

US007303746B2

(12) United States Patent Wiegand et al.

(54) METHODS OF TREATING EYE DISORDERS WITH MODIFIED CHIMERIC POLYPEPTIDES

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- (*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 425 days.
- (21) Appl. No.: 10/988,243
- (22) Filed: Nov. 12, 2004

(65) Prior Publication Data

US 2005/0175610 A1 Aug. 11, 2005

Related U.S. Application Data

- (63) Continuation-in-part of application No. 10/009,852, filed as application No. PCT/US00/14142 on May 23, 2000, now Pat. No. 7,070,959.
- (60) Provisional application No. 60/138,133, filed on Jun. 8, 1999.
- (51) Int. Cl.

A61K 38/18	(2006.01)
C07K 14/71	(2006.01)
C12N 15/62	(2006.01)

- (52) **U.S. Cl.** 424/134.1; 424/192.1; 514/2; 514/12; 530/350; 530/387.3; 536/23.4
- (58) **Field of Classification Search** None See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,712,380 A 1/1998 Kendall et al.

(10) Patent No.: US 7,303,746 B2

(45) **Date of Patent: Dec. 4, 2007**

6,011,003	Α	1/2000	Charnock-Jones et al.
2005/0260203	A1 $*$	11/2005	Wiegand et al 424/145.1
2006/0030529	A1 $*$	2/2006	Wiegand et al 514/12
2006/0172944	$\Delta 1 *$	8/2006	Wiegand et al 514/12

FOREIGN PATENT DOCUMENTS

WO	WO97/44453	11/1997
WO	WO98/13071	4/1998
WO	WO99/03996	1/1999

OTHER PUBLICATIONS

Witmer et al. (2003). Vascular endothelial growth factors and angiogenesis in eye disease. Prog. Retin. Eye Res. 22:1-29.* Terman, B. I., et al, "Identification of a new endothelial cell growth

factor receptor tyrosine kinase", Oncogene (1991) 6:1677-1683. Terman, B.I., et al, "Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor", Biochem Biophys Res Comm (1992) 187(3):1579-1586.

Davis-Smyth, T., et al., 1996, "The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1 determines ligand binding and may initiate a signal transduction cascade", The EMBO Journal 15(18):4919-4927.

Holash, J., et al., (2002) PNAS 99(17):11393-11398.

Heidaran, M.A., et al., (1990) J. Bio. Chem. 265(31):18741-18744. Cunningham, S.A., et al., (1997) Biochem. Biophys. Res. Comm. 231:596-599.

Fuh, G., et al., (1998) J. Bio. Chem. 273(18):11197-11204.

Wiesmann, C., et al., (1997) Cell 91:695-704.

Barleon, B., et al., (1997) J. Bio. Chem. 272(16):10382-10388. Davis-Smyth, T., et al., (1998) J. Bio. Chem. 273(6):3216-3222.

* cited by examiner

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(57) ABSTRACT

Modified chimeric polypeptides with improved pharmacokinetics are disclosed useful for treating eye disorders, including age-related macular degeneration and diabetic retinopathy.

5 Claims, 21 Drawing Sheets

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Fig. 1

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Fig. 2

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Fig. 3

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Fig. 4

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Fig. 5

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Fig. 6

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Binding Stoichiometry of hVEGF165 to Flt1D2Flk1D3.FcΔC1(a) & VEGFR1R2-FcΔC1(a)	VEGF/VEGFR1R2-FcΔC1(a)	0.98	0.94	66.0	0.97 ± 0.02	
	VEGF/FIt1D2FIk1D3.FcAC1(a)	0.93	0.97	-	0.96 ± 0.03	
	hVEGF165 (nM)	+	10	50	Average ± StDev	

Fig. 7



Fig. 8

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Fig. 9

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Fig. 10

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Fig. 11

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Fig. 12

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Fig. 13A

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Fig. 13B

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FIG. 14

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FIG. 16

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FIG. 17

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FIG. 18A



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FIG. 19

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% Grade 4 Lesions

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METHODS OF TREATING EYE DISORDERS WITH MODIFIED CHIMERIC POLYPEPTIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Ser. No. 10/009,852 filed 6 Dec. 2001, now U.S. Pat. No. 7,070,959, which is the National Stage of International ¹⁰ Application No. PCT/US00/14142 filed 23 May 2000, which claims the benefit under 35 USC § 119(e) of U.S. Provisional 60/138,133 filed 8 Jun. 1999, which applications are herein specifically incorporated by reference in their entireties. ¹⁵

BACKGROUND

STATEMENT REGARDING RELATED ART

A class of cell-derived dimeric mitogens with selectivity for vascular endothelial cells has been identified and designated vascular endothelial cell growth factor (VEGF). VEGF is a dimer with an apparent molecular mass of about 46 kDa with each subunit having an apparent molecular mass of about 23 kDa. The membrane-bound tyrosine kinase receptor, known as Flt (also known as VEGFR2), was shown to be a VEGF receptor (DeVries et al. (1992) Science 255:989991). Another form of the VEGF receptor, designated KDR or Flk-1 (also known as VEGFR3), is also known to bind VEGF and induce mitogenesis (Terman et al. (1991) Oncogene 6:1677-1683; Terman et al. (1992) Biochem. Biophys. Res. Comm. 187:1579-1586).

U.S. Pat. No. 6,011,003 describes an altered, soluble form of Flt polypeptide capable of binding to VEGF comprising five or fewer complete immunoglobulin domains. WO 97/44453 describes chimeric VEGF receptor proteins comprising amino acid sequences derived from VEGF receptors Flt1 and KDR.

BRIEF SUMMARY OF THE INVENTION

In a first aspect, the invention provides an isolated nucleic acid molecule, comprising (a) a nucleotide sequence encod- 45 ing a vascular endothelial growth factor (VEGF) receptor component consisting essentially of an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor and Ig domain 3 of a second VEGF receptor; and (b) a nucleotide sequence encoding a multimerizing component, wherein the first 50 VEGF receptor is Flt1, the second VEGF receptor is Flk1 or Flt4, and the VEGF receptor component is the only VEGF receptor component of the fusion polypeptide. In one embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is 55 upstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor. In another embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding 60 Ig domain 3 of the extracellular domain of the second VEGF receptor. In one embodiment, the multimerizing component comprises an immunoglobulin domain. Preferably, the immunoglobulin domain is the Fc domain of IgG or the heavy chain of IgG. In specific embodiments, the nucleotide 65 sequence is selected from the group consisting of the nucleotide sequence of SEQ ID NO:11, 13, and 15, or a nucleotide

sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of SEQ ID NO:11, 13, and 15.

The components of the fusion polypeptide encoded by the nucleic acid molecule of the invention are arranged as 1,2,3; 1,3,2; 2,1,3; 2,3,1; 3,1,2; or 3,2,1, wherein 1 is the first VEGF receptor component, 2 is the second VEGF receptor component, and 3 is the multimerizing component.

In a second aspect, the invention features a vector comprises a nucleic acid molecule of the invention. In a more specific embodiment, the vector is an expression vector comprising the nucleic acid molecule of the invention operatively linked to an expression control sequence.

In a third aspect, the invention features a host-vector system for the production of a fusion polypeptide which comprises the expression vector of the invention in a suitable host cell. The suitable host cell may be a bacterial cell, yeast cell, insect cell, or mammalian cell. In a preferred embodiment, the host cell is an $E.\ coli$ cell or a CHO cell. In a fourth aspect, the invention features a method of producing a fusion polypeptide which comprises growing cells of the host-vector system of the invention under

conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

In a fifth aspect, the invention features a dimeric vascular endothelial growth factor (VEGF) antagonist, comprising two fusion polypeptides, each fusion polypeptide comprising (a) a VEGF receptor component consisting essentially of an immunoglobulin-like (Ig) domain 2 of an Flt-1 VEGF receptor and Ig domain 3 of an Flk-1 or Flt-4 VEGF receptor; and (b) a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component of each fusion protein. In specific embodiments, the dimeric VEGF antagonist is modified by acetylation or pegylation.

In a sixth aspect, the invention features a fusion polypeptide, comprising (a) a VEGF receptor component consisting essentially of an immunoglobulin-like (Ig) domain 2 of an Flt-1 VEGF receptor and Ig domain 3 of an Flk-1 or Flt-4 VEGF receptor; and (b) a multimerizing component, wherein the VEGF receptor component is the only VEGF 40 receptor component of the fusion polypeptide. In one embodiment, the multimerizing component comprises an immunoglobulin domain. More specifically, the multimerizing component is an immunoglobulin domain which is one of the Fc domain of IgG or the heavy chain of IgG. In specific embodiments, the fusion polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:12 (Flt1D2. Flk1D3FcΔC1(a)), SEQ ID NO:14 (Flt1D2VEGFR3D3Fc Δ C1(a)), and SEQ ID NO:16 (VEGFR1R2 $Fc\Delta C1(a)$).

In a seventh aspect, the invention features a pharmaceutical composition comprising the fusion polypeptide of the invention and a pharmaceutically acceptable carrier.

In an eighth aspect, the invention features a therapeutic method for treating or ameliorating an eye disorder, comprising administering the pharmaceutical composition of the invention to a patient in need thereof. In one embodiment, the eye disorder treated is age related macular degeneration. In another embodiment, the eye disorder treated is diabetic retinopathy

Other objects and advantages will become apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Binding of unmodified Flt1(1-3)-Fc, basic region deletion mutant Flt1(1-3)-Fc, and Flt1(1-³)_{$R \to N$} mutant proteins in a Biacore-based assay.

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FIG. 2. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1 $(1-3_{\Delta B})$ -Fc, Mut2: Flt1 $(2-3_{\Delta B})$ -Fc, and Flt1(2-3) mutant proteins to Matrigel® coated plates.

FIG. 3. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1 $(1-3_{\Delta B})$ -Fc, Mut2: Flt1 $(2-3_{\Delta B})$ -Fc, and Flt1(2-3) mutant proteins in an ELISA-based assay

FIG. 4. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3_{ΔB})-Fc, Mut2: Flt1(2-3_{ΔB})-Fc, and Flt1(2-3) mutant proteins.

FIG. 5. Extra cellular matrix (ECM) assay of Flt1D2. 10 Flk1D3. FcΔC1(a) and Flt1D2. VEGFR3D3. FcΔC1(a).

FIG. 6. MG/R2 Cell proliferation assay. Modified Flt receptors Flt1(1-3)-Fc, Flt1D2. Flk1D3. Fc∆C1(a) and Flt1D2. VEGFR3D3. FcΔC1(a), plus an irrelevant receptor termed Tie2-Fc as a negative control, were titrated from 40 15 nM to 20 pM and incubated on the cells for 1 hr at 37° C.

FIG. 6. Biacore analysis of binding stoichiometry. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3. Fc∆C1(a) or VEGFR1R2-Fc Δ C1(a), using the conversion factor of 1000 20 RU equivalent to 1 ng/ml.

FIG. 8. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2. Flk1D3. Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a).

FIG. 9. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2. Flk1D3. Fc Δ C1(a) and Flt1D2. VEGFR3D3. Fc Δ C1(a). 25

FIG. 10. The ability of Flt1D2. Flk1D3. $Fc\Delta C1(a)$ to inhibit HT-1080 fibrosarcoma tumor growth in vivo.

FIG. 11. The ability of Flt1D2. Flk1D3. Fc∆C1(a) to inhibit C6 glioma tumor growth in vivo.

FIG. 12. VEGF-induced uterine hyperpermeability. FIGS. 13A-B. Assessment of corpus luteum angiogenesis using progesterone as a readout.

FIG. 14. VEGFR1R2-FcΔC1(a) prevents Evans Blue leakage in streptozotocin-treated rats.

FIG. 15. VEGFR1R2-FcΔC1(a) prevents neovasculariza- 35 tion induced by retinal ischemia. Serial 10 um cross sections were collected and stained with hematoxylin and eosin. For each animal, nuclei in preretinal neovessels were counted in a series of ten sections within 300 microns of the optic nerve head and averaged. Counts were obtained in three indepen- 40 dent experiments, n≥4 for each treatment group in each study

FIG. 16. Effect of subcutaneous VEGFR1R2-Fc Δ C1(a) injections on choroidal neovascularization area. The size of CNV lesions was measured in choroidal flat mounts. The 45 Nucleic Acid Constructs images were digitized using an Axioskop microscope equipped with a video camera, and the total area of choroidal neovascularization associated with each laser burn was measured using Image-Pro Plus software.

FIG. 17. VEGFR1R2-Fc Δ C1(a) inhibits subretinal 50 neovascularization in Rho/VEGF transgenic mice.

FIGS. 18A-B. VEGF-Induced breakdown of the blood retinal barrier. A. Following intravitreal injections of VEGF, adult mice (C57BL/6) treated with injections of VEGFR1R2-Fc Δ C1(a) had a significantly smaller retina to 55 lung leakage ration than mice treated with Fc fragment, indicating less breakdown of BRB. B. Double transgenic mice treated with injections of VEGFR1R2-Fc Δ C1(a) had a significant reduction in the retina to lung leakage ration compared to mice treated with Fc fragment. 60

FIG. 19. Effect of VEGFR1R2-Fc Δ C1(a) administration on corneal thickness in suture and alkali burn models of corneal trauma. Corneas were injured by suture placement or application of NaOH as described, and a single dose of VEGFR1R2-Fc∆C1(a) (25 mg/kg, ip) or saline (n=5 per 65 group) was administered immediately following injury. The contralateral cornea served as normal, undamaged controls.

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Corneas were collected 7 days later and cross-sections were cut and stained with hematoxylin and eosin. Corneal thickness was measured as an index of corneal edema.

FIG. 20. System or intravitreal VEGF trap administration prevents laser-induced choroidal neovascularization (CNV) and reverses vascular leak in established lesions

DETAILED DESCRIPTION OF THE INVENTION

It has been a long standing problem in the art to produce a receptor based VEGF antagonist that has a pharmacokinetic profile that is appropriate for consideration of the antagonist as a therapeutic candidate. Applicants describe herein, for the first time, a chimeric polypeptide molecule, capable of antagonizing VEGF activity, that exhibits improved pharmacokinetic properties as compared to other known receptor-based VEGF antagonists. The chimeric polypeptide molecules described herein thus provide for the first time appropriate molecules for use in therapies in which antagonism of VEGF is a desired result.

The extracellular ligand binding domain is defined as the portion of a receptor that, in its native conformation in the cell membrane, is oriented extracellularly where it can contact with its cognate ligand. The extracellular ligand binding domain does not include the hydrophobic amino acids associated with the receptor's transmembrane domain or any amino acids associated with the receptor's intracellular domain. Generally, the intracellular or cytoplasmic domain of a receptor is usually composed of positively charged or polar amino acids (i.e. lysine, arginine, histidine, glutamic acid, aspartic acid). The preceding 15-30, predominantly hydrophobic or apolar amino acids (i.e. leucine, valine, isoleucine, and phenylalanine) comprise the transmembrane domain. The extracellular domain comprises the amino acids that precede the hydrophobic transmembrane stretch of amino acids. Usually the transmembrane domain is flanked by positively charged or polar amino acids such as lysine or arginine. von Heijne has published detailed rules that are commonly referred to by skilled artisans when determining which amino acids of a given receptor belong to the extracellular, transmembrane, or intracellular domains (See, von Heijne (1995) BioEssays 17:25.

N.Y.).

The present invention provides for the construction of nucleic acid molecules encoding chimeric polypeptide molecules that are inserted into a vector that is able to express the chimeric polypeptide molecules when introduced into an appropriate host cell. Appropriate host cells include, but are not limited to, bacterial cells, yeast cells, insect cells, and mammalian cells. Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the chimeric polypeptide molecules under control of transcriptional/translational control signals. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (See Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience,

Expression of nucleic acid molecules encoding the chimeric polypeptide molecules may be regulated by a second nucleic acid sequence so that the chimeric polypeptide molecule is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the

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chimeric polypeptide molecules described herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of the chimeric polypeptide molecules include, but are not limited to, the long terminal repeat as described in Squinto et al., (1991, Cell 65:1-20); the SV40 early promoter region (Bernoist et al. (1981) Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al. (1980) Cell 22:787-797), the herpes 10 thymidine kinase promoter (Wagner et al. (1981) Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothionine gene (Brinster et al. (1982) Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff et al. (1978) Proc. 15 Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer et al. (1983) Proc. Natl. Acad. Sci. U.S.A. 80:21-25): promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline 20 phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (see for example, Swift et al. (1984) Cell 38:639-646); insulin 25 gene control region which is active in pancreatic beta cells (Hanahan (1985) Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al. (1984) Cell 38:647-658), mouse mammary tumor virus control region which is active in testicular, 30 breast, lymphoid and mast cells (Leder et al. (1986) Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al. (1987) Genes Devel. 1:268-276), alphafetoprotein gene control region which is active in liver (Krumlauf et al. (1985) Mol. Cell. Biol. 5:1639-1648); alpha 3 1-antitrypsin gene control region which is active in the liver (Kelsey et al. (1987) Genes Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al. (1985) Nature 315:338-340); myelin basic protein gene control region which is active in oligodendrocyte 40 cells in the brain (Readhead et al. (1987) Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani (1985) Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al. (1986) Science 45 234:1372-1378)

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising chimeric polypeptide molecule-encoding nucleic acid as described herein, are used to transfect the 50 macular degeneration and diabetic retinopathy. host and thereby direct expression of such nucleic acids to produce the chimeric polypeptide molecules, which may then be recovered in a biologically active form. As used herein, a biologically active form includes a form capable of binding to VEGF. Expression vectors containing the chi- 55 meric nucleic acid molecules described herein can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an 60 expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to the inserted chimeric polypeptide molecule sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or 65 absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phe-

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notype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the chimeric polypeptide molecule DNA sequence is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the chimeric polypeptide molecules

Cells of the present invention may transiently or, preferably, constitutively and permanently express the chimeric polypeptide molecules.

The chimeric polypeptide molecules may be purified by any technique which allows for the subsequent formation of a stable, biologically active chimeric polypeptide molecule. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis (see, for example, U.S. Pat. No. 5,663,304). In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

Therapeutic Methods

The present invention also has diagnostic and therapeutic utilities. In particular embodiments of the invention, methods of detecting aberrancies in the function or expression of the chimeric polypeptide molecules described herein may be used in the diagnosis of disorders. In other embodiments, manipulation of the chimeric polypeptide molecules or agonists or antagonists which bind the chimeric polypeptide molecules may be used in the treatment of diseases. In further embodiments, the chimeric polypeptide molecule is utilized as an agent to block the binding of a binding agent to its target.

By way of example, but not limitation, the method of the invention may be useful in treating clinical conditions that are characterized by vascular permeability, edema or inflammation such as brain edema associated with injury, stroke or tumor; edema associated with inflammatory disorders such as psoriasis or arthritis, including rheumatoid arthritis; asthma: generalized edema associated with burns: ascites and pleural effusion associated with tumors, inflammation or trauma; chronic airway inflammation; capillary leak syndrome; sepsis; kidney disease associated with increased leakage of protein; and eye disorders such as age related

Specific Embodiments

The eye comprises several structurally and functionally distinct vascular beds, which supply ocular components critical to the maintenance of vision. These include the retinal and choroidal vasculatures, which supply the inner and outer portions of the retina, respectively, and the limbal vasculature located at the periphery of the cornea. Injuries and diseases that impair the normal structure or function of these vascular beds are among the leading causes of visual impairment and blindness. For example, diabetic retinopathy is the most common disease affecting the retinal vasculature, and is the leading cause of vision loss among the working age population in the United States, while the wet form of age-related macular degeneration (AMD) is the most common form of choroidal neovascularization and a

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leading cause of blindness in the elderly. Vascularization of the cornea secondary to injury or disease is yet another category of ocular vascular disease that can lead to severe impairment of vision.

Each of the above conditions is characterized by patho-5 logical neovascularization, associated with or preceded by abnormal, excessive vascular permeability that often leads to pronounced edema in the affected tissue (cornea or retina). The production of abnormally high levels of VEGF has been implicated as a principal cause of the increased 10 vascular permeability, as well as pathological angiogenesis (Aiello et al. (1994) N. Engl. J. Med. 331:1480-1487). Moreover, both the edema associated with abnormal vascular permeability and pathological neovascularization contribute directly to the impairments of vision. Therefore, 15 inhibition of VEGF action is one strategy now being explored for the treatment of ocular vascular diseases such as diabetic retinopathy and AMD.

Other features of the invention will become apparent in the course of the following descriptions of exemplary 20 embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

Example 1

Expression of Flt1(1-3)-Fc Protein in CHO K1 Cells

Using standard molecular biology techniques (see Sambrook et al. supra), the gene encoding Flt1(1-3)-Fc was inserted into the expression vector pEE14.1 (Lonza Biologics) at a multiple cloning site downstream of the CMV promoter. CHO K1 cells were transfected with the pEE14.1/ 35 Flt1(1-3)-Fc DNA construct using lipofectamine (Gaithersburg, Md.). The transfected CHO K1 cells were grown in glutamine-free DMEM (JRH, Kansas City, Mo.) containing 25 µM methionine sulfoximine (MSX) from Sigma Inc., St. Louis, Mo., and high recombinant protein expressors were $\ \ _{40}$ obtained by screening the CHO K1 cell supernatants from over 100 hand-picked colony isolates using a standard immunoassay which captures and detects human Fc. The selected hand-picked clone was amplified in the presence of 100 µM MSX followed by a second round of screening of 45 the amplified clones. The highest producing clone had a specific productivity of recombinant Flt1(1-3)-Fc protein of 55 pg/cell/day.

The selected clone was expanded in 225 cm² T-flasks (Corning, Acton, Mass.) and then into 8.5 L roller bottles 50 ford, Ill., Cat.#26777). (Corning, Acton, Mass.) using the cell culture media described supra. Cells were removed from the roller bottles by standard trypsinization and put into 3.5 L of suspension medium. The suspension medium is comprised of glutamine-free ISCHO medium (Irvine Scientific, Santa 55 Ana, Calif.) containing 5% fetal bovine serum (FBS from Hyclone Labs, Logan, Utah), 100 µM MSX and GS supplement (JRH Scientific, Kansas City, Mo.) in a 5 L Celligen bioreactor (New Brunswick Scientific, New Brunswick, N.J.) at a density of 0.3×10^6 cells/mL. After the cells reached 60 a density of $3.6{\times}10^6/\text{mL}$ and were adapted to suspension they were transferred to a 60 L bioreactor (ABEC, Allentown, Pa.) at a density of 0.5×106 cells/mL in 20 L of ISCHO medium with 5% fetal bovine serum. After two days an additional 20 L of ISCHO+5% fetal bovine serum was added 65 to the bioreactor. The cells were allowed to grow for an additional two days reaching a final density of 3.1×106

cells/mL, and a final Flt1(1-3)-Fc concentration at harvest was 95 mg/L. At harvest the cells were removed by tangential flow filtration using 0.45 μ m Prostak Filters (Millipore, Inc., Bedford, Mass.).

Example 2

Purification of Flt1(1-3)-Fc Protein Obtained from CHO K1 Cells

Flt1(1-3)-Fc protein was initially purified by affinity chromatography. A Protein A column was used to bind, with high specificity, the Fc portion of the molecule. This affinitypurified protein was then concentrated and passed over a SEC column. The protein was then eluted into the formulation buffer. The following describes these procedures in detail.

Materials and Methods. All chemicals were obtained from J. T. Baker, Phillipsburg, N.J. with the exception of PBS, ²⁰ which was obtained as a 10× concentrate from Life Technologies, Gaithersburg, Md. Protein A Fast Flow and Superdex 200 preparation grade resins were obtained from Pharmacia, Piscataway, N.J. Equipment and membranes for protein concentration were obtained from Millipore, Bed-²⁵ ford, Mass.

Approximately 40 L of 0.45 µm-filtered CHO conditioned media containing Flt1(1-3)-Fc protein was applied to a 290 mL Protein A Fast Flow column (10 cm diameter) that had been equilibrated with PBS. The column was washed with PBS containing 350 mM NaCl and 0.02% CHAPS and the bound protein was eluted with 20 mM Citric Acid containing 10 m \hat{M} Na₂HPO₄. The single peak in the elution was collected and its pH was raised to neutrality with 1M NaOH. The eluate fractions was concentrated to approximately 9 mg/mL using 10K regenerated cellulose membranes by both tangential flow filtration and by stirred cell concentration. To remove aggregates and other contaminants, the concentrated protein was applied to a column packed with Superdex 200 preparation grade resin (10 cm×55 cm) and run in PBS containing 5% glycerol. The main peak fractions were pooled, sterile filtered, aliquoted and stored at -80° C.

Example 3

Acetylation of Flt1(1-3)-Fc Protein

Two milligrams of Flt1(1-3)-Fc protein were acetylated as described in the instruction manual provided with the sulfo-NHS-acetate modification kit (Pierce Chemical Co., Rockford, Ill., Cat.#26777).

Example 4

Characterization of Acetylation of Flt1(1-3)-Fc Protein

IEF analysis: Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc were analyzed by standard IEF analysis. Flt1(1-3)-Fc protein is not able to migrate into the gel and therefore must have a pl greater than 9.3, the highest pl in the standard. However, acetylated Flt1(1-3)-Fc is able to migrate into the gel and equilibrate at a pl of approximately 5.2. This result demonstrates that acetylation reduces the net positive charge of the protein and therefore its pl considerably.

Binding to extracellular matrix components. To test for binding to extracellular matrix components, Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc where tested in an assay

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designed to mimic the interaction with extracellular matrix components. In this assay, 96-well tissue culture plates are coated with Matrigel (Biocoat MATRIGEL® matrix thin layer 96 well plate, Catalog #40607, Becton Dickinson Labware, Bedford, Mass.). The plates are incubated with varying concentrations of either Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, or rTie2-Fc (an irrelevant control) protein are added to the wells. The plates are incubated for 1-2 hours at either room temperature or 37° C. degrees and then detection of bound proteins is accomplished by adding a secondary 10 alkaline phosphatase-conjugated anti-human Fc antibody to the wells. Finally, alkaline phosphatase substrate is added to the wells and optical density is measured. Like the irrelevant control protein rTie2-Fc, acetylated Flt1(1-3)-Fc does not exhibit any binding to the Matrigel coated plate, whereas the 15 non-acetylated Flt1(1-3)-Fc protein exhibits significant binding. This result indicates that acetylation of basic amino acid residues is an effective way to interfere with the charge interactions that exist between positively charged proteins and the negatively charged extracellular matrix components 20 they are exposed to in vivo.

Example 5

Pegylation of Flt1(1-3)-Fc Protein

Materials and Methods. Purified Flt1(1-3)-Fc derived from CHO cells (see supra) was used in the following pegylation experiments. Functionalized PEGs were obtained from Shearwater Polymers, Huntsville, Ala.; Bicine from ³⁰ Sigma, St Louis, Mo.; Superose 6 column from Pharmacia, Piscataway, N.J.; PBS as a 10x concentrate from Life Technologies, Gaithersburg, Md.; Glycerol from J. T. Baker, Phillipsburg, N.J.; and Bis-Tris precast gels from Novex, Calif. ³⁵

20K PEG strands functionalized with amine-specific terminal moieties were used in small-scale reaction studies that were set-up to evaluate different reaction conditions in which the PEG:protein stoichiometry was varied. Based on these reactions and the analyses of samples on standard $^{\rm 40}$ SDS-PAGE, Flt1(1-3)-Fc at a concentration of 1.5 mg/mL was reacted at pH 8.1 with 20K SPA-PEG (PEG succinimidyl propionate) molecules at a PEG-to-Flt1(1-3)-Fc monomer molar ratio of 1:6. The reaction was allowed to proceed at 8° C. overnight. For initial purification, the reaction 45 products were applied to a 10 mm×30 cm Superose 6 column equilibrated with PBS containing 5% Glycerol. The column appeared to separate pegylated Flt1(1-3)-Fc molecules based on the extent of pegylation. Fractions corresponding to what appeared to be primarily mono-pegylated 50 and di-pegylated dimeric Flt1(1-3)-Fc, as judged by banding patterns on reducing and non-reducing SDS-PAGE gels were pooled. The protein concentration was determined by measuring absorbance at 280 nm. The pegylated Flt1(1-3)-Fc protein was sterile filtered, aliquoted and stored at -40° 55 С.

Example 6

Binding of Unmodified, Acetylated, and Pegylated Flt1(1-3)-Fc

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a Biacore-based assay to evaluate their ability to bind to the Flt1 ligand, VEGF. In this assay, 65 unmodified Flt1(1-3)-Fc protein was immobilized on the surface of a Biacore chip (see Biacore Instruction Manual,

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Pharmacia, Inc., Piscataway, N.J., for standard procedures) and a sample containing 0.2 µg/ml VEGF and either unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc or pegylated Flt1(1-3)-Fc (each at 25 µg/ml) was passed over the Flt1(1-3)-Fc-coated chip. To minimize the effects of nonspecific binding, the bound samples were washed with a 0.5M NaCl wash. In one sample, unmodified Flt1(1-3)-Fc was mixed with heparin. Heparin is a negatively charged molecule and the Flt1(1-3)-Fc protein is a positively charged molecule, so when the two molecules are mixed together, they should interact through their respective charges. This essentially neutralizes Flt1(1-3)-Fc's inherent positive charge making the molecule behave as if it has been chemically or genetically modified so as to reduce its charge and its tendency to bind via charge interactions. Acetylated, pegylated, and heparin-treated Flt1(1-3)-Fc are each able to completely compete with the Biacore chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control and irrelevant protein. Unmodified Flt1(1-3)-Fc appeared to only partially compete with Biacore chip-bound Flt1(1-3)-Fc for VEGF binding. However, washing the bound samples with 0.5M NaCl resulted in a binding profile similar to the modified forms of Flt1(1-3)-Fc, indicating that the unmodified protein was exhibiting non-specific binding to the chip that could be eliminated by the salt wash.

Example 7

Binding of Unmodified, Acetylated, and Pegylated Flt1(1-3)-Fc in an ELISA-Based Assay

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a standard ELISA-based assay to evaluate their ability to bind the Flt1 receptor ligand VEGF. Both pegylated and acetylated Flt1(1-3)-Fc proteins are capable of binding to VEGF, demonstrating that modifying the protein either by pegylation or acetylation does not destroy its ability to bind its ligand.

Example 8

Pharmacokinetic Analysis of Unmodified Flt1(1-3)-Fc, Acetylated Flt1(1-3)-Fc, and Pegylated Flt1(1-3)-Fc

In vivo experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc protein. Balb/c mice (23-28 g; 3 mice/group) were injected subcutaneously with 4 mg/kg of unmodified, acetylated, or pegylated Flt1 (1-3)-Fc. The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, and 3 days after injection of protein. The sera were assayed in a standard ELISA-based assay designed to detect Flt1(1-3)-Fc protein. Briefly, the assay involves coating an ELISA plate with VEGF, binding the unmodified, acetylated, or pegylated Flt1(1-3)-Fc-containing sera, and reporting with an anti-Fc antibody linked to alkaline phosphatase. The Tmax for all of the Flt1(1-3)-Fc proteins was between the 6 hour and 24 hour time points. The Cmax for the different proteins was as follows: Unmodified: 0.06 $\mu/\text{ml-}$ $0.15~\mu\text{g/ml};$ acetylated: 1.5 $\mu\text{g/ml-4.0}~\mu\text{g/ml};$ and pegylated: approximately 5 µg/ml.

Example 9

Step-Acetylation of Flt1(1-3)-Fc

To determine what minimal amount of acetylation is necessary to eliminate binding to extracellular matrix components, an experiment was designed that acetylated the

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Flt1(1-3)-Fc protein in a step-wise fashion by using increasing amounts of molar excess of acetylation reagent in the acetylation reaction mixture. The range of molar excess was as follows: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 moles of acetylation reagent per 1 mole of Flt1(1-3)-Fc monomer. The reactions were performed as detailed in the instruction manual provided with the sulfo-NHS-Acetate modification kit (Pierce Chemical Co., Rockford, Ill., Cat. #26777).

Example 10

Characterization of Step-Acetylated Flt1(1-3)-Fc

IEF analysis. Unmodified Flt1(1-3)-Fc and step-acety-¹⁵ lated Flt1(1-3)-Fc proteins were analyzed by standard IEF analysis. Unmodified Flt1(1-3)-Fc protein was not able to migrate into the gel due to its extremely high pl (greater than 9.3). However, most of the step-acetylated Flt1(1-3)-Fc samples (30-100 fold molar excess samples) were able to ²⁰ migrate into the gel and equilibrate at pls ranging between 4.55-8.43, depending on the degree of acetylation of the protein. This result demonstrates that acetylation can change the positive charge of the protein in a dose-dependent manner and that reduction of the pl can be controlled by ²⁵ controlling the degree of acetylation.

Binding of step-acetylated Flt1(1-3)-Fc to extracellular matrix components. To test for binding to extracellular matrix components, Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc where tested in the above-described assay designed to mimic the interaction with extracellular matrix components. Varying concentrations of either unmodified Flt1(1-3)-Fc, step-acetylated Flt1(1-3)-Fc (10, 20, and 30 fold molar excess samples), or rTie2-Fc (an irrelevant control) protein were added to the wells. The plates were incubated for 1-2 hours at room temperature or 37° C. and then detection of bound proteins was accomplished by adding a secondary alkaline phosphatase-conjugated anti-human Fc antibody to the wells. Alkaline phosphatase substrate was subsequently added to the wells and optical density measured. Like the 40 irrelevant control protein rTie2-Fc, step-acetylated Flt1(1-3)-Fc (20 and 30 fold molar excess samples) did not exhibit any significant binding to the Matrigel coated plate, whereas the non-acetylated Flt1(1-3)-Fc protein exhibited significant binding. The binding is saturable, indicating that the Flt1 45 (1-3)-Fc protein may be binding to specific sites, rather than a more general charge-mediated interaction that might not be saturable. The 10 fold molar excess sample showed reduced binding, but the degree of acetylation was not enough to completely block binding to extracellular matrix 50 components. The 20 fold molar excess and higher samples displayed no detectable binding, despite the fact that by IEF analysis the lower molar excess samples still had a large net positive charge. This result demonstrates that it is not necessary to completely acetylate all available basic amino 55 acids in order to eliminate binding to extracellular matrix components.

Binding of step-acetylated Flt1(1-3)-Fc in a Biacorebased assay. Unmodified and step-acetylated Flt1(1-3)-Fc proteins where tested in a Biacore-based assay to evaluate 60 their ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein (0.5, 1.0, or $5.0 \mu g/ml$) was immobilized on the surface of a Biacore chip (see Biacore Instruction Manual, Pharmacia, Inc., Piscataway, N.J., for standard procedures) and a solution containing 0.2 $\mu g/ml$ 65 VEGF and either unmodified Flt1(1-3)-Fc (at either 0.5, 1.0, or $5.0 \mu g/ml$) or 10 different step-acetylated Flt1(1-3)-Fc

samples (at 0.5, 1.0, or 5.0 µg/ml each) were passed over the Flt1(1-3)-Fc-coated chip. At a sub-stoichiometric ratio (0.5 µg/ml of either unmodified Flt1(1-3) or step-acetylated Flt1(1-3)-Fc vs. 0.2 µg/ml VEGF), there is not enough Flt1(1-3)-Fc (either unmodified or step-acetylated) in the solution to completely bind the VEGF. At 1.0 µg/ml, which approximates a 1:1 stoichiometric ratio, both unmodified and step-acetylated Flt1(1-3)-Fc are better able to compete for VEGF binding, but there is still insufficient Flt1(1-3)-Fc protein (either unmodified or step-acetylated) to completely bind the available VEGF. However, at 5.0 µg/ml, which is several times greater than a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to bind the VEGF, regardless of the degree of acetylation. This clearly demonstrates that acetylation does not alter Flt1(1-3)-Fc's ability to bind VEGF.

Pharmacokinetic analysis of step-acetylated Flt1(1-3)-Fc. In vivo experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc protein. Balb/c mice (23-28 g) were injected subcutaneously with 4 mg/kg of unmodified or 10, 20, 40, 60 and 100 fold molar excess samples of stepacetylated Flt1(1-3)-Fc (3 mice for unmodified, 10, 20 and 40 fold molar excess samples and 2 mice for 60 and 100 fold molar excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1 (1-3)-Fc (described supra). The Tmax for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the Cmax was as follows: unmodified Flt1(1-3)-Fc: 0.06 µg/ml; 10 fold molar excess sample: -0.7 µg/ml; 20 fold molar excess sample: 2 µg/ml; 40 fold molar excess sample: 4 µg/ml; 60 fold molar excess sample: 2 µg/ml; 100 fold molar excess sample: 1 µg/ml. This results demonstrates that acetylation or pegylation of Flt1(1-3)-Fc significantly improves its pharmacokinetic profile.

Example 11

Construction of Flt1 (1-3)-Fc Basic Region Deletion Mutant Designated Mut1: Flt1($1-3_{AB}$)-Fc

Based on the observation that acetylated Flt1(1-3)-Fc, which has a pl below 6, has much better pharmacokinetics than the highly positive unmodified Flt1(1-3)-Fc (pl>9.3), it was asked whether the difference in pharmacokinetics could be attributed to the net charge of the protein, which made it stick to negatively charged extracellular matrix components, or whether there were perhaps specific locations on the surface of the Flt1(1-3)-Fc protein that constituted specific binding sites for extracellular matrix components. Referring to the nucleic acid and amino acid sequence of SEQ ID NO:1 and 2, the signal sequence for secretion is located at the beginning of the sequence and extends to the glycine coded for by nucleotides 76-78. The mature protein begins with Ser-Lys-Leu-Lys (SEQ ID NO:35), starting at nucleotide 79 of the nucleic acid sequence. Flt1 Ig domain 1 extends from nucleotide 79 to 393, ending with the amino acids Ser-Asp-Thr. Flt1 Ig domain 2 extends from nucleotide 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1 Ig domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val to Asp-Lys-Ala). There is a bridging amino acid sequence, Gly-Pro-Gly, encoded by nucleotides 997-1005, followed by the nucleotide sequence encoding human Fc (nucleotides 1006-1701 or amino acids Glu-Pro-Lys to Pro-Gly-Lys-stop).

