shows persistent sub- and intraretinal fluid.

may follow a more aggressive course similar to type 2 vessels, or these vessels may erode through the RPE becoming type 2 neovascularization within 1 year leading to more rapid vision loss.[13–15]

Given a more variable natural course, a rigid monthly, "one-size-fits-all" dosing regimen may be less suitable for eyes with type 1 vessels than for eyes with other neovascular patterns and (in theory) could inhibit an important compensatory mechanism aimed at preventing loss of overlying neurosensory elements. We believe that aggressive strategies aimed at eliminating type 1 neovascularization could ultimately prove to be detrimental by accelerating GA in some patients. This concern is limited not only to a fixed-schedule continuous monthly regimen, but also to combination strategies using verteporfin photodynamic therapy or radiation that aim to completely occlude the neovascular lesion. It is well known that photodynamic therapy monotherapy affects pathologic neovascularization and vessels perfusing the normal choriocapillaris.[16–18] Combination strategies that add an antiangiogenic agent[19] or an anti-inflammatory agent seem to enhance and prolong choroidal hypoperfusion, occasionally resulting in profound visual loss.[20,21] It is unknown what effects this hypoperfusion may have on RPE and the outer retina, but obvious concerns are GA and photoreceptor damage.

The "treat and extend" dosing regimen is a tailored maintenance regimen intended to achieve optimal visual results with two additional goals.[10] One goal is to reduce the treatment burden by reducing the number of patient visits and the number of imaging studies performed by eliminating the need for the monthly visits necessitated by alternative dosing strategies. We recently reported success in achieving this goal in a small cohort of eyes with newly diagnosed type 3 neovascularization.[11] In this previous report, patients experienced a sustained visual improvement of ~2 Snellen lines with nearly half the number of office visits and injections compared with a monthly dosing regimen.

A second goal of the "treat and extend" dosing regimen is to reduce the risk of new sight-threatening submacular hemorrhages. We recently showed a statistically significant increase in macular hemorrhages when patients in the PIER trial were switched from a monthly to quarterly dosing regimen.[8] Unfortunately, large and potentially devastating submacular hemorrhages may occur almost immediately after a high-quality OCT examination showing an absence of fluid.[7,9] Theoretically, eyes treated with an OCT-guided as-needed regimen in which patients may go long intervals without VEGF suppression could be at greater risk for sight-threatening submacular hemorrhages compared with eyes receiving more frequent and regular anti-VEGF treatments. Because of our concern regarding the risk of new hemorrhages with long intervals between treatments, we limited the interval between anti-VEGF injections to no longer than 10 weeks.

In applying the "treat and extend" strategy to eyes with type 1 neovascularization, we elected to modify the regimen used in our previous report in which the dosing interval was extended only in the absence of intraretinal fluid, subretinal fluid, and PED.[11] In contrast to eyes with type 2 and 3 vessels, eyes with type 1 neovascularization often continue to manifest extrafoveolar subretinal fluid (83% in this series) and/or PED (83% in this series) after a loading sequence of 3 monthly intravitreal injections of an anti-VEGF agent. The type 1 neovascular lesions are typically larger and may represent a more mature neovascular phenotype that is less responsive to anti-VEGF treatment. In this study, after three monthly treatments, we extended the dosing interval even in the presence of a persistent PED and provided any remaining fluid spared the foveola and was judged not to be affecting visual acuity.

In our series, patients following this modified "treat and extend" regimen were seen on average 12 times (range, 8–19) during the first 24 months reflecting the variable course of these eyes and a reduced need for retreatment. Although patients on a "treat and extend" dosing regimen receive a mandated injection at each visit, their eyes received a similar number of injections as received by the 37 patients who completed 24-month follow-up in the PrONTO study (average, 9.9; range, 3–25) during the first 24 months.[6] The significant reduction in patient visits of nearly 50% without an increase in the number of treatments could potentially decrease the burden on patients, practitioners, and the healthcare system as a whole.

None of the 18 eyes in our series experienced a sight-threatening hemorrhage during a cumulative observation period of 540 months. This finding seemed to support our hypothesis that more frequent and consistent dosing of anti-VEGF treatment may help reduce the occurrence of new macular hemorrhages.