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A more detailed analysis of the Flt1 amino acid sequence reveals that there is a cluster, namely, amino acid residues 272-281 of SEQ ID NO:2, in which 6 out of 10 amino acid residues are basic. This sequence is located in Flt1 Ig domain 3 of the receptor, which is not itself essential for binding of VEGF ligand, but which confers a higher affinity binding to ligand. An alignment of the sequence of Ig domain 3 with that of Ig domain 2 reveals that in this region, there is very poor alignment between the two Ig domains, and that there are about 10 additional amino acids in Ig domain 3. An 10 analysis of the hydrophilicity profiles (MacVector computer software) of these two domains clearly indicates the presence of a hydrophilic region in the protein. These observations raised the possibility that the actual three dimensional conformation of Flt1 Ig domain 3 allowed for some type of protrusion that is not in Flt1 Ig domain 2. To test this hypothesis, the 10 additional amino acids were deleted and the resulting protein was tested to see whether the deletion would affect the pharmacokinetics favorably without seriously compromising the affinity of the receptor for VEGF. 20 This DNA construct, which was constructed using standard molecular biology techniques in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, Mass.), is referred to as Mut1: Flt1(1-3 $_{\Delta B}$)-Fc. The Mut1: Flt1(1- 3_{AB})-Fc construct (SEQ ID NO:3-4) was derived from 25 Flt1(1-3)-Fc by deletion of nucleotides 814-843 of SEQ ID NO:1, which deletes the highly basic 10-amino acid residue sequence from Flt1 Ig domain 3. The final DNA construct was sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied 30 Biosystems, Inc., Foster City, Calif.).

Example 12

Construction of Flt1(1-3)-Fc Basic Region Deletion Mutant Designated Mut2: Flt1(2-3_{AB)-Fc}

A second deletion mutant construct, designated Mut2: Flt1(2-3_{ΔB})-Fc (SEQ ID NO:5-6), was derived from the Mut1: Flt1(1-3_{ΔB})-Fc construct by deletion of Flt1 Ig 40 domain 1 encoded by nucleotides 79-393 (SEQ ID NO:1); for convenience, nucleotides 73-78 were changed (TC-CGGA; SEQ ID NO:26). This introduced a restriction site (BspE1) without altering the associated amino acid sequence, Ser-Gly.

Example 13

Construction of Flt1(1-3)-Fc Deletion Mutant Designated Mut3: Flt1(2-3)-Fc

A third deletion mutate construct, designated Mut3: Flt1 (2-3)-Fc (SEQ ID NO:7-8), was constructed the same way as the Mut2: Flt1(2-3 $_{AB}$)-Fc construct, except that Flt1 Ig domain 3 was left intact (the basic region amino acids were 55 genetically modified Flt1(1-3)-Fc proteins, which is not deleted).

Example 14

Construction of Flt(1-3)-Fc Basic Region N-Glycosylation Mutant Designated Mut4: Flt1(1-3_{R->N})-Fc

A construct was made in which a N-glycosylation site was introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1(1-3_{R_{-}} 65 >N)-Fc (SEQ ID NO:9-10) and was made by changing nucleotides 824-825 of SEQ ID NO:1 from GA to AC,

consequently changing the coded Arg residue (AGA) into an Asn residue (AAC). The resulting amino acid sequence is therefore changed from Arg-Ala-Ser to Asn-Ala-Ser, which matches the canonical signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue.

Example 15

Characterization of Acetylated Flt1(1-3)-Fc, Mut1: Flt1(1-3_{ΔB})-Fc, and Mut4: Flt1(1-3_{R->N})-Fc Mutants

Binding to extracellular matrix components. To determine whether the three modified proteins were more or less likely to have improved pharmacokinetic properties, Matrigel coated 96-well dishes were incubated with varying concentrations of the mutant proteins and detected with anti-human Fc/alkaline-phosphatase conjugated antibodies. As shown in FIG. 2, this experiment showed that while the unmodified Flt1(1-3)-Fc protein could bind avidly to these wells, the Mut3: Flt1(2-3)-Fc protein bound somewhat more weakly, the Mut1: Flt1(1-3_{AB})-Fc protein bound more weakly still, and the Mut2: $Flt1(2-3_{\Delta B})$ -Fc protein showed the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1(1-3_{R->N})-Fc glycosylation mutant protein showed only marginal benefit on the Matrigel assay. These results confirm the hypothesis that a linear sequence of positive amino acids can be deleted from the primary sequence resulting in a decrease in charge interaction with extracellular matrix components.

Binding of Mut1 and Mut4 in a Biacore-based assay. Unmodified and acetylated Flt1(1-3)-Fc and genetically modified Mut1: Flt1(1-3_{ΔB})-Fc and Mut4: Flt1(1-3_{R->N})-Fc proteins where tested in a Biacore-based assay to evaluate 35 their ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein (0.25, 0.5, or 1.0 µg/ml) was immobilized on the surface of a Biacore chip and a solution containing 0.1 µg/ml VEGF and either purified or COS cell supernatant containing unmodified Flt1(1-3)-Fc (at approximately (0.25, 0.5, or 1.0 µg/ml), purified acetylated Flt1(1-3)-Fc (at (0.25, 0.5, or 1.0 µg/ml), COS cell supernatant containing Mut1: Flt1(1-3_{ΔB})-Fc (at approximately (0.25, 0.5, or 1.0 µg/ml), or COS cell supernatant containing Mut4: Flt1(1-3_{*R*->*N*})-Fc (at approximately (0.25, 0.5, or 1.0) 45 µg/ml) were passed over the Flt1(1-3)-Fc-coated chip. As shown in FIG. 1, at the sub-stoichiometric ratio (0.25 µg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. µg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF to the 50 Flt1(1-3)-Fc immobilized on the Biacore chip. At 0.5 µg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the Biacore chip. At 1.0 µg/ml of unmodified, acetylated or approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the Biacore chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3_{AB})-Fc are essentially equal in their 60 ability to block VEGF binding, whereas Mut4: $Flt1(1-3_{R-1})$ >N)-Fc is somewhat less efficient at blocking binding. These results confirm the hypothesis that it is possible to reduce the non-specific binding of a positively charged molecule by genetically removing a linear sequence of predominantly negatively charged amino acids.

Binding of Mut1. Mut2 and Mut3 in an ELISA-based assay. To determine whether the three mutant proteins could

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bind the Flt1 ligand VEGF, binding experiments were done in which 96-well plates coated with VEGF were incubated with varying concentrations of the respective mutant protein, and after washing, the amount bound was detected by incubating with an alkaline phosphatase conjugated antihuman Fc antibody and quantitated colorimetrically by the addition of an appropriate alkaline phosphatase substrate. As shown in FIG. 3, this experiment showed that all the mutant proteins could bind VEGF similarly, at the concentrations tested.

Example 16

Pharmacokinetic Analysis of Acetylated Flt1(1-3)-Fc, Mut1, and Unmodified Flt1(1-3)-Fc

In vivo experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1- $3_{\Delta B}$)-Fc, and 40 fold molar excess acetylated Flt1(1-3)-Fc protein. Balb/c mice (25-30 g) were injected subcutaneously 20 with 4 mg/kg of unmodified Flt1(1-3)-Fc, 40 fold molar excess acetylated Flt1(1-3)-Fc, and Mut1: Flt1(1- 3_{AB})-Fc proteins (4 mice each). These mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, 3 days, and 5 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1- 25 3)-Fc protein which involves coating an ELISA plate with VEGF, binding the Flt1(1-3)-Fc and reporting with an anti-Fc antibody linked to alkaline phosphatase. As shown in FIG. 4, the Cmax for these reagents was as follows: Unmodified Flt1(1-3)-Fc -0.15 µg/ml; 40 fold molar excess 30 acetylated Flt1(1-3)-Fc—1.5 μ g/ml; and Mut1: Flt1(1-3_{AB})-Fc-0.7 µg/ml.

Example 17

Modified Flt1 Receptor Vector Construction

Chimeric molecules were constructed, denoted R1R2 (Flt1. D2. Flk1D3. $Fc\Delta C1(a)$ and VEGFR1R2-Fc\DeltaC1(a) and R1R3(Flt1D2. VEGFR3D3-Fc\DeltaC1(a) and VEGFR1R3- 40 Fc\DeltaC1(a) respectively, wherein R1 and Flt1D2=Ig domain 2 of Flt1 (VEGFR1); R2 and Flk1D3=Ig domain 3 of Flt4 (VEGFR2); and R3 and VEGFR3D3=Ig domain 3 of Flt4 (VEGFR3)) were much less sticky to ECM, as judged by an in vitro ECM binding assay and had greatly improved PK as 45 described herein. In addition, these molecules were able to bind VEGF tightly and block phosphorylation of the native Flk1 receptor expressed in endothelial cells.

Construction of the expression plasmid pFlt1D2. Flk1D3. Fc Δ C1(a). Expression plasmids pMT21. Flt1(1-3).Fc (6519 50 bp) and pMT21. Flk-1(1-3).Fc (5230 bp) are plasmids that encode ampicillin resistance and Fc-tagged versions of Ig domains 1-3 of human Flt1 and human Flk1, respectively. These plasmids were used to construct a DNA fragment consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 55 3 of Flk1, using PCR amplification of the respective Ig domains followed by further rounds of PCR to achieve fusion of the two domains into a single fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows: 5': bsp/flt1D2 (5'-GACTAGCAGTCCGG-AGG- 60 TAGACCTTTCGTAGAGATG-3') (SEQ ID NO:18), 3': Flt1D2-Flk1D3. as (5'-CGGACTCAGAACCACATCTAT-GATTGTATTGGT-3') (SEQ ID NO:19). The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid 65 sequence GRPFVEM (SEQ ID NO:20) corresponding to amino acids 27-33 of SEQ ID NO:12. The 3' primer encodes

the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of SEQ ID NO:12) and continuing into VVLS (SEQ ID NO:17) (corresponding to amino acids 127-130 of SEQ ID NO:12) of Flk1.

For Ig domain 3 of Fk1, the 5' and 3' amplification primers were as follows:5': Flt1D2-Flk1D3. s (5—-ACAAT-CATAGATGTGGTTCTGAGTCCGTCTCATGG-3') (SEQ

ID NO:21); 3': Flk1D3/apa/srf.as (5'-GATAATGC-CCGGGCCCTTTTCATGGACCCTGACAAATG-3') (SEQ ID NO:22). The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer
encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (SEQ ID NO:23) (corresponding to amino acids 223-228 of SEQ ID NO:12), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The
bridging sequence corresponds to amino acids 229-231 of SEQ ID NO:12.

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described supra) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEl and Smal and the resulting 614 bp fragment was subcloned into the BspEl to Srfl restriction sites of the vector pMT21/ Δ B2. Fc, to create the plasmid pMT21/Flt1D2. Flk1D3. Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRl and Srfl and the resulting 702 bp fragment was transferred into the 35 EcoRl to Srfl restriction sites of the plasmid pFlt1(1-3)B2- $Fc\Delta C1(a)$ to produce the plasmid pFlt1D2. Flk1D3. Fc $\Delta C1$ (a). The complete DNA and deduced amino acid sequences

of the Flt1D2. Flk1D3. FcΔC1(a) chimeric molecule is shown in SEQ ID NO:11-12. Construction of the expression plasmid

expression pFlt1D2VEGFR3D3Fc∆C1(a). The expression plasmid pMT21. Flt1(1-3).Fc (6519 bp) encodes ampicillin resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1 receptor. This plasmid was used to produce a DNA fragment containing Ig domain 2 of Flt1 by PCR. RNA from the cell line HEL921.7 was used to produce Ig domain 3 of Flk1, using standard RT-PCR methodology. A further round of PCR amplification was used to achieve fusion of the two Ig domains into a single fused fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows: 5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGAC-CTTTCGTAGAGATG-3') (SEO ID NO:24), 3': Flt1D2, VEGFR3D3. as(TTCCTGGGCAACAGCTGGATATCTAT-GATTGTATTGGT) (SEQ ID NO:25). The 5' amplification primer encodes a BspE1 restriction site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO:20) (corresponding to amino acids 27-33 of SEQ ID NO:11-12). The 3' amplification primer encodes the reverse complement of the end of Flt1 Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of SEQ ID NO:14) and continuing into IQLL of VEGFR3 (corresponding to amino acids 127-130 of SEQ ID NO:14).

For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as follows: 5': R3D3. s (ATCCAGCTGT-TGCCCAGGAAGTCGCTGGAGCTGCTGGTA) (SEQ ID

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NO:27), 3': R3D3. as (ATTTTCATGCACAATGACCTCG-GTGCTCTCCCGAAATCG) (SEQ ID NO:28). Both the 5' and 3' amplification primers match the sequence of VEGFR3. The 296 bp amplification product of this RT-PCR reaction was isolated by standard techniques and subjected 5 to a second round of PCR to add suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The amplification primers were as follows: 5': Flt1D2. VEGFR3D3. s(TCATAGATATCCAGCTGTTGCCCAG- 10 GAAGTCGCTGGAG) (SEQ ID NO:29), 3': VEGFR3D3/ (GATAATGCCCGGGGCCATTTTCATGCAsrf.as CAATGACCTCGGT) (SEQ ID NO:30). The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (SEQ ID NO:31) (corresponding to amino acids 221-226 of SEQ ID NO:14), followed by a bridging sequence that includes a recognition sequence for 20 Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of SEQ ID NO:14.

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig 25 domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described supra, to produce the fusion product. This PCR product was subsequently digested with the restriction 30 enzymes BspEl and Smal and the resulting 625 bp fragment was subcloned into the BspEl to Srfl restriction sites of the vector pMT21/Flt1AB2. Fc (described supra), to create the plasmid pMT21/Flt1D2. VEGFR3D3. Fc. The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by 35 standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRl and Srfl and the resulting 693 bp fragment was subcloned into the EcoR1 to Srfl restriction sites of the plasmid pFlt1(1-3) Δ B2-Fc Δ C1(a) to produce the plasmid designated pFlt1D2. VEGFR3D3. 40 Fc C1(a). The complete DNA deduced amino acid sequence of the Flt1D2. VEGFR3D3. FcΔC1(a) chimeric molecule is shown in SEQ ID NO:13-14.

Example 18

Extracellular Matrix Binding (ECM) Binding Assay

ECM-coated plates (Becton Dickinson catalog #35-4607) were rehydrated with warm DME supplemented with 50 glutamine (2 mM), 100 U penicillin, 100 U streptomycin, and 10% BCS for at least 1 hr. before adding samples. The plates were then incubated for 1 hr. at room temperature with varying concentrations of Flt1D2. Flk1D3. Fc∆C1(a) and Flt1D2. VEGFR3D3. FcAC1(a) starting at 10 nM with 55 subsequent 2-fold dilutions in PBS plus 10% BCS. The plates were then washed 3 times with PBS plus 0.1% Triton-X and incubated with alkaline phosphatase-conjugated anti-human Fc antibody (Promega, 1:4000 in PBS plus 10% BCS) for 1 hr. at room temperature. The plates were 60 then washed 4 times with PBS 0.1% Triton-X and alkaline phosphatase buffer/pNPP solution (Sigma) was added for color development. Plates were read at I=405-570 nm. The results of this experiment are shown in FIG. 5 and demonstrate that the Flt1D2. Flk1D3. FcAC1(a) and Flt1D2. 65 VEGFR3D3. $Fc\Delta C1(a)$ proteins are considerably less sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

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Example 19

Transient Expression of pFlt1D2. Flk1D3. Fc∆C1(a) in CHO-K1 (E1A) Cells

A large scale (2 L) culture of *E. coli* DH10B cells carrying the pFlt1D2. Flk1D3. Fc Δ C1(a) plasmid described supra in Example 17(a) was grown overnight in Terrific Broth (TB) plus 100 µg/ml ampicillin. The next day, the plasmid DNA was extracted using a QlAgen Endofree Megaprep kit following the manufacturer's protocol. The concentration of the purified plasmid DNA was determined by standard techniques using a UV spectrophotometer and fluorometer. The plasmid DNA was verified by standard restriction enzyme digestion of aliquots using the restriction enzymes EcoRI plus Notl and Asel. All restriction enzyme digest fragments corresponded to the predicted sizes when analyzed on a 1% agarose gel.

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4×106 cells/plate. Plating media was Gibco Ham's F-12 supplemented with 10% Hyclone Fetal Bovine Serum (FBS), 100 U penicillin/100 U streptomycin and glutamine (2 mM). The following day each plate of cells was transfected with 6 μ g of the pFlt1D2. Flk1D3. Fc Δ C1(a) plasmid DNA using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells, 12 ml/plate of Optimem supplemented with 10% FBS was added. Plates were incubated at 37° C. in a 5% CO2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II supplemented with glutamine (2 mM) and 1 mM sodium butyrate) was added. The plates were incubated at 37° C. for 3 days. After 3 days of incubation, the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet cells. The supernatant was decanted into sterile 1 L bottles and purification of the expressed protein was performed as described infra.

Example 20

Construction pVEGFR1R2-Fc∆C1(a) Expression Vector

The pVEGFR1R2. Fc Δ C1(a) (SEQ ID NO:15-16) expression plasmid was constructed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of SEQ ID NO:16) between Flt1d2-Flk1d3-Fc Δ C1(a) amino acids 26 and 27 of SEQ ID NO:12 (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231. The SDT amino acid sequence is native to the Flt1 receptor and was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2. Fc Δ C1(a) chimeric molecule is shown in SEQ ID NO:15-16.

Example 21

Cell Culture Process Used to Produce Modified Flt1 Receptors

Cell Culture Process Used to Produce Flt1D2. Flk1D3. $Fc\Delta C1(a)$. The process for production of Flt1D2. Flk1D3.

Mylan Exhibit 1016 Mylan v. Regeneron, IPR2021-00880 Page 31 Joining Petitioner: Apotex $Fc\Delta C1(a)$ protein using the expression plasmid pFlt1D2. Flk1D3. $Fc\Delta C1(a)$ described supra in Example 1 involves suspension culture of recombinant Chinese hamster ovary (CHO K1/E1A) cells which constitutively express the protein product. The cells are grown in bioreactors and the protein product is isolated and purified by affinity and size exclusion chromatography.

Cell Expansion. Two confluent T-225 cm² flasks containing the Ft1 D2. Flk1D3. Fc Δ C1(a) expressing cell line were expanded by passaging cells into eight T-225 cm² flasks in 10 medium (GMEM+10% serum, GIBCO) and incubated at 37° C. and 5% CO₂. When the flasks approached confluence (approximately 3 to 4 days) the cells were detached using trypsin. Fresh medium was added to protect the cells from further exposure to the trypsin. The cells were centrifuged 15 and resuspended in fresh medium then transferred to eight 850 cm² roller bottles and incubated at 37° C. and 5% CO₂ until confluent.

Suspension Culture in Bioreactors. Cells grown in roller bottles were trypsinized to detach them from the surface and 20 washed with suspension culture medium. The cells are aseptically transferred to a 5 L bioreactor (New Brunswick Celligen Plus) where the cells are grown in 3.5 L of suspension culture. The suspension culture medium was a glutamine-free low glucose modification of IS-CHO (Irvine 2 Scientific) to which 5% fetal bovine serum (Hyclone), GS supplement (Life Technologies) and 25 μM methionine sulfoximine (Sigma) was added. The pH was controlled at 7.2 by addition of carbon dioxide to the inlet gas or by addition of a liquid solution of sodium carbonate to the 30 bioreactor. Dissolved oxygen level was maintained at 30% of saturation by addition of oxygen or nitrogen to the inlet gas and temperature controlled at 37° C. When a density of 4×10^6 cells/mL was reached the cells were transferred to a 40 L bioreactor containing the same medium and setpoints for controlling the bioreactor. The temperature setpoint was reduced to 34° C. to slow cell growth and increase the relative rate of protein expression.

Cell Culture Process Used to Produce Flt1D2. VEGFR3D3. $Fc\Delta C1(a)$. The same methodologies as 40 described supra for Flt1D2. Flk1D3. $Fc\Delta C1(a)$ were used to produce Flt1D2. VEGFR3D3. $Fc\Delta C1(a)$.

Example 22

Harvest and Purification of Modified Flt1 Receptors

Harvest and Purification of Flt1D2. Flk1D3. Fc Δ C1(a). The product protein was aseptically harvested from the bioreactor while retaining cells using Millipore Prostak 50 tangential-flow filtration modules and a low-shear mechanical pump (Fristam). Fresh medium was added to the bioreactor to replace that removed during the harvest filtration. Approximately 40 L of harvest filtrate was then loaded onto a 400 mL column containing Protein A Sepharose resin 55 (Amersham Pharmacia). After loading the resin was washed with buffer containing 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2 to remove any unbound contaminating proteins. Flt1D2. Flk1D3. Fc Δ C1(a) protein was eluted with a pH 3.0 citrate buffer. The eluted protein was 60 neutralized by addition of Tris base and frozen at -20° C.

Several frozen lots of Flt1D2. Flk1D3. Fc Δ C1(a) protein from the Protein A step above were thawed, pooled and concentrated using a Millipore 30 kD nominal molecular weight cutoff (NMWCO) tangential flow filtration mem-5 brane. The protein was transferred to a stirred cell concentrator (Millipore) and further concentrated to 30 mg/mL

using a 30 kD NMWCO membrane. The concentrated protein was loaded onto a size exclusion column packed with Superdex 200 resin (Amersham Pharmacia) that was equilibrated with phosphate buffered saline plus 5% glycerol. The same buffer was used to run the column. The fractions corresponding to Flt1D2. Flk1D3. Fc Δ C1(a) dimer were pooled, sterile filtered through a 0.22 micron filter, aliquoted and frozen.

Harvest and Purification of Flt1D2. VEGFR3D3. Fc Δ C1 (a). The same methodologies as described supra for Flt1D2. Flk1D3. Fc Δ C1(a) were used to harvest and purify Flt1D2. VEGFR3D3. Fc Δ C1(a).

Example 23

Phosphorylation Assay for Transiently Expressed VEGFR2

Primary human umbilical vein endothelial cells (HU-VECs), passage 4-6, were starved for 2 hrs in serum-free DME high glucose media. Samples containing 40 ng/ml (1 nM) human VEGF165, which is a ligand for the VEGF receptors Flt1, Flk1 and Flt4(VEGFR3) were prepared and were preincubated for 1 hr. at room temperature with varying amounts of the modified Flt1 receptors Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), Flt1D2Flk1D3. Fc\DeltaC1(a) and Flt1D2VEGFR3D3. Fc∆C1(a) in serum-free DME-high glucose media containing 0.1% BSA. Cells were challenged for 5 minutes with the samples prepared above +/-VEGF165, followed by whole cell lysis using complete lysis buffer. Cell lysates were immunoprecipitated with an antibody directed against the C-terminus of VEGFR2 receptor. The immunoprecipitated lysates were loaded onto 4-12% SDS-PAGE Novex gel and then transferred to PVDF membrane using standard transfer methodologies. Detection of phosphorylated VEGFR2 was done by immunoblotting with the anti-phospho Tyrosine mAb called 4G10 (UBI) and developed using ECL-reagent (Amersham). Detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are phosphorylated to varying levels depending on which modified Flt1receptor is used during the preincubations with VEGF. At a 1.5 molar excess of either Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3. FcΔC1 45 (a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3. $Fc\Delta C1(a)$ does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand and modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3. Fc Δ C1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-

surface receptors. Detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are not phosphorylated by challenge samples which have VEGF165 preincubated with 1 and 2 fold molar excess or 3 and 4 fold molar excess of either transient Flt1D2Flk1D3Fc\DeltaC1(a), stable Flt1D2Flk1D3Fc\DeltaC1(a), or transient VEGFR1R2-Fc\DeltaC1 (a). At all modified Flt1 receptor concentrations tested there is complete binding of VEGF165 ligand during the preincubation, resulting in no detectable stimulation of cell-surface receptors by unbound VEGF165 as compared to control media challenge.

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21 Example 24

Cell Proliferation Bioassay

The test cell population is MG87 cells that have been 5 stably transfected with a expression plasmid that contains a DNA insert encoding the VEGFR2(Flk1) extracellular domain fused to the TrkB intracellular kinase domain, thus producing a chimeric molecule. The reason the TrkB intracellular kinase domain was used rather than the native 10 VEGFR2(Flk1) intracellular kinase domain is that the intracellular kinase domain of VEGFR2(Flk1) does not cause a strong proliferative response when stimulated by VEGF165 in these cells. It is known that MG87 cells containing full length TrkB receptor give a robust proliferative response 15 when stimulated with BDNF, so the TrkB intracellular kinase domain was engineered to replace the intracellular kinase domain of VEGFR2(Flk1) to take advantage of this proliferative response capability.

 5×10^3 cells/well were plated in a 96 well plate and 20 allowed to settle for 2 hrs at 37° C. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2Flk1D3Fc Δ C1(a) and Flt1D2. VEGFR3D3. FcΔC1(a), plus an irrelevant receptor termed Tie2-Fc as a negative control, were titrated from 40 nM to 20 pM and incubated on the cells for 1 hr at 37° C. 25 Human recombinant VEGF165 in defined media was then added to all the wells at a concentration of 1.56 nM. The plates were incubated for 72 hrs at 37° C. and then MTS (Owen's reagent, Promega) added and the plates were incubated for an additional for 4 hrs. Finally, the plates were read on a spectrophotometer at 450/570 nm. The results of this experiment are shown in FIG. 6. The control receptor Tie2-Fc does not block VEGF165-induced cell proliferation at any concentration whereas Flt1D2. Flk1D3. $Fc\Delta C1(a)$ blocks 1.56 nM VEGF165 with a half maximal dose of 0.8 35 nM. Flt1(1-3)-Fc and Flt1D2. VEGFR3D3. Fc\DeltaC1(a) are less effective in blocking VEGF165 in this assay with a half maximal dose of ~2 nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units.

Example 25

Binding Stoichiometry of Modified Flt Receptors to VEGF165

BIAcore Analysis. The stoichiometry of Flt1D2Flk1D3. Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a) interaction with human VEGF165 was determined by measuring either the level of VEGF saturation binding to the Flt1D2Flk1D3. 50 Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a) surfaces or measuring concentration of VEGF165 needed to completely prevent binding of Flt1D2Flk1D3. Fc Δ C1(a) or VEGFR1R2-Fc Δ C1 (a) to VEGF BIAcore chip surface.

Modified Flt receptors Flt1D2Flk1D3. $Fc\Delta C1(a)$ and 55 VEGFR1R2-Fc $\Delta C1(a)$, were captured with an anti-Fc specific antibody that was first immobilized on a Biacore chip (BIACORE) using amine-coupling chemistry. A blank antibody surface was used as a negative control. VEGF165 was injected at a concentration of 1 nM, 10 nM, and 50 nM over 60 the Flt1D2Flk1D3. Fc $\Delta C1(a)$ and VEGFR1R2-Fc $\Delta C1(a)$ surfaces at 10 µl/min for one hour. A real-time binding signal was recorded and saturation binding was achieved at the end of each injection. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized 65 Flt1D2Flk1D3. Fc $\Delta C1(a)$ or VEGFR1R2-Fc $\Delta C1(a)$, using the conversion factor of 1000 RU equivalent to 1 ng/ml. The

results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3. $Fc\Delta C1(a)$ or VEGFR1R2-Fc $\Delta C1(a)$ molecule (FIG. 7).

In solution, Flt1D2Flk1D3. FcAC1(a) or VEGFR1R2- $Fc\Delta C1(a)$ at a concentration of 1 nM (estimated to be 1000) times higher than the KD of the Flt1D2Flk1D3. FcΔC1(a) or VEGFR1R2-Fc Δ C1(a)/VEGF165 interaction) were mixed with varied concentrations of VEGF165. After one hour incubation, concentrations of the free Flt1D2Flk1D3. $Fc\Delta C1(a)$ in solution were measured as a binding signal to an amine-coupled VEGF165 surface. A calibration curve was used to convert the Flt1D2Flk1D3. Fc∆C1(a) BIAcore binding signal to its molar concentration. The data showed that the addition of 1 nM VEGF165 into the Flt1D2Flk1D3. Fc\DeltaC1(a) solution completely blocked Flt1D2Flk1D3. FcΔC1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3. $Fc\Delta C1(a)$ molecule. When the concentration of Flt1D2Flk1D3. $Fc\Delta C1(a)$ was plotted as a function of added concentration of VEGF165, the slope of the linear portion was -1.06 for Flt1D2Flk1D3. Fc Δ C1(a) and -1.07 for VEGFR1R2-Fc Δ C1(a). The magnitude of the slope, very close to negative one, was indicative that one molecule of VEGF165 bound to one molecule of either Flt1D2Flk1D3. FcΔC1(a) or VEGFR1R2-FcΔC1(a)

Size Exclusion Chromatography. Flt1D2Flk1D3. Fc\DeltaC1 (a) was mixed with a 3-fold excess of VEGF165 and the receptor-ligand complex was purified using a Pharmacia Superose 6 size exclusion chromatography column. The receptor-ligand complex was then incubated in a buffer containing 6M guanidine hydrochloride in order to dissociate it into its component proteins. Flt1D2Flk1D3. Fc Δ C1(a) was separated from VEGF165 using Superose 6 size exclusion chromatography column run in 6M guanidium chloride. In order to determine complex stoichiometry, several injections of Flt1D2Flk1D3. Fc∆C1(a) and VEGF165 were made and peak height or peak integrated intensity was plotted as a function of the concentration of injected protein. The calibration was done under condition identical to one used in 40 separating components of Flt1D2Flk1D3. Fc\DeltaC1(a)/VEGF complex. Quantification of the Flt1D2Flk1D3. Fc∆C1(a)/ VEGF complex composition was based on the calibration curves. The results of this experiment (FIG. 7) shows the ratio of VEGF165 to Flt1D2Flk1D3. Fc∆C1(a) in a complex 45 to be 1:1.

Determination of the Binding Stoichiometry of Flt1D2Flk1D3. FcΔC1(a)/VEGF165 Complex by Size Exclusion Chromatography

Example 26

Flt1D2Flk1D3. FcΔC1(a)/VEGF165 Complex Preparation. VEGF165 (concentration=3.61 mg/ml) was mixed with CHO cell transiently expressed Flt1D2. Flk1D3. FcΔC1(a) (concentration=0.9 mg/ml) in molar ratio of 3:1 (VEGF165: Flt1D2. Flk1D3. FcΔC1(a)) and incubated overnight at 4° C. Size Exclusion Chromatography (SEC) under native conditions. To separate the complex from excess of unbound VEGF165, 50 µl of the complex was loaded on a Pharmacia Superose 12 PC 3.2/30 which was equilibrated in PBS buffer. The sample was eluted with the same buffer at flow rate 40 µl/min. at room temperature. Peak #1 represents the complex and peak #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2 ml were combined and guanidinium hydrochloride (GuHCI) was added to a final concentration 4.5M to dissociate the complex.

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Size Exclusion Chromatography (SEC) under dissociative conditions. To separate the components of the receptor-ligand complex and to determine their molar ratio, 50 μ l of dissociated complex as described supra was loaded onto a Superose 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted with the same solution at a flow rate 40 μ l/min. at room temperature.

Calculation of Flt1D2Flk1D3. Fc∆C1(a):VEGF165 Complex Stoichiometry. The stoichiometry of the receptor-ligand complex was determined from the peak area or the peak height of the components. Concentrations of VEGF165 and Flt1D2Flk1D3. Fc Δ C1(a) corresponding to the peak height or peak area, respectively, were obtained from the standard curves for VEGF165 and Flt1D2Flk1D3. Fc∆C1(a). To obtain a standard curve, four different concentrations (0.04 mg/ml -0.3 mg/ml) of either component were injected onto a Pharmacia Superose 12 PC 3.2/30 column equilibrated in 6M guanidinium chloride and eluted with the same solution at flow rate 40 µl/min. at room temperature. The standard curve was obtained by plotting peak area or peak height vs protein concentration. The molar ratio of VEGF165: 20 Flt1D2Flk1D3. FcΔC1(a) determined from the peak area of the components was 1.16. The molar ratio of VEGF165: Flt1D2Flk1D3. Fc∆C1(a) determined from the peak height of the components was 1.10.

Example 27

Determination of the Stoichiometry of the Flt1D2Flk1D3. Fc∆C1(a)/VEGF165 Complex by Size Exclusion Chromatography with On-Line Light Scattering

Complex preparation. VEGF165 was mixed with CHO transiently expressed Flt1D2. Flk1D3. $Fc\Delta C1(a)$ protein in molar ratio of 3:1 (VEGF165:Flt1D2Flk1D3. $Fc\Delta C1(a)$) and incubated overnight at 4° C.

Size Exclusion Chromatography (SEC) with On-Line Light Scattering. Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa Barbara, Calif.) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine 40 the molecular weight (MW) of the receptor-ligand complex. Samples were injected onto a Superose 12 HR 10/30 column (Pharmacia) equilibrated in PBS buffer and eluted with the same buffer at flow rate 0.5 ml/min. at room temperature. The elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3. Fc Δ C1(a)/VEGF165 complex at the ⁵⁰ peak position is 157 300, the MW of VEGF165 at the peak position is 44 390 and the MW of R1R2 at the peak is 113 300

These data indicated that the stoichiometry of the Flt1D2Flk1D3. Fc Δ C1(a)/VEGF complex is 1:1 as its corresponds to the sum of molecular weights for Flt1D2Flk1D3. Fc Δ C1(a) and VEGF165. Importantly, this method conclusively proved that the Flt1D2Flk1D3. Fc Δ C1 (a)/VEGF165 complex was indeed composed of only one molecule of VEGF165 ligand and only one molecule of the flt1D2Flk1D3. Fc Δ C1(a).

Example 28

Peptide Mapping of Flt1D2. Flk1D3. $Fc\Delta C1(a)$

The disulfide structures and glycosylation sites in Flt1D2. Flk1D3. $Fc\Delta C1(a)$ were determined by a peptide mapping

method. In this method, the protein was first cleaved with trypsin. Tryptic fragments were analyzed and identified by HPLC coupled with mass spectrometry, in addition to an N-terminal sequencing technique. Reduction of the tryptic digest was employed to help identify disulfide-bond-containing fragments. Treatment of the tryptic digest with PNGase F (Glyko, Novato, Calif.) was employed to help identify fragments with N-linked glycosylation sites.

There are a total of ten cysteines in Flt1D2. Flk1D3. Fc Δ C1(a); six of them belong to the Fc region. Cys27 has-been confirmed to be disulfide bonded to Cys76. Cys121 is confirmed to be disulfide bonded to Cys182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, because these two cysteines can not be separated enzymatically from each other, it can not be determined whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is confirmed to be disulfide bonded to Cys306. Cys 352 is confirmed to be disulfide bonded to Cys410.

There are five possible N-linked glycosylation sites in Flt1D2. Flk1D3. Fc Δ C1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino acid sequence NIT), Asn193

(amino acid sequence NST), and Asn282 (amino acid sequence NST). In addition, partial glycosylation is observed on Asn65 and Asn120.

Example 29

Pharmacokinetic Analysis of Modified Flt Receptors

Pharmacokinetic analysis of Flt1(1-3)-Fc (A40). Flt1D2. Flk1D3. $Fc\Delta C1(a)$ and VEGFR1R2-Fc $\Delta C1(a)$. Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2. Flk1D3. FcΔC1(a), CHO stably expressed Flt1D2. Flk1D3. FcΔC1(a), and CHO transiently expressed VEGFR1R2- $Fc\Delta C1(a)$. The mice were tail bled at 1, 2, 4, 6, 24 hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2. Flk1D3. $Fc\Delta C1(a)$ or VEGFR1R2-Fc $\Delta C1(a)$. The ELISA involves coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc (A40), Flt1D2. Flk1D3. $Fc\Delta C1(a)$ or VEGFR1R2-Fc $\Delta C1(a)$ and reporting with an anti-Fc antibody linked to horse radish peroxidase. The results of this experiments are shown in FIG. 8. The T_{max} for Flt1(1-3)-Fc (A40) was at 6 hrs while the T_{max} for the transient and stable Flt1D2. Flk1D3. Fc∆C1(a) and the transient VEGFR1R2-Fc Δ C1(a) was 24 hrs. The C_{max} for Flt1(1-3)-Fc (A40) was 8 µg/ml. For both transients (Flt1D2. Flk1D3Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a)) the C_{max} was 18 µg/ml and the C_{max} for the stable VEGFR1R2-Fc Δ C1(a) was 30 µg/ml.

Pharmacokinetic analysis of Flt1(1-3)-Fc (A40). Flt1D2.
Flk1D3. FcΔC1(a) and Flt1D2. VEGFR3D3. FcΔC1(a).
Balb/c mice (25-30 g) were injected subcutaneously with 4
60 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed
Flt1D2Flk1D3FcΔC1(a) and CHO transiently expressed
Flt1D2. VEGFR3D3. FcΔC1(a). The mice were tail bled at
1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera
were assayed in an ELISA designed to detect Flt1(1-3)-Fc,
65 Flt1D2. Flk1D3. FcΔC1(a) and Flt1D2. VEGFR3D3.
FcΔC1(a). The ELISA involves coating an ELISA plate with
165, binding the Flt1(1-3)-Fc, Flt1D2. Flk1D3. FcΔC1(a) or

Mylan Exhibit 1016 Mylan v. Regeneron, IPR2021-00880 Page 34 Joining Petitioner: Apotex Flt1D2. VEGFR3D3. Fc Δ C1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5 whereas, Flt1D2. Flk1D3. Fc Δ C1(a) and Flt1D2VEGFR3D3Fc Δ C1(a) were detectable for 15 days or 5 more. The results of this experiment are shown in FIG. 9.

Example 30

Evaluation of the Ability of Flt1D2. Flk1D3. Fc Δ C1(a) to Inhibit Tumor Growth In Vivo

To evaluate the ability of Flt1D2. Flk1D3. Fc\DeltaC1(a) to inhibit tumor growth in vivo a model in which tumor cell suspensions are implanted subcutaneously on the right flank 1 of male severe combined immunodeficiency (SCID) mice was employed. Two cell lines, the human HT-1080 fibrosarcoma cell line (ATCC accession no. CCL-121) and the rat C6 glioma cell line (ATCC accession no. CCL-107), each of which exhibit distinctly different morphologies and growth 20 characteristics, were used in the assay. The first dose of Flt1D2. Flk1D3. Fc Δ C1(a) (at 25 mg/Kg or as indicated in FIG. 10-11) was given on the day of tumor implantation. Animals subsequently received subcutaneous injections of Flt1(1-3)-Fc (A40), Flt1D2. Flk1D3. FcAC1(a) or vehicle 25 either every other day (EOD) or two times per week (2×/wk) for a period of 2 weeks. After 2 weeks, animals were perfused with fixative, tumors were removed and samples were blinded. Tumor volume was determined by measuring the length and width of visible subcutaneous tumors. Both of Flt1(1-3)-Fc (A40) and Flt1D2. Flk1D3. FcΔC1(a) significantly reduced the growth of tumors formed by HT-1080 and C6 cells

Example 31

The Effect of VEGF165 and Modified Flt Receptors in Female Reproductive System

The stereotypic pattern of vascular remodeling which 40 occur in the uterus and ovary over the course of the reproductive cycle has been well characterized, making these tissues particularly well suited to the study of mechanisms which regulate angiogenesis, vascular remodeling and vascular regression. Indeed, in situ hybridization studies in 45 the reproductive tissues provided the first clear evidence that VEGF acts as a mediator of physiological angiogenesis in mature rodents, as well as humans and non-human primates. As cyclic angiogenesis and vascular remodeling are prominent features of the normal ovary and uterus, it is not 50 surprising that abnormal blood vessel growth and/or vascular dysfunction have been found to characterize many pathological conditions which affect these organs. Furthermore, these pathogenic vascular abnormalities are thought to be caused or perpetuated by the dysregulated expression of one 55 or more angiogenic or anti-angiogenic factors, most prominently VEGF.

For example, abnormal angiogenesis is characteristic of polycystic ovary disease, endometriosis and endometrial carcinoma, and in each case VEGF is over expressed in the 60 affected tissue. Overexpression of VEGF is also thought to play a pathogenic role in the establishment of systemic vascular hyperpermeability in ovarian hyperstimulation syndrome and preeclampsia. In addition, VEGF has been implicated as the permeability factor responsible for the production of ascites associated with ovarian carcinoma and other tumors. Agents which effectively neutralize the biological

actions of VEGF can reasonably be anticipated to be of therapeutic benefit in the above and related conditions.

Angiogenesis and vascular remodeling are also hallmarks of blastocyst implantation and placental development. VEGF is strongly expressed both in the maternal decidua and in embryonic trophoblasts, where it is thought to first stimulate expansion and hyperpermeability of the uterine vasculature during the peri-implantation period and subsequently mediate formation of both the maternal and embry-

onic components of the placental vasculature. VEGF is also required for luteal angiogenesis and associated progesterone secretion necessary to prepare the uterus for implantation. Thus, agents which inhibit the biological actions of VEGF may prove to be useful as contraceptive agents (by prevent-15 ing implantation), or as an abortifacients in the early stages of gestation. The latter application might find particular use as a non-surgical intervention for the termination of ectopic pregnancies.

While the expression of VEGF receptors is largely confined to the vascular endothelium in normal reproductive tissues, Flt1is also expressed by trophoblasts in the placenta in both humans and animals where it has been proposed to play a role in trophoblast invasion. Interestingly, both Flt1 and KDR (Flk1) are expressed by choriocarcinoma cell line BeWo, and VEGF has been shown to promote DNA synthesis and tyrosine phosphorylation of MAP kinase in these cells. Furthermore, primary and metastatic ovarian carcinomas not only to express high levels of VEGF, but-in addition to the vascular endothelium-the tumor cells themselves express KDR and/or Flt1. These findings suggest that VEGF may not only be critically involved in the generation and maintenance of tumor vasculature, but that at least in some tumors of reproductive origin VEGF may subserve an autocrine role, directly supporting the survival and prolif-35 eration of the tumor cells. Thus agents which block the actions of VEGF may have particularly beneficial applications to the treatment of tumors of reproductive origin.

Assessment of VEGF-Induced Uterine Hyperpermeability. Pregnant mare's serum gonadotrophin (PMSG) was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a surge of estradiol after 2 days which in turn causes an induction of VEGF in the uterus. It is reported that this induction results in hyperpermeability of the uterus and an increase in uterine wet weight 6 hrs. later and, therefore, could potentially be blocked by the modified Flt receptors Flt1(1-3)-Fc (A40), Flt1D2. Flk1D3Fc Δ C1(a) and Flt1D2VEGFR3D3Fc Δ C1(a). In this in vivo model, the normal weight of the rat uterus is about 50 mg and this can be induced to 300-350 mg by PMSG. Desiccation of the tissue reveals that this is all water weight. Subcutaneous injection of Flt1(1-3)-Fc (A40), Flt1D2. Flk1D3, $Fc\Delta C1(a)$ and Flt1D2, VEGFR3D3, Fc1C1(a) at 25 mg/kg at 1 hr. after PMSG injection results in about a 50% inhibition of the increase in uterine wet weight. Increasing the dose of modified Flt receptor does not further reduce the increase in wet weight suggesting that there is a VEGFindependent component to this model (FIG. 12).

Assessment of corpus luteum angiogenesis using progesterone as a readout. Pregnant mare's serum gonadotrophin (PMSG) is injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a fully functioning corpus luteum containing a dense network of blood vessels after 4 days that allows for the secretion of progesterone into the blood stream in order to prepare the uterus for implantation. The induction of angiogenesis in the corpus luteum requires VEGF; therefore, blocking VEGF would result in a lack of new blood vessels and thus a lack

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of progesterone secreted into the blood stream. In this in vivo model, resting levels of progesterone are about 5 ng/ml and this can be induced to a level of 25-40 ng/ml after PMSG. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2. Flk1D3. Fc Δ C1(a) at 25 mg/kg or 5 mg/kg at 1 hr. 5 after PMSG injection results in a complete inhibition of the progesterone induction on day 4 (FIGS. 13A-B).

Example 33

Pharmacokinetic Analysis of Flt1(1-3)-Fc (A40) and Pegylated Flt1(1-3)-Fc

Flt1(1-3)-Fc was PEGvlated with either 10 kD PEG or 20 kD PEG and tested in balb/c mice for their pharmacokinetic 15 profile. Both PEGylated forms of Flt1(1-3)-Fc were found to have much better PK profiles than Flt1(1-3)-Fc (A40), with the Tmax occurring at 24 hrs. for the PEGylated molecules as opposed to 6 hrs. for Flt1(1-3)-Fc (A40).

Example 34

VEGF165 ELISA to Test Affinity of Modified Flt1 Receptor Variants

10 pM of VEGF165 was incubated overnight at room temperature with modified Flt1 receptor variants ranging from 160 pM to 0.1 pM. The modified Flt1 receptor variants used in this experiment were Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), transiently expressed Flt1D2Flk1D3FcΔC1(a), transiently expressed Flt1D2VEFGFR3D3-Fc C1(a), Flt1-(1- 3_{NAS})-Fc, Flt1(1- $3_{R->C}$)-Fc and Tie2-Fc. Flt1(1- 3_{NAS})-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic amino acid sequence KNKRASVRRR (SEQ ID NO:32) is replaced by NASVNGSR (SEQ ID NO:33), resulting in the 35 incorporation of two new glycosylation sites and a net reduction of five positive charges, both with the purpose of reducing the unfavorable effects of this sequence on PK. $Flt1(1-3_{R->C})$ -Fc is a modification in which a single arginine (R) residue within the same basic amino acid sequence is 40 changed to a cysteine (C) (KNKRASVRRR (SEQ ID NO:32)→KNKCASVRRR (SEQ ID NO:34)) to allow for pegylation at that residue, which could then shield the basic region from exerting its unfavorable effects on PK. After incubation the solution was transferred to a plate containing 45 a capture antibody for VEGF165 (R&D). The amount of free VEGF165 was then determined using an antibody to report free VEGF165. This showed that the modified Flt1 receptor variant with the highest affinity for VEGF165 (determined as the lowest amount of free VEGF165) was Flt1D2Flk1D3. 50 $Fc\Delta C1(a)$, followed by Flt1(1-3)-Fc and Flt1(1-3)-Fc (A40) and then by $Flt1(1-3_{R->C})$ -Fc, $Flt1(1-3_{NAS})$ -Fc and Flt1D2VEFGFR3D3-Fc∆C1(a). Tie2Fc has no affinity for VEGF165.

Example 35

Breakdown of Blood-retinal Barrier in Diabetes is Reversed by Inhibition of VEGF

Rats received a single injection of VEGFR1R2-Fc Δ C1(a) (SEQ ID NO:16) (25 mg/kg, i.p.) or PBS 4 weeks after induction of diabetes by streptozotocin (65 mg/kg, i.v.). The permeability of retinal vessels was assessed 24 hours later by measuring the extravasation of Evans Blue dye, which 65 binds to albumin in the circulation. Under deep anesthesia, Evans Blue dye (45 mg/kg) was injected intravenously, and

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allowed to circulate for 60 minutes, and blood samples were taken periodically to assess the concentration of dve in the circulation. The animals were then perfused to flush dye and blood from the vasculature, the eye enucleated and the retinas removed. Evans blue was extracted, and the concentration of dye in the retina was normalized to retinal weight and the time-averaged concentration of Evans blue in the circulation (mL plasma×g retina weight⁻¹×hr⁻¹) to provide an index of vascular leak. VEGFR1R2-Fc\DeltaC1(a) normal-10 ized retinal vascular permeability to levels evident in nondiabetic rats.

Example 36

VEGFR1R2-Fc∆C1(a) Prevents Neovascularization Induced by Retinal Ischemia

Excessive upregulation of VEGF expression is responsible for pathologic neovascularization in many retinal dis- $_{20}$ eases. The anti-angiogenic properties of VEGFR1R2-Fc Δ C1 (a) were investigated in a mouse model of oxygen-induced ischemic retinopathy (OIR). OIR was produced by transiently exposing mouse pups to increased ambient oxygen (hyperoxia), resulting in obliteration of the developing microvasculature within the central retina. Subsequent return of the animals to room air results in relatively hypoxic conditions in the retina, which in turn stimulates an angiogenic response that shares features with both diabetic retinopathy, retinopathy of prematurity and other ischemic retinopathies. VEGFR1R2-FcAC1(a) (25 mg/kg, ip) was administered every other day beginning 12-24 hours after returning the mice from hyperoxia to room air. Littermate controls received injections of human Fc following the same schedule. Retinas were collected 1 week following return to room air. Flat mounts were prepared from one retina obtained from each animal, and the retinal vessels stained with fluoresceinated lectin (griffonia simplicifolia). The other retina was embedded and cross-sections were cut and stained with hematoxylin and eosin.

Retinas of all control mice exposed to hyperoxia exhibited marked pathologic angiogenesis, characterized by the presence of vascular tufts penetrating the inner limiting membrane and chaotic sprouting of vessels on the surface of the retina, particularly around the optic nerve head. Administration of VEGFR1R2-Fc Δ C1(a) almost completely blocked the development of these vascular abnormalities as quantitated by counting endothelial cell nuclei in the abnormal pre-retinal vessels (FIG. 15). Although pathologic angiogenesis was dramatically inhibited, systemic administration of VEGFR1R2-Fc∆C1(a) did not block the growth of normalappearing intraretinal vessels in these animals.

Example 37

Subcutaneous Administration of VEGFR1R2-Fc∆C1(a) Suppresses Choroidal Neovascularization

Though animals do not develop age related macular 60 degeneration (AMD) per se, choroidal neovascularization resembling that seen in AMD can be produced by using a laser to produce focal disruptions in Bruch's membrane and the overlying retinal pigment epithelium (RPE). This injury stimulates the abnormal growth of underlying choroidal capillaries into the rpe layer and subretinal space. Disruption of Bruch's membrane is common to all forms of choroidal neovascularization (CNV), including that which character-

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izes the wet form of AMD. In the laser-induced model of choroidal neovascularization, groups of 9 or 10 mice were treated with sc injections of 25 mg/kg of VEGFR1R2-Fc Δ C1(a) or human Fc one day prior to laser injury and on days 2, 5, 8, and 11 after laser. At 14 days after laser injury, 5 the mice were injected intravenously with fluorescein-labeled dextran (50 mg), euthanized, and eyes were rapidly dissected for choroidal flat mounts or frozen in optimum cutting temperature embedding compound and sectioned for evaluation of the lesions. VEGFR1R2-Fc Δ C1(a) administration reduced the area of CNV lesions by approximately 70% (FIG. **16**).

The effect of VEGFR1R2-Fc∆C1(a) on laser-induced choroidal neovascularization also was evaluated in adult cynomolgus monkeys. In this experiment, VEGFR1R2- $Fc\Delta C1(a)$ was administered by intravenous or intravitreal injection. Each animal received nine or ten laser burns to each retina, and the development of active choroidal neovascular lesions was assessed by fluorescein angiography, once 20 before the initiation of treatment and 15, 20 and 19 days postlaser. VEGFR1R2-Fc\DeltaC1(a) was administered intravenously once per week, beginning one week before laser injury, at a dose of 3 mg/kg or 10 mg/kg. Intravitreal injections were made once every two weeks, at a dose of 50, 25 250 or 500 mcg/eye beginning one week before laser, or once, two weeks following laser (500 mcg dose only), at which time active CNV lesions had already formed. Control animals received weekly intravenous or biweekly intravitreal injections of placebo, beginning one week before laser. Each of the above experimental and control groups comprised six animals, 3 males and 3 females. CNV lesions were visualized by fluorescein angiography and graded. Active CNV lesions characterized bright hyperflourescence, with late leakage beyond the borders of the laser spot (Grade 4). developed at 32% and 48% of the laser burn sites, in animals receiving intravitreal or intravenous administration of placebo. In contrast, the development of grade 4 lesions was completely or nearly completely prevented in all groups of animals receiving intravenous or intravitreal injections of 40 VEGFR1R2-Fc\DeltaC1(a) (FIG. 20). Moreover a single intravitreal injection (500 mcg) of VEGFR1R2-Fc∆C1(a) made following the laser injury reduced the incidence of grade 4 lesions from 44% to 0% within 10 days of treatment (FIG. 20). 45

Example 38

Subcutaneous Administration of VEGFR1R2-Fc∆C1(a) Inhibits Subretinal Neovascularization in Rho/VEGF Transgenic Mice

Transgenic mice expressing a recombinant human VEGF transgene under the control of the rhodopsin promoter (Rho/VEGF) were used in these experiments. These animals 55 begin to express VEGF in photoreceptors on about postnatal day (P) 7, which typically results in pronounced subretinal neovascularization by P21. At P7, mice were divided into 2 groups and treated with 25 mg/kg of VEGFR1R2-Fc Δ C1(a) (9 mice, 17 eyes) or human Fc (10 mice, 19 eyes) on P7, 60 P10, P13, P16, and P19. On P21, the mice were anesthetized and perfused with fluorescein-labeled dextran. Retinal whole mounts from mice treated with VEGFR1R2-Fc Δ C1 (a) showed few areas of neovascularization while many new vessels were present in the subretinal space of mice that had 65 been treated with Fc. Measurement of the total area of neovascularization per retina by image analysis showed

significantly less neovascularization in VEGFR1R2-Fc Δ C1 (a)-treated mice, compared to those treated with Fc (FIG. 17).

Example 39

Subcutaneous Injections of VEGFR1R2-Fc∆C1(a) Suppress VEGF-Induced Breakdown of the Blood-retinal Barrier

Adult C57BL/6 mice were given a sc injection of 25 mg/kg of VEGFR1R2-Fc Δ C1(a) or Fc fragment and on the following day received an intravitreous injection of 1 µl of 10⁻⁶M VEGF. Six hours later, retinal vascular permeability was measured using [³H]-mannitol as a tracer. Mice treated with VEGFR1R2-Fc Δ C1(a) (9 mice, 18 eyes) had a significantly smaller retina to lung leakage ratio (the ratio of radioactivity in the retina compared to excised lung) than mice treated with Fc fragment (9 mice, 18 eyes) indicating less breakdown of the blood retinal barrier (FIG. **18**A).

The effect of VEGFR1R2-Fc∆C1(a) on VEGF-mediated vascular leak was also evaluated in a second experiment, which employed double transgenic mice (rtTA/rho-TRE/ VEGF). These mice are characterized by photoreceptorspecific expression of the VEGF transgene that is inducible by administration of doxycycline. Adult rtTA/rho-TRE/ VEGF mice were injected sc with 25 mg/kg VEGFR1R2-Fc\DeltaC1(a) (10 mice, 20 eyes) or Fc fragment (10 mice, 20 eyes). On the following day, doxycycline (2 mg/mL) was placed in their drinking water to stimulate over-expression of VEGF within the retina. Two days later, they were given a second sc injection of VEGFR1R2-Fc\DeltaC1(a) or Fc fragment and then the next day retinal vascular permeability was measured with [3H]-mannitol. Mice treated with VEGFR1R2-Fc Δ C1(a) exhibited a significant reduction in the retina to lung leakage ratio compared to mice treated with Fc (FIG. 18B), indicating that impairment in the blood-retinal barrier was ameliorated.

Example 40

VEGFR1R2-FcΔC1(a) Inhibits Injury-induced Corneal Neovascularization, Edema, and Inflammation

Corneal neovascularization was induced in male C57BI/6 mice by intrastromal placement of 3 nylon sutures, or by chemical injury (NaOH) and mechanical debridement of the corneal epithelium. Multiple experiments were conducted in 50 which VEGFR1R2-Fc∆C1(a) was administered intraperitoneally once or at multiple time points immediately before or following injury. The growth of corneal neovessels was evaluated by slit-lamp microscopy and histological evaluation. The vasculature was labeled with an endothelial cell specific fluorescein-conjugated lectin, and neovascularization was evaluated in corneal flat-mounts, as well as in cross sections using PECAM immunohistochemistry. The presence of corneal edema was evaluated, using slit lamp microscopy, and corneal thickness was measured in crosssections; increases in corneal thickness reflect the amount of edema. The numbers of polymorphonuclear leukocytes (PMN) and macrophages were determined by staining crosssections with HEMA-3 or rat anti-mouse F4/80 monoclonal antibody, respectively

Dosing regimens which employed multiple injections of VEGFR1R2-Fc Δ C1(a) (25 mg/kg, ip) completely inhibited corneal neovascularization in both the suture and chemical

Mylan Exhibit 1016 Mylan v. Regeneron, IPR2021-00880 Page 37 Joining Petitioner: Apotex injury models. Moreover, single injections of 25 or 12.5 mg/kg VEGFR1R2-Fc Δ C1(a) given immediately after suture injury effectively blocked corneal neovascularization for at least 9 days, while injections of 6.25 and 2.5 mg/kg ameliorated but did not block corneal neovascularization. 5 The lowest dose of VEGFR1R2-Fc Δ C1(a) tested (0.5 mg/kg) had no evident effect. Corneal thickness, reflecting the amount of edema present, was significantly reduced in VEGFR1R2-Fc Δ C1(a)-treated animals compared to vehicle-treated controls (FIG. **19**). Histological analyses 10 showed that the infiltration of neutrophils and macrophages into the damaged cornea also was dramatically reduced by VEGFR1R2-Fc Δ C1(a) treatment.

Example 41

Transient Treatment with VEGFR1R2-Fc∆C1(a) Produces Long-lasting Inhibition of Corneal Neovascularization and Conjunctivalization Following Alkali Burn Injury

Corneas were injured by application of NaOH and mechanical debridement of the corneal epithelium in adult, male C57BI/6 mice. VEGFR1R2-Fc Δ C1(a) or a control protein (human Fc) was administered subcutaneously (12.5 25 mg/kg) on days 0 (the day of injury), 7 and 14, at which time re-epithelialization of the cornea was complete. The animals were euthanized on days 28 or 42 (14 or 28 days following

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the last injection of VEGFR1R2-Fc Δ C1(a) and corneas taken for histological evaluation. Tissues were processed as described above.

Treatment with VEGFR1R2-FcΔC1(a) inhibited corneal neovascularization during the period of active treatment (as determined by slit-lamp microscopy), as well as 2 and 4 weeks following treatment cessation. In eyes evaluated on day 28 (14 days after the last injection of VEGFR1R2-10 FcΔC1(a), the neovascular response to injury remained completely suppressed and conjunctivalization of the cornea was also inhibited as evidenced by a more normal appearing morphology of the re-epithelialized cornea and a substantial reduction in goblet cell number (~30% relative to controls).
 Corneal inflammation and edema also were reduced sub-

stantially. Evaluation of flat-mounted corneas taken at Day 42 showed that neovascularization was still largely suppressed, though limited, focal sprouting of vessels at the corneal margin was observed in some cases.

The data show that when administered at the time of injury, VEGFR1R2-Fc Δ C1(a) improves corneal healing by potently inhibiting the development of corneal neovascularization, inflammation, edema and conjunctivalization of the corneal epithelium. These effects persisted for several weeks following cessation of treatment, suggesting that acute inhibition of VEGF following corneal injury may have long-term benefits.

SEQUENCE LISTING

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Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro 20 Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr 35 Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro 50 Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala	1 5	10	15
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr 35 40 45 Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro 50 55 60 Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala	Cys Leu Leu Thr Gly Se 20	er Ser Ser Gly Ser Lys Leu 25	Lys Asp Pro 30
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro 50 55 60 Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala	Glu Leu Ser Leu Lys Gly Th 35	hr Gln His Ile Met Gln Ala 40 45	Gly Gln Thr
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala	Leu His Leu Gln Cys Arg Gl 50 5!	ly Glu Ala Ala His Lys Trp 5	Ser Leu Pro
65 70 75 80	Glu Met Val Ser Lys Glu Se 65 70	er Glu Arg Leu Ser Ile Thr 75	Lys Ser Ala 80
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr 85 90 95	Cys Gly Arg Asn Gly Lys Gl 85	ln Phe Cys Ser Thr Leu Thr 90	Leu Asn Thr 95

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Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val 100 105 110 Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
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 Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val

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 Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe 180 185 190 Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu 195 200 205 Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg 210 215 220
 Gin Thr Asn Thr Ile Ile Asp Val Gin Ile Ser Thr Pro Arg Pro Val

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 Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp 385
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 Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Glu Gln Tyr 405 410 415 Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu 435 440 445 Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg 450 455 460
 Glu Pro Gln Val Tyr
 Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys

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 Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp 485 490 495 Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys 500 505 510

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We claim:

1. A method of treating retinal neovascularization, comprising administering a fusion polypeptide capable of binding vascular endothelial growth factor (VEGF) to a patient in need thereof, wherein the fusion polypeptide comprises the amino acid sequence of SEQ ID NO:16.

2. The method of claim **1**, wherein neovascularization is ⁵⁵ induced by ischemia.

3. A method of treating, ameliorating, or inhibiting choroidal neovascularization, comprising administering a fusion polypeptide capable of binding vascular endothelial growth factor (VEGE) to a patient in need thereof, wherein ⁶⁰ the fusion polypeptide comprises the amino acid sequence of SEQ ID NO:16.

4. A method of treating, ameliorating, or inhibiting vascular leak in the retina, comprising administering a fusion polypeptide capable of binding vascular endothelial growth factor (VEGF) to a patient in need thereof, wherein the fusion polypeptide comprises the amino acid sequence of SEQ ID NO:16.

5. A method of treating, ameliorating, or inhibiting retinal edema, comprising administering a fusion polypeptide capable of binding vascular endothelial growth factor (VEGF) to a patient in need thereof, wherein the fusion polypeptide comprises the amino acid sequence of SEQ ID NO:16.

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(12) United States Patent Wiegand et al.

- (54) USE OF VEGF INHIBITORS FOR TREATMENT OF EYE DISORDERS
- (75) Inventors: Stanley J. Wiegand, Croton-on-Hudson, NY (US); Nicholas J. Papadopoulos, LaGrangeville, NY (US); George D. Yancopoulos, Yorktown Heights, NY (US); James P. Fandl, LaGrangeville, NY (US); Thomas J. Daly, New City, NY (US)
- (73) Assignee: Regeneron Pharmaceuticals, Inc., Tarrytown, NY (US)
- Subject to any disclaimer, the term of this (*) Notice: patent is extended or adjusted under 35 U.S.C. 154(b) by 239 days.

This patent is subject to a terminal disclaimer.

- (21) Appl. No.: 11/218,234
- (22) Filed: Sep. 1, 2005

(65)**Prior Publication Data**

US 2006/0030529 A1 Feb. 9. 2006

Related U.S. Application Data

- Continuation-in-part of application No. 11/089,803, (63) filed on Mar. 25, 2005, and a continuation-in-part of application No. 10/880,021, filed on Jun. 29, 2004, now Pat. No. 7,279,159, which is a continuation-inpart of application No. 10/988,243, filed on Nov. 12, 2004, which is a continuation-in-part of application No. 10/609,775, filed on Jun. 30, 2003, now Pat. No. 7,087,411, which is a continuation-in-part of application No. 10/009,852, filed as application No. PCT/ US00/14142 on May 23, 2000, now Pat. No. 7,070, 959.
- (60) Provisional application No. 60/138,133, filed on Jun. 8, 1999.

US 7,303,747 B2 (10) Patent No.: *Dec. 4, 2007

(45) Date of Patent:

- (51) Int. Cl. A61K 38/18 (2006.01)C07K 14/71 (2006.01) C12N 15/62 (2006.01)
- (52) U.S. Cl. 424/134.1; 424/192.1; 514/2; 514/12; 530/350; 536/23.4
- Field of Classification Search None (58)See application file for complete search history.

(56)**References** Cited

U.S. PATENT DOCUMENTS

6,100,071 A 8/2000 Davis-Smyth

FOREIGN PATENT DOCUMENTS

WO	WO97/44453	11/1997
wo	WO98/13071	4/1998
WO	WO99/03996	1/1999
	OTHER PU	JBLICATIONS

Terman, B.I., et al., (1991) Oncogene 6:1677-1683.

Terman, B.I., et al., (1992) Biochem. Biophys. Res. Comm. 187(3):1579-1586.

Davis-Smyth, T., et al., (1996) The EMBO Journal 15(18):4919-4927

Holash, J., et al., (2002) PNAS 99(17):11393-11398

Heidaran, M.A., et al., (1990) J. Bio. Chem. 265(31):18741-18744. Cunningham, S.A., et al., (1997) Biochem. Biophys. Res. Comm. 231:596-599

Fuh, G., et al., (1998) J. Bio. Chem. 273(18):11197-11204. Wiesmann, C., et al., (1997) Cell 91:695-704.

Barleon, B., et al., (1997) J. Bio. Chem. 272(16):10382-10388. Davis-Smyth, T., et al., (1998) J. Bio. Chem. 273(6):3216-3222.

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(57)ABSTRACT

Modified chimeric polypeptides with improved pharmacokinetics and improved tissue penetration are disclosed useful for treating eye disorders, including age-related macular degeneration and diabetic retinopathy.

6 Claims, 11 Drawing Sheets

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		-		-	-	
Flk1D3.FcΔC1(a) & VEGFR1R2-FcΔC1(a)	VEGF/VEGFR1R2-FcdC1(a)	0.98	0.94	0.99		0.97 ± 0.02
oichiometry of hVEGF165 to Flt1D2	VEGF/FI11D2Fik1D3.FcAC1(a)	0.93	0.97			0.96 ± 0.03
Binding St	hVEGF165 (nM)	-	10	50		Average ± StDev

Fig. I

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Fig. 2

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Fig. 3

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Fig. 4

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Fig. 5

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Fig. 6

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Fig. 7A

Fig. 7B

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Fig. 8

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USE OF VEGF INHIBITORS FOR TREATMENT OF EYE DISORDERS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Ser. No. 11/089,803 filed 25 Mar. 2005, which is a continuation-in-part of application Ser. No. 10/988,243 filed 12 Nov. 2004, which is a continuation-in-part of application 10 Ser. No. 10/009,852 filed 6 Dec. 2001, now U.S. Pat. No. 7,070,959 which is the National Stage of International Application No. PCT/US00/14142 filed 23 May 2000, which claims the benefit under 35 USC § 119(e) of U.S. Provisional 60/138,133 filed 8 Jun. 1999, and this applica-15 tion is a continuation-in-part of application Ser. No. 10/880, 021 filed 29 Jun. 2004, now U.S. Pat. No. 7,279,159 which is a continuation-in-part of application Ser. No. 10/609,775 filed 30 Jun. 2003, now U.S. Pat. No. 7,087,411 which applications are herein specifically incorporated by refer- 20 ence in their entireties.

BACKGROUND

Statement Regarding Related Art

A class of cell-derived dimeric mitogens with selectivity for vascular endothelial cells has been identified and designated vascular endothelial cell growth factor (VEGF). VEGF is a dimer with an apparent molecular mass of about 46 kDa with each subunit having an apparent molecular mass of about 23 kDa. The membrane-bound tyrosine kinase receptor, known as Ft (also known as VEGFR2), was shown to be a VEGF receptor (DeVries et al. (1992) Science 255:989-991). Another form of the VEGF receptor, desig- 35 nated KDR or Flk-1 (also known as VEGFR3), is also known to bind VEGF and induce mitogenesis (Terman et al. (1991) Oncogene 6:1677-1683; Terman et al. (1992) Biochem. Biophys. Res. Comm. 187:1579-1586).

U.S. Pat. No. 6,011,003 describes an altered, soluble form 40 of Flt polypeptide capable of binding to VEGF comprising five or fewer complete immunoglobulin domains. WO 97/44453 describes chimeric VEGF receptor proteins comprising amino acid sequences derived from VEGF receptors Flt1 and KDR.

BRIEF SUMMARY OF THE INVENTION

The invention features a therapeutic method for treating or ameliorating an eye disorder, comprising administering a 50 vascular endothelial growth factor (VEGF) inhibitor to a patient in need thereof. In one embodiment, the eve disorder treated is age related macular degeneration. In another embodiment, the eye disorder treated is diabetic retinopathy.

Preferably, the VEGF inhibitor used in the method of the 55 invention comprises an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor and Ig domain 3 of a second VEGF receptor; and a multimerizing component, wherein the first VEGF receptor is Flt1, the second VEGF receptor is Flk1 or Flt4, and the multimerizing component is selected from the 60 group consisting of (i) an amino acid sequence between 1 to about 200 amino acids in length having at least one cysteine residue, and (ii) an immunoglobulin domain, or fragment of an immunoglobulin domain. In specific embodiments, the VEGF inhibitor is a fusion polypeptide "VEGF trap" 65 measured using Image-Pro Plus software. selected from the group consisting of SEQ ID NO:2 $(Flt1D2.Flk1D3Fc\Delta C1(a)),$ SEQ ID NO:4

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(Flt1D2.VEGFR3D3.Fc Δ C1(a)), SEQ ID NO:6 (VEGFR1R2 Fc∆C1(a)), and SEQ ID NO:23. In another embodiment, the VEGF inhibitor is a fusion polypeptide encoded by a nucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 22, and a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of SEQ ID NO:1, 3, 5, and 22.

In a second aspect, the invention features a method for the treatment of a human subject diagnosed with an eye disorder, comprising administering an effective amount of a vascular endothelial growth factor (VEGF) inhibitor to the human subject, the method comprising administering to the subject an initial dose of at least approximately 25-4000 micrograms VEGF inhibitor protein to an affected eye, and administering to the subject a plurality of subsequent doses of the VEGF inhibitor protein in an amount that is approximately the same or less than the initial dose, wherein the subsequent doses are separated in time from each other by at least two weeks. The eye disorder is one of age-related macular degeneration or diabetic retinopathy. In various embodiments, the initial dose is at least approximately 25 to 4000 micrograms of VEGF inhibitor protein. In various embodiments, the subsequent doses are separated in time 25 from each other by at least two weeks to 12 months; more preferably, the subsequent doses are separated in time from each other by at least 3-6 months. The VEGF inhibitor protein is administered directly to the affected eye, including by use of eye drops or intravitreal injection. Preferably, the VEGF inhibitor is a dimer having two fusion polypeptides consisting essentially of an immunoglobulin-like (Ig) domain 2 of Flt1 and Ig domain 3 of Flk1 or Flt4, and a multimerizing component. In specific embodiments, the VEGF inhibitor is a dimer comprising the fusion polypeptide of SEQ ID NO:2, 4, 6, or 23.

Other objects and advantages will become apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Biacore analysis of binding stoichiometry. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.Fc∆C1(a) or VEGFR1R2-Fc Δ C1(a), using the conversion factor of 1000 45 RU equivalent to 1 ng/ml.

FIG. 2. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a).

FIG. 3. Pharmacokinetics of Flt1(1 -3)-Fc (A40), Flt1D2. Flk1D3. FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a).

FIG. 4. VEGFR1R2-FcΔC1(a) prevents neovascularization induced by retinal ischemia. Serial 10 um cross sections were collected and stained with hematoxylin and eosin. For each animal, nuclei in preretinal neovessels were counted in a series of ten sections within 300 microns of the optic nerve head and averaged. Counts were obtained in three independent experiments, $n \ge 4$ for each treatment group in each study

FIG. 5. Effect of subcutaneous VEGFR1 R2-FcΔC1(a) injections on choroidal neovascularization area. The size of CNV lesions was measured in choroidal flat mounts. The images were digitized using an Axioskop microscope equipped with a video camera, and the total area of choroidal neovascularization associated with each laser burn was

FIG. 6. VEGFR1R2-FcΔC1(a) inhibits subretinal neovascularization in Rho/VEGF transgenic mice.

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FIG. 7A-B. VEGF-Induced breakdown of the blood retinal barrier. A. Following intravitreal injections of VEGF, adult mice (C57BL/6) treated with injections of VEGFR1R2-Fc Δ C1(a) had a significantly smaller retina to lung leakage ratio than mice treated with Fc fragment, s indicating less breakdown of BRB. B. Double transgenic mice treated with injections of VEGFR1R2-Fc Δ C1(a) had a significant reduction in the retina to lung leakage ratio compared to mice treated with Fc fragment.

FIG. 8. Effect of VEGFR1R2-Fc Δ C1(a) administration on 10 corneal thickness in suture and alkali burn models of corneal trauma. Corneas were injured by suture placement or application of NaOH as described, and a single dose of VEGFR1R2-Fc Δ C1(a) (25 mg/kg, ip) or saline (n=5 per group) was administered immediately following injury. The 15 contralateral cornea served as normal, undamaged controls. Corneas were collected 7 days later and cross-sections were cut and stained with hematoxylin and eosin. Corneal thickness was measured as an index of corneal edema.

FIG. **9**. System or intravitreal VEGF trap protein admin-²⁰ istration prevents laser-induced choroidal neovascularization (CNV) and reverses vascular leak in established lesions. FIG. **10**. Dose response curve of Baf/Flt cells grown in

VEGF. FIG. 11. Inhibition of VEGF growth response by VEGF ²⁵

trap VEGFR1R2-Fc Δ C1(a) or anti-VEGF antibody.

DETAILED DESCRIPTION OF THE INVENTION

It has been a longstanding problem in the art to produce a receptor-based VEGF antagonist that has a pharmacokinetic profile that is appropriate for consideration of the antagonist as a therapeutic candidate. Applicants describe herein, for the first time, a chimeric polypeptide molecule, ³⁵ capable of antagonizing VEGF activity, that exhibits improved pharmacokinetic properties as compared to other known receptor-based VEGF antagonists. The chimeric polypeptide molecules described herein thus provide appropriate molecules for use in therapies in which antagonism of ⁴⁰ VEGF is a desired result.

The extracellular ligand binding domain is defined as the portion of a receptor that, in its native conformation in the cell membrane, is oriented extracellularly where it can 45 contact with its cognate ligand. The extracellular ligand binding domain does not include the hydrophobic amino acids associated with the receptor's transmembrane domain or any amino acids associated with the receptor's intracellular domain. Generally, the intracellular or cytoplasmic domain of a receptor is usually composed of positively charged or polar amino acids (i.e. lysine, arginine, histidine, glutamic acid, aspartic acid). The preceding 15-30, predominantly hydrophobic or apolar amino acids (i.e. leucine, valine, isoleucine, and phenylalanine) comprise the transmembrane domain. The extracellular domain comprises the 55 amino acids that precede the hydrophobic transmembrane stretch of amino acids. Usually the transmembrane domain is flanked by positively charged or polar amino acids such as lysine or arginine. von Heijne has published detailed rules that are commonly referred to by skilled artisans when determining which amino acids of a given receptor belong to the extracellular, transmembrane, or intracellular domains (See, von Heijne (1995) BioEssays 17:25

Nucleic Acid Constructs and Encoded Fusion Polypeptides 65 The present invention provides for the construction of nucleic acid molecules encoding chimeric polypeptide mol4

ecules that are inserted into a vector that is able to express the chimeric polypeptide molecules when introduced into an appropriate host cell. Appropriate host cells include, but are not limited to, bacterial cells, yeast cells, insect cells, and mammalian cells. Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the chimeric polypeptide molecules under control of transcriptional/translational control signals. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (See Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NYD.

Expression of nucleic acid molecules encoding the chimeric polypeptide molecules may be regulated by a second nucleic acid sequence so that the chimeric polypeptide molecule is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the chimeric polypeptide molecules described herein may be controlled by any promoter/enhancer element known in the art.

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising chimeric polypeptide molecule-encoding nucleic acids as described herein, are used to transfect the host and thereby direct expression of such nucleic acids to produce the chimeric polypeptide molecules, which may then be recovered in a biologically active form. As used herein, a biologically active form includes a form capable of binding to VEGF. Expression vectors containing the chimeric nucleic acid molecules described herein can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to the inserted chimeric polypeptide molecule sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the chimeric polypeptide molecule DNA sequence is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the chimeric polypeptide molecules.

Cells of the present invention may transiently or, preferably, constitutively and permanently express the chimeric polypeptide molecules.