In our goal of evaluating the long-term results of the "treat and extend" dosing regimen, we wanted to investigate whether visual acuity could be improved and maintained with this anti-VEGF treatment regimen and how any such effect observed would compare historically with outcomes reported for the large randomized MARINA[1] trial, which examined almost exclusively type 1 neovascular lesions. Our patients' baseline visual acuity of 20/69 was somewhat

better than the mean baseline Early Treatment Diabetic Retinopathy Study letter score in the MARINA study, which was 53.6 letters (Snellen equivalent of 20/80⁻). Although, unlike in the MARINA trial, there was not a statistically significant visual improvement at 24 months, the mean visual acuity of 20/70 at this time point was similar to that of the MARINA trial. In addition, the reported results of the MARINA trial apply only to those patients who were willing and able to complete 2 years of monthly visits and injections. In the MARINA trial, 14.1% of enrolled patients did not complete the 24-month follow-up visit. Similarly, in our experience, it is often difficult to sustain monthly visits in the population with neovascular AMD.

It is problematic to compare our visual acuity data with the PrONTO study[5,6] in which patients gained an average of 11.3 letters because the lesion compositions in the 2 studies were dissimilar. In the PrONTO study, 75% of eyes had lesions with at least some classic choroidal neovascularization (type 2 neovascularization) and 25% had retinal angiomatous proliferation (type 3 neovasularization). These more aggressive lesions tend to present with more active exudation and worse visual acuity (20/80+ in the PrONTO study) compared with type 1 lesions. This may offer such patients a greater chance for visual improvement after resolution of exudation. We have recently reported on type 3 lesions treated according to the "treat and extend" regimen in which an average of ~2 lines of improvement was found after 24 months.[11] This is similar to the ANCHOR study in which patients with exclusively classic lesions gained 11.3 letters. The superior visual results in eyes with type 3 neovascularization may relate to a smaller size and a more robust response to treatment than are typical for type 1 lesions. In addition, in our previous study, we used a more aggressive treatment regimen in which an absence of PED and fluid both centrally and in the extrafoveolar macula was required before the interval between injections was extended.

Although historical comparisons between different studies may be relevant, they are hampered by the impossibility of statistical analysis. Conclusions about the superiority of one treatment protocol or the other remain speculative at best, particularly when the numbers in the studies being compared are low.

Unlike the MARINA and PrONTO studies, which are limited to 24-month follow-up, 9 of our 17 patients completed 36-month follow-up with a mean of 20 injections. Visual acuity remained stable at the 24-month level, suggesting true long-term stabilization. We are not aware of any other dosing regimen of anti-VEGF therapy, which has showed stable visual acuity >3 years. Furthermore, the absence of sight-threatening macular hemorrhages and rare progression of GA overlying the neovascular lesions suggest additional long-term benefits of the "treat and extend" dosing regimen.

Our study is limited by its retrospective, noncomparative nature and relatively few patients. Another limitation is a possible ascertainment bias as a result of a methodology that excluded patients with a follow-up of <24 months and those who were noncompliant with the "treat and extend" dosing regimen. It is possible that patients who did not have 24-month follow-up may have discontinued the treatment regimen as a result of poor outcomes. However, few such cases could be recalled.

Despite these limitations, our study supports that the modified "treat and extend" dosing regimen used in this study for type 1 neovascularization may be a safe and effective way to reduce the number of follow-up visits and injections required in these eyes for up to 36 months. Despite persistent PEDs and extrafoveolar fluid in most eyes, visual acuity remained stable with no eyes experiencing sight-threatening submacular hemorrhages. Given these encouraging long-term results of a treatment strategy designed to control but not eliminate a potentially protective compensatory form of neovascularization, we suggest that future studies aimed at improving visual outcomes in eyes harboring type 1 neovascularization explore similar nondestructive treatment modalities.

Key words: type 1 neovascularization, bevacizumab, ranibizumab, Lucentis, Avastin, treat and extend.

References

1. Rosenfeld PJ, Brown DM, Heier JS, et al; MARINA Study Group. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–1431.
2. Brown DM, Kaiser PM, Michels M, et al; ANCHOR Study Group. Ranibizumab versus verteporforin for neovascular age-related macular degeneration. N Engl J Med 2006;355: 1432–1444.
3. Grossniklaus HE, Green WR. Choroidal neovascularization. Am J Ophthalmol 2004;137:496–503.
4. Regillo CD, Brown DM, Abraham P, et al. Randomized, double-masked, sham-controlled trial of ranibizumab for neovascular age-related macular degeneration: PIER Study year 1. Am J Ophthalmol 2008;145:239–248.
5. Fung AE, Lalwani GA, Rosenfeld PJ, et al. An optical coherence tomography-guided, variable dosing regimen with intra-vitreal ranibizumab (Lucentis) for neovascular age-related macular degeneration. Am J Ophthalmol 2007;143:566–583.
6. Lalwani GA, Rosenfeld PJ, Fung AE, et al. A variable-dosing regimen with intravitreal ranibizumab for neovascular age-related macular degeneration: year 2 of the PrONTO Study. Am J Ophthalmol 2009;148:43–58.el.
7. Levine JP, Marcus I, Sorenson JA, Spaide RF, Cooney MJ, Freund KB. Macular hemorrhage in neovascular age-related