The chimeric polypeptide molecules may be purified by any technique which allows for the subsequent formation of a stable, biologically active chimeric polypeptide molecule. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis (see, for example, U.S. Pat. No. 5,663,304). In order to further purify the factors, conventional ion exchange chro-

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matography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

The method of the invention encompasses the use of a fusion protein consisting essentially of first and second vascular endothelial growth factor (VEGF) receptor components and a multimerizing component, wherein the first VEGF receptor component is an immunoglobulin-like (Ig) domain 2 of Flt1, the second VEGF receptor component is an Ig domain 3 of a Flk1 or Flt4, and the multimerizing component is selected from the group consisting of (i) a 10 multimerizing component comprising a cleavable region (C-region), (ii) a truncated multimerizing component, (iii) an amino acid sequence between 1 to about 200 amino acids in length having at least one cysteine residue, (iv) a leucine zipper, (v) a helix loop motif, (vi) a coil-coil motif, and (vii) 15 an immunoglobulin domain. Examples of the VEGF inhibitors useful in the method of the invention include fusion proteins encoded by a nucleotide sequence selected from the group consisting of the nucleotide sequence of SEQ ID NO:1, 3, 5, 22, and a nucleotide sequence which, as a result 20 of the degeneracy of the genetic code, differs from the nucleotide sequence of SEQ ID NO:1, 3, 5, or 22, and fusion protein selected from the group consisting of SEQ ID NO:2 (Flt1D2.Flk1D3Fc Δ C1(a)), SEO ID NO:4 (Flt1D2.VEGFR3D3.FcΔC1(a)), SEQ (VEGFR1R2Fc Δ C1(a)) and (SEQ ID NO:23).

Therapeutic Methods

The present invention also has diagnostic and therapeutic utilities. In particular embodiments of the invention, methods of detecting aberrancies in the function or expression of the chimeric polypeptide molecules described herein may be used in the diagnosis of disorders. In other embodiments, manipulation of the chimeric polypeptide molecules or agonists or antagonists which bind the chimeric polypeptide molecules may be used in the treatment of diseases. In further embodiments, the chimeric polypeptide molecule is utilized as an agent to block the binding of a binding agent to its target.

By way of example, but not limitation, the method of the invention may be useful in treating clinical conditions that are characterized by vascular permeability, edema or inflammation such as brain edema associated with injury, stroke or tumor; edema associated with inflammatory disorders such as psoriasis or arthritis, including rheumatoid arthritis; asthma; generalized edema associated with burns; ascites and pleural effusion associated with tumors, inflammation or trauma; chronic airway inflammation; capillary leak syndrome; sepsis; kidney disease associated with increased leakage of protein; and eye disorders such as age related have diabetic retinopathy.

Combination Therapies

In numerous embodiments, a VEGF inhibitor may be administered in combination with one or more additional compounds or therapies, including a second VEGF inhibitor. 55 Combination therapy includes administration of a single pharmaceutical dosage formulation which contains a VEGF inhibitor molecule and one or more additional agents; as well as administration of a VEGF inhibitor and one or more additional agent(s) in its own separate pharmaceutical dosage formulation. For example, a VEGF inhibitor and a cytotoxic agent, a chemotherapeutic agent or a growth inhibitory agent can be administered to the patient together in a single dosage composition such as a combined formulation, or each agent can be administered in a separate 65 dosage formulation. Where separate dosage formulations are used, the VEGF-specific fusion protein of the invention and

one or more additional agents can be administered concurrently, or at separately staggered times, i.e., sequentially. The therapeutic methods of the invention may also be combined with other agents or medical procedures used for treatment of eye disorders.

Treatment Population

The eye comprises several structurally and functionally distinct vascular beds, which supply ocular components critical to the maintenance of vision. These include the retinal and choroidal vasculatures, which supply the inner and outer portions of the retina, respectively, and the limbal vasculature located at the periphery of the cornea. Injuries and diseases that impair the normal structure or function of these vascular beds are among the leading causes of visual impairment and blindness. For example, diabetic retinopathy is the most common disease affecting the retinal vasculature, and is the leading cause of vision loss among the working age population in the United States. Vascularization of the cornea secondary to injury or disease is yet another category of ocular vascular disease that can lead to severe impairment of vision.

"Macular degeneration" is a medical term that applies to ID 25 any of several disease syndromes which involve a gradual loss or impairment of eyesight due to cell and tissue degeneration of the yellow macular region in the center of the retina. Macular degeneration is often characterized as one of two types, non-exudative (dry form) or exudative (wet form). Although both types are bilateral and progressive, each type may reflect different pathological processes. The wet form of age-related macular degeneration (AMD) is the most common form of choroidal neovascularization and a leading cause of blindness in the elderly. AMD affects millions of Americans over the age of 60, and is the leading cause of new blindness among the elderly. It is characterized and usually diagnosed by the presence of elevated levels of two types of cellular debris within the retina, called drusen and lipofuscin.

> There are several types of symptomatic treatment, however, that have been used with limited and isolated success. depending on the particular condition of the patient, to treat exudative (wet form) macular degeneration. Laser photocoagulation therapy may benefit certain patients with macular degeneration. However, there are high recurrence rates for selected choroidal neovascular membranes which may initially respond to laser therapy. Vision loss may also result from the laser therapy. Low dose radiation (teletherapy) has also been hypothesized as a possible treatment to induce regression of choroidal neovascularization. Surgical removal of neovascular membranes is another possible treatment, but it is a highly specialized procedure and reportedly has not had promising results to date. There is presently no effective treatment for non-exudative (dry form) macular degeneration. Management of non-exudative macular degeneration is limited to early diagnosis and careful follow-up to determine if the patient develops choroidal neovascularization. Protection against exposure to ultraviolet light and prescribed dosages of anti-oxidant vitamins (e.g., vitamin A, \beta-carotene, lutein, zeaxanthin, vitamin C and vitamin E) and zinc may also be of some benefit, but as yet these treatments remain unproven.

> Accordingly, the population to be treated by the method of the invention is preferably one of (i) a human subject diagnosed as suffering from macular degeneration, (ii) a human subject diagnosed as suffering from diabetes-related

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retinopathy, and (iii) a human subject suffering from pathological vascularization of the cornea secondary to injury or disease.

Methods of Administration and Compositions

Preferably, administration of the VEGF inhibitor will be directly to the eye, e.g., topical. Topical methods of administration include, for example, by eye drops, subconjunctival injections or implants, intravitreal injections or implants, 10 sub-Tenon's injections or implants, incorporation in surgical irrigating solutions, etc.

Compositions suitable for topical administration are known to the art (see, for example, US Patent Application 2005/0059639). In various embodiments, compositions of $^{\ 15}$ the invention can comprise a liquid comprising an active agent in solution, in suspension, or both. As used herein, liquid compositions include gels. Preferably the liquid composition is aqueous. Alternatively, the composition can take $_{20}$ form of an ointment. In a preferred embodiment, the composition is an in situ gellable aqueous composition, more preferably an in situ gellable aqueous solution. Such a composition can comprise a gelling agent in a concentration effective to promote gelling upon contact with the eye or $\ ^{25}$ lacrimal fluid in the exterior of the eye. Aqueous compositions of the invention have ophthalmically compatible pH and osmolality. The composition can comprise an ophthalmic depot formulation comprising an active agent for subconjunctival administration. The microparticles comprising active agent can be embedded in a biocompatible pharmaceutically acceptable polymer or a lipid encapsulating agent. The depot formulations may be adapted to release all or substantially all the active material over an extended 35 period of time. The polymer or lipid matrix, if present, may be adapted to degrade sufficiently to be transported from the site of administration after release of all or substantially all the active agent. The depot formulation can be a liquid 40 formulation, comprising a pharmaceutical acceptable polymer and a dissolved or dispersed active agent, Upon injection, the polymer forms a depot at the injections site, e.g. by gelifying or precipitating. The composition can comprise a solid article that can be inserted in a suitable location in the 45 eye, such as between the eye and eyelid or in the conjuctival sac, where the article releases the active agent. Solid articles suitable for implantation in the eye in such fashion generally comprise polymers and can be bioerodible or non-bioerod-50 ible.

In one embodiment of the method of the invention, a human subject with at least one visually impaired eye is treated with 25-4000 micrograms of a VEGF inhibitor protein via intravitreal injection. Improvement of clinical ⁵⁵ symptoms are monitored by one or more methods known to the art, for example, indirect ophthalmoscopy, fundus photography, fluorescein angiopathy, electroretinography, external eye examination, slit lamp biomicroscopy, applanation tonometry, pachymetry, and autorefaction. Subsequent doses may be administered weekly or monthly, e.g., with a frequency of 2-8 weeks or 1-12 months apart.

Other features of the invention will become apparent in the course of the following descriptions of exemplary 65 embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

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EXAMPLES

Example 1

Modified Flt1 Receptor Vector Construction

Chimeric molecules were constructed, denoted R1R2 (Flt1.D2.Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a) and R1R3 (Flt1D2.VEGFR3D3-Fc Δ C1(a) and VEGFR1R3respectively, wherein R1 and Flt1D2=Ig domain 2 of Flt1 (VEGFR1); R2 and Flk1D3=Ig of Flk1 (VEGFR2); and R3 and VEGFR3D3=Ig domain 3 of Flt4 (VEGFR3)) were much less sticky to ECM, as judged by an in vitro ECM binding assay and had greatly improved PK as described herein. In addition, these molecules were able to bind VEGF tightly and block phosphorylation of the native Flk1 receptor expressed in endothelial cells.

- Construction of the expression plasmid pFlt1D2.Flk1D3.Fc Δ C1(a). Expression plasmids pMT21.Flt1(1-3).Fc (6519 bp) and pMT21.Flk-1(1-3).Fc (5230 bp) are plasmids that en ampicillin resistance and Fc-tagged versions of Ig domains 1-3 of human Flt1 and human Flk1, respectively. These plasmids were used to construct a DNA fragment consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 3 of Flk1, using PCR amplification of the respective Ig domains followed by further rounds of PCR to achieve fusion of the two domains into a single fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows: 5': bsp/flt1D2 (5'-GACTAGCAGTCCGG-AGGTAGACCTTTCGTA-
- GAGATG-3') (SEQ ID NO:8), 3': Flt1D2-Flk1D3.as (5'-CGGACTCAGMCCACATCTATGATTGTATTGGT-3') (SEQ ID NO:9). The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of 5 Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO:10) corresponding to amino acids 27-33 of SEQ ID NO:2. The 3' primer encodes the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID 0 of Flt1 (corresponding to amino acids 123-126 of SEQ ID
- NO:2) and continuing into VVLS (SEQ ID NO:7) (corresponding to amino acids 127-130 of SEQ ID NO:7) (corresponding to amino acids 127-130 of SEQ ID NO:2) of Flk1.

For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as follows:5': Flt1D2-Flk1D3.s (5'-ACMT-CATAGATGTGGTTCTGAGTCCGTCTCATGG-3') (SEQ ID NO: 11); 3': Flk1D3/apa/srf.as (5'-GATAATGC-CCGGGCCCTTTTCATGGACCCTGACAAATG-3') (SEQ ID NO:12). The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (SEQ ID NO:13) (corresponding to amino acids 223-228 of SEQ ID NO:2), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of SEO ID NO:2.

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described supra) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEl and Smal and the resulting 614 bp fragment was subcloned into the BspEl to Srfl restriction sites of the vector pMT21/ΔB2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was

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verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoR1 and Srfl and the resulting 702 bp fragment was transferred into the EcoR1 to Srfl restriction sites of the plasmid pFlt1(1-3)B2-Fc Δ C1(a) to produce the plasmid pFlt1D2.Flk1D3.Fc Δ C1 (a). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.Fc Δ C1(a) chimeric molecule is shown in SEQ ID NO:1-2.

Construction of the plasmid expression pFlt1D2VEGFR3D3Fc Δ C1(a). The expression plasmid 10 pMT21.Flt1(1-3).Fc (6519 bp) encodes ampicillin resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1 receptor. This plasmid was used to produce a DNA fragment containing Ig domain 2 of Flt1 by PCR. RNA from the cell line HEL921.7 was used to produce Ig domain 3 of Flk1, 15 using standard RT-PCR methodology. A further round of PCR amplification was used to achieve fusion of the two Ig domains into a single fused fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows: 5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTT- 20 CGTAGAGATG-3') NO:14), (SEQ ID 3': (TTCCTGGGCAACAGCTG-Flt1D2.VEGFR3D3.as GATA-TCTATGATTGTATTGGT) (SEQ ID NO:15). The 5' amplification primer encodes a BspE1 restriction site upstream of Ig domain 2 of Flt1, defined by the amino acid 25 sequence GRPFVEM (SEQ ID NO:10) (corresponding to amino acids 27-33 of SEQ ID NO:1-2). The 3' amplification primer encodes the reverse complement of the end of Flt1 Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of SEQ ID NO:4) and continuing into IQLL of VEGFR3 (corresponding to amino acids 127-130 of SEQ ID NO:4).

For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as follows: 5': R3D3.s (ATCCAGCTGT- 35 TGCCCAGGAAGTCGCTGGAGCTGCTGGTA) (SEQ ID NO:17), 3': R3D3.as (ATTTTCATGCACAATGACCTCG-GTGCTCTCCCGAAATCG) (SEQ ID NO:18). Both the 5' and 3' amplification primers match the sequence of VEGFR3. The 296 bp amplification product of this RT-PCR 40 reaction was isolated by standard techniques and subjected to a second round of PCR to add suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). primers amplification were The as 5':Flt1D2.VEGFR3D3.s(TCATAGATATCCAGCTGTTGC-CCAGGAAGTCGCTGGAG) (SEQ ID NO: 19), 3': VEG FR3D3/srf.as (GATAATGCCCGGGCCATTTTCATGCA-CAATGACCTCGGT) (SEQ ID NO:20). The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused 50 directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (SEQ ID NO:21) (corresponding to amino acids 221-226 of SEQ ID NO:4), followed by a bridging sequence 55 that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of SEQ ID NO:4.

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig 60 domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described supra, to produce the fusion product. This PCR product was subsequently digested with the restriction 65 enzymes BspEl and Smal and the resulting 625 bp fragment was subcloned into the BspEl to Srfl restriction sites of the

vector pMT21/Flt1AB2.Fc (described supra), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc. The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRl and Srfl and the resulting 693 bp fragment was subcloned into the EcoRl to Srfl restriction sites of the plasmid pFlt1(1-3) Δ B2-Fc Δ C1(a) to produce the plasmid designated pFlt1D2.VEGFR3D3.Fc∆C1(a). The complete DNA deduced acid amino sequence of the Flt1D2.VEGFR3D3.Fc Δ C1(a) chimeric molecule is shown in SEQ ID NO:3-4.

Example 2

Construction pVEGFR1R2-Fc∆C1(a) Expression Vector

The pVEGFR1R2.Fc Δ C1(a) (SEQ ID NO:15-16) expression plasmid was constructed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of SEQ ID NO:6) between Flt1d2-Flk1d3-Fc Δ C1(a) amino acids 26 and 27 of SEQ ID NO:2 (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231. The SDT amino acid sequence is native to the Flt1 receptor and was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2.Fc Δ C1 (a) chimeric molecule is shown in SEQ ID NO:5-6.

Example 3

Cell Culture Process Used to Produce Modified Flt1 Receptors

Cell Expansion. Two confluent T-225 cm² flasks containing the Flt1D2.Flk1D3.Fc Δ C1(a) expressing cell line were expanded by passaging cells into eight T-225 cm² flasks in medium (GMEM+10% serum, GIBCO) and incubated at 37° C. and 5% CO₂. When the flasks approached confluence (approximately 3 to 4 days) the cells were detached using trypsin. Fresh medium was added to protect the cells from further exposure to the trypsin. The cells were centrifuged and resuspended in fresh medium then transferred to eight 850 cm² roller bottles and incubated at 37° C. and 5% CO₂ until confluent.

Suspension Culture in Bioreactors. Cells grown in roller bottles were trypsinized to detach them from the surface and washed with suspension culture medium. The cells are aseptically transferred to a 5 L bioreactor (New Brunswick Celligen Plus) where the cells are grown in 3.5 L of suspension culture. The suspension culture medium was a glutamine-free low glucose modification of IS-CHO (Irvine Scientific) to which 5% fetal bovine serum (Hyclone), GS supplement (Life Technologies) and 25 μ M methionine sulfoximine (Sigma) was added. The pH was controlled at 7.2 by addition of carbon dioxide to the inlet gas or by

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addition of a liquid solution of sodium carbonate to the bioreactor. Dissolved oxygen level was maintained at 30% of saturation by addition of oxygen or nitrogen to the inlet gas and temperature controlled at 37° C. When a density of 4×10^6 cells/mL was reached the cells were transferred to a 540 L bioreactor containing the same medium and setpoints for controlling the bioreactor. The temperature setpoint was reduced to 34° C. to slow cell growth and increase the relative rate of protein expression.

Cell Culture Process Used to Produce Flt1D2.VEGFR3D3.Fc Δ C1(a). The same methodologies as described supra for Flt1D2.Flk1D3.Fc Δ C1(a) were used to produce Flt1D2.VEGFR3D3.Fc Δ C1(a).

Example 4

Harvest and Purification of Modified Flt1 Receptors

Harvest and Purification of Flt1D2.Flk1D3.Fc Δ C1(a). The product protein was aseptically harvested from the ²⁰ bioreactor while retaining cells using Millipore Prostak tangential-flow filtration modules and a low-shear mechanical pump (Fristam). Fresh medium was added to the bioreactor to replace that removed during the harvest filtration. Approximately 40 L of harvest filtrate was then loaded onto ²⁵ a 400 mL column containing Protein A Sepharose resin (Amersham Pharmacia). After loading the resin was washed with buffer containing 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2 to remove any unbound contaminating proteins. Flt1D2.Flk1D3.Fc Δ C1(a) protein was a luted with a pH 3.0 citrate buffer. The eluted protein was neutralized by addition of Tris base and frozen at -20° C.

Several frozen lots of Flt1D2.Flk1D3.Fc Δ C1(a) protein from the Protein A step above were thawed, pooled and concentrated using a Millipore 30 kD nominal molecular 35 weight cutoff (NMWCO) tangential flow filtration membrane. The protein was transferred to a stirred cell concentrator (Millipore) and further concentrated to 30 mg/mL using a 30 kD NMWCO membrane. The concentrated protein was loaded onto a size exclusion column packed 40 with Superdex 200 resin (Amersham Pharmacia) that was equilibrated with phosphate buffered saline plus 5% glycerol. The same buffer was used to run the column. The fractions corresponding to Flt1D2.Flk1D3.Fc Δ C1(a) dimer were pooled, sterile filtered through a 0.22 micron filter, 45 aliquoted and frozen.

Harvest and Purification of Flt1D2.VEGFR3D3.Fc Δ C1 (a). The same methodologies as described supra for Flt1D2.Flk1D3.Fc Δ C1(a) were used to harvest and purify Flt1D2.VEGFR3D3.Fc Δ C1(a).

Example 5

Binding Stoichiometry of Modified Fit Receptors to VEGF165

Biacore Analysis. The stoichiometry of Flt1D2Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a) interaction with human VEGF165 was determined by measuring either the level of VEGF saturation binding to the 60 Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a) surfaces or measuring concentration of VEGF165 needed to completely prevent binding of Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a) to VEGF Biacore chip surface.

Modified Flt receptors Flt1D2Flk1D3.Fc Δ C1(a) and 65 VEGFR1R2-Fc Δ C1(a), were captured with an anti-Fc specific antibody that was first immobilized on a Biacore chip

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te to the (BIACORE) using amine-coupling chemistry. A blank antibody surface was used as a negative control. VEGF165 was injected at a concentration of 1 nM, 10 nM, and 50 nM over the Flt1D2Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a) surfaces at 10 µl/min for one hour. A real-time binding signal setpoint was recorded and saturation binding was achieved at the end of each injection. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a), using Produce 10 the conversion factor of 1000 RU equivalent to 1 ng/ml. The logies as eused to vertice was reached binding stoichiometry of one VEGF1C3 for the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF1C3 or VEGFR1R2-Fc Δ C1(a) molecule (FIG. 1).

In solution, Flt1D2Flk1D3.FcAC1(a) or VEGFR1R2-15 Fc Δ C1(a) at a concentration of 1 nM (estimated to be 1000 times higher than the KD of the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a)/VEGF165 interaction) were mixed with varied concentrations of VEGF165. After one hour incubation, concentrations of the free Flt1D2Flk1D3.Fc Δ C1 (a) in solution were measured as a binding signal to an amine-coupled VEGF165 surface. A calibration curve was used to convert the Flt1D2Flk1D3.Fc\DeltaC1(a) Biacore binding signal to its molar concentration. The data showed that addition of 1 nM VEGF165 into the the Flt1D2Flk1D3.Fc\DeltaC1(a) solution completely blocked Flt1D2Flk1D3.Fc Δ C1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.FcΔC1(a) molecule. When the concentration of Flt1D2Flk1D3.Fc Δ C1(a) was plotted as a function of added concentration of VEGF165, the slope of the linear portion was -1.06 for Flt1D2Flk1D3.Fc Δ C1(a) and -1.07 VEGFR1R2-Fc Δ C1(a). The magnitude of the slope, very close to negative one, was indicative that one molecule of VEGF165 bound to one molecule of either Flt1D2Flk1D3.Fc∆C1(a) or VEGFR1R2- $Fc\Delta C1(a)$.

Size Exclusion Chromatography. Flt1D2Flk1D3.Fc∆C1 (a) was mixed with a 3-fold excess of VEGF165 and the receptor-ligand complex was purified using a Pharmacia Superose 6 size exclusion chromatography column. The receptor-ligand complex was then incubated in a buffer containing 6 M guanidine hydrochloride in order to dissociate it into its component proteins. Flt1D2Flk1D3.Fc∆C1 (a) was separated from VEGF165 using Superose 6 size exclusion chromatography column run in 6 M guanidium chloride. In order to determine complex stoichiometry, several injections of Flt1D2Flk1D3.Fc∆C1(a) and VEGF165 were made and peak height or peak integrated intensity was plotted as a function of the concentration of injected protein. The calibration was done under conditions identical to those 50 used in separating components of Flt1D2Flk1D3.Fc\DeltaC1(a)/ VEGF complex. Quantification of the Flt1D2Flk1D3.FcΔC1 (a)/VEGF complex composition was based on the calibration curves. The results of this experiment (FIG. 1) shows 55 the ratio of VEGF165 to Flt1D2Flk1D3.FcΔC1(a) in a complex to be 1:1.

Example 6

Pharmacokinetic Analysis of Modified Fit Receptors

Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a). Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.Fc Δ C1(a), CHO stably expressed

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Flt1D2.Flk1D3.Fc\DeltaC1(a), and CHO transiently expressed VEGFR1R2-Fc Δ C1(a). The mice were tail bled at 1, 2, 4, 6, 24 hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a). The ELISA involves coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. The results of this experiments are shown in 10 FIG. 2. The T_{max} for Flt1(1-3)-Fc (A40) was at 6 hrs while the T_{max} for the transient and stable Flt1D2.Flk1D3.Fc Δ C1 (a) and the transient VEGFR1R2-Fc Δ C1(a) was 24 hrs. The , for Flt1(1-3)-Fc (A40) was 8 µg/ml. For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the 15 C_{max} was 18 µg/ml and the C_{max} for the stable VEGFR1R2-Fc Δ C1(a) was 30 µg/ml.

Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VÈGFR3D3.FcΔC1 (a). Balb/c mice (25-30 g) were injected subcutaneously 20 with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.Fc\DeltaC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.Fc Δ C1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect 25 Flt1D2.Flk1D3.FC Δ C1(a) Flt1(1-3)-Fc, and Flt1D2.VEGFR3D3.FCΔC1(a). The ELISA involves coating an ELISA plate with 165, binding the Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) or Flt1D2.VEGFR3D3.FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5, whereas, Flt1D2.Flk1D3.Fc Δ C1(a) and Flt1D2.VEGFR3D3.Fc\DeltaC1(a) were detectable for 15 days or more. The results of this experiment are shown in FIG. 3. 35

Example 7

Breakdown of Blood-retinal Barrier Reversed by Inhibition of VEGF

Rats received a single injection of VEGFR1R2-Fc Δ C1(a) (SEQ ID NO:6) (25 mg/kg, i.p.) or PBS 4 weeks after induction of diabetes by streptozotocin (65 mg/kg, i.v.). The permeability of retinal vessels was assessed 24 hours later 45 by measuring the extravasation of Evans Blue dye, which binds to albumin in the circulation. Under deep anesthesia, Evans Blue dye (45 mg/kg) was injected intravenously, and allowed to circulate for 60 minutes, and blood samples were taken periodically to assess the concentration of dye in the 50 circulation. The animals were then perfused to flush dye and blood from the vasculature, the eve enucleated and the retinas removed. Evans blue was extracted, and the concentration of dye in the retina was normalized to retinal weight and the time-averaged concentration of Evans blue in the 55 circulation (L plasma×g retina weight⁻¹×hr⁻¹) to provide an index of vascular leak. VEGFR1R2-Fc\DeltaC1(a) normalized retinal vascular permeability to levels evident in non-diabetic rats.

Example 8

VEGFR1R2-Fc∆C1(a) Prevents Neovascularization Induced by Retinal Ischemia

Excessive upregulation of VEGF expression is responsible for pathologic neovascularization in many retinal dis14

eases. The anti-angiogenic properties of VEGFR1R2-Fc\DeltaC1 (a) were investigated in a mouse model of oxygen-induced ischemic retinopathy (OIR). OIR was produced by transiently exposing mouse pups to increased ambient oxygen (hyperoxia), resulting in obliteration of the developing microvasculature within the central retina. Subsequent return of the animals to room air results in relatively hypoxic conditions in the retina, which in turn stimulates an angiogenic response that shares features with both diabetic retinopathy, retinopathy of prematurity and other ischemic retinopathies. VEGFR1R2-Fc\DeltaC1(a) (25 mg/kg, ip) was administered every other day beginning 12-24 hours after returning the mice from hyperoxia to room air. Littermate controls received injections of human Fc following the same schedule. Retinas were collected 1 week following return to room air. Flat mounts were prepared from one retina obtained from each animal, and the retinal vessels stained with fluoresceinated lectin (Griffonia simplicifolia). The other retina was embedded and cross-sections were cut and stained with hematoxylin and eosin.

Retinas of all control mice exposed to hyperoxia exhibited marked pathologic angiogenesis, characterized by the presence of vascular tufts penetrating the inner limiting membrane and chaotic sprouting of vessels on the surface of the retina, particularly around the optic nerve head. Administration of VEGFR1R2-Fc Δ C1(a) almost completely blocked the development of these vascular abnormalities as quantitated by counting endothelial cell nuclei in the abnormal pre-retinal vessels (FIG. 4). Although pathologic angiogenesis was dramatically inhibited, systemic administration of VEGFR1R2-Fc Δ C1(a) did not block the growth of normalappearing intraretinal vessels in these animals.

Example 9

Suppression of Choroidal Neovascularization

Though animals do not develop age related macular degeneration (AMD) per se, choroidal neovascularization 40 resembling that seen in AMD can be produced by using a laser to produce focal disruptions in Bruch's membrane and the overlying retinal pigment epithelium (RPE). This injury stimulates the abnormal growth of underlying choroidal capillaries into the RPE layer and subretinal space. Disruption of Bruch's membrane is common to all forms of choroidal neovascularization (CNV), including that which characterizes the wet form of AMD. In the laser-induced model of choroidal neovascularization, groups of 9 or 10 mice were treated with subcutaneous (sc) injections of 25 mg/kg of VEGFR1R2-Fc Δ C1(a) or human Fc one day prior to laser injury and on days 2, 5, 8, and 11 after laser. At 14 days after laser injury, the mice were injected intravenously with fluorescein-labeled dextran (50 mg), euthanized, and eyes were rapidly dissected for choroidal flat mounts or frozen in optimum cutting temperature embedding compound and sectioned for evaluation of the lesions. VEGFR1R2-Fc∆C1(a) administration reduced the area of CNV lesions by approximately 70% (FIG. 5).

The effect of VEGFR1R2-FcΔC1(a) on laser-induced 60 choroidal neovascularization also was evaluated in adult cynomolgus monkeys. In this experiment, VEGFR1R2-FcΔC1(a) was administered by intravenous or intravitreal injection. Each animal received nine or ten laser burns to each retina, and the development of active choroidal neovas-65 cular lesions was assessed by fluorescein angiography, once before the initiation of treatment and 15, 20 and 29 days postlaser. VEGFR1R2-FcΔC1(a) was administered intrave-

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nously once per week, beginning one week before laser injury, at a dose of 3 mg/kg or 10 mg/kg. Intravitreal injections were made once every two weeks, at a dose of 50, 250 or 500 mcg/eye beginning one week before laser, or once, two weeks following laser (500 mcg dose only), at which time active CNV lesions had already formed. Control animals received weekly intravenous or biweekly intravitreal injections of placebo, beginning one week before laser. Each of the above experimental and control groups comprised six animals, 3 males and 3 females. CNV lesions were 10 visualized by fluorescein angiography and graded. Active CNV lesions characterized bright hyperfluorescence, with late leakage beyond the borders of the laser spot (Grade 4), developed at 32% and 48% of the laser burn sites, in animals receiving intravitreal or intravenous administration of pla-15 cebo. In contrast, the development of grade 4 lesions was completely or nearly completely prevented in all groups of animals receiving intravenous or intravitreal injections of VEGFR1R2-Fc∆C1(a) (FIG. 9). Moreover a single intravitreal injection (500 mcg) of VEGFR1R2-Fc\DeltaC1(a) made 20 following the laser injury reduced the incidence of grade 4 lesions from 44% to 0% within 10 days of treatment (FIG. 9).

Example 10

Inhibition of Subretinal Neovascularization in rho/VEGF Transgenic Mice

Transgenic mice expressing a recombinant human VEGF transgene under the control of the rhodopsin promoter (Rho/VEGF) were used in these experiments. These animals begin to express VEGF in photoreceptors on about postnatal day (P) 7, which typically results in pronounced subretinal neovascularization by P21. At P7, mice were divided into 2 35 groups and treated with 25 mg/kg of VEGFR1R2-Fc Δ C1(a) (9 mice, 17 eyes) or human Fc (10 mice, 19 eyes) on P7, P10, P13, P16, and P19. On P21, the mice were anesthetized and perfused with fluorescein-labeled dextran. Retinal whole mounts from mice treated with VEGFR1R2-FcΔC1 40 (a) showed few areas of neovascularization while many new vessels were present in the subretinal space of mice that had been treated with Fc. Measurement of the total area of neovascularization per retina by image analysis showed significantly less neovascularization in VEGFR1R2-Fc $\Delta C1$ $_{45}$ (a)-treated mice, compared to those treated with Fc (FIG. 6).

Example 11

Suppression of VEGF-induced Breakdown of the Blood-Retinal Barrier

Adult C57BL/6 mice were given a sc injection of 25 mg/kg of VEGFR1R2-Fc Δ C1(a) or Fc fragment and on the following day received an intravitreous injection of 1 µl of 55 10⁻⁶ M VEGF. Six hours later, retinal vascular permeability was measured using [³H]-mannitol as a tracer. Mice treated with VEGFR1R2-Fc Δ C1(a) (9 mice, 18 eyes) had a significantly smaller retina to lung leakage ratio (the ratio of radioactivity in the retina compared to excised lung) than 60 mice treated with Fc fragment (9 mice, 18 eyes) indicating less breakdown of the blood retinal barrier (FIG. 7A).

The effect of VEGFR1R2-Fc Δ C1(a) on VEGF-mediated vascular leak was also evaluated in a second experiment, which employed double transgenic mice (rtTA/rho-TRE/ 65 VEGF). These mice are characterized by photoreceptor-specific expression of the VEGF transgene that is inducible

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by administration of doxycycline. Adult rtTA/rho-TRE/ VEGF mice were injected sc with 25 mg/kg VEGFR1R2-Fc\DeltaC1(a) (10 mice, 20 eyes) or Fc fragment (10 mice, 20 eyes). On the following day, doxycycline (2 mg/mL) was placed in their drinking water to stimulate over-expression of VEGF within the retina. Two days later, they were given a second sc injection of VEGFR1R2-Fc\DeltaC1(a) or Fc fragment and then the next day retinal vascular permeability was measured with [³H]-mannitol. Mice treated with VEGFR1R2-Fc\DeltaC1(a) exhibited a significant reduction in the retina to lung leakage ratio compared to mice treated with Fc (FIG. 7B), indicating that impairment in the bloodretinal barrier was ameliorated.

Example 12

Inhibition Injury-Induced Corneal Neovascularization

Corneal neovascularization was induced in male C57B1/6 mice by intrastromal placement of 3 nylon sutures, or by chemical injury (NaOH) and mechanical debridement of the corneal epithelium. Multiple experiments were conducted in which VEGFR1R2-Fc\DeltaC1(a) was administered intraperito-25 neally once or at multiple time points immediately before or following injury. The growth of corneal neovessels was evaluated by slit-lamp microscopy and histological evaluation. The vasculature was labeled with an endothelial cell specific fluorescein-conjugated lectin, and neovascularization was evaluated in corneal flat-mounts, as well as in cross sections using PECAM immunohistochemistry. The presence of corneal edema was evaluated, using slit lamp microscopy, and corneal thickness was measured in crosssections; increases in corneal thickness reflect the amount of edema. The numbers of polymorphonuclear leukocytes (PMN) and macrophages were determined by staining crosssections with HEMA-3 or rat anti-mouse F4/80 monoclonal antibody, respectively

Dosing regimens which employed multiple injections of
VEGFR1R2-FcΔC1(a) (25 mg/kg, ip) completely inhibited corneal neovascularization in both the suture and chemical injury models. Moreover, single injections of 25 or 12.5 mg/kg VEGFR1R2-FcΔC1(a) given immediately after suture injury effectively blocked corneal neovascularization
for at least 9 days, while injections of 6.25 and 2.5 mg/kg ameliorated but did not block corneal neovascularization. The lowest dose of VEGFR1R2-FcΔC1(a) tested (0.5 mg/kg) had no evident effect. Corneal thickness, reflecting the amount of edema present, was significantly reduced in VEGFR1R2-FcΔC1(a)-treated animals compared to vehicle-treated controls (FIG. 8). Histological analyses showed that the infiltration of neutrophils and macrophages into the damaged cornea also was dramatically reduced by VEGFR1R2-FcΔC1(a) treatment.

Example 13

Inhibition of Corneal Neovascularization and Conjunctivalization Following Alkali Burn Injury

Corneas were injured by application of NaOH and mechanical debridement of the corneal epithelium in adult, male C57Bl/6 mice. VEGFR1R2-Fc Δ C1(a) or a control protein (human Fc) was administered subcutaneously (12.5 mg/kg) on days 0 (the day of injury), 7 and 14, at which time re-epithelialization of the cornea was complete. The animals were euthanized on days 28 or 42 (14 or 28 days following

Mylan Exhibit 1016 Mylan v. Regeneron, IPR2021-00880 Page 77 Joining Petitioner: Apotex the last injection of VEGFR1R2-Fc∆C1(a) and corneas taken for histological evaluation. Tissues were processed as described above.

Treatment with VEGFR1R2-Fc∆C1(a) inhibited corneal neovascularization during the period of active treatment (as 5 determined by slit-lamp microscopy), as well as 2 and 4 weeks following treatment cessation. In eyes evaluated on day 28 (14 days after the last injection of VEGFR1R2- $Fc\Delta C1(a)$, the neovascular response to injury remained completely suppressed and conjunctivalization of the cornea 10 used to measure the effect of two different VEGF inhibitors was also inhibited as evidenced by a more normal appearing morphology of the re-epithelialized cornea and a substantial reduction in goblet cell number (~30% relative to controls). Corneal inflammation and edema also were reduced substantially. Evaluation of flat-mounted corneas taken at Day 42 showed that neovascularization was still largely suppressed, though limited, focal sprouting of vessels at the corneal margin was observed in some cases.

The data show that when administered at the time of injury, VEGFR1R2-Fc\DeltaC1(a) improves corneal healing by 20 potently inhibiting the development of corneal neovascularization, inflammation, edema and conjunctivalization of the corneal epithelium. These effects persisted for several weeks following cessation of treatment, suggesting that acute inhibition of VEGF following corneal injury may have long- 25 term benefits.

Example 14

In Vitro Assay with Baf/3 Cells Expressing a Chimeric VEGF Receptor

Materials. Cells: Baf/Flt(1-7)-EpoR, clone C1H. Media: RPMI 1640, 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml) and L-glutamine (2 mM). Growth 35 factor: IL-3 (1 ng/ml). VEGF: VEGF 121 (R&D Biosystems). Detection: WST-8, CCK-8 kit from Dojindo Molecular Technologies. Instruments and analysis: Wallac Victor II Multilabel counter. All data analysed using Graphpad Prizm software with the four parameter logistic equation. 40

To create a reproducible bioassay having a K_D close to the of the VEGF inhibitor or fusion protein "trap" of SEQ ID NO:6, a chimeric receptor containing the VEGFR1 extracellular domain fused to the cytoplasmic and transmembrane domains of human EpoR via a PGL peptide bridge was 45 constructed. EpoR is able to potently drive proliferation of the mouse pro-B cell line, Baf/3. VEGF binding to the VEGFR1 extracellular domain causing receptor dimerization and activation of EpoR signaling. Neither VEGFR1 nor VEGFR2 native sequence receptors are capable of driving 50 Baf/3 proliferation.

The receptor construct was inserted into a retroviral vector (CMV promotor-chimeric receptor-IRES-GFP) and used to infect Baf/3 cells. Cells expressing GFP (green fluorescent protein) were isolated by 2 rounds of fluores- 55 cence activated cell sorting (FACS). This pool was further sorted for expression of VEGFR1. A clonal line was subsequently isolated and used for assay development.

The derived cell line proliferates in response to VEGF₁₂₁ with an EC50 of approximately 18 pM after 3 days of 60 growth. The growth response is measured by the bioreduction of the tetrazolium salt WST-8 provided in the CCK-8 kit. The growth response induced by the addition of 70 pM VEGF₁₂₁ is blocked by the VEGF trap protein (SEQ ID NO:6) with an IC₅₀ of approximately 40 pM. The IC₅₀ in this 65 bioassay is 25 times larger than the biochemically determined Kd of 1.5 pM. FIG. 10 shows the growth response of

Baf/Flt cells grown in 0-900 pM VEGF measured by the bioreduction of a tetrazolium salt.

Example 15

Inhibition of VEGF Growth Response by Two Different VEGF Inhibitors

The in vitro Baf/Flt cell line assay described above was on the response to VEGF. Cells were incubated for 3 days in 70 pM VEGF and exposed to varying concentrations of VEGF trap (SEQ ID $\hat{N}O$:6) (0-500 pM) or an anti-VEGF antibody (AvastinTM, Genentech) (0-500 nM). The results are shown in FIG. 11. The IC_{50} for the VEGF trap was 44 pM and for the anti-VEGF antibody 1.4 nM.

Example 16

Pharmacokinetic Analysis of Intravitreal Delivery of Two VEGF Inhibitors

Ocular and systemic levels of two VEGF inhibitors were determined after a single intravitreal administration to male Pigmented New Zealand Cross Bred rabbits. At various time points following the injection, the rabbits were sacrificed and vitreous, retina, and choroid tissues were collected, as well as blood samples for plasma and serum. All samples were analyzed in order to determine tissue and circulating levels of the VEGF trap protein of SEQ ID NO:6 or a 30 truncated version termed a "mini-VEGF trap" lacking the human Fc component (SEQ ID NO:23) (described in US 2004/0014667 and US 2005/0043236, herein incorporated by reference in their entirety), as well as to determine the appropriate pharmacokinetic parameters for the proteins in ocular tissue and plasma. This information allows determination of the ability of an intravitreally administered protein to reach the desired site of action, i.e. the macula in the case of macular degeneration.

Sixty-six male Pigmented New Zealand Cross Bred rabbits (F1 cross New Zealand White and New Zealand Red) were randomly divided into 2 groups with each group consisting of 33 rabbits. The animals in Group 1 were given a single intravitreal injection of full length VEGF trap protein (SEQ ID NO:6) into each eye at a dose of 500 micrograms/eye. The rabbits in Group 2 were given a single intravitreal administration of mini-VEGF trap into each eye at a dose of 250 eye micrograms/eye. At each time point (pre-dose, 0.25, 1, 6, 24, 72, 168, 336, 504, and 672 hrs post-dose), three animals were anesthetized and blood was collected via cardiac puncture in order to obtain plasma and serum. At the time of sacrifice, both eyes were enucleated from each animal and retina, choroids, and vitreous humor were collected.

Sample Processing. Generally, vitreal samples were thawed at room temperature and transferred to individual 5 mL polypropylene tubes. An equivalent weight per volume of RIPA buffer (20 MM Tris HCl, pH 7.5, 5 mM benzamidine, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM EDTA) was added to each sample, and homogenized (Cyclone I.Q. Microprocessor, Sentry) for 2, 45 second cycles at 5,500 rpm. The samples were then incubated for 20 minutes on ice and then centrifuged for 30 minute at 5,500 rpm at 4° C. The supernatant was removed and stored at -80° C. for analysis. Retinal and choroid samples were similarly processed the samples were homogenized for 30-60 seconds at the highest

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speed setting (Ultra Tunax T8 Homogenizer with S8N-5G Disposing Element, IKA Laboratoies). The samples were transferred to individual 1.5 mL eppendorf tubes and incubated for 20 minutes on ice. They were then centrifuged for 30 minutes at 5,500 rpm, 4° C. The supernatant was removed, transferred to a new 1.5 mL eppendorf tube and stored at -80° C. for analysis.

Sample Analysis. In general, VEGF trap protein levels in the samples were measured using an enzyme-linked immunosorbent assay (ELISA) system where micro-titer plates were coated with human VEGF_{165} antigen.

Results. After a single intravitreal injection of the full length or truncated VEGF trap protein into both rabbit eyes, the protein can be detected in both ocular tissue (vitreous 15 humor, retina and choroid) and plasma for up to 672 hrs. These results demonstrated that if a compound is delivered into the vitreous humor, it can be cleared from that region and be distributed into the surrounding tissue, i.e. retina and choroid, before reaching the circulation from which it is 20 eliminated from the body. This is supported not only by the ability to detected and measure the amount of the two traps in the various tissues and plasma, but also by the time it takes for the protein to reach its Cmax in that particular tissue. For mini-VEGF trap protein, it reaches its maximal concentration in the vitreous humor 1 hr after injection. The protein then passes into the retina where the Cmax occurs 6.00 hr after the initial injection. The choroid, which is adjacent to the retina, is with a Tmax of 24.0 hr, after which the protein can reach the circulation and achieve peak levels 72.0 hr after the injections. The full length VEGF trap also displayed a similar tissue progression, although the time frame for reaching the maximal concentrations was longer, in most cases, than that observed for mini-VEGF trap. Peak vitreous humor concentrations of VEGF trap were reached 6 hr after injection; retina followed with a Tmax at 24.0 hr. Choroid tissue had a Tmax of 15 min (0.250 hr), however, this result appears to be driven by a particular sample having an extremely high level of the protein at that time. As 40 observed with the mini-VEGF trap, peak plasma concentrations were reached 72.0 hr after the injections. Since animals injected with mini-VEGF trap received a dose that was half that of the full length protein (250 g/eye vs. 500 g/eye, respectively), the Cmax and AUC values in tissue and 45 plasma tended to be less than that observed for VEGF trap. In the vitreous humor, the Cmax for the mini-VEGF trap was almost half that of the full length protein, 253 g/mL vs 491 g/mL. In addition, the AUC for the mini-VEGF trap was half that of VEGF trap; there was no apparent difference between 50 the proteins in terms of t1/2 (115 hr vs. 112 hr). In choroid tissue obtained from rabbits which received mini VEGF trap, both the Cmax and AUC values were substantially lower (values were a third (AUC) to an eighth (Cmax) lower) than that observed in samples from VEGF trap 55 treated animals. This difference, especially with regards to AUC, could be accounted for by the decreased elimination t1/2 in the mini VEGF trap samples. The larger protein had a t1/2 of 131 hr while the t1/2 of the smaller protein was 70.9 hr. This same scenario was observed with regards to the 60 plasma samples. The full length VEGF trap samples had a greater Cmax, AUC and t1/2 than samples obtained from the smaller protein. In contrast to these other tissues, in retinal homogenates, both VEGF trap and mini VEGF trap had similar pharmacokinetic profiles. Despite receiving signifi- 65 cantly different intravitreal doses, retinal homogenates had Cmax and AUC measurements that were nearly identical.

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The elimination half-life was shorter, however, in retinal tissue obtained from mini1VEGF trap injected rabbits (132 hr vs. 114 hr).

The results of this study demonstrate that both full-length VEGF trap and mini-VEGF trap can be injected intravitreally and that the proteins penetrate to the desired site of action, i.e. retina or related structure. The results show that the protein is present in the eye tissue for up to 672 hrs, thus allowing for monthly treatment paradigms. Further, once the mini-VEGF trap moves out of the eye tissue into the systemic circulation, it is cleared more quickly from the body than the full-length VEGF trap, thus reducing unwanted systemic action.

Example 17

Treatment of Age-Related Macular Degeneration

A patient manifesting age-related macular degeneration is treated with an intravitreal injection of the VEGF trap protein of SEQ ID NO:6 or 23. The purpose of this treatment is to reduce or prevent the development of neovascularization, macular disease, and retinal damage. Once a patient reaches the age of 60, increased ophthalmic surveillance is performed to detect the presence of AMD. This increased surveillance should include periodic retinal examinations and fluorescein angiograms to monitor for the presence of subretinal fluid, blood, exudates, RPE detachment, cvstic retinal changes, or the presence of grayish green subretinal neovascular membrane. When AMD is diagnosed, a regime of VEGF trap protein treatment is commenced coupled with or without other treatments such as photocoagulation. As the first step of treatment, the patient is to receive a full ophthalmic examination to establish a baseline of ocular health. The ophthalmic examination includes indirect ophthalmoscopy, slit-lamp biomicroscopy, peripheral retinal examination, intraocular pressure measurements, visual acuity (unaided and best corrected) symptomatology, fund us photography, fluorescein angiography, electroretinography and A-scan measurements. Following the preliminary examination, an intravitreal injection of VEGF trap protein is given to the patient's affected eye manifesting AMD. If both eyes are affected, they may be treated separately. The eye to be treated is injected with 25-4000 micrograms of VEGF trap protein in an ophthalmic solution.

After treatment, the patients' eyes are to be examined on days one (1), two (2), seven (7), fifteen (15), thirty (30) and sixty (60). Because of the possibility of reoccurrence, the patient should return for periodic examinations on a monthly basis thereafter. On each examination day the patient is monitored for vitreous liquefaction. Additionally, the patient is monitored for posterior vitreous detachments using indirect ophthalmoscopy with scleral depression. Finally, the extent of AMD presented by the patient is continuously monitored through periodic retinal examinations and fluorescein angiograms to monitor for the presence of subretinal fluid, blood, exudates, RPE detachment, cystic retinal changes, or the presence of grayish green subretinal neovascular membrane. Additional VEGF trap protein treatments may be required if indicia of reoccurring neovascularization are observed. Additional treatments may be given on weekly or monthly basis. In a preferred embodiment, an initial treatment is followed by subsequent treatments between 1-6 months apart.

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21 Example 18

A Double-Masked, Placebo-Controlled, Dose Escalation, Phase I Study of Intravenous VEGF Trap in Patients with Neovascular Age-Related Macular Degeneration

A study was conducted to obtain preliminary assessments of the safety, pharmacokinetics (PK), and biological activity of single and repeated intravenous (IV) doses of the VEGF trap antagonist (SEQ ID NO:6) in patients with neovascular age-related macular degeneration (AMD).

Methods. Successive cohorts of patients with neovascular AMD (≤ 12 disc areas, $\geq 50\%$ active choroidal neovascularization (CNV), ETDRS best-corrected visual acuity 15 (BCVA)≦20/40) were randomized (3:1) to receive either VEGF trap or placebo at dose levels of 0.3, 1.0, or 3.0 mg/kg. Patients received a single IV dose, followed by a 4-week safety observation/PK evaluation period, followed by 3 biweekly IV doses. Safety assessments included labo- 20 ratory assessments (hematology, chemistry, urinalysis, anti-VEGF trap antibody measurements), vital signs, and ophthalmic exams. Measures of biological activity included mean percent change in excess retinal thickness (ERT) as assessed by optical coherence tomography (OCT), and 25 ETDRS BCVA. Adverse events (AEs) were graded using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE v. 3.0). Dose-limiting toxicity (DLT) was defined as Grade 2 or 3 ocular AEs, or Grade 3 or 4 systemic AEs with modified criteria for hypertension 30 and proteinuria. The maximum tolerated dose (MTD) was defined as the dose level below that at which ≥ 2 patients experienced DLT.

Results. Twenty-five patients were enrolled (11 male, 14 female; mean age 76 years). Nineteen patients received 35 VEGF trap (7 at 0.3 mg/kg; 7 at 1.0 mg/kg; 5 at 3.0 mg/kg), and 6 patients received placebo. The majority of AEs encountered on VEGF Trap treatment were mild to moderate in severity. Two of 5 patients encountered protocol-defined DLT at the 3.0 mg/kg dose level: Grade 4 hypertension 40 (n=1); Grade 2 proteinuria (n=1). Therefore, all of the patients in the 3.0 mg/kg dose group were prematurely

withdrawn from study. None of the patients in the study developed anti-VEGF trap antibodies. The mean percent changes in ERT were: -12%, -10%, -66%, -60% for the placebo, 0.3, 1.0, and 3.0 mg/kg dose groups at Day 15 5 (ANOVA p<0.02), and -5.6%, +47.1%, -63.3% for the placebo, 0.3, and 1.0 mg/kg dose groups at Day 71 (ANOVA p<0.02). The changes in BCVA were: +1.9, +1.8, +3.4, and +4.6, for the placebo, 0.3, 1.0, and 3.0 mg/kg dose groups at Day 15 and were: +2.8, +3.9, and +3.9 for the placebo, 0.3, 10 and 1.0 mg/kg dose groups at Day 71. The BCVA results were not statistically significant.

Optical coherence tomography scans (temporal to nasal and inferior to superior transverse) and maps were obtained. In one example patient, at baseline the foveal thickness was 348 μ m with a pocket of subretinal fluid beneath the fovea. On day 29, there was little anatomic evidence of improvement, but at day 71 (two weeks after the fourth infusion of VEGF trap), the pocket of subretinal fluid had resolved, foveal thickness had improved to 232 μ m, and macular volume was within the normal range at 6.69 mm³. At day 99, 6 weeks after the last infusion of VEGF trap, there was deterioration, with recurrence of the pocket of subfoveal fluid, increase in foveal thickness to 248 μ m, and increase in macular volume to 7.31 mm³.

Digital fluorescein angiography was conducted at baseline and 3 time points after initiation of treatment with 1. mg/kg of VEGF trap. At baseline, there was a large area of occult subfoveal CNV that leaked during the mid- and late-phases of the angiogram so that there was white dye pooled beneath the entire macula. At day 29, there was mildly reduced leakage, but by day 71, there was a substantial reduction in leakage and pooling of dye. At day 99, there was some increase in leakage compasted to day 71, but less than seen at baseline.

Conclusions: The maximum tolerated dose of intravenous VEGF trap in this study of neovascular AMD patients was 1.0 mg/kg. A dose-dependent improvement in ERT as evaluated by OCT was suggested in this small number of patients, with a longer initial duration in improvement at the 3.0 mg/kg as compared to the 1.0 mg/kg dose level. A trend towards a dose-related improvement in BCVA was also suggested.

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Lys	Leu	Thr	Val 420	Asp	Lys	Ser	Arg	Trp 425	Gln	Gln	Gly	Asn	Val 430	Phe	Ser		
Cys	Ser	Val 435	Met	His	Glu	Ala	Leu 440	His	Asn	His	Tyr	Thr 445	Gln	Lys	Ser		
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aaqa	acad	jca (catt	tgtc	ag a	gtcca	atgaa	a aa	ggaca	aaa	ctca	acaca	atq o	ccca	cqtqc	720	
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Pro	Ala	Pro	Glu	Leu 245	Leu	Gly	Gly	Pro	Ser 250	Val	Phe	Leu	Phe	Pro 255	Pro	

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Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys 260 265 270 Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp 275 280 285 Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu 290 295 300 Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu 305 310 315 320 His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn 325 330 335 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
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We claim:

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1. A therapeutic method for treating or ameliorating an eye disorder, comprising administering a fusion polypeptide comprising the amino acid sequence of SEQ ID NO:6, wherein the eye disorder is associated with choroidal $_{60}$ neovascularization, vascular leak, or retinal edema, and wherein administration is systemic.

2. The therapeutic method of claim 1, wherein the eye disorder is age related macular degeneration or diabetic retinopathy. 65

3. A method for the treatment of a human subject diagnosed with age-related macular degeneration, comprising

administering an effective amount of a vascular endothelial growth factor (VEGF) inhibitor to the human subject, the method comprising: (a) administering to the subject an initial dose of at least approximately 25-4000 micrograms VEGF inhibitor protein per eye; and (b) administering to the subject a plurality of subsequent doses of the VEGF inhibitor protein in an amount that is approximately the same or less than the initial dose, wherein the subsequent doses are separated in time from each other by at least two weeks, wherein the VEGF inhibitor is a fusion polypeptide comprising the amino acid sequence of SEQ ID NO:6 and administration of the VEGF inhibitor is systemic.

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4. The method of claim **3**, wherein the initial dose is at least approximately 1000 micrograms of VEGF inhibitor protein.

5. The method of claim 4, wherein the subsequent doses are separated in time from each other by at least four weeks.

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6. The method of claim **5**, wherein the subsequent doses are separated in time from each other by at least 3 to 6 months.

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US007306799B2

(12) United States Patent Wiegand et al.

- (54) USE OF VEGF INHIBITORS FOR TREATMENT OF EYE DISORDERS
- (75) Inventors: Stanley J. Wiegand, Croton on Hudson, NY (US); Nicholas J. Papadopoulos, LaGrangeville, NY (US); George D. Yancopoulos, Yorktown Heights, NY (US); James P. Fandl, LaGrangeville, NY (US); Thomas J. Daly, New City, NY (US)
- (73) Assignee: Regeneron Pharmaceuticals, Inc., Tarrytown, NY (US)
- (*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 285 days.

This patent is subject to a terminal disclaimer.

- (21) Appl. No.: 11/089,803
- (22) Filed: Mar. 25, 2005

(65) **Prior Publication Data**

US 2005/0260203 A1 Nov. 24, 2005

Related U.S. Application Data

- (63) Continuation-in-part of application No. 10/988,243, filed on Nov. 12, 2004, which is a continuation-in-part of application No. 10/009,852, filed as application No. PCT/US00/14142 on May 23, 2000, now Pat. No. 7,070,959, which is a continuation-in-part of application No. 10/880,021, filed on Jun. 29, 2004, now Pat. No. 7,279,159, which is a continuation-in-part of application No. 10/609,775, filed on Jun. 30, 2003, now Pat. No. 7,087,411.
- (60) Provisional application No. 60/138,133, filed on Jun. 8, 1999.
- (51) Int. Cl.

A61K 38/18	(2006.01)
C07K 14/71	(2006.01)
C12N 15/62	(2006.01)

- (58) **Field of Classification Search** None See application file for complete search history.
- (56) References Cited

U.S. PATENT DOCUMENTS

6,100,071 A 8/2000 Davis-Smyth

(10) Patent No.: US 7,306,799 B2

(45) **Date of Patent:** *Dec. 11, 2007

2005/0175610	A1*	8/2005	Wiegand et al 424/145.1	
2006/0030529	A1*	2/2006	Wiegand et al 514/12	
2006/0058234	A1*	3/2006	Daly et al 514/12	
2006/0148705	A1*	7/2006	Daly et al 514/12	
2006/0172944	A1*	8/2006	Wiegand et al 514/12	

FOREIGN PATENT DOCUMENTS

WO	WO97/44453	11/1997
WO	WO98/13071	4/1998
WO	WO99/03996	1/1999

OTHER PUBLICATIONS

Herley et al. (1999). Characterization of the VEGF binding site on the Flt-1 receptor. Biochem Biophys Res Commun. 262(3):731-738.*

Witmer et al. (2003). Vascular endothelial growth factors and angiogenesis in eye disease. Prog. Retin. Eye Res. 22(1):1-29.* Terman, B.I., et al, "Identification of a new endothelial cell growth factor receptor tyrosine kinase", Oncogene (1991) 6:1677-1683. Terman, B.I., et al., "Identification of teh KDR tyrosine kinase as a

receptor for vascular endothelial cell growth factor", Biochem Biophys Res Comm (1992) 187(3):1579-1586. Davis-Smyth, T., et al., 1996, The EMBO Journal 15(18):4919-

4927.

Holash, J., et al., (2002) PNAS 99(17):11393-11398.

Heidaran, M.A., et al., (1990) J. Bio. Chem. 265(31):18741-18744. Cunningham, S.A., et al., (1997) Biochem. Biophys. Res. Comm. 231:596-599.

Fuh, G., et al., (1998) J. Bio. Chem. 273(18):11197-11204.

Wiesmann, C., et al., (1997) Cell 91:695-704.

Barleon, B., et al., (1997) J. Bio. Chem. 272(16):10382-10388. Davis-Smyth, T., et al., (1998) J. Bio. Chem. 273(6):3216-3222.

* cited by examiner

Primary Examiner—Christine J. Saoud Assistant Examiner—Jon M Lockard (74) Attorney, Agent, or Firm—Valeta Gregg, Esq.

(57) ABSTRACT

Modified chimeric polypeptides with improved pharmacokinetics and improved tissue penetration are disclosed useful for treating eye disorders, including age-related macular degeneration and diabetic retinopathy.

11 Claims, 11 Drawing Sheets

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hVEGF165 (nM) VEGF/FIt1D2FIk1D3.FcΔC1(a) VEGF/ 1 0.93 10 0.97 50 1	
1 0.93 10 0.97 50 1	GF/VEGFR1R2-FcΔC1(a)
10 0.97 50 1	0.98
50 1	0.94
	0.99
· · · · · · · · · · · · · · · · · · ·	
Average ± StDev 0.96 ± 0.03	0.97 ± 0.02

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Fig. 2

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Fig. 3

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Fig. 4

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Fig. 5

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Fig. 6

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Fig. 7A



Fig. 7B

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Fig. 8

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% Grade 4 Lesions

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Fig.

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USE OF VEGF INHIBITORS FOR TREATMENT OF EYE DISORDERS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Ser. No. 10/988,243 filed 12 Nov. 2004, which is a continuation-in-part of application Ser. No. 10/009,852 filed 6 Dec. 2001, now U.S. Pat. No. 7,070,959, which is the National 10 Stage of International Application No. PCT/US00/14142 filed 23 May 2000, which claims the benefit under 35 USC § 119(e) of U.S. Provisional 60/138,133 filed 8 Jun. 1999, and this application is a continuation-in-part of application Ser. No. 10/880,021 filed 29 Jun. 2004, now U.S. Pat. No. 15 7,279,159 which is a continuation-in-part of application Ser. No. 10/609,775 filed 30 Jun. 2003, now U.S. Pat. No. 7,087,411, which applications are herein specifically incorporated by reference in their entireties.

BACKGROUND

Statement Regarding Related Art

A class of cell-derived dimeric mitogens with selectivity for vascular endothelial cells has been identified and designated vascular endothelial cell growth factor (VEGF). VEGF is a dimer with an apparent molecular mass of about 46 kDa with each subunit having an apparent molecular mass of about 23 kDa. The membrane-bound tyrosine kinase receptor, known as Flt (also known as VEGFR2), was shown to be a VEGF receptor (DeVries et al. (1992) Science 255:989-991). Another form of the VEGF receptor, designated KDR or Flk-1 (also known as VEGFR3), is also known to bind VEGF and induce mitogenesis (Terman et al. (1991) Oncogene 6:1677-1683; Terman et al. (1992) Bioschem. Biophys. Res. Comm. 187:1579-1586).

U.S. Pat. No. 6,011,003 describes an altered, soluble form of Fit polypeptide capable of binding to VEGF comprising five or fewer complete immunoglobulin domains. WO 97/44453 describes chimeric VEGF receptor proteins comprising amino acid sequences derived from VEGF receptors Flt1 and KDR.

BRIEF SUMMARY OF THE INVENTION

The invention features a therapeutic method for treating or ameliorating an eye disorder, comprising administering a vascular endothelial growth factor (VEGF) inhibitor to a patient in need thereof. In one embodiment, the eye disorder treated is age related macular degeneration. In another 50 embodiment, the eye disorder treated is diabetic retinopathy.

Preferably, the VEGF inhibitor used in the method of the invention comprises an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor and Ig domain 3 of a second VEGF receptor; and a multimerizing component, wherein the first 55 VEGF receptor is Flt1, the second VEGF receptor is Flk1 or Flt4, and the multimerizing component is selected from the group consisting of (i) an amino acid sequence between 1 to about 200 amino acids in length having at least one cysteine residue, and (ii) an immunoglobulin domain, or fragment of 60 an immunoglobulin domain. In specific embodiments, the VEGF inhibitor is a fusion polypeptide "VEGF trap' selected from the group consisting of SEQ ID NO:2 (Flt1D2.Flk1D3Fc Δ C1(a)), ID SEQ NO:4 $(Flt1D2.VEGFR3D3.FC\DeltaC1(a)),$ SEQ ID NO:6 65 (VEGFR1R2 Fc Δ C1(a)), and SEQ ID NO:23. In another embodiment, the VEGF inhibitor is a fusion polypeptide

encoded by a nucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 22, and a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of SEQ ID NO:1, 3, 5, and 22.

In a second aspect, the invention features a method for the treatment of a human subject diagnosed with an eye disorder, comprising administering an effective amount of a vascular endothelial growth factor (VEGF) inhibitor to the human subject, the method comprising administering to the subject an initial dose of at least approximately 25-4000 micrograms VEGF inhibitor protein to an affected eye, and administering to the subject a plurality of subsequent doses of the VEGF inhibitor protein in an amount that is approximately the same or less than the initial dose, wherein the subsequent doses are separated in time from each other by at least two weeks. The eye disorder is one of age-related macular degeneration or diabetic retinopathy. In various embodiments, the initial dose is at least approximately 25 to 4000 micrograms of VEGF inhibitor protein. In various embodiments, the subsequent doses are separated in time from each other by at least two weeks to 12 months; more preferably, the subsequent doses are separated in time from each other by at least 3-6 months. The VEGF inhibitor protein is administered directly to the affected eye, including by use of eye drops or intravitreal injection. Preferably, the VEGF inhibitor is a dimer having two fusion polypeptides consisting essentially of an immunoglobulin-like (Ig) domain 2 of Flt1 and Ig domain 3 of Flk1 or Flt4, and a multimerizing component. In specific embodiments, the VEGF inhibitor is a dimer comprising the fusion polypeptide of SEQ ID NO:2, 4, 6, or 23.

Other objects and advantages will become apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Biacore analysis of binding stoichiometry. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml.

FIG. 2. Pharmacokinetics of Flt1 (1-3)-Fc (A40), Flt1D2.Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a).

FIG. 3. Pharmacokinetics of Flt1 (1-3)-Fc (A40), Flt1D2.Flk1D3.Fc Δ C1(a) and Flt1D2.VEGFR3D3.Fc Δ C1 (a).

FIG. 4. VEGFR1R2-Fc Δ C1(a) prevents neovascularization induced by retinal ischemia. Serial 10 µm cross sections were collected and stained with hematoxylin and eosin. For each animal, nuclei in preretinal neovessels were counted in a series of ten sections within 300 microns of the optic nerve head and averaged. Counts were obtained in three independent experiments, n \geq 4 for each treatment group in each study.

FIG. 5. Effect of subcutaneous VEGFR1R2-Fc Δ C1(a) injections on choroidal neovascularization area. The size of CNV lesions was measured in choroidal flat mounts. The images were digitized using an Axioskop microscope equipped with a video camera, and the total area of choroidal neovascularization associated with each laser burn was measured using Image-Pro Plus software.

FIG. 6. VEGFR1R2-Fc Δ C1(a) inhibits subretinal neovascularization in Rho/VEGF transgenic mice.

FIG. 7A-B. VEGF-Induced breakdown of the blood retinal barrier. A. Following intravitreal injections of VEGF, adult mice (C57BL/6) treated with injections of

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VEGFR1R2-Fc Δ C1(a) had a significantly smaller retina to lung leakage ratio than mice treated with Fc fragment, indicating less breakdown of BRB. B. Double transgenic mice treated with injections of VEGFR1R2-Fc Δ C1(a) had a significant reduction in the retina to lung leakage ratio s compared to mice treated with Fc fragment.

FIG. 8. Effect of VEGFR1R2-Fc Δ C1(a) administration on corneal thickness in suture and alkali burn models of corneal trauma. Corneas were injured by suture placement or application of NaOH as described, and a single dose of 10 VEGFR1R2-Fc Δ C1(a) (25 mg/kg, ip) or saline (n=5 per group) was administered immediately following injury. The contralateral cornea served as normal, undamaged controls. Corneas were collected 7 days later and cross-sections were cut and stained with hematoxylin and eosin. Corneal thick- 15 ness was measured as an index of corneal edema.

FIG. 9. System or intravitreal VEGF trap protein administration prevents laser-induced choroidal neovascularization (CNV) and reverses vascular leak in established lesions.

FIG. 10. Dose response curve of Baf/Flt cells grown in $\ ^{20}$ VEGF.

FIG. 11. Inhibition of VEGF growth response by VEGF trap VEGFR1R2-Fc Δ C1(a) or anti-VEGF antibody.

DETAILED DESCRIPTION OF THE INVENTION

It has been a longstanding problem in the art to produce a receptor-based VEGF antagonist that has a pharmacokinetic profile that is appropriate for consideration of the ³⁰ antagonist as a therapeutic candidate. Applicants describe herein, for the first time, a chimeric polypeptide molecule, capable of antagonizing VEGF activity, that exhibits improved pharmacokinetic properties as compared to other known receptor-based VEGF antagonists. The chimeric ³⁵ polypeptide molecules described herein thus provide appropriate molecules for use in therapies in which antagonism of VEGF is a desired result.

The extracellular ligand binding domain is defined as the portion of a receptor that, in its native conformation in the cell membrane, is oriented extracellularly where it can contact with its cognate ligand. The extracellular ligand binding domain does not include the hydrophobic amino acids associated with the receptor's transmembrane domain or any amino acids associated with the receptor's intracellular domain. Generally, the intracellular or cytoplasmic domain of a receptor is usually composed of positively charged or polar amino acids (i.e., lysine, arginine, histidine, glutamic acid, aspartic acid). The preceding 15-30, predominantly hydrophobic or apolar amino acids (i.e., leucine, valine, isoleucine, and phenylalanine) comprise the transmembrane domain. The extracellular domain comprises the amino acids that precede the hydrophobic transmembrane stretch of amino acids. Usually the transmembrane domain is flanked by positively charged or polar amino acids such as lysine or arginine. von Heijne has published detailed rules that are commonly referred to by skilled artisans when determining which amino acids of a given receptor belong to the extracellular, transmembrane, or intracellular domains (See, von Heijne (1995) BioEssays 17:25).

Nucleic Acid Constructs and Encoded Fusion Polypeptides

The present invention provides for the construction of nucleic acid molecules encoding chimeric polypeptide molecules that are inserted into a vector that is able to express 65 the chimeric polypeptide molecules when introduced into an appropriate host cell. Appropriate host cells include, but are 4

not limited to, bacterial cells, yeast cells, insect cells, and mammalian cells. Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the chimeric polypeptide molecules under control of transcriptional/translational control signals. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (See Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY).

Expression of nucleic acid molecules encoding the chimeric polypeptide molecules may be regulated by a second nucleic acid sequence so that the chimeric polypeptide molecule is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the chimeric polypeptide molecules described herein may be controlled by any promoter/enhancer element known in the art.

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising chimeric polypeptide molecule-encoding nucleic acids as described herein, are used to transfect the host and thereby direct expression of such nucleic acids to produce the chimeric polypeptide molecules, which may then be recovered in a biologically active form. As used herein, a biologically active form includes a form capable of binding to VEGF. Expression vectors containing the chimeric nucleic acid molecules described herein can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to the inserted chimeric polypeptide molecule sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the chimeric polypeptide molecule DNA sequence is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the chimeric polypeptide molecules.

Cells of the present invention may transiently or, preferably, constitutively and permanently express the chimeric polypeptide molecules.

The chimeric polypeptide molecules may be purified by any technique which allows for the subsequent formation of a stable, biologically active chimeric polypeptide molecule. 60 For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis (see, for example, U.S. Pat. No. 5,663,304). In order to 65 further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

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The method of the invention encompasses the use of a fusion protein consisting essentially of first and second vascular endothelial growth factor (VEGF) receptor components and a multimerizing component, wherein the first VEGF receptor component is an immunoglobulin-like (Ig) domain 2 of Flt1, the second VEGF receptor component is an Ig domain 3 of a Flk1 or Flt4, and the multimerizing component is selected from the group consisting of (i) a multimerizing component comprising a cleavable region (C-region), (ii) a truncated multimerizing component, (iii) 10 an amino acid sequence between 1 to about 200 amino acids in length having at least one cysteine residue, (iv) a leucine zipper, (v) a helix loop motif, (vi) a coil-coil motif, and (vii) an immunoglobulin domain. Examples of the VEGF inhibitors useful in the method of the invention include fusion 15 proteins encoded by a nucleotide sequence selected from the group consisting of the nucleotide sequence of SEQ ID NO:1, 3, 5, 22, and a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of SEQ ID NO:1, 3, 5, or 22, and fusion 20 protein selected from the group consisting of SEQ ID NO:2 (Flt1D2.Flk1D3Fc Δ C1(a)), SEO ID $NO \cdot 4$ (Flt1D2.VEGFR3D3.FcΔC1(a)), **SEO** ID NO:6 (VEGFR1R2 $Fc\Delta C1(a)$) and (SEQ ID NO:23).

Therapeutic Methods

The present invention also has diagnostic and therapeutic utilities. In particular embodiments of the invention, methods of detecting aberrancies in the function or expression of the chimeric polypeptide molecules described herein may be used in the diagnosis of disorders. In other embodiments, manipulation of the chimeric polypeptide molecules or agonists or antagonists which bind the chimeric polypeptide molecules may be used in the treatment of diseases. In further embodiments, the chimeric polypeptide molecule is utilized as an agent to block the binding of a binding agent to its target.

By way of example, but not limitation, the method of the invention may be useful in treating clinical conditions that are characterized by vascular permeability, edema or inflammation such as brain edema associated with injury, stroke or tumor; edema associated with inflammatory disorders such as psoriasis or arthritis, including rheumatoid arthritis; asthma; generalized edema associated with burns; ascites and pleural effusion associated with tumors, inflammation or trauma; chronic airway inflammation; capillary leak syndrome; sepsis; kidney disease associated with inflammation are related leakage of protein; and eye disorders such as age related macular degeneration and diabetic retinopathy.

Combination Therapies

In numerous embodiments, a VEGF inhibitor may be administered in combination with one or more additional compounds or therapies, including a second VEGF inhibitor. Combination therapy includes administration of a single pharmaceutical dosage formulation which contains a VEGF 55 inhibitor molecule and one or more additional agents; as well as administration of a VEGF inhibitor and one or more additional agent(s) in its own separate pharmaceutical dosage formulation. For example, a VEGF inhibitor and a cytotoxic agent, a chemotherapeutic agent or a growth 60 inhibitory agent can be administered to the patient together in a single dosage composition such as a combined formulation, or each agent can be administered in a separate dosage formulation. Where separate dosage formulations are used, the VEGF-specific fusion protein of the invention and a one or more additional agents can be administered concurrently, or at separately staggered times, i.e., sequentially. The

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therapeutic methods of the invention may also be combined with other agents or medical procedures used for treatment of eve disorders.

Treatment Population

The eye comprises several structurally and functionally distinct vascular beds, which supply ocular components critical to the maintenance of vision. These include the retinal and choroidal vasculatures, which supply the inner and outer portions of the retina, respectively, and the limbal vasculature located at the periphery of the cornea. Injuries and diseases that impair the normal structure or function of these vascular beds are among the leading causes of visual impairment and blindness. For example, diabetic retinopathy is the most common disease affecting the retinal vasculature, and is the leading cause of vision loss among the working age population in the United States. Vascularization of the cornea secondary to injury or disease is yet another category of ocular vascular disease that can lead to severe impairment of vision.

"Macular degeneration" is a medical term that applies to any of several disease syndromes which involve a gradual loss or impairment of eyesight due to cell and tissue degeneration of the yellow macular region in the center of the retina. Macular degeneration is often characterized as one of two types, non-exudative (dry form) or exudative (wet form). Although both types are bilateral and progressive, each type may reflect different pathological processes. The wet form of age-related macular degeneration (AMD) is the most common form of choroidal neovascularization and a leading cause of blindness in the elderly. AMD affects millions of Americans over the age of 60, and is the leading cause of new blindness among the elderly. It is characterized and usually diagnosed by the presence of elevated levels of two types of cellular debris within the retina, called drusen and lipofuscin.

There are several types of symptomatic treatment, however, that have been used with limited and isolated success, depending on the particular condition of the patient, to treat exudative (wet form) macular degeneration. Laser photocoagulation therapy may benefit certain patients with macular degeneration. However, there are high recurrence rates for selected choroidal neovascular membranes which may initially respond to laser therapy. Vision loss may also result from the laser therapy. Low dose radiation (teletherapy) has also been hypothesized as a possible treatment to induce regression of choroidal neovascularization. Surgical removal of neovascular membranes is another possible treatment, but it is a highly specialized procedure and reportedly has not had promising results to date. There is presently no effective treatment for non-exudative (dry form) macular degeneration. Management of non-exudative macular degeneration is limited to early diagnosis and careful follow-up to determine if the patient develops choroidal neovascularization. Protection against exposure to ultraviolet light and prescribed dosages of anti-oxidant vitamins (e.g., vitamin A, β-carotene, lutein, zeaxanthin, vitamin C and vitamin E) and zinc may also be of some benefit, but as yet these treatments remain unproven.

Accordingly, the population to be treated by the method of the invention is preferably one of (i) a human subject diagnosed as suffering from macular degeneration, (ii) a human subject diagnosed as suffering from diabetes-related retinopathy, and (iii) a human subject suffering from pathological vascularization of the cornea secondary to injury or disease.

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Methods of Administration and Compositions

Preferably, administration of the VEGF inhibitor will be directly to the eye, e.g., topical. Topical methods of administration include, for example, by eye drops, subconjunctival injections or implants, intravitreal injections or implants, 5 sub-Tenon's injections or implants, incorporation in surgical irrigating solutions, etc.

Compositions suitable for topical administration are known to the art (see, for example, U.S. Patent Application 2005/0059639). In various embodiments, compositions of 10 the invention can comprise a liquid comprising an active agent in solution, in suspension, or both. As used herein, liquid compositions include gels. Preferably the liquid composition is aqueous. Alternatively, the composition can take form of an ointment. In a preferred embodiment, the com- 15 position is an in situ gellable aqueous composition, more preferably an in situ gellable aqueous solution. Such a composition can comprise a gelling agent in a concentration effective to promote gelling upon contact with the eye or lacrimal fluid in the exterior of the eye. Aqueous composi- 20 tions of the invention have ophthalmically compatible pH and osmolality. The composition can comprise an ophthalmic depot formulation comprising an active agent for subconjunctival administration. The microparticles comprising active agent can be embedded in a biocompatible 25 pharmaceutically acceptable polymer or a lipid encapsulating agent. The depot formulations may be adapted to release all or substantially all the active material over an extended period of time. The polymer or lipid matrix, if present, may be adapted to degrade sufficiently to be transported from the site of administration after release of all or substantially all the active agent. The depot formulation can be a liquid formulation, comprising a pharmaceutical acceptable polymer and a dissolved or dispersed active agent, Upon injection, the polymer forms a depot at the injections site, e.g. by 35 gelifying or precipitating. The composition can comprise a solid article that can be inserted in a suitable location in the eye, such as between the eye and eyelid or in the conjuctival sac, where the article releases the active agent. Solid articles suitable for implantation in the eye in such fashion generally 40 comprise polymers and can be bioerodible or non-bioerodible.