macular degeneration after stabilization with antiangiogenic therapy. Retina 2009;29:1074–1079.
8. Barbazetto I, Saroj N, Freund KB. Dosing regimen and the frequency of macular hemorrhages in neovascular age-related macular degeneration treated with ribizumab. Presented at the Annual Meeting of the Retina Society, New York, NY, September 2008.
9. Margolis R, Freund KB. Hemorrhagic recurrence of neovascular age-related macular degeneration not predicted by spectral domain optical coherence tomography. Retin Cases Brief Rep In press.
10. Spaide R. Ranibizumab according to need: a treatment for age-related macular degeneration. Am J Ophthalmol 2007;143: 679–680.
11. Engelbert M, Zweifel S, Freund KB."Treat and Extend" dosing of intravitreal anti-vascular endothelial growth factor therapy for type 3 neovascularization/retinal angiomatous proliferation. Retina In press.
12. Blinder KJ, Bradley S, Bressler NM, et al; Treatment of Age-Related Macular Degeneration with Photodynamic Therapy study group; Verteporfin in Photodynamic Therapy study group. Effect of lesion size, visual acuity, and lesion composition on visual acuity change with and without verteporfin therapy for choroidal neovascularization secondary to age-related macular degeneration: TAP and VIP report no. 1. Am J Ophthalmol 2003;136:407–418.
13. Bressler NM, Arnold J, Benchaboune M, et al; Treatment of Age-Related Macular Degeneration with Photodynamic Therapy (TAP) study group. Verteporfin therapy of subfoveal choroidal neovascularization in patients with age-related macular degeneration: additional information regarding baseline lesion composition's impact on vision outcomes—TAP report No. 3. Arch Ophthalmol 2002;120:1443–1454.
14. Bressler SB, Pieramici DJ, Koester JM, Bressler NM. Natural history of minimally classic subfoveal choroidal neovascular lesions in the treatment of age-related macular degeneration with photodynamic therapy (TAP) investigation: outcomes potentially relevant to management—TAP report No. 6. Arch Ophthalmol 2004;122:325–329.
15. Macular Photocoagulation Study Group. Occult choroidal neovascularization. Influence on visual outcome in patients with age-related 400–412; Erratumin Arch Ophthalmol 1996; 114: 400–412; Erratum in Arch Ophthalmol 1996;114:1023.
16. Cardillo PF, Eandi CM, Ventre L, Rigault de la Longrais RC, Grignolo FM. Photodynamic therapy for chronic central serous chorioretinopathy. Retina 2003;23:752–763.
17. Gelisken F, Lafaut BA, Inhoffen W, Voelker M, Grisanti S, Bartz-Schmidt KU. Clinicopathological findings of choroidal neovascularization following verteporforin-photodynamic therapy. Br J Ophthalmol 2004;88:207–211.
18. Recchia FM, Greenbaum S, Recchia CA, Ruby AJ, Alldredge CD, Hassan TS. Self-reported acute decrease in visual acuity after photodynamic therapy for age-related macular degeneration. Retina 2006;26:1042–1048.
19. Kiss CG, Wagner J, Simader C, Michels S, Schmidt-Erfurth U. Effect of photodynamic therapy (PDT) and ranibizumab on vascular anatomy and retinal function in neovascular age-related macular degeneration. Invest Ophthalmol Vis Sci 2007;48:4544.
20. Lo Giudice G, De Belvis V, Piermarocchi S, Galan A, Prosdocimo G. Acute visual loss and chorioretinal infarction after photodynamic therapy combined with intravitreal triamcino-lone. Eur J Ophthalmol 2008;18:652–655.
21. Blaha GR, Wertz FD, Marx JL. Profound choroidal hypoperfusion after combined photodynamic therapy and intravitreal triamcinolone acetonide. Ophthalmic Surg Lasers Imaging 2008;39:6–11.