In one embodiment of the method of the invention, a human subject with at least one visually impaired eye is treated with 25-4000 micrograms of a VEGF inhibitor 45 protein via intravitreal injection. Improvement of clinical symptoms are monitored by one or more methods known to the art, for example, indirect ophthalmoscopy, fundus photography, fluorescein angiopathy, electroretinography, external eye examination, slit lamp biomicroscopy, applanation 50 tonometry, pachymetry, and autorefaction. Subsequent doses may be administered weekly or monthly, e.g., with a frequency of 2-8 weeks or 1-12 months apart.

Other features of the invention will become apparent in the course of the following descriptions of exemplary 55 embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

Example 1

Modified Flt1 Receptor Vector Construction

Chimeric molecules were constructed, denoted R1R2 65 (Flt1.D2.Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a) and R1R3 (Flt1D2.VEGFR3D3-Fc Δ C1(a) and VEGFR1R3-

 $Fc\Delta C1(a)$ respectively, wherein R1 and Flt1D2=Ig domain 2 of Flt1 (VEGFR1); R2 and Flk1D3=Ig domain 3 of Flk1 (VEGFR2); and R3 and VEGFR3D3=Ig domain 3 of Flt4 (VEGFR3)) were much less sticky to ECM, as judged by an in vitro ECM binding assay and had greatly improved PK as described herein. In addition, these molecules were able to bind VEGF tightly and block phosphorylation of the native Flk1 receptor expressed in endothelial cells.

Construction of the expression plasmid pFlt1D2.Flk1D3.Fc Δ C1(a). Expression plasmids pMT21.Flt1(1-3).Fc (6519 bp) and pMT21.Flk-1(1-3).Fc (5230 bp) are plasmids that encode ampicillin resistance and Fc-tagged versions of Ig domains 1-3 of human Flt1 and human Flk1, respectively. These plasmids were used to construct a DNA fragment consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 3 of Flk1, using PCR amplification of the respective Ig domains followed by further rounds of PCR to achieve fusion of the two domains into a single fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows: 5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCG-

TAGAGATG-3') (SEQ ID NO:8), 3': Flt1D2-Flk1D3.as (5'-CGGACTCAGMCCACATCTATGATTGTATTGGT-3') (SEQ ID NO:9). The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO:10) corresponding to amino acids 27-33 of SEQ ID NO:2. The 3' primer encodes the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of SEQ ID NO:7) (corresponding to amino acids 127-130 of SEQ ID NO:2) of Flk1.

For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as follows: 5': Flt1D2-Flk1D3.s (5'-ACMT-CATAGATGTGGTTCTGAGTCCGTCTCATGG-3') (SEQ NO:11); 3': Flk1D3/apa/srf.as (5'-GATAATGC-ID CCGGGCCCTTTTCATGGACCCTGACAAATG-3') (SEQ ID NO:12). The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (SEQ ID NO:13) (corresponding to amino acids 223-228 of SEQ ID NO:2), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of SEQ ID NO:2.

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described supra) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 614 bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ Δ B2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was 60 verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 702 bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1 (1-3)B2- $Fc\Delta C1(a)$ to produce the plasmid pFlt1D2.Flk1D3.Fc $\Delta C1$ (a). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.Fc Δ C1(a) chimeric molecule is shown in SEQ ID NO:1-2.

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Construction of the plasmid expression pFlt1D2VEGFR3D3Fc∆C1(a). The expression plasmid pMT21.Flt1(1-3).Fc (6519 bp) encodes ampicillin resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1 receptor. This plasmid was used to produce a DNA fragment 5 containing Ig domain 2 of Flt1 by PCR. RNA from the cell line HEL921.7 was used to produce Ig domain 3 of Flk1, using standard RT-PCR methodology. A further round of PCR amplification was used to achieve fusion of the two Ig domains into a single fused fragment. For Ig domain 2 of 10 Flt1, the 5' and 3' amplification primers were as follows: 5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGAC-CTTTCGTAGAGATG-3') (SEQ ID NO:14), 3': TTCCTGGGCMCAGCTG-Flt1D2.VEGFR3D3.as GATATCTATGATTGTATTGGT) (SEQ ID NO:15). The 5' 15 amplification primer encodes a BspE1 restriction site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO:10) (corresponding to amino acids 27-33 of SEQ ID NO:1-2). The 3' amplification primer encodes the reverse complement of the end of Flt1 Ig 20 domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of SEQ ID NO:4) and continuing into IQLL of VEGFR3 (corresponding to amino acids 127-130 of SEQ ID NO:4). 25

For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as follows: 5': R3D3.s (ATCCAGCTGT-TGCCCAGGMGTCGCTGGAGCTGCTGGTA) (SEQ ID NO:17), 3': R3D3.as (ATTTTCATGCACMTGACCTCG-GTGCTCTCCCGAAATCG) (SEQ ID NO:18). Both the 5' and 3' amplification primers match the sequence of VEGFR3. The 296 bp amplification product of this RT-PCR reaction was isolated by standard techniques and subjected to a second round of PCR to add suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion 35 of the Flk1D3 and Fc domains via a GPG bridge (see below). amplification The primers were as follows: 5':Flt1D2.VEGFR3D3.s(TCATAGATATCCAGCTGTTGC-CCAGGMGTCGCTGGAG) (SEQ ID NO:19), 3': VEGFR3D3/srf.as (GATMTGCCCGGGCCATTTTCATG- 40 CACMTGACCTCGGT) (SEQ ID NO:20). The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the amino acids 45 VIVHEN (SEQ ID NO:21) (corresponding to amino acids 221-226 of SEQ ID NO:4), followed by a bridging sequence that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of SEQ ID NO:4.

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as 55 described supra, to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 625 bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/Flt1AB2.Fc (described supra), to create the 60 plasmid pMT21/Flt1D2.VEGFR3D3.Fc. The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 693 bp fragment was subcloned into the EcoRI to SrfI 65 restriction sites of the plasmid pFlt1(1-3) Δ B2-Fc Δ C1(a) to plasmid produce the designated

pFlt1D2.VEGFR3D3.Fc Δ C1(a). The complete DNA deduced amino acid sequence of the Flt1D2.VEGFR3D3.Fc Δ C1(a) chimeric molecule is shown in SEQ ID NO:3-4.

Example 2

Construction pVEGFR1R2-Fc\DeltaC1(a) Expression Vector

The pVEGFR1R2.Fc Δ C1(a) (SEQ ID NO:15-16) expression plasmid was constructed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of SEQ ID NO:6) between Flt1d2-Flk1d3-Fc Δ C1(a) amino acids 26 and 27 of SEQ ID NO:2 (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231. The SDT amino acid sequence is native to the Flt1 receptor and was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2.Fc Δ C1 (a) chimeric molecule is shown in SEQ ID NO:5-6.

Example 3

Cell Culture Process Used to Produce Modified Flt1 Receptors

Cell Culture Process Used to Produce Flt1D2.Flk1D3.Fc Δ C1(a). The process for production of Flt1D2.Flk1D3.Fc Δ C1(a) protein using the expression plasmid pFlt1D2.Flk1D3.Fc Δ C1(a) involves suspension culture of recombinant Chinese hamster ovary (CHO K1/E1A) cells which constitutively express the protein product. The cells are grown in bioreactors and the protein product is isolated and purified by affinity and size exclusion chromatography.

Cell Expansion. Two confluent T-225 cm² flasks containing the Flt1D2.Flk1D3.Fc Δ C1(a) expressing cell line were expanded by passaging cells into eight T-225 cm² flasks in medium (GMEM+10% serum, GIBCO) and incubated at 37° C. and 5% CO₂. When the flasks approached confluence (approximately 3 to 4 days) the cells were detached using trypsin. Fresh medium was added to protect the cells from further exposure to the trypsin. The cells were centrifuged and resuspended in fresh medium then transferred to eight 850 cm² roller bottles and incubated at 37° C. and 5% CO₂ until confluent.

Suspension Culture in Bioreactors. Cells grown in roller bottles were trypsinized to detach them from the surface and washed with suspension culture medium. The cells are aseptically transferred to a 5 L bioreactor (New Brunswick Celligen Plus) where the cells are grown in 3.5 L of suspension culture. The suspension culture medium was a glutamine-free low glucose modification of IS-CHO (Irvine Scientific) to which 5% fetal bovine serum (Hyclone), GS supplement (Life Technologies) and 25 µM methionine sulfoximine (Sigma) was added. The pH was controlled at 7.2 by addition of carbon dioxide to the inlet gas or by addition of a liquid solution of sodium carbonate to the bioreactor. Dissolved oxygen level was maintained at 30% of saturation by addition of oxygen or nitrogen to the inlet gas and temperature controlled at 37° C. When a density of 4×10^6 cells/mL was reached the cells were transferred to a 40 L bioreactor containing the same medium and setpoints for controlling the bioreactor. The temperature setpoint was

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 $Fc\Delta C1(a)$.

reduced to 34° C. to slow cell growth and increase the relative rate of protein expression.

Cell Culture Process Used to Produce Flt1D2.VEGFR3D3.Fc Δ C1(a). The same methodologies as described supra for Flt1D2.Flk1D3.Fc Δ C1(a) were used to 5 produce Flt1D2.VEGFR3D3.Fc Δ C1(a).

Example 4

Harvest and Purification of Modified Flt1 Receptors

Harvest and Purification of Flt1D2.Flk1D3.Fc Δ C1(a). The product protein was aseptically harvested from the bioreactor while retaining cells using Millipore Prostak tangential-flow filtration modules and a low-shear mechanical pump (Fristam). Fresh medium was added to the bioreactor to replace that removed during the harvest filtration. Approximately 40 L of harvest filtrate was then loaded onto a 400 mL column containing Protein A Sepharose resin (Amersham Pharmacia). After loading the resin was washed with buffer containing 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2 to remove any unbound contaminating proteins. Flt1D2.Flk1D3.Fc Δ C1(a) protein was eluted with a pH 3.0 citrate buffer. The eluted protein was neutralized by addition of Tris base and frozen at -20° C. 25

Several frozen lots of Flt1D2.Flk1D3.Fc Δ C1(a) protein from the Protein A step above were thawed, pooled and concentrated using a Millipore 30 kD nominal molecular weight cutoff (NMWCO) tangential flow filtration membrane. The protein was transferred to a stirred cell concentrator (Millipore) and further concentrated to 30 mg/mL using a 30 kD NMWCO membrane. The concentrated protein was loaded onto a size exclusion column packed with Superdex 200 resin (Amersham Pharmacia) that was equilibrated with phosphate buffered saline plus 5% glycerol. The same buffer was used to run the column. The fractions corresponding to Flt1D2.Flk1D3.Fc Δ C1(a) dimer were pooled, sterile filtered through a 0.22 micron filter, aliquoted and frozen.

Harvest and Purification of Flt1D2.VEGFR3D3.Fc Δ C1 40 (a). The same methodologies as described supra for Flt1D2.Flk1D3.Fc Δ C1(a) were used to harvest and purify Flt1D2.VEGFR3D3.Fc Δ C1(a).

Example 5

Binding Stoichiometry of Modified Flt Receptors to VEGF165

Biacore Analysis. The stoichiometry of 50 Flt1D2Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a) interaction with human VEGF165 was determined by measuring either the level of VEGF saturation binding to the Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a) surfaces or measuring concentration of VEGF165 needed to com-55 pletely prevent binding of Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a) to VEGF Biacore chip surface.

Modified Flt receptors Flt1D2Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a), were captured with an anti-Fc specific antibody that was first immobilized on a Biacore chip 60 (BIACORE) using amine-coupling chemistry. A blank antibody surface was used as a negative control. VEGF165 was injected at a concentration of 1 nM, 10 nM, and 50 nM over the Flt1D2Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a) surfaces at 10 µl/min for one hour. A real-time binding signal 65 was recorded and saturation binding was achieved at the end of each injection. Binding stoichiometry was calculated as a

molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a) molecule (FIG. 1).

In solution, Flt1D2Flk1D3.Fc\DeltaC1(a) or VEGFR1R2- $Fc\Delta C1(a)$ at a concentration of 1 nM (estimated to be 1000 times higher than the KD of the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-Fc Δ C1(a)/VEGF165 interaction) were mixed 10 with varied concentrations of VEGF165. After one hour incubation, concentrations of the free Flt1D2Flk1D3.Fc∆C1 (a) in solution were measured as a binding signal to an amine-coupled VEGF165 surface. A calibration curve was used to convert the Flt1D2Flk1D3.Fc\DeltaC1(a) Biacore binding signal to its molar concentration. The data showed that addition of 1 nM VEGF165 into the Flt1D2Flk1D3.Fc Δ C1(a) solution completely blocked Flt1D2Flk1D3.Fc∆C1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.FcΔC1(a) molecule. When the concentration of Flt1D2Flk1D3.Fc Δ C1(a) was plotted as a function of added concentration of VEGF165, the slope of the linear portion was -1.06 for Flt1D2Flk1D3.FcΔC1(a) and -1.07 for VEGFR1R2-FcΔC1 (a). The magnitude of the slope, very close to negative one, was indicative that one molecule of VEGF165 bound to one molecule of either Flt1D2Flk1D3.Fc\DeltaC1(a) or VEGFR1R2-

Size Exclusion Chromatography. Flt1D2Flk1D3.Fc Δ C1 (a) was mixed with a 3-fold excess of VEGF165 and the receptor-ligand complex was purified using a Pharmacia Superose 6 size exclusion chromatography column. The receptor-ligand complex was then incubated in a buffer containing 6M guanidine hydrochloride in order to dissociate it into its component proteins. Flt1D2Flk1D3.Fc Δ C1(a) was separated from VEGF165 using Superose 6 size exclusion chromatography column run in 6M guanidium chloride. In order to determine complex stoichiometry, several injections of Flt1D2Flk1D3.Fc Δ C1(a) and VEGF165 were made and peak height or peak integrated intensity was plotted as a function of the concentration of injected protein. The calibration was done under conditions identical to those used in separating components of Flt1D2Flk1D3.Fc Δ C1(a)/

45 VEGF complex. Quantification of the Flt1D2Flk1D3.FcΔC1 (a)/VEGF complex composition was based on the calibration curves. The results of this experiment (FIG. 1) shows the ratio of VEGF165 to Flt1D2Flk1D3.FcΔC1(a) in a complex to be 1:1.

Example 6

Pharmacokinetic Analysis of Modified Fit Receptors

Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a). Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.Fc Δ C1(a), CHO transiently expressed Flt1D2.Flk1D3.Fc Δ C1(a), and CHO transiently expressed VEGFR1R2-Fc Δ C1(a). The mice were tail bled at 1, 2, 4, 6, 24 hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a). The ELISA involves coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc (A40),

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Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) and reporting with an anti-Fc antibody linked to horseradish peroxidase. The results of this experiments are shown in FIG. **2**. The T_{max} for Flt1(1-3)-Fc (A40) was at 6 hrs while the T_{max} for the transient and stable Flt1D2.Flk1D3.FcΔC1 (a) and the transient VEGFR1R2-FcΔC1(a) was 24 hrs. The C_{max} for Flt1(1-3)-Fc (A40) was 8 µg/ml. For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the C_{max} was 18 µg/ml and the C_{max} for the stable VEGFR1R2-FcΔC1(a) was 30 µg/ml.

Pharmacokinetic analysis of Flt1(1-3)-Fc (A40). Flt1D2.Flk1D3.Fc\DeltaC1(a) and Flt1D2.VEGFR3D3.Fc\DeltaC1 (a). Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.Fc\DeltaC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.Fc∆C1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.Fc∆C1(a) and Flt1D2.VEGFR3D3.Fc Δ C1(a). The ELISA involves coating 20 an ELISA plate with 165, binding the Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) or Flt1D2.VEGFR3D3.FcΔC1(a) and reporting with an anti-Fc antibody linked to horseradish peroxidase. Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5, whereas Flt1D2.Flk1D3.Fc\DeltaC1(a) 25 and Flt1D2.VEGFR3D3.Fc Δ C1(a) were detectable for 15 days or more. The results of this experiment are shown in FIG. 3.

Example 7

Breakdown of Blood-Retinal Barrier Reversed by Inhibition of VEGF

Rats received a single injection of VEGFR1R2-Fc Δ C1(a) 35 (SEQ ID NO:6) (25 mg/kg, i.p.) or PBS 4 weeks after induction of diabetes by streptozotocin (65 mg/kg, i.v.). The permeability of retinal vessels was assessed 24 hours later by measuring the extravasation of Evans Blue dye, which binds to albumin in the circulation. Under deep anesthesia, 40 Evans Blue dye (45 mg/kg) was injected intravenously, and allowed to circulate for 60 minutes, and blood samples were taken periodically to assess the concentration of dye in the circulation. The animals were then perfused to flush dye and blood from the vasculature, the eye enucleated and the 45 retinas removed. Evans blue was extracted, and the concentration of dye in the retina was normalized to retinal weight and the time-averaged concentration of Evans blue in the circulation (mL plasma×g retina weight⁻¹×hr⁻¹) to provide an index of vascular leak. VEGFR1R2-Fc AC1(a) normal- 50 ized retinal vascular permeability to levels evident in nondiabetic rats.

Example 8

VEGFR1R2-FcΔC1(a) Prevents Neovascularization Induced by Retinal Ischemia

Excessive upregulation of VEGF expression is responsible for pathologic neovascularization in many retinal dis-60 eases. The anti-angiogenic properties of VEGFR1R2-Fc Δ C1 (a) were investigated in a mouse model of oxygen-induced ischemic retinopathy (OIR). OIR was produced by transiently exposing mouse pups to increased ambient oxygen (hyperoxia), resulting in obliteration of the developing 65 microvasculature within the central retina. Subsequent return of the animals to room air results in relatively hypoxic

conditions in the retina, which in turn stimulates an angiogenic response that shares features with both diabetic retinopathy, retinopathy of prematurity and other ischemic retinopathies. VEGFR1R2-Fc∆C1(a) (25 mg/kg, ip) was
administered every other day beginning 12-24 hours after returning the mice from hyperoxia to room air. Litternate controls received injections of human Fc following the same schedule. Retinas were collected 1 week following return to room air. Flat mounts were prepared from one retina
obtained from each animal, and the retinal vessels stained with fluoresceinated lectin (*Griffonia simplicifolia*). The other retina was embedded and cross-sections were cut and stained with hematoxylin and eosin.

Retinas of all control mice exposed to hyperoxia exhibited marked pathologic angiogenesis, characterized by the presence of vascular tufts penetrating the inner limiting membrane and chaotic sprouting of vessels on the surface of the retina, particularly around the optic nerve head. Administration of VEGFR1R2-FcAC1(a) almost completely blocked the development of these vascular abnormalities as quantitated by counting endothelial cell nuclei in the abnormal pre-retinal vessels (FIG. 4). Although pathologic angiogenesis was dramatically inhibited, systemic administration of VEGFR1R2-FcAC1(a) did not block the growth of normal appearing intraretinal vessels in these animals.

Example 9

Suppression of Choroidal Neovascularization

Though animals do not develop age related macular degeneration (AMD) per se, choroidal neovascularization resembling that seen in AMD can be produced by using a laser to produce focal disruptions in Bruch's membrane and the overlying retinal pigment epithelium (RPE). This injury stimulates the abnormal growth of underlying choroidal capillaries into the RPE layer and subretinal space. Disruption of Bruch's membrane is common to all forms of choroidal neovascularization (CNV), including that which characterizes the wet form of AMD. In the laser-induced model of choroidal neovascularization, groups of 9 or 10 mice were treated with subcutaneous (sc) injections of 25 mg/kg of VEGFR1R2-Fc Δ C1(a) or human Fc one day prior to laser injury and on days 2, 5, 8, and 11 after laser. At 14 days after laser injury, the mice were injected intravenously with fluorescein-labeled dextran (50 mg), euthanized, and eyes were rapidly dissected for choroidal flat mounts or frozen in optimum cutting temperature embedding compound and sectioned for evaluation of the lesions. VEGFR1R2-Fc∆C1(a) administration reduced the area of CNV lesions by approximately 70% (FIG. 5).

The effect of VEGFR1R2-FcAC1(a) on laser-induced choroidal neovascularization also was evaluated in adult cynomolgus monkeys. In this experiment, VEGFR1R2-55 FcΔC1(a) was administered by intravenous or intravitreal injection. Each animal received nine or ten laser burns to each retina, and the development of active choroidal neovascular lesions was assessed by fluorescein angiography, once before the initiation of treatment and 15, 20 and 29 days postlaser. VEGFR1R2-Fc\DeltaC1(a) was administered intravenously once per week, beginning one week before laser injury, at a dose of 3 mg/kg or 10 mg/kg. Intravitreal injections were made once every two weeks, at a dose of 50, 250 or 500 mcg/eye beginning one week before laser, or once, two weeks following laser (500 mcg dose only), at which time active CNV lesions had already formed. Control animals received weekly intravenous or biweekly intravit-

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real injections of placebo, beginning one week before laser. Each of the above experimental and control groups comprised six animals, 3 males and 3 females. CNV lesions were visualized by fluorescein angiography and graded. Active CNV lesions characterized bright hyperfluorescence, with late leakage beyond the borders of the laser spot (Grade 4), developed at 32% and 48% of the laser burn sites, in animals receiving intravitreal or intravenous administration of placebo. In contrast, the development of grade 4 lesions was completely or nearly completely prevented in all groups of 10 animals receiving intravenous or intravitreal injections of VEGFR1R2-Fc Δ C1(a) (FIG. 9). Moreover a single intravitreal injection (500 mcg) of VEGFR1R2-Fc\DeltaC1(a) made following the laser injury reduced the incidence of grade 4 lesions from 44% to 0% within 10 days of treatment (FIG. 15 chemical injury (NaOH) and mechanical debridement of the 9).

Example 10

Inhibition of Subretinal Neovascularization in rho/VEGF Transgenic Mice

Transgenic mice expressing a recombinant human VEGF transgene under the control of the rhodopsin promoter (Rho/VEGF) were used in these experiments. These animals 25 begin to express VEGF in photoreceptors on about postnatal day (P) 7, which typically results in pronounced subretinal neovascularization by P21. At P7, mice were divided into 2 groups and treated with 25 mg/kg of VEGFR1R2-Fc\DeltaC1(a) (9 mice, 17 eyes) or human Fc (10 mice, 19 eyes) on P7, 30 P10, P13, P16, and P19. On P21, the mice were anesthetized and perfused with fluorescein-labeled dextran. Retinal whole mounts from mice treated with VEGFR1R2-Fc∆C1 (a) showed few areas of neovascularization while many new vessels were present in the subretinal space of mice that had 35 been treated with Fc. Measurement of the total area of neovascularization per retina by image analysis showed significantly less neovascularization in VEGFR1R2-FcΔC1 (a)-treated mice, compared to those treated with Fc (FIG. 6).

Example 11

Suppression of VEGF-Induced Breakdown of the Blood-Retinal Barrier

Adult C57BL/6 mice were given a sc injection of 25 mg/kg of VEGFR1R2-Fc Δ C1(a) or Fc fragment and on the following day received an intravitreous injection of 1 ul of 10⁻⁶ M VEGF. Six hours later, retinal vascular permeability was measured using [³H]-mannitol as a tracer. Mice treated 50 with VEGFR1R2-Fc Δ C1(a) (9 mice, 18 eyes) had a significantly smaller retina to lung leakage ratio (the ratio of radioactivity in the retina compared to excised lung) than mice treated with Fc fragment (9 mice, 18 eyes) indicating less breakdown of the blood retinal barrier (FIG. 7A).

The effect of VEGFR1R2-Fc∆C1(a) on VEGF-mediated vascular leak was also evaluated in a second experiment, which employed double transgenic mice (rtTA/rho-TRE/ VEGF). These mice are characterized by photoreceptorspecific expression of the VEGF transgene that is inducible 60 by administration of doxycycline. Adult rtTA/rho-TRE/ VEGF mice were injected sc with 25 mg/kg VEGFR1R2-FcΔC1(a) (10 mice, 20 eyes) or Fc fragment (10 mice, 20 eyes). On the following day, doxycycline (2 mg/mL) was placed in their drinking water to stimulate over-expression 65 of VEGF within the retina. Two days later, they were given a second sc injection of VEGFR1R2-Fc∆C1(a) or Fc frag-

ment and then the next day retinal vascular permeability was measured with [3H]-mannitol. Mice treated with VEGFR1R2-Fc Δ C1(a) exhibited a significant reduction in the retina to lung leakage ratio compared to mice treated with Fc (FIG. 7B), indicating that impairment in the bloodretinal barrier was ameliorated.

Example 12

Inhibition Injury-Induced Corneal Neovascularization

Corneal neovascularization was induced in male C57BI/6 mice by intrastromal placement of 3 nylon sutures, or by corneal epithelium. Multiple experiments were conducted in which VEGFR1R2-Fc\DeltaC1(a) was administered intraperitoneally once or at multiple time points immediately before or following injury. The growth of corneal neovessels was 20 evaluated by slit-lamp microscopy and histological evaluation. The vasculature was labeled with an endothelial cell specific fluorescein-conjugated lectin, and neovascularization was evaluated in corneal flat-mounts, as well as in cross sections using PECAM immunohistochemistry. The presence of corneal edema was evaluated, using slit lamp microscopy, and corneal thickness was measured in crosssections; increases in corneal thickness reflect the amount of edema. The numbers of polymorphonuclear leukocytes (PMN) and macrophages were determined by staining crosssections with HEMA-3 or rat anti-mouse F4/80 monoclonal antibody, respectively.

Dosing regimens which employed multiple injections of VEGFR1R2-Fc∆C1(a) (25 mg/kg, ip) completely inhibited corneal neovascularization in both the suture and chemical injury models. Moreover, single injections of 25 or 12.5 mg/kg VEGFR1R2-Fc\DeltaC1(a) given immediately after suture injury effectively blocked corneal neovascularization for at least 9 days, while injections of 6.25 and 2.5 mg/kg ameliorated but did not block corneal neovascularization. 40 The lowest dose of VEGFR1R2-Fc Δ C1(a) tested (0.5 mg/kg) had no evident effect. Corneal thickness, reflecting the amount of edema present, was significantly reduced in VEGFR1R2-Fc∆C1(a)-treated animals compared to vehicle-treated controls (FIG. 8). Histological analyses 45 showed that the infiltration of neutrophils and macrophages into the damaged cornea also was dramatically reduced by VEGFR1R2-Fc Δ C1(a) treatment.

Example 13

Inhibition of Corneal Neovascularization and Conjunctivalization Following Alkali Burn Injury

Corneas were injured by application of NaOH and 55 mechanical debridement of the corneal epithelium in adult, male C57BI/6 mice. VEGFR1R2-FcAC1(a) or a control protein (human Fc) was administered subcutaneously (12.5 mg/kg) on days 0 (the day of injury), 7 and 14, at which time reepithelialization of the cornea was complete. The animals were euthanized on days 28 or 42 (14 or 28 days following the last injection of VEGFR1R2-Fc∆C1(a) and corneas taken for histological evaluation. Tissues were processed as described above.

Treatment with VEGFR1R2-Fc∆C1(a) inhibited corneal neovascularization during the period of active treatment (as determined by slit-lamp microscopy), as well as 2 and 4 weeks following treatment cessation. In eyes evaluated on

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day 28 (14 days after the last injection of VEGFR1R2-Fc Δ C1(a), the neovascular response to injury remained completely suppressed and conjunctivalization of the cornea was also inhibited as evidenced by a more normal appearing morphology of the re-epithelialized cornea and a substantial reduction in goblet cell number (~30% relative to controls). Corneal inflammation and edema also were reduced substantially. Evaluation of flat-mounted corneas taken at Day 42 showed that neovascularization was still largely suppressed, though limited, focal sprouting of vessels at the corneal margin was observed in some cases.

The data show that when administered at the time of injury, VEGFR1R2-Fc Δ C1 (a) improves corneal healing by potently inhibiting the development of corneal neovascularization, inflammation, edema and conjunctivalization of the corneal epithelium. These effects persisted for several weeks following cessation of treatment, suggesting that acute inhibition of VEGF following corneal injury may have long- $_{20}$ term benefits.

Example 14

In Vitro Assay with Baf/3 Cells Expressing a Chimeric VEGF Receptor

Materials. Cells: Baf/Flt(1-7)-EpoR, clone C1H. Media: RPMI 1640, 10% fetal bovine serum, penicillin (100 U/ml), 30 streptomycin (100 U/ml) and L-glutamine (2 mM). Growth factor: IL-3 (1 ng/ml). VEGF: VEGF 121 (R&D Biosystems). Detection: WST-8, CCK-8 kit from Dojindo Molecular Technologies. Instruments and analysis: Wallac Victor II Multilabel counter. All data analysed using Graphpad Prizm 35 software with the four parameter logistic equation.

To create a reproducible bioassay having a K_D close to the of the VEGF inhibitor or fusion protein "trap" of SEQ ID NO:6, a chimeric receptor containing the VEGFR1 extracellular domain fused to the cytoplasmic and transmembrane domains of human EpoR via a PGL peptide bridge was constructed. EpoR is able to potently drive proliferation of the mouse pro-B cell line, Baf/3. VEGF binding to the VEGFR1 extracellular domain causing receptor dimerization and activation of EpoR signaling. Neither VEGFR1 nor VEGFR2 native sequence receptors are capable of driving Baf/3 proliferation.

The receptor construct was inserted into a retroviral vector (CMV promotor-chimeric receptor-IRES-GFP) and 50 used to infect Baf/3 cells. Cells expressing GFP (green fluorescent protein) were isolated by 2 rounds of fluorescence activated cell sorting (FACS). This pool was further sorted for expression of VEGFR1. A clonal line was subsequently isolated and used for assay development. ⁵⁵

The derived cell line proliferates in response to VEGF₁₂₁ with an EC50 of approximately 18 pM after 3 days of growth. The growth response is measured by the bioreduction of the tetrazolium salt WST-8 provided in the CCK-8 kit. The growth response induced by the addition of 70 pM VEGF₁₂₁ is blocked by the VEGF trap protein (SEQ ID NO:6) with an IC₅₀ of approximately 40 pM. The IC₅₀ in this bioassay is 25 times larger than the biochemically determined Kd of 1.5 pM. FIG. **10** shows the growth response of 65 Bat/FIt cells grown in 0-900 pM VEGF measured by the bioreduction of a tetrazolium salt.

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Example 15

Inhibition of VEGF Growth Response by Two Different VEGF Inhibitors

The in vitro Baf/Flt cell line assay described above was used to measure the effect of two different VEGF inhibitors on the response to VEGF. Cells were incubated for 3 days in 70 pM VEGF and exposed to varying concentrations of VEGF trap (SEQ ID NO:6) (0-500 pM) or an anti-VEGF antibody (AvastinTM, Genentech) (0-500 nM). The results are shown in FIG. **11**. The IC₅₀ for the VEGF trap was 44 pM and for the anti-VEGF antibody 1.4 nM.

Example 16

Pharmacokinetic Analysis of Intravitreal Delivery of Two VEGF Inhibitors

Ocular and systemic levels of two VEGF inhibitors were determined after a single intravitreal administration to male Pigmented New Zealand Cross Bred rabbits. At various time points following the injection, the rabbits were sacrificed and vitreous, retina, and choroid tissues were collected, as well as blood samples for plasma and serum. All samples were analyzed in order to determine tissue and circulating levels of the VEGF trap protein of SEQ ID NO:6 or a truncated version termed a "mini-VEGF trap" lacking the human Fc component (SEQ ID NO:23) (described in U.S. 2004/0014667 and U.S. 2005/0043236, herein incorporated by reference in their entirety), as well as to determine the appropriate pharmacokinetic parameters for the proteins in ocular tissue and plasma. This information allows determination of the ability of an intravitreally administered protein to reach the desired site of action, i.e. the macula in the case of macular degeneration.

Sixty-six male Pigmented New Zealand Cross Bred rabbits (F1 cross New Zealand White and New Zealand Red) were randomly divided into 2 groups with each group 40 consisting of 33 rabbits. The animals in Group 1 were given a single intravitreal injection of full length VEGF trap protein (SEQ ID NO:6) into each eye at a dose of 500 micrograms/eye. The rabbits in Group 2 were given a single intravitreal administration of mini-VEGF trap into each eye 45 at a dose of 250 micrograms/eye. At each time point (predose, 0.25, 1, 6, 24, 72, 168, 336, 504, and 672 hrs postdose), three animals were anesthetized and blood was collected via cardiac puncture in order to obtain plasma and serum. At the time of sacrifice, both eyes were enucleated 50 from each animal and retina, choroids, and vitreous humor were collected.

Sample Processing. Generally, vitreal samples were thawed at room temperature and transferred to individual 5 mL polypropylene tubes. An equivalent weight per volume 55 of RIPA buffer (20 MM Tris HCl, pH 7.5, 5 mM benzamidine, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM EDTA) was added to each sample, and homogenized (Cyclone I.Q. Microprocessor, Sentry) for two 45 second cycles at 5,500 rpm. The samples were then incubated for 20 minutes on ice and then 60 centrifuged for 30 minute at 5,500 rpm at 4° C. The supernatant was removed and stored at -80° C. for analysis. Retinal and choroid samples were similarly processed the samples were homogenized for 30-60 seconds at the highest speed setting (Ultra Tunax T8 Homogenizer with S8N-5G Disposing Element, IKA Laboratories). The samples were transferred to individual 1.5 mL eppendorf tubes and incu-

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bated for 20 minutes on ice. They were then centrifuged for 30 minutes at 5,500 rpm, 4° C. The supernatant was removed, transferred to a new 1.5 mL eppendorf tube and stored at -80° C. for analysis.

Sample Analysis. In general, VEGF trap protein levels in the samples were measured using an enzyme-linked immunosorbent assay (ELISA) system where micro-titer plates were coated with human $VEGF_{165}$ antigen.

length or truncated VEGF trap protein into both rabbit eyes, the protein can be detected in both ocular tissue (vitreous humor, retina and choroid) and plasma for up to 672 hrs. These results demonstrated that if a compound is delivered into the vitreous humor, it can be cleared from that region 15 and be distributed into the surrounding tissue, i.e. retina and choroid, before reaching the circulation from which it is eliminated from the body. This is supported not only by the ability to detected and measure the amount of the two traps in the various tissues and plasma, but also by the time it 20 protein of SEQ ID NO:6 or 23. The purpose of this treatment takes for the protein to reach its Cmax in that particular tissue. For mini-VEGF trap protein, it reaches its maximal concentration in the vitreous humor 1 hr after injection. The protein then passes into the retina where the Cmax occurs 6.00 hr after the initial injection. The choroid, which is 25 adjacent to the retina, is with a Tmax of 24.0 hr, after which the protein can reach the circulation and achieve peak levels 72.0 hr after the injections. The full length VEGF trap also displayed a similar tissue progression, although the time frame for reaching the maximal concentrations was longer, in most cases, than that observed for mini-VEGF trap. Peak vitreous humor concentrations of VEGF trap were reached 6 hr after injection; retina followed with a Tmax at 24.0 hr. Choroid tissue had a Tmax of 15 min (0.250 hr), however, this result appears to be driven by a particular sample having 35 an extremely high level of the protein at that time. As observed with the mini-VEGF trap, peak plasma concentrations were reached 72.0 hr after the injections. Since animals injected with mini-VEGF trap received a dose that was half that of the full length protein (250 micrograms/eye vs. 500 40 micrograms/eye, respectively), the Cmax and AUC values in tissue and plasma tended to be less than that observed for VEGF trap. In the vitreous humor, the Cmax for the mini-VEGF trap was almost half that of the full length protein. 253 micrograms/eye vs. 491 micrograms/mL. In addition, 45 the AUC for the mini-VEGF trap was half that of VEGF trap; there was no apparent difference between the proteins in terms of t1/2 (115 hr vs. 112 hr). In choroid tissue obtained from rabbits which received mini VEGF trap, both the Cmax and AUC values were substantially lower (values 50 were a third (AUC) to an eighth (Cmax) lower) than that observed in samples from VEGF trap treated animals. This difference, especially with regards to AUC, could be accounted for by the decreased elimination t1/2 in the mini VEGF trap samples. The larger protein had a t1/2 of 131 hr 55 while the t1/2 of the smaller protein was 70.9 hr. This same scenario was observed with regards to the plasma samples. The full length VEGF trap samples had a greater Cmax, AUC and t1/2 than samples obtained from the smaller protein. In contrast to these other tissues, in retinal homo- 60 genates, both VEGF trap and mini VEGF trap had similar pharmacokinetic profiles. Despite receiving significantly different intravitreal doses, retinal homogenates had Cmax and AUC measurements that were nearly identical. The elimination half-life was shorter, however, in retinal tissue 65 obtained from mini1VEGF trap injected rabbits (132 hr vs. 114 hr).

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The results of this study demonstrate that both full-length VEGF trap and mini-VEGF trap can be injected intravitreally and that the proteins penetrate to the desired site of action, i.e. retina or related structure. The results show that the protein is present in the eye tissue for up to 672 hrs, thus allowing for monthly treatment paradigms. Further, once the mini-VEGF trap moves out of the eye tissue into the systemic circulation, it is cleared more quickly from the Results. After a single intravitreal injection of the full 10 body than the full-length VEGF trap, thus reducing unwanted systemic action.

Example 17

Treatment of Age-Related Macular Degeneration

A patient manifesting age-related macular degeneration is treated with an intravitreal injection of the VEGF trap is to reduce or prevent the development of neovascularization, macular disease, and retinal damage. Once a patient reaches the age of 60, increased ophthalmic surveillance is performed to detect the presence of AMD. This increased surveillance should include periodic retinal examinations and fluorescein angiograms to monitor for the presence of subretinal fluid, blood, exudates, RPE detachment, cvstic retinal changes, or the presence of grayish green subretinal neovascular membrane. When AMD is diagnosed, a regime of VEGF trap protein treatment is commenced coupled with or without other treatments such as photocoagulation. As the first step of treatment, the patient is to receive a full ophthalmic examination to establish a baseline of ocular health. The ophthalmic examination includes indirect ophthalmoscopy, slit-lamp biomicroscopy, peripheral retinal examination, intraocular pressure measurements, visual acuity (unaided and best corrected) symptomatology, fundus photography, fluorescein angiography, electroretinography and A-scan measurements. Following the preliminary examination, an intravitreal injection of VEGF trap protein is given to the patient's affected eye manifesting AMD. If both eyes are affected, they may be treated separately. The eye to be treated is injected with 25-4000 micorgrams of VEGF trap protein in an ophthalmic solution.

After treatment, the patients' eyes are to be examined on days one (1), two (2), seven (7), fifteen (15), thirty (30) and sixty (60). Because of the possibility of reoccurrence, the patient should return for periodic examinations on a monthly basis thereafter. On each examination day the patient is monitored for vitreous liquefaction. Additionally, the patient is monitored for posterior vitreous detachments using indirect ophthalmoscopy with scleral depression. Finally, the extent of AMD presented by the patient is continuously monitored through periodic retinal examinations and fluorescein angiograms to monitor for the presence of subretinal fluid, blood, exudates, RPE detachment, cystic retinal changes, or the presence of grayish green subretinal neovascular membrane. Additional VEGF trap protein treatments may be required if indicia of reoccurring neovascularization are observed. Additional treatments may be given on weekly or monthly basis. In a preferred embodiment, an initial treatment is followed by subsequent treatments between 1-6 months apart.

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Met Tyr Ser Glu Ile Pro Glu Ile His Met Thr Glu Gly Arg Glu 35 40 45

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Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu 50 55 60 LysLysPheProLeuAspThrLeuIleProAspGlyLysArgIleIle6570707580 Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu 85 90 95 Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys 100 105 110 Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Val 115 120 125 Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu Lys Leu Val 130 135 140 Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile Asp Phe Asn 145 150 155 160 Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu Val Asn Arg 165 170 175 Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe Leu Ser Thr 180 185 190 Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu Tyr Thr Cys 195 200 205 Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr Phe Val Arg 210 215 220 Val His Glu Lys Gly Pro Gly Asp Lys Thr His Thr Cys Pro Cys 225 230 230 235 240
 Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro

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 255
 Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys 260 265 270 Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp 275 280 285 Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu 290 295 300
 Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu

 305
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 320
 His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn 325 330 333 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 Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys 450 455

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1440

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aggetetgea caaccactac acgeagaaga geeteteeet gteteegggt aaatgagegg

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Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu 85 90 95 Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys 100 105 110 Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Ile Gln 115 120 120 Leu Leu Pro Arg Lys Ser Leu Glu Leu Leu Val Gly Glu Lys Leu Val 130 135 140 Trp Asp Tyr Pro Gly Lys Gln Ala Glu Arg Gly Lys Trp Val Pro Glu 165 170 175 Arg Arg Ser Gln Gln Thr His Thr Glu Leu Ser Ser Ile Leu Thr Ile 180 185 190 His Asn Val Ser Gln His Asp Leu Gly Ser Tyr Val Cys Lys Ala Asn 195 200 205 Asn Gly Ile Gln Arg Phe Arg Glu Ser Thr Glu Val Ile Val His Glu 210 215 220 Asn Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro225230240 Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys 245 250 250 255
 Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val 260
 265
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 Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp 275 280 285 Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr 290 295 300 Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp 305 310 315 320 Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu 325 330 335 Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg 340 345 350 Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys 355 360 365 Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp 370 375 380 Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys385390395400 Thr Thr Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser 405 410 415Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser 420 425 430Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser 435 440 445 Leu Ser Leu Ser Pro Gly Lys 450 455 <210> SEQ ID NO 5 <211> LENGTH: 1377 <212> TYPE: DNA <213> ORGANISM: homo sapien

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Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met 35 40 45	Thr Glu
Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn 50 55 60	Ile Thr
Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp 65 70 75	Gly Lys 80
Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn 85 90	Ala Thr 95

 Tyr
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 Glu
 Ile
 Gly
 Leu
 Leu
 Thr
 Cys
 Glu
 Ala
 Thr
 Val
 Asn
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 Tyr
 Lys
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 Tyr
 Leu
 Thr
 His
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 Glu
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Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu 130 135 140 Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Leu 165 170 170 175 Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe 180 185 190 Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu 195 200 205 Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr 210 215 220
 Phe Val Arg Val His Glu Lys Asp Lys Thr His Thr Cys Pro Cys

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 Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro 245 250 255 Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys 260 265 270 Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp 275 280 285 Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu 290 295 300 Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu 305 310 315 320 His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn 325 330 330 335 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
 Pro
 Pro
 Val
 Leu
 Asp
 Ser
 Asp
 Ser
 Phe
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Leu	Ser	Thr 195	Leu	Thr	Ile	Asp	Gly 200	Val	Thr	Arg	Ser	Asp 205	Gln	Gly	Leu
Tyr	Thr 210	Cys	Ala	Ala	Ser	Ser 215	Gly	Leu	Met	Thr	L y s 220	Lys	Asn	Ser	Thr
Phe 225	Val	Arg	Val	His	Glu 230	Lys	Asp	Lys	Thr	His 235	Thr	Cys	Pro	Pro	Cys 240

We claim:

1. A therapeutic method for treating or ameliorating an eye disorder, comprising administering a fusion polypeptide capable of binding vascular endothelial growth factor (VEGF) to a patient in need thereof, wherein the fusion polypeptide consists of an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor Flt1 and Ig domain 3 of a second VEGF receptor Flt4 or Flt4; and a multimerizing component, wherein the multimerizing component is an immunoglobulin domain or fragment of an immunoglobulin domain, wherein the eye disorder is selected from the group consisting of eye drops, subconjunctival injection, intravitreal implant, sub-Tenon's injection, and sub-Ten

2. The therapeutic method of claim **1**, wherein the fusion polypeptide comprises SEQ ID NO:6.

3. A therapeutic method for treating or ameliorating an eye disorder, comprising administering a dimeric protein comprising two fusion polypeptides, wherein each fusion polypeptide consists of the amino acid sequence of SEQ ID NO:6, wherein the eye disorder is selected from the group consisting of age related macular degeneration and diabetic retinopathy, and wherein administration is selected from the group consisting of eye drops, subconjunctival injection, subconjunctival implant, intravitreal injection, implant.

4. A method for the treatment of a human subject diagnosed with age-related macular degeneration, comprising administering an effective amount of a vascular endothelial growth factor (VEGF) inhibitor to the human subject, the method comprising:

- (a) administering to the subject an initial dose of at least approximately 25-4000 micrograms VEGF inhibitor protein per eye; and
- (b) administering to the subject a plurality of subsequent doses of the VEGF inhibitor protein in an amount that

is approximately the same or less than the initial dose, wherein the subsequent doses are separated in time from each other by at least two weeks, wherein the VEGF inhibitor is a dimeric protein comprising two fusion polypeptides, wherein each fusion polypeptide consists of an immunoglobulin-like (lg) domain 2 of a first VEGF receptor Flt1 and Ig domain 3 of a second VEGF receptor Flt1 or Flt4, and a multimerizing component, and wherein the multimerizing component is an immunoglobulin domain or fragment of an immunoglobulin domain, and wherein administration is selected from the group consiting of eye drops, subconjunctival injection, subconjunctival implant, intravitreal injection, intravitreal implant, sub-Tenon's injection, and sub-Tenon's implant.

5. The method of claim **4**, wherein the initial dose is at least approximately 50 micrograms of VEGF inhibitor protein.

6. The method of claim **5**, wherein the initial dose is at least approximately 100 micrograms of VEGF inhibitor protein.

7. The method of claim 6, wherein the initial dose is at least approximately 1000 micrograms of VEGF inhibitor protein.

8. The method of claim 4, wherein the subsequent doses are separated in time from each other by at least four weeks.

9. The method of claim **8**, wherein the subsequent doses are separated in time from each other by at least 3 to 6 months.

10. The method of claim 4, wherein the initial dose and at least one subsequent dose is administered by intravitreal injection.

11. The method of claim **4**, wherein the VEGF inhibitor is a dimer having two fusion polypeptides, wherein each fusion polypeptide comprises the amino acid sequence of SEQ ID NO:6.

* * * * *

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US007521049B2

(12) United States Patent Wiegand et al.

(54) USE OF VEGF INHIBITORS FOR TREATMENT OF EYE DISORDERS

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- (*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.
- (21) Appl. No.: 11/998,709
- (22) Filed: Nov. 30, 2007

(65) Prior Publication Data

US 2008/0220004 A1 Sep. 11, 2008

Related U.S. Application Data

- (60) Division of application No. 11/218,234, filed on Sep. 1, 2005, now Pat. No. 7,303,747, which is a continuation-in-part of application No. 11/089,803, filed on Mar. 25, 2005, now Pat. No. 7,306,799, which is a continuation-in-part of application No. 10/988,243, filed on Nov. 12, 2004, now Pat. No. 7,303,746, which is a continuation-in-part of application No. 10/009, 852, filed as application No. PCT/US00/14142 on May 23, 2000, now Pat. No. 7,070,959, said application No. 11/218,234 is a continuation-in-part of application No. 11/218,234 is a continuation-in-part of application No. 10/88,0021, filed on Jun. 29, 2004, now Pat. No. 7,279, 159, which is a continuation-in-part of application No. 10/609,775, filed on Jun. 30, 2003, now Pat. No. 7,087, 411.
- (60) Provisional application No. 60/138,133, filed on Jun. 8, 1999.
- (51) Int. Cl.

A61K 38/18	(2006.01)
C07K 14/71	(2006.01)
C12N 15/62	(2006.01)

(10) Patent No.: US 7,521,049 B2

(45) **Date of Patent:** Apr. 21, 2009

- (52) U.S. Cl. 424/134.1; 424/192.1; 514/2; 514/12; 530/350; 536/23.4
- (58) **Field of Classification Search** None See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,100,071	Α	8/2000	Davis-Smyth et al.
6,897,294	B2	5/2005	Davis-Smyth et al.
2005/0281831	A1	12/2005	Davis-Smyth et al.

FOREIGN PATENT DOCUMENTS

WO	WO97/44453	11/1997
WO	WO98/13071	4/1998
WO	WO99/03996	1/1999

OTHER PUBLICATIONS

Terman, B.I., et al., (1991) Oncogene 6:1677-1683.

Terman, B.I., et al., (1992) Biochem. Biophys. Res. Comm. 187(3):1579-1586.

Davis-Smyth, T., et al., (1996) The EMBO Journal 15(18):4919-4927.

Holash, J., et al., (2002) PNAS 99(17):11393-11398.

Heidaran, M.A., et al., (1990) J. Bio. Chem. 265(31):18741-18744. Cunningham, S.A., et al., (1997) Biochem. Biophys. Res. Comm. 231:596-599.

Fuh, G., et al., (1998) J. Bio. Chem. 273(18):11197-11204.

Wiesmann, C., et al., (1997) Cell 91:695-704.

Barleon, B., et al., (1997) J. Bio. Chem. 272(16):10382-10388. Davis-Smyth, T., et al., (1998) J. Bio. Chem. 273(6):3216-3222.

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(57) ABSTRACT

Modified chimeric polypeptides with improved pharmacokinetics and improved tissue penetration are disclosed useful for treating eye disorders, including age-related macular degeneration and diabetic retinopathy.

15 Claims, 11 Drawing Sheets

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Binding Stoichiomet	ry of hVEGF165 to Flt1D2Flk1D3. F	с∆С1(a) & VEGFR1R2- Fc∆С1(a)
hVEGF165 (nM)	VEGF/Flt1D2FlkD3.FcAC1(a)	VEGF/VEGFR1R2- FcAC1(a)
1	0.93	0.98
10	0.97	0.94
50	-	0.99
Average ± StDev	0.96 ± 0.03	0.97 ± 0.02

Figure 1

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Figure 2

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Figure 3

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Figure 4

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Figure 8



% Grade 4 Lesions

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USE OF VEGF INHIBITORS FOR TREATMENT OF EYE DISORDERS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of U.S. patent application Ser. No. 11/218,234 filed 1 Sep. 2005, now U.S. Pat. No. 7,303,747, which is a continuation-in-part of application Ser. No. 11/089.803 filed 25 Mar. 2005, now U.S. Pat. No. 7.306, 10 799, which is a continuation-in-part of application Ser. No. 10/988,243 filed 12 Nov. 2004, now U.S. Pat. No. 7,303,746, which is a continuation-in-part of application Ser. No. 10/009.852 filed 6 Dec. 2001, now U.S. Pat. No. 7.070,959, which is the National Stage of International Application No. 15 PCT/US00/14142 filed 23 May 2000, which claims the benefit under 35 USC § 119(e) of U.S. Provisional 60/138,133 filed 8 Jun. 1999, and U.S. patent application Ser. No. 11/218, 234 filed 1 Sep. 2005, now U.S. Pat. No. 7,303,747 is a continuation-in-part of application Ser. No. 10/880,021 filed 20 29 Jun. 2004, now U.S. Pat. No. 7,279,159, which is a continuation-in-part of application Ser. No. 10/609,775 filed 30 Jun. 2003, now U.S. Pat. No. 7,087,411, which applications are herein specifically incorporated by reference in their entireties 25

BACKGROUND

Statement Regarding Related Art

A class of cell-derived dimeric mitogens with selectivity for vascular endothelial cells has been identified and designated vascular endothelial cell growth factor (VEGF). VEGF is a dimer with an apparent molecular mass of about 26 kDa with each subunit having an apparent molecular mass of 35 about 23 kDa. The membrane-bound tyrosine kinase receptor, known as Flt (also known as VEGFR2), was shown to be a VEGF receptor (DeVries et al. (1992) Science 255:989-991). Another form of the VEGF receptor, designated KDR or Flk-1 (also known as VEGFR3), is also known to bind VEGF and induce mitogenesis (Terman et al. (1991) Oncogene 6:1677-1683; Terman et al. (1992) Biochem. Biophys. Res. Comm. 187:1579-1586).

U.S. Pat. No. 6,011,003 describes an altered, soluble form of Flt polypeptide capable of binding to VEGF comprising 45 five or fewer complete immunoglobulin domains. WO 97/44453 describes chimeric VEGF receptor proteins comprising amino acid sequences derived from VEGF receptors Flt1 and KDR.

BRIEF SUMMARY OF THE INVENTION

The invention features a therapeutic method for treating or ameliorating an eye disorder, comprising administering a vascular endothelial growth factor (VEGF) inhibitor to a patient in need thereof. In one embodiment, the eye disorder treated is age related macular degeneration. In another embodiment, the eye disorder treated is diabetic retinopathy.

Preferably, the VEGF inhibitor used in the method of the invention comprises an immunoglobulin-like (Ig) domain 2 60 of a first VEGF receptor and Ig domain 3 of a second VEGF receptor; and a multimerizing component, wherein the first VEGF receptor is Flt1, the second VEGF receptor is Flk1 or Flt4, and the multimerizing component is selected from the group consisting of (i) an amino acid sequence between 1 to 65 about 200 amino acids in length having at least one cysteine residue, and (ii) an immunoglobulin domain, or fragment of

an immunoglobulin domain. In specific embodiments, the VEGF inhibitor is a fusion polypeptide "VEGF trap" selected from the group consisting of SEQ ID NO:2 (Flt1D2.Flk1D3Fc Δ C1(a)), SEQ ÌD NO:4 (Flt1D2.VEGFR3D3.Fc Δ C1(a)), SEO ID NO:6 (VEGFR1R2 Fc Δ C1(a)), and SEQ ID NO:23. In another embodiment, the VEGF inhibitor is a fusion polypeptide encoded by a nucleotide sequence selected from the group consisting of SEQ ID NO:1, 3, 5, 22, and a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of SEQ ID NO:1, 3, 5, and 22

In a second aspect, the invention features a method for the treatment of a human subject diagnosed with an eye disorder, comprising administering an effective amount of a vascular endothelial growth factor (VEGF) inhibitor to the human subject, the method comprising administering to the subject an initial dose of at least approximately 25-4000 ug VEGF inhibitor protein to an affected eye, and administering to the subject a plurality of subsequent doses of the VEGF inhibitor protein in an amount that is approximately the same or less than the initial dose, wherein the subsequent doses are separated in time from each other by at least two weeks. The eye disorder is one of age-related macular degeneration or diabetic retinopathy. In various embodiments, the initial dose is at least approximately 25 to 4000 ug of VEGF inhibitor protein. In various embodiments, the subsequent doses are separated in time from each other by at least two weeks to 12 months; more preferably, the subsequent doses are separated in time from each other by at least 3-6 months. The VEGF inhibitor protein is administered directly to the affected eye, including by use of eye drops or intravitreal injection. Preferably, the VEGF inhibitor is a dimer having two fusion polypeptides consisting essentially of an immunoglobulinlike (Ig) domain 2 of Flt1 and Ig domain 3 of Flk1 or Flt4, and a multimerizing component. In specific embodiments, the VEGF inhibitor is a dimer comprising the fusion polypeptide of SEO ID NO:2, 4, 6, or 23.

Other objects and advantages will become apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Biacore analysis of binding stoichiometry. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml.

⁵⁰ FIG. **2**. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a).

FIG. **3**. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc Δ C1(a) and Flt1D2.VEGFR3D3.Fc Δ C1 (a).

FIG. 4. VEGFR1R2-Fc Δ C1(a) prevents neovascularization induced by retinal ischemia. Serial 10 µm cross sections were collected and stained with hematoxylin and eosin. For each animal, nuclei in preretinal neovessels were counted in a series of ten sections within 300 microns of the optic nerve head and averaged. Counts were obtained in three independent experiments, n \geq 4 for each treatment group in each study.

FIG. 5. Effect of subcutaneous VEGFR1R2-Fc Δ C1(a) injections on choroidal neovascularization area. The size of CNV lesions was measured in choroidal flat mounts. The images were digitized using an Axioskop microscope equipped with a video camera, and the total area of choroidal

Mylan Exhibit 1016 Mylan v. Regeneron, IPR2021-00880 Page 135 Joining Petitioner: Apotex neovascularization associated with each laser burn was measured using Image-Pro Plus software.

FIG. 6. VEGFR1R2-Fc Δ C1(a) inhibits subretinal neovascularization in Rho/VEGF transgenic mice.

FIGS. 7A-B. VEGF-Induced breakdown of the blood retinal barrier. A. Following intravitreal injections of VEGF, adult mice (C57BL/6) treated with injections of VEGFR1R2-Fc Δ C1(a) had a significantly smaller retina to lung leakage ratio than mice treated with Fc fragment, indicating less breakdown of BRB. B. Double transgenic mice treated with injections of VEGFR1R2-Fc Δ C1(a) had a significant reduction in the retina to lung leakage ratio compared to mice treated with Fc fragment.

FIG. **8**. Effect of VEGFR1R2-Fc Δ C1(a) administration on corneal thickness in suture and alkali burn models of corneal ¹⁵ trauma. Corneas were injured by suture placement or application of NaOH as described, and a single dose of VEGFR1R2-Fc Δ C1(a) (25 mg/kg, ip) or saline (n=5 per group) was administered immediately following injury. The contralateral cornea served as normal, undamaged controls. ²⁰ Corneas were collected 7 days later and cross-sections were cut and stained with hematoxylin and eosin. Corneal thickness was measured as an index of corneal edema.

FIG. **9**. System or intravitreal VEGF trap protein administration prevents laser-induced choroidal neovascularization ²⁵ (CNV) and reverses vascular leak in established lesions.

FIG. **10**. Dose response curve of Baf/Flt cells grown in VEGF.

FIG. **11**. Inhibition of VEGF growth response by VEGF $_{30}$ trap VEGFR1R2-Fc Δ C1(a) or anti-VEGF antibody.

DETAILED DESCRIPTION OF THE INVENTION

It has been a longstanding problem in the art to produce a 35 receptor-based VEGF antagonist that has a pharmacokinetic profile that is appropriate for consideration of the antagonist as a therapeutic candidate. Applicants describe herein, for the first time, a chimeric polypeptide molecule, capable of antagonizing VEGF activity, that exhibits improved pharmacokinetic properties as compared to other known receptorbased VEGF antagonists. The chimeric polypeptide molecules described herein thus provide appropriate molecules for use in therapies in which antagonism of VEGF is a desired result. 45

The extracellular ligand binding domain is defined as the portion of a receptor that, in its native conformation in the cell membrane, is oriented extracellularly where it can contact with its cognate ligand. The extracellular ligand binding domain does not include the hydrophobic amino acids associated with the receptor's transmembrane domain or any amino acids associated with the receptor's intracellular domain. Generally, the intracellular or cytoplasmic domain of a receptor is usually composed of positively charged or polar amino acids (i.e. lysine, arginine, histidine, glutamic acid, 55 aspartic acid). The preceding 15-30, predominantly hydrophobic or apolar amino acids (i.e. leucine, valine, isoleucine, and phenylalanine) comprise the transmembrane domain. The extracellular domain comprises the amino acids that precede the hydrophobic transmembrane stretch of amino 60 acids. Usually the transmembrane domain is flanked by positively charged or polar amino acids such as lysine or arginine. von Heijne has published detailed rules that are commonly referred to by skilled artisans when determining which amino acids of a given receptor belong to the extracellular, trans- 65 membrane, or intracellular domains (See, von Heijne (1995) BioEssays 17:25.

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Nucleic Acid Constructs and Encoded Fusion Polypeptides

The present invention provides for the construction of nucleic acid molecules encoding chimeric polypeptide molecules that are inserted into a vector that is able to express the chimeric polypeptide molecules when introduced into an appropriate host cell. Appropriate host cells include, but are not limited to, bacterial cells, yeast cells, insect cells, and mammalian cells. Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the chimeric polypeptide molecules under control of transcriptional/translational control signals. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (See Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, N.Y.).

Expression of nucleic acid molecules encoding the chimeric polypeptide molecules may be regulated by a second nucleic acid sequence so that the chimeric polypeptide molecule is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the chimeric polypeptide molecules described herein may be controlled by any promoter/enhancer element known in the art.

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising chimeric polypeptide molecule-encoding nucleic acids as described herein, are used to transfect the host and thereby direct expression of such nucleic acids to produce the chimeric polypeptide molecules, which may then be recovered in a biologically active form. As used herein, a biologically active form includes a form capable of binding to VEGF. Expression vectors containing the chimeric nucleic acid molecules described herein can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to the inserted chimeric polypeptide molecule sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the chimeric polypeptide molecule DNA sequence is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaving the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the chimeric polypeptide molecules.

Cells of the present invention may transiently or, preferably, constitutively and permanently express the chimeric polypeptide molecules.

The chimeric polypeptide molecules may be purified by any technique which allows for the subsequent formation of a stable, biologically active chimeric polypeptide molecule. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis (see, for example, U.S. Pat. No. 5,663,304). In order to further purify the factors, conventional ion exchange chromatography,

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hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

The method of the invention encompasses the use of a fusion protein consisting essentially of first and second vascular endothelial growth factor (VEGF) receptor components and a multimerizing component, wherein the first VEGF receptor component is an immunoglobulin-like (Ig) domain 2 of Flt1, the second VEGF receptor component is an Ig domain 3 of a Flk1 or Flt4, and the multimerizing component is selected from the group consisting of (i) a multimerizing 10 component comprising a cleavable region (C-region), (ii) a truncated multimerizing component, (iii) an amino acid sequence between 1 to about 200 amino acids in length having at least one cysteine residue, (iv) a leucine zipper, (v) a helix loop motif, (vi) a coil-coil motif, and (vii) an immuno- 15 globulin domain. Examples of the VEGF inhibitors useful in the method of the invention include fusion proteins encoded by a nucleotide sequence selected from the group consisting of the nucleotide sequence of SEQ ID NO:1, 3, 5, 22, and a nucleotide sequence which, as a result of the degeneracy of 20 the genetic code, differs from the nucleotide sequence of SEQ ID NO:1, 3, 5, or 22, and fusion protein selected from the group consisting of SEQ ID NO:2 (Flt1D2.Flk1D3Fc∆C1 (a)), SEQ ID NO:4 (Flt1D2.VEGFR3D3.FcΔC1(a)), SEQ ID NO:6 (VEGFR1R2 Fc\DeltaC1(a)) and (SEQ ID NO:23).

Therapeutic Methods

The present invention also has diagnostic and therapeutic utilities. In particular embodiments of the invention, methods of detecting aberrancies in the function or expression of the chimeric polypeptide molecules described herein may be used in the diagnosis of disorders. In other embodiments, manipulation of the chimeric polypeptide molecules or agonists or antagonists which bind the chimeric polypeptide molecules may be used in the treatment of diseases. In further embodiments, the chimeric polypeptide molecule is utilized as an agent to block the binding of a binding agent to its target.

By way of example, but not limitation, the method of the invention may be useful in treating clinical conditions that are characterized by vascular permeability, edema or inflammation such as brain edema associated with injury, stroke or tumor; edema associated with inflammatory disorders such as psoriasis or arthritis, including rheumatoid arthritis; asthma; generalized edema associated with burns; ascites and pleural effusion associated with tumors, inflammation or trauma; chronic airway inflammation; capillary leak syndrome; sepsis; kidney disease associated with increased leakage of protein; and eye disorders such as age related macular degeneration and diabetic retinopathy.

Combination Therapies

In numerous embodiments, a VEGF inhibitor may be administered in combination with one or more additional compounds or therapies, including a second VEGF inhibitor. Combination therapy includes administration of a single pharmaceutical dosage formulation which contains a VEGF inhibitor molecule and one or more additional agents; as well as administration of a VEGF inhibitor and one or more additional agent(s) in its own separate pharmaceutical dosage formulation. For example, a VEGF inhibitor and a cytotoxic agent, a chemotherapeutic agent or a growth inhibitory agent 60 can be administered to the patient together in a single dosage composition such as a combined formulation, or each agent can be administered in a separate dosage formulation. Where separate dosage formulations are used, the VEGF-specific fusion protein of the invention and one or more additional 65 agents can be administered concurrently, or at separately staggered times, i.e., sequentially. The therapeutic methods of

the invention may also be combined with other agents or medical procedures used for treatment of eye disorders.

Treatment Population

The eye comprises several structurally and functionally distinct vascular beds, which supply ocular components critical to the maintenance of vision. These include the retinal and choroidal vasculatures, which supply the inner and outer portions of the retina, respectively, and the limbal vasculature located at the periphery of the cornea. Injuries and diseases that impair the normal structure or function of these vascular beds are among the leading causes of visual impairment and blindness. For example, diabetic retinopathy is the most common disease affecting the retinal vasculature, and is the leading cause of vision loss among the working age population in the United States. Vascularization of the cornea secondary to injury or disease is yet another category of ocular vascular disease that can lead to severe impairment of vision.

"Macular degeneration" is a medical term that applies to any of several disease syndromes which involve a gradual loss or impairment of eyesight due to cell and tissue degeneration of the yellow macular region in the center of the retina. Macular degeneration is often characterized as one of two types, non-exudative (dry form) or exudative (wet form). Although both types are bilateral and progressive, each type may reflect different pathological processes. The wet form of age-related macular degeneration (AMD) is the most common form of choroidal neovascularization and a leading cause of blindness in the elderly. AMD affects millions of Americans over the age of 60, and is the leading cause of new blindness among the elderly. It is characterized and usually diagnosed by the presence of elevated levels of two types of cellular debris within the retina, called drusen and lipofuscin.

There are several types of symptomatic treatment, however, that have been used with limited and isolated success, depending on the particular condition of the patient, to treat exudative (wet form) macular degeneration. Laser photocoagulation therapy may benefit certain patients with macular degeneration. However, there are high recurrence rates for selected choroidal neovascular membranes which may initially respond to laser therapy. Vision loss may also result from the laser therapy. Low dose radiation (teletherapy) has also been hypothesized as a possible treatment to induce regression of choroidal neovascularization. Surgical removal of neovascular membranes is another possible treatment, but it is a highly specialized procedure and reportedly has not had promising results to date. There is presently no effective treatment for non-exudative (dry form) macular degeneration. Management of non-exudative macular degeneration is 50 limited to early diagnosis and careful follow-up to determine if the patient develops choroidal neovascularization. Protection against exposure to ultraviolet light and prescribed dosages of anti-oxidant vitamins (e.g., vitamin A, β-carotene, lutein, zeaxanthin, vitamin C and vitamin E) and zinc may 55 also be of some benefit, but as yet these treatments remain unproven.

Accordingly, the population to be treated by the method of the invention is preferably one of (i) a human subject diagnosed as suffering from macular degeneration, (ii) a human subject diagnosed as suffering from diabetes-related retinopathy, and (iii) a human subject suffering from pathological vascularization of the cornea secondary to injury or disease.

Methods of Administration and Compositions

Preferably, administration of the VEGF inhibitor will be directly to the eye, e.g., topical. Topical methods of administration include, for example, by eye drops, subconjunctival

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injections or implants, intravitreal injections or implants, sub-Tenon's injections or implants, incorporation in surgical irrigating solutions, etc.

Compositions suitable for topical administration are known to the art (see, for example, US Patent Application 2005/0059639). In various embodiments, compositions of the invention can comprise a liquid comprising an active agent in solution, in suspension, or both. As used herein, liquid compositions include gels. Preferably the liquid composition is aqueous. Alternatively, the composition can take 10 form of an ointment. In a preferred embodiment, the composition is an in situ gellable aqueous composition, more preferably an in situ gellable aqueous solution. Such a composition can comprise a gelling agent in a concentration effective to promote gelling upon contact with the eye or lacrimal fluid in the exterior of the eye. Aqueous compositions of the invention have ophthalmically compatible pH and osmolality. The composition can comprise an ophthalmic depot formulation comprising an active agent for subconjunctival administration. The microparticles comprising active agent can be embedded in a biocompatible pharmaceutically acceptable polymer or a lipid encapsulating agent. The depot formulations may be adapted to release all or substantially all the active material over an extended period of time. The polymer or lipid matrix, if present, may be adapted to degrade sufficiently to be transported from the site of administration after release of all or substantially all the active agent. The depot formulation can be a liquid formulation, comprising a pharmaceutical acceptable polymer and a dissolved or dispersed active agent, Upon injection, the polymer forms a depot at the injections site, e.g. by gelifying or precipitating. The composition can comprise a solid article that can be inserted in a suitable location in the eye, such as between the eye and eyelid or in the conjuctival sac, where the article releases the active agent. Solid articles suitable for implantation in the eye 35 in such fashion generally comprise polymers and can be bioerodible or non-bioerodible.

In one embodiment of the method of the invention, a human subject with at least one visually impaired eye is treated with 25-4000 ug of a VEGF inhibitor protein via 40 intravitreal injection. Improvement of clinical symptoms are monitored by one or more methods known to the art, for example, indirect ophthalmoscopy, fundus photography, fluorescein angiopathy, electroretinography, external eye examination, slit lamp biomicroscopy, applanation tonom-45 etry, pachymetry, and autorefaction. Subsequent doses may be administered weekly or monthly, e.g., with a frequency of 2-8 weeks or 1-12 months apart.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodi- 50 ments which are given for illustration of the invention and are not intended to be limiting thereof.

EXAMPLES

Example 1

Modified Flt1 Receptor Vector Construction

Chimeric molecules were constructed, denoted R1R2 60 (Flt1.D2.Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a) and R1R3 (Flt1D2.VEGFR3D3-Fc Δ C1(a) and VEGFR1R3-Fc Δ C1(a) respectively, wherein R1 and Flt1D2=Ig domain 2 of Flt1 (VEGFR1); R2 and Flk1D3=Ig domain 3 of Flk1 (VEGFR2); and R3 and VEGFR3D3=Ig domain 3 of Flt4 65 (VEGFR3)) were much less sticky to ECM, as judged by an in vitro ECM binding assay and had greatly improved PK as

described herein. In addition, these molecules were able to bind VEGF tightly and block phosphorylation of the native Flk1 receptor expressed in endothelial cells.

Construction of the expression plasmid $pFlt1D2.Flk1D3.Fc\DeltaC1(a).$ Expression plasmids pMT21.Flt1(1-3).Fc (6519 bp) and pMT21.Flk-1(1-3).Fc (5230 bp) are plasmids that encode ampicillin resistance and Fc-tagged versions of Ig domains 1-3 of human Flt1 and human Flk1, respectively. These plasmids were used to construct a DNA fragment consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 3 of Flk1, using PCR amplification of the respective Ig domains followed by further rounds of PCR to achieve fusion of the two domains into a single fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows: 5': bsp/flt1D2 (5'-GACTAGCAGTCCGG-AGGTAGACCTTTCGTAGAGATG-3') (SEQ ID NO:8), 3': Flt1D2-Flk1D3.as (5'-CGGACTCAGAACCACATCTAT-GATTGTATTGGT-3') (SEQ ID NO:9). The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO:10) corresponding to amino acids 27-33 of SEQ ID NO:2. The 3' primer encodes the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of SEQ ID NO:2) and continuing into VVLS (SEQ ID NO:7) (corresponding to amino acids 127-130 of SEQ ID NO:2) of Flk1

For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as follows: 5': Flt1D2-Flk1D3.s (5'-ACAATCATAGAT-GTGGTTCTGAGTCCGTCTCATGG-3') (SEQ ID NO:11); Flk1D3/apa/srf.as (5'-GATAATGCCCGGGC-CCTTTTCATGGACCCTGACAAATG-3') (SEQ ID NO:12). The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (SEQ ID NO:13) (corresponding to amino acids 223-228 of SEQ ID NO:2), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of SEQ ID NO:2.

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/ flt1D2 and Flk1D3/apa/srf.as (described supra) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 614 bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ Δ B2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then 55 digested with the restriction enzymes EcoRI and SrfI and the resulting 702 bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-Fc Δ C1(a) to produce the plasmid pFlt1D2.Flk1D3.Fc\DeltaC1(a). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.FcΔC1(a) chimeric molecule is shown in SEO ID NO:1-2.

Construction of the expression plasmid pFlt1D2VEGFR3D3Fc Δ C1(a). The expression plasmid pMT21.Flt1(1-3).Fc (6519 bp) encodes ampicillin resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1 receptor. This plasmid was used to produce a DNA fragment containing Ig domain 2 of Flt1 by PCR. RNA from the cell

Mylan Exhibit 1016 Mylan v. Regeneron, IPR2021-00880 Page 138 Joining Petitioner: Apotex line HEL921.7 was used to produce Ig domain 3 of Flk1, using standard RT-PCR methodology. A further round of PCR amplification was used to achieve fusion of the two Ig domains into a single fused fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows: 5': bsp/ 5 flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTT-CG-TAGAGATG-3') (SEQ ID NO:14). Flt1D2.VEGFR3D3.as (TTCCTGGGCAACAGCTG-GATA-TCTATGATTGTATTGGT) (SEQ ID NO:15). The 5' amplification primer encodes a BspE1 restriction site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO:10) (corresponding to amino acids 27-33 of SEQ ID NO:1-2). The 3' amplification primer encodes the reverse complement of the end of Flt1 Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of SEQ ID NO:4) and continuing into IQLL of VEGFR3 (corresponding to amino acids 127-130 of SEO ID NO:4). 20

For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as follows: 5': R3D3.s (ATCCAGCTGTTGC-CCAGGAAGTCGCTGGAGCTGCTGGTA) (SEQ ID NO:17), 3': R3D3.as (ATTTTCATGCACAATGACCTCG-GTGCTCTCCCGAAATCG) (SEQ ID NO:18). Both the 5' ²⁵ and 3' amplification primers match the sequence of VEGFR3. The 296 bp amplification product of this RT-PCR reaction was isolated by standard techniques and subjected to a second round of PCR to add suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The follows: amplification primers were as 5':Flt1D2.VEGFR3D3.s(TCATAGATATCCAGCTGTTGC-CCAGGAAGTCGCTGGAG) (SEQ ID NO:19), 3': VEGFR3D3/srf.as (GATAATGCCCGGGCCATTTTCATG-CACAATGACCTCGGT) (SEQ ID NO:20). The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' 40 end of VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (SEQ ID NO:21) (corresponding to amino acids 221-226 of SEQ ID NO:4), followed by a bridging sequence that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to $\ ^{45}$ amino acids 227-229 of SEQ ID NO:4.

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described supra, to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 625 bp fragment 55 was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/Flt1AB2.Fc (described supra), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc. The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested 60 with the restriction enzymes EcoRI and SrfI and the resulting 693 bp fragment was subcloned into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3) Δ B2-Fc Δ C1(a) to produce the plasmid designated pFlt1D2.VEGFR3D3.FcΔC1(a). The complete DNA deduced amino acid sequence of the 65 Flt1D2.VEGFR3D3.Fc∆C1(a) chimeric molecule is shown in SEQ ID NO:3-4.

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Example 2

Construction pVEGFR1R2-Fc\DeltaC1(a) Expression Vector

The pVEGFR1R2.Fc Δ C1(a) (SEQ ID NO:15-16) expression plasmid was constructed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of SEQ ID NO:6) between Flt1d2-Flk1d3-Fc Δ C1(a) amino acids 26 and 27 of SEQ ID NO:2 (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231. The SDT amino acid sequence is native to the Flt1 receptor and was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2.Fc Δ C1 (a) chimeric molecule is shown in SEQ ID NO:5-6.

Example 3

Cell Culture Process Used to Produce Modified Flt1 Receptors

Cell Culture Process Used to Produce Flt1D2.Flk1D3.Fc Δ C1(a). The process for production of Flt1D2.Flk1D3.Fc Δ C1(a) protein using the expression plasmid pFlt1D2.Flk1D3.Fc Δ C1(a) involves suspension culture of recombinant Chinese hamster ovary (CHO K1/E1A) cells which constitutively express the protein product. The cells are grown in bioreactors and the protein product is isolated and purified by affinity and size exclusion chromatography.

Cell Expansion. Two confluent T-225 cm² flasks containing the Flt1D2.Flk1D3.Fc Δ C1(a) expressing cell line were expanded by passaging cells into eight T-225 cm² flasks in medium (GMEM+10% serum, GIBCO) and incubated at 37° C. and 5% CO₂. When the flasks approached confluence (approximately 3 to 4 days) the cells were detached using trypsin. Fresh medium was added to protect the cells from further exposure to the trypsin. The cells were centrifuged and resuspended in fresh medium then transferred to eight 850 cm² roller bottles and incubated at 37° C. and 5% CO₂ until confluent.

Suspension Culture in Bioreactors. Cells grown in roller bottles were trypsinized to detach them from the surface and washed with suspension culture medium. The cells are aseptically transferred to a 5 L bioreactor (New Brunswick Celligen Plus) where the cells are grown in 3.5 L of suspension culture. The suspension culture medium was a glutamine-free low glucose modification of IS-CHO (Irvine Scientific) to which 5% fetal bovine serum (Hyclone), GS supplement (Life Technologies) and 25 µM methionine sulfoximine (Sigma) was added. The pH was controlled at 7.2 by addition of carbon dioxide to the inlet gas or by addition of a liquid solution of sodium carbonate to the bioreactor. Dissolved oxygen level was maintained at 30% of saturation by addition of oxygen or nitrogen to the inlet gas and temperature controlled at 37° C. When a density of 4×10⁶ cells/mL was reached the cells were transferred to a 40 L bioreactor containing the same medium and setpoints for controlling the bioreactor. The temperature setpoint was reduced to 34° C. to slow cell growth and increase the relative rate of protein expression.

Cell Culture Process Used to Produce Flt1D2.VEGFR3D3.Fc Δ C1(a). The same methodologies as described supra for Flt1D2.Flk1D3.Fc Δ C1(a) were used to produce Flt1D2.VEGFR3D3.Fc Δ C1(a).

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Harvest and Purification of Modified Flt1 Receptors

Harvest and Purification of Flt1D2.Flk1D3.Fc Δ C1(a). The sproduct protein was aseptically harvested from the bioreactor while retaining cells using Millipore Prostak tangential-flow filtration modules and a low-shear mechanical pump (Fristam). Fresh medium was added to the bioreactor to replace that removed during the harvest filtration. Approximately 140L of harvest filtrate was then loaded onto a 400 mL column containing Protein A Sepharose resin (Amersham Pharmacia). After loading the resin was washed with buffer containing 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2 to remove any unbound contaminating proteins. 1 Flt1D2.Flk1D3.Fc Δ C1(a) protein was eluted with a pH 3.0 citrate buffer. The eluted protein was neutralized by addition of Tris base and frozen at -20° C.

Several frozen lots of Flt1D2.Flk1D3.Fc Δ C1(a) protein from the Protein A step above were thawed, pooled and concentrated using a Millipore 30 kD nominal molecular weight cutoff (NMWCO) tangential flow filtration membrane. The protein was transferred to a stirred cell concentrator (Millipore) and further concentrated to 30 mg/mL using a 30 kD NMWCO membrane. The concentrated protein was loaded 25 onto a size exclusion column packed with Superdex 200 resin (Amersham Pharmacia) that was equilibrated with phosphate buffered saline plus 5% glycerol. The same buffer was used to run the column. The fractions corresponding to Flt1D2.Flk1D3.Fc Δ C1(a) dimer were pooled, sterile filtered 30 through a 0.22 micron filter, aliquoted and frozen.

Harvest and Purification of Flt1D2.VEGFR3D3.Fc Δ C1 (a). The same methodologies as described supra for Flt1D2.Flk1D3.Fc Δ C1(a) were used to harvest and purify Flt1D2.VEGFR3D3.Fc Δ C1(a).

Example 5

Binding Stoichiometry of Modified Flt Receptors to VEGF165

Biacore Analysis. The stoichiometry of Flt1D2Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a) interaction with human VEGF165 was determined by measuring either the level of VEGF saturation binding to the 45 Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a) surfaces or measuring concentration of VEGF165 needed to completely prevent binding of Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a) to VEGF Biacore chip surface.

Modified Flt receptors Flt1D2Flk1D3.Fc\DeltaC1(a) and 50 VEGFR1R2-Fc Δ C1(a), were captured with an anti-Fc specific antibody that was first immobilized on a Biacore chip (BIACORE) using amine-coupling chemistry. A blank antibody surface was used as a negative control. VEGF165 was injected at a concentration of 1 nM, 10 nM, and 50 nM over 55 the Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a) surfaces at 10 µl/min for one hour. A real-time binding signal was recorded and saturation binding was achieved at the end of each injection. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized 60 Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3.Fc∆C1(a) or VEGFR1R2-Fc Δ C1(a) molecule (FIG. 1).

In solution, Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a) at a concentration of 1 nM (estimated to be 1000

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times higher than the KD of the Flt1D2Flk1D3.Fc\DeltaC1(a) or VEGFRIR2-Fc∆C1(a)/VEGF165 interaction) were mixed with varied concentrations of VEGF165. After one hour incubation, concentrations of the free Flt1D2Flk1D3.FcΔC1(a) in solution were measured as a binding signal to an aminecoupled VEGF165 surface. A calibration curve was used to convert the Flt1D2Flk1D3.Fc∆C1(a) Biacore binding signal to its molar concentration. The data showed that the addition of 1 nMVEGF165 into the Flt1D2Flk1D3.Fc\DeltaC1(a) solution completely blocked Flt1D2Flk1D3.Fc Δ C1(a) binding to the VEGF165 surface. This result suggested the binding stoichiof one VEGF165 molecule per ometrv one Flt1D2Flk1D3.Fc Δ C1(a) molecule. When the concentration of Flt1D2Flk1D3.Fc Δ C1(a) was plotted as a function of added concentration of VEGF165, the slope of the linear portion was -1.06 for Flt1D2Flk1D3.Fc\DeltaC1(a) and -1.07 for VEGFR1R2-Fc Δ C1(a). The magnitude of the slope, very close to negative one, was indicative that one molecule of VEGF165 bound to one molecule of either

Flt1D2Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a). Size Exclusion Chromatography. Flt1D2Flk1D3.Fc∆C1 (a) was mixed with a 3-fold excess of VEGF165 and the receptor-ligand complex was purified using a Pharmacia Superose 6 size exclusion chromatography column. The receptor-ligand complex was then incubated in a buffer containing 6M guanidine hydrochloride in order to dissociate it into its component proteins. Flt1D2Flk1D3.Fc Δ C1(a) was separated from VEGF165 using Superose 6 size exclusion chromatography column run in 6M guanidium chloride. In order to determine complex stoichiometry, several injections of Flt1D2Flk1D3.Fc\DeltaC1(a) and VEGF165 were made and peak height or peak integrated intensity was plotted as a function of the concentration of injected protein. The calibration was done under conditions identical to those used in separating components of Flt1D2Flk1D3.Fc\DeltaC1(a)/VEGF 35 complex. Quantification of the Flt1D2Flk1D3.Fc Δ C1(a)/ VEGF complex composition was based on the calibration curves. The results of this experiment (FIG. 1) shows the ratio of VEGF165 to Flt1D2Flk1D3.Fc Δ C1(a) in a complex to be 40 1:1.

Example 6

Pharmacokinetic Analysis of Modified Flt Receptors

Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a). Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed $Flt1D2.Flk1D3.Fc\DeltaC1(a),$ CHO stably expressed Flt1D2.Flk1D3.Fc∆C1(a), and CHO transiently expressed VEGFR1R2-Fc Δ C1(a). The mice were tail bled at 1, 2, 4, 6. 24 hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a). The ELISA involves coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc (A40). Flt1D2.Flk1D3.Fc Δ C1(a) or VEGFR1R2-Fc Δ C1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. The results of this experiments are shown in FIG. 2. The T_{max} for Flt1(1-3)-Fc (A40) was at 6 hrs while the T_{max} for the transient and stable Flt1D2.Flk1D3.Fc Δ C1(a) and the transient VEGFR1R2-Fc Δ C1(a) was 24 hrs. The C_{max} for Flt1(1-3)-Fc (A40) was 8 µg/ml. For both transients (Flt1D2.Flk1D3.Fc Δ C1(a) and VEGFR1R2-Fc Δ C1(a)) the C_{max} was 18 µg/ml and the C_{max} for the stable VEGFR1R2-Fc Δ C1(a) was 30 µg/ml.

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Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1 (a). Balb/c mice (25-30 g) were injected subcutaneously with 4 mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.Fc Δ C1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.Fc Δ C1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1 (a). The ELISA involves coating an ELISA plate with 165, 10 binding the Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) or Flt1D2.VEGFR3D3.FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5, whereas. Flt1D2.Flk1D3.Fc Δ C1(a) and 15 Flt1D2.VEGFR3D3.Fc Δ C1(a) were detectable for 15 days or more. The results of this experiment are shown in FIG. 3.

Example 7

Breakdown of Blood-retinal Barrier Reversed by Inhibition of VEGF

Rats received a single injection of VEGFR1R2-FcΔC1(a) (SEQ ID NO:6) (25 mg/kg, i.p.) or PBS 4 weeks after induc- 25 tion of diabetes by streptozotocin (65 mg/kg, i.v.). The permeability of retinal vessels was assessed 24 hours later by measuring the extravasation of Evans Blue dye, which binds to albumin in the circulation. Under deep anesthesia, Evans Blue dye (45 mg/kg) was injected intravenously, and allowed to circulate for 60 minutes, and blood samples were taken periodically to assess the concentration of dye in the circulation. The animals were then perfused to flush dye and blood from the vasculature, the eye enucleated and the retinas removed. Evans blue was extracted, and the concentration of 35 dye in the retina was normalized to retinal weight and the time-averaged concentration of Evans blue in the circulation $(\Box L \text{ plasma×g retina weight}^{-1} \times hr^{-1})$ to provide an index of vascular leak. VEGFR1R2-Fc Δ C1(a) normalized retinal vascular permeability to levels evident in non-diabetic rats. 40

Example 8

VEGFR1R2-Fc∆C1(a) Prevents Neovascularization Induced by Retinal Ischemia

Excessive upregulation of VEGF expression is responsible for pathologic neovascularization in many retinal diseases. The anti-angiogenic properties of VEGFR1R2-Fc∆C1(a) were investigated in a mouse model of oxygen-induced 50 ischemic retinopathy (OIR). OIR was produced by transiently exposing mouse pups to increased ambient oxygen (hyperoxia), resulting in obliteration of the developing microvasculature within the central retina. Subsequent return of the animals to room air results in relatively hypoxic conditions in the 55 retina, which in turn stimulates an angiogenic response that shares features with both diabetic retinopathy, retinopathy of prematurity and other ischemic retinopathies. VEGFR1R2- $Fc\Delta C1(a)$ (25 mg/kg, ip) was administered every other day beginning 12-24 hours after returning the mice from hyper- 60 oxia to room air. Littermate controls received injections of human Fc following the same schedule. Retinas were collected 1 week following return to room air. Flat mounts were prepared from one retina obtained from each animal, and the retinal vessels stained with fluoresceinated lectin (Griffonia 65 simplicifolia). The other retina was embedded and crosssections were cut and stained with hematoxylin and eosin.

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Retinas of all control mice exposed to hyperoxia exhibited marked pathologic angiogenesis, characterized by the presence of vascular tufts penetrating the inner limiting membrane and chaotic sprouting of vessels on the surface of the retina, particularly around the optic nerve head. Administration of VEGFR1R2-Fc Δ C1(a) almost completely blocked the development of these vascular abnormalities as quantitated by counting endothelial cell nuclei in the abnormal pre-retinal vessels (FIG. 4). Although pathologic angiogenesis was dramatically inhibited, systemic administration of VEGFR1R2-Fc Δ C1(a) did not block the growth of normal-appearing intraretinal vessels in these animals.

Example 9

Suppression of Choroidal Neovascularization

Though animals do not develop age related macular degen-20 eration (AMD) per se, choroidal neovascularization resembling that seen in AMD can be produced by using a laser to produce focal disruptions in Bruch's membrane and the overlying retinal pigment epithelium (RPE). This injury stimulates the abnormal growth of underlying choroidal capillaries into the RPE layer and subretinal space. Disruption of Bruch's membrane is common to all forms of choroidal neovascularization (CNV), including that which characterizes the wet form of AMD. In the laser-induced model of choroidal neovascularization, groups of 9 or 10 mice were treated with subcutaneous (sc) injections of 25 mg/kg of VEGFR1R2-Fc Δ C1(a) or human Fc one day prior to laser injury and on days 2, 5, 8, and 11 after laser. At 14 days after laser injury, the mice were injected intravenously with fluorescein-labeled dextran (50 mg), euthanized, and eyes were rapidly dissected for choroidal flat mounts or frozen in optimum cutting temperature embedding compound and sectioned for evaluation of the lesions. VEGFR1R2-Fc Δ C1(a) administration reduced the area of CNV lesions by approximately 70% (FIG. 5).

The effect of VEGFR1R2-Fc Δ C1(a) on laser-induced choroidal neovascularization also was evaluated in adult cynomolgus monkeys. In this experiment, VEGFR1R2-Fc\DeltaC1(a) was administered by intravenous or intravitreal injection. Each animal received nine or ten laser burns to each retina, 45 and the development of active choroidal neovascular lesions was assessed by fluorescein angiography, once before the initiation of treatment and 15, 20 and 29 days postlaser. VEGFR1R2-Fc Δ C1(a) was administered intravenously once per week, beginning one week before laser injury, at a dose of 3 mg/kg or 10 mg/kg. Intravitreal injections were made once every two weeks, at a dose of 50, 250 or 500 mcg/eye beginning one week before laser, or once, two weeks following laser (500 mcg dose only), at which time active CNV lesions had already formed. Control animals received weekly intravenous or biweekly intravitreal injections of placebo, beginning one week before laser. Each of the above experimental and control groups comprised six animals, 3 males and 3 females. CNV lesions were visualized by fluorescein angiography and graded. Active CNV lesions characterized bright hyperfluorescence, with late leakage beyond the borders of the laser spot (Grade 4), developed at 32% and 48% of the laser burn sites, in animals receiving intravitreal or intravenous administration of placebo. In contrast, the development of grade 4 lesions was completely or nearly completely prevented in all groups of animals receiving intravenous or intravitreal injections of VEGFR1R2-Fc\DeltaC1(a) (FIG. 9). Moreover a single intravitreal injection (500 mcg) of VEGFR1R2-

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 $Fc\Delta C1(a)$ made following the laser injury reduced the incidence of grade 4 lesions from 44% to 0% within 10 days of treatment (FIG. 9).

Example 10

Inhibition of Subretinal Neovascularization in rho/VEGF Transgenic Mice

Transgenic mice expressing a recombinant human VEGF 10 transgene under the control of the rhodopsin promoter (Rho/ VEGF) were used in these experiments. These animals begin to express VEGF in photoreceptors on about postnatal day (P) 7, which typically results in pronounced subretinal neovascularization by P21. At P7, mice were divided into 2 groups 15 and treated with 25 mg/kg of VEGFR1R2-Fc Δ C1(a) (9 mice, 17 eyes) or human Fc (10 mice, 19 eyes) on P7, P10, P13, P16, and P19. On P21, the mice were anesthetized and perfused with fluorescein-labeled dextran. Retinal whole mounts from mice treated with VEGFR1R2-Fc Δ C1(a) showed few areas of 20 neovascularization while many new vessels were present in the subretinal space of mice that had been treated with Fc. Measurement of the total area of neovascularization per retina by image analysis showed significantly less neovascularization in VEGFR1R2-Fc∆C1(a)-treated mice, compared to 25 those treated with Fc (FIG. 6).

Example 11

Suppression of VEGF-induced Breakdown of the Blood-Retinal Barrier

Adult C57BL/6 mice were given a sc injection of 25 mg/kg of VEGFR1R2-Fc Δ C1(a) or Fc fragment and on the following day received an intravitreous injection of 1 μ l of 10⁻⁶ M ₃₅ VEGF. Six hours later, retinal vascular permeability was measured using [³H]-mannitol as a tracer. Mice treated with VEGFR1R2-Fc Δ C1(a) (9 mice, 18 eyes) had a significantly smaller retina to lung leakage ratio (the ratio of radioactivity in the retina compared to excised lung) than mice treated with 40 Fc fragment (9 mice, 18 eyes) indicating less breakdown of the blood retinal barrier (FIG. 7A).

The effect of VEGFR1R2-Fc∆C1(a) on VEGF-mediated vascular leak was also evaluated in a second experiment, which employed double transgenic mice (rtTA/rho-TRE/ 45 VEGF). These mice are characterized by photoreceptor-specific expression of the VEGF transgene that is inducible by administration of doxycycline. Adult rtTA/rho-TRE/VEGF mice were injected sc with 25 mg/kg VEGFR1R2-Fc∆C1(a) (10 mice, 20 eyes) or Fc fragment (10 mice, 20 eyes). On the 50 following day, doxycycline (2 mg/mL) was placed in their drinking water to stimulate over-expression of VEGF within the retina. Two days later, they were given a second sc injection of VEGFR1R2-Fc\DeltaC1(a) or Fc fragment and then the next day retinal vascular permeability was measured with 55 [³H]-mannitol. Mice treated with VEGFR1R2-Fc Δ C1(a) exhibited a significant reduction in the retina to lung leakage ratio compared to mice treated with Fc (FIG. 7B), indicating that impairment in the blood-retinal barrier was ameliorated.

Example 12

Inhibition Injury-Induced Corneal Neovascularization

Corneal neovascularization was induced in male C57BI/6 mice by intrastromal placement of 3 nylon sutures, or by

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chemical injury (NaOH) and mechanical debridement of the corneal epithelium. Multiple experiments were conducted in which VEGFR1R2-Fc∆C1(a) was administered intraperitoneally once or at multiple time points immediately before or following injury. The growth of corneal neovessels was evaluated by slit-lamp microscopy and histological evaluation. The vasculature was labeled with an endothelial cell specific fluorescein-conjugated lectin, and neovascularization was evaluated in corneal flat-mounts, as well as in cross sections using PECAM immunohistochemistry. The presence of corneal edema was evaluated, using slit lamp microscopy, and corneal thickness was measured in cross-sections; increases in corneal thickness reflect the amount of edema. The numbers of polymorphonuclear leukocytes (PMN) and macrophages were determined by staining cross-sections with HEMA-3 or rat anti-mouse F4/80 monoclonal antibody, respectively.

Dosing regimens which employed multiple injections of VEGFR1R2-FcΔC1(a) (25 mg/kg, ip) completely inhibited corneal neovascularization in both the suture and chemical injury models. Moreover, single injections of 25 or 12.5 mg/kgVEGFR1R2-Fc Δ C1(a) given immediately after suture injury effectively blocked corneal neovascularization for at least 9 days, while injections of 6.25 and 2.5 mg/kg ameliorated but did not block corneal neovascularization. The lowest dose of VEGFR1R2-Fc\DeltaC1(a) tested (0.5 mg/kg) had no evident effect. Corneal thickness, reflecting the amount of edema present, was significantly reduced in VEGFR1R2-Fc C1(a)-treated animals compared to vehicle-treated controls (FIG. 8). Histological analyses showed that the infiltration of neutrophils and macrophages into the damaged cornea also was dramatically reduced by VEGFR1R2-Fc\DeltaC1(a) treatment.

Example 13

Inhibition of Corneal Neovascularization and Conjunctivalization Following Alkali Burn Injury

Corneas were injured by application of NaOH and mechanical debridement of the corneal epithelium in adult, male C57BI/6 mice. VEGFR1R2-Fc Δ C1(a) or a control protein (human Fc) was administered subcutaneously (12.5 mg/kg) on days 0 (the day of injury), 7 and 14, at which time re-epithelialization of the cornea was complete. The animals were euthanized on days 28 or 42 (14 or 28 days following the last injection of VEGFR1R2-Fc Δ C1(a) and corneas taken for histological evaluation. Tissues were processed as described above.

Treatment with VEGFR1R2-FcΔC1(a) inhibited corneal
neovascularization during the period of active treatment (as determined by slit-lamp microscopy), as well as 2 and 4 weeks following treatment cessation. In eyes evaluated on day 28 (14 days after the last injection of VEGFR1R2-FcΔC1 (a), the neovascular response to injury remained completely
suppressed and conjunctivalization of the cornea was also inhibited as evidenced by a more normal appearing morphology of the re-epithelialized cornea and a substantial reduction in goblet cell number (~30% relative to controls). Corneal inflammation and edema also were reduced substantially.
Evaluation of flat-mounted corneas taken at Day 42 showed that neovascularization was still largely suppressed, though limited, focal sprouting of vessels at the corneal margin was observed in some cases.

The data show that when administered at the time of injury, VEGFR1R2-Fc Δ C1(a) improves corneal healing by potently inhibiting the development of corneal neovascularization, inflammation, edema and conjunctivalization of the corneal

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epithelium. These effects persisted for several weeks following cessation of treatment, suggesting that acute inhibition of VEGF following corneal injury may have long-term benefits.

Example 14

In Vitro Assay with Baf/3 Cells Expressing a Chimeric VEGF Receptor

Materials. Cells: Baf/Flt(1-7)-EpoR, clone C1H. Media: RPMI 1640, 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 U/ml) and L-glutamine (2 mM). Growth factor: IL-3 (1 ng/ml). VEGF: VEGF 121 (R&D Biosystems). Detection: WST-8, CCK-8 kit from Dojindo Molecular Technologies. Instruments and analysis: Wallac Victor II Multilabel counter. All data analysed using Graphpad Prizm software with the four parameter logistic equation.

To create a reproducible bioassay having a K_D close to the of the VEGF inhibitor or fusion protein "trap" of SEQ ID NO:6, a chimeric receptor containing the VEGFR1 extracellular domain fused to the cytoplasmic and transmembrane domains of human EpoR via a PGL peptide bridge was constructed. EpoR is able to potently drive proliferation of the mouse pro-B cell line, Baf/3. VEGF binding to the VEGFR1 extracellular domain causing receptor dimerization and activation of EpoR signaling. Neither VEGFR1 nor VEGFR2 native sequence receptors are capable of driving Baf/3 proliferation.

The receptor construct was inserted into a retroviral vector (CMV promotor-chimeric receptor-IRES-GFP) and used to 30 infect Baf/3 cells. Cells expressing GFP (green fluorescent protein) were isolated by 2 rounds of fluorescence activated cell sorting (FACS). This pool was further sorted for expression of VEGFR1. A clonal line was subsequently isolated and used for assay development.

The derived cell line proliferates in response to VEGF₁₂₁ with an EC50 of approximately 18 pM after 3 days of growth. The growth response is measured by the bioreduction of the tetrazolium salt WST-8 provided in the CCK-8 kit. The growth response induced by the addition of 70 pM VEGF₁₂₁ 40 is blocked by the VEGF trap protein (SEQ ID NO:6) with an IC₅₀ of approximately 40 pM. The IC₅₀ in this bioassay is 25 times larger than the biochemically determined Kd of 1.5 pM. FIG. **10** shows the growth response of Baf/Flt cells grown in 0-900 pM VEGF measured by the bioreduction of a tetrazo-45 lium salt.

Example 15

Inhibition of VEGF Growth Response by Two Different VEGF Inhibitors

The in vitro Baf/Flt cell line assay described above was used to measure the effect of two different VEGF inhibitors on the response to VEGF. Cells were incubated for 3 days in 70 pM VEGF and exposed to varying concentrations of ⁵⁵ VEGF trap (SEQ ID NO:6) (0-500 pM) or an anti-VEGF antibody (AvastinTM, Genentech) (0-500 nM). The results are shown in FIG. **11**. The IC₅₀ for the VEGF trap was 44 pM and for the anti-VEGF antibody 1.4 nM.

Example 16

Pharmacokinetic Analysis of Intravitreal Delivery of Two VEGF Inhibitors

Ocular and systemic levels of two VEGF inhibitors were determined after a single intravitreal administration to male

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Pigmented New Zealand Cross Bred rabbits. At various time points following the injection, the rabbits were sacrificed and vitreous, retina, and choroid tissues were collected, as well as blood samples for plasma and serum. All samples were analyzed in order to determine tissue and circulating levels of the VEGF trap protein of SEQ ID NO:6 or a truncated version termed a "mini-VEGF trap" lacking the human Fc component (SEQ ID NO:23) (described in US 2004/0014667 and US 2005/0043236, herein incorporated by reference in their entirety), as well as to determine the appropriate pharmacokinetic parameters for the proteins in ocular tissue and plasma. This information allows determination of the ability of an intravitreally administered protein to reach the desired site of action, i.e. the macula in the case of macular degeneration.

66 male Pigmented New Zealand Cross Bred rabbits (F1 cross New Zealand White and New Zealand Red) were randomly divided into 2 groups with each group consisting of 33 rabbits. The animals in Group 1 were given a single intravitreal injection of full length VEGF trap protein (SEQ ID NO:6) into each eye at a dose of 500 ug/eye. The rabbits in Group 2 were given a single intravitreal administration of mini-VEGF trap into each eye at a dose of 250 ug/eye. At each time point (predose, 0.25, 1, 6, 24, 72, 168, 336, 504, and 672 hrs postdose), three animals were anesthetized and blood was collected via cardiac puncture in order to obtain plasma and serum. At the time of sacrifice, both eyes were enucleated from each animal and retina, choroids, and vitreous humor were collected.

Sample Processing. Generally, vitreal samples were thawed at room temperature and transferred to individual 5 mL polypropylene tubes. An equivalent weight per volume of RIPA buffer (20 MM Tris HCl, pH 7.5, 5 mM benzamidine, 150 mM sodium chloride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM EDTA) was added to each sample, and homogenized (Cyclone I.Q. Microprocessor, Sentry) for 2, 45 second cycles at 5,500 rpm. The samples were then incubated for 20 minutes on ice and then centrifuged for 30 minute at 5,500 rpm at 4° C. The supernatant was removed and stored at -80° C. for analysis. Retinal and choroid samples were similarly processed the samples were homogenized for 30-60 seconds at the highest speed setting (Ultra Tunax T8 Homogenizer with S8N-5G Disposing Element, IKA Laboratoies). The samples were transferred to individual 1.5 mL eppendorf tubes and incubated for 20 minutes on ice. They were then centrifuged for 30 minutes at 5,500 rpm, 4° C. The supernatant was removed, transferred to

a new 1.5 mL eppendorf tube and stored at -80° C. for analysis.
 Sample Analysis. In general, VEGF trap protein levels in

the samples were measured using an enzyme-linked immunosorbent assay (ELISA) system where micro-titer plates were coated with human VEGF165 antigen.

Results. After a single intravitreal injection of the full 55 length or truncated VEGF trap protein into both rabbit eyes, the protein can be detected in both ocular tissue (vitreous humor, retina and choroid) and plasma for up to 672 hrs. These results demonstrated that if a compound is delivered into the vitreous humor, it can be cleared from that region and 60 be distributed into the surrounding tissue, i.e. retina and choroid, before reaching the circulation from which it is eliminated from the body. This is supported not only by the ability to detected and measure the amount of the two traps in the various tissues and plasma, but also by the time it takes for the 65 protein to reach its Cmax in that particular tissue. For mini-VEGF trap protein, it reaches its maximal concentration in the vitreous humor 1 hr after injection. The protein then

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passes into the retina where the Cmax occurs 6.00 hr after the initial injection. The choroid, which is adjacent to the retina. is with a Tmax of 24.0 hr, after which the protein can reach the circulation and achieve peak levels 72.0 hr after the injections. The full length VEGF trap also displayed a similar tissue progression, although the time frame for reaching the maximal concentrations was longer, in most cases, than that observed for mini-VEGF trap. Peak vitreous humor concentrations of VEGF trap were reached 6 hr after injection; retina followed with a Tmax at 24.0 hr. Choroid tissue had a Tmax 10 of 15 min (0.250 hr), however, this result appears to be driven by a particular sample having an extremely high level of the protein at that time. As observed with the mini-VEGF trap, peak plasma concentrations were reached 72.0 hr after the injections. Since animals injected with mini-VEGF trap received a dose that was half that of the full length protein (250 g/eye vs. 500 g/eye, respectively), the Cmax and AUC values in tissue and plasma tended to be less than that observed for VEGF trap. In the vitreous humor, the Cmax for the mini-VEGF trap was almost half that of the full length 20 protein, 253 g/mL vs 491 g/mL. In addition, the AUC for the mini-VEGF trap was half that of VEGF trap; there was no apparent difference between the proteins in terms of $t^{1/2}$ (115 hr vs. 112 hr). In choroid tissue obtained from rabbits which received mini VEGF trap, both the Cmax and AUC values 25 were substantially lower (values were a third (AUC) to an eighth (Cmax) lower) than that observed in samples from VEGF trap treated animals. This difference, especially with regards to AUC, could be accounted for by the decreased elimination t¹/₂ in the mini VEGF trap samples. The larger protein had a $t^{1/2}$ of 131 hr while the $t^{1/2}$ of the smaller protein was 70.9 hr. This same scenario was observed with regards to the plasma samples. The full length VEGF trap samples had a greater Cmax, AUC and t1/2 than samples obtained from the smaller protein. In contrast to these other tissues, in retinal 35 homogenates, both VEGF trap and mini VEGF trap had similar pharmacokinetic profiles. Despite receiving significantly different intravitreal doses, retinal homogenates had Cmax and AUC measurements that were nearly identical. The elimination half-life was shorter, however, in retinal tissue 40 obtained from mini1VEGF trap injected rabbits (132 hr vs. 114 hr).

The results of this study demonstrate that both full-length VEGF trap and mini-VEGF trap can be injected intravitreally and that the proteins penetrate to the desired site of action, i.e. 45 retina or related structure. The results show that the protein is present in the eye tissue for up to 672 hrs, thus allowing for monthly treatment paradigms. Further, once the mini-VEGF trap moves out of the eye tissue into the systemic circulation, it is cleared more quickly from the body than the full-length 50 VEGF trap, thus reducing unwanted systemic action.

Example 17

Treatment of Age-Related Macular Degeneration

A patient manifesting age-related macular degeneration is treated with an intravitreal injection of the VEGF trap protein of SEQ ID NO:6 or 23. The purpose of this treatment is to reduce or prevent the development of neovascularization, 60 macular disease, and retinal damage. Once a patient reaches the age of 60, increased ophthalmic surveillance is performed to detect the presence of AMD. This increased surveillance should include periodic retinal examinations and fluorescein angiograms to monitor for the presence of subretinal fluid, 65 blood, exudates, RPE detachment, cystic retinal changes, or the presence of grayish green subretinal neovascular mem20

brane. When AMD is diagnosed, a regime of VEGF trap protein treatment is commenced coupled with or without other treatments such as photocoagulation. As the first step of treatment, the patient is to receive a full ophthalmic examination to establish a baseline of ocular health. The ophthalmic examination includes indirect ophthalmoscopy, slit-lamp biomicroscopy, peripheral retinal examination, intraocular pressure measurements, visual acuity (unaided and best corrected) symptomatology, fundus photography, fluorescein angiography, electroretinography and A-scan measurements. Following the preliminary examination, an intravitreal injection of VEGF trap protein is given to the patient's affected eye manifesting AMD. If both eyes are affected, they may be treated separately. The eye to be treated is injected with 25-4000 ug of VEGF trap protein in an ophthalmic solution.

After treatment, the patients' eyes are to be examined on days one (1), two (2), seven (7), fifteen (15), thirty (30) and sixty (60). Because of the possibility of reoccurrence, the patient should return for periodic examinations on a monthly basis thereafter. On each examination day the patient is monitored for vitreous liquefaction. Additionally, the patient is monitored for posterior vitreous detachments using indirect ophthalmoscopy with scleral depression. Finally, the extent of AMD presented by the patient is continuously monitored through periodic retinal examinations and fluorescein angiograms to monitor for the presence of subretinal fluid, blood, exudates, RPE detachment, cystic retinal changes, or the presence of grayish green subretinal neovascular membrane. Additional VEGF trap protein treatments may be required if indicia of reoccurring neovascularization are observed. Additional treatments may be given on weekly or monthly basis. In a preferred embodiment, an initial treatment is followed by subsequent treatments between 1-6 months apart.

Example 18

A Double-Masked, Placebo-Controlled, Dose Escalation, Phase I Study of Intravenous VEGF Trap in Patients with Neovascular Age-Related Macular Degeneration

A study was conducted to obtain preliminary assessments of the safety, pharmacokinetics (PK), and biological activity of single and repeated intravenous (IV) doses of the VEGF trap antagonist (SEQ ID NO:6) in patients with neovascular age-related macular degeneration (AMD).

Methods. Successive cohorts of patients with neovascular AMD (≦12 disc areas, ≧50% active choroidal neovascularization (CNV), ETDRS best-corrected visual acuity (BCVA) $\leq 20/40$) were randomized (3:1) to receive either VEGF trap or placebo at dose levels of 0.3, 1.0, or 3.0 mg/kg. Patients received a single IV dose, followed by a 4-week safety observation/PK evaluation period, followed by 3 biweekly IV 55 doses. Safety assessments included laboratory assessments (hematology, chemistry, urinalysis, anti-VEGF trap antibody measurements), vital signs, and ophthalmic exams. Measures of biological activity included mean percent change in excess retinal thickness (ERT) as assessed by optical coherence tomography (OCT), and ETDRS BCVA. Adverse events (AEs) were graded using the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE v. 3.0). Dose-limiting toxicity (DLT) was defined as Grade 2 or 3 ocular AEs, or Grade 3 or 4 systemic AEs with modified criteria for hypertension and proteinuria. The maximum tolerated dose (MTD) was defined as the dose level below that at which ≥ 2 patients experienced DLT.

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Results. Twenty-five patients were enrolled (11 male, 14 female; mean age 76 years). Nineteen patients received VEGF trap (7 at 0.3 mg/kg; 7 at 1.0 mg/kg; 5 at 3.0 mg/kg), and 6 patients received placebo. The majority of AEs encountered on VEGF Trap treatment were mild to moderate in severity. Two of 5 patients encountered protocol-defined DLT at the 3.0 mg/kg dose level: Grade 4 hypertension (n=1); Grade 2 proteinuria (n=1). Therefore, all of the patients in the 3.0 mg/kg dose group were prematurely withdrawn from study. None of the patients in the study developed anti-VEGF 10 trap antibodies. The mean percent changes in ERT were: -12%, -10%, -66%, -60% for the placebo, 0.3, 1.0, and 3.0 mg/kg dose groups at Day 15 (ANOVA p<0.02), and -5.6%, +47.1%, -63.3% for the placebo, 0.3, and 1.0 mg/kg dose groups at Day 71 (ANOVA p<0.02). The changes in BCVA were: +1.9, +1.8, +3.4, and +4.6, for the placebo, 0.3, 1.0, and 3.0 mg/kg dose groups at Day 15 and were: +2.8, +3.9, and +3.9 for the placebo, 0.3, and 1.0 mg/kg dose groups at Day 71. The BCVA results were not statistically significant.

Optical coherence tomography scans (temporal to nasal 20 and inferior to superior transverse) and maps were obtained. In one example patient, at baseline the foveal thickness was 348 µm with a pocket of subretinal fluid beneath the fovea. On day 29, there was little anatomic evidence of improvement,

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but at day 71 (two weeks after the fourth infusion of VEGF trap), the pocket of subretinal fluid had resolved, foveal thickness had improved to 232 μ m, and macular volume was within the normal range at 6.69 mm³. At day 99, 6 weeks after the last infusion of VEGF trap, there was deterioration, with recurrence of the pocket of subfoveal fluid, increase in foveal thickness to 248 μ m, and increase in macular volume to 7.31 mm³.

Digital fluorescein angiography was conducted at baseline and 3 time points after initiation of treatment with 1. mg/kg of VEGF trap. At baseline, there was a large area of occult subfoveal CNV that leaked during the mid- and late-phases of the angiogram so that there was white dye pooled beneath the entire macula. At day 29, there was mildly reduced leakage, but by day 71, there was a substantial reduction in leakage and pooling of dye. At day 99, there was some increase in leakage compasted to day 71, but less than seen at baseline.

Conclusions: The maximum tolerated dose of intravenous VEGF trap in this study of neovascular AMD patients was 1.0 mg/kg. A dose-dependent improvement in ERT as evaluated by OCT was suggested in this small number of patients, with a longer initial duration in improvement at the 3.0 mg/kg as compared to the 1.0 mg/kg dose level. A trend towards a dose-related improvement in BCVA was also suggested.

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CROHENCE LICTING

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 385
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 395
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Lys 145	Leu	Val	Leu	Asn	Cys 150	Thr	Ala	Arg	Thr	Glu 155	Leu	Asn	Val	Gly	Ile 160
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We claim:

1. A therapeutic method for treating or ameliorating an eye disorder, comprising administering a fusion polypeptide capable of binding vascular endothelial growth factor (VEGF) to a patient in need thereof, wherein the fusion polypeptide consists essentially of an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor Flt1 and Ig domain 3 of a second VEGF receptor Flk1 or Flt4; and a multimerizing 45 component, wherein the multimerizing component is an amino acid sequence between 1 and 200 amino acids in length having at least one cysteine residue.

2. The therapeutic method of claim **1**, wherein the Ig domain 2 and Ig domain 3 are the only VEGF receptor com- ⁵⁰ ponents of the fusion polypeptide.

3. The therapeutic method of claim **2**, wherein the fusion polypeptide comprises the amino acid sequence of SEQ ID NO: 23.

4. The therapeutic method of claim **1**, wherein the eye disorder is age-related macular degeneration or diabetic ret-inopathy.

5. The therapeutic method of claim **4**, wherein the eye disorder is associated with choroidal neovascularization, vas- $_{60}$ cular leak, and/or retinal edema.

6. A therapeutic method for treating or ameliorating an eye disorder, comprising administering a fusion polypeptide capable of binding vascular endothelial growth factor (VEGF) to a patient in need thereof, wherein the fusion 65 polypeptide consists essentially of an immunoglobulin-like (Ig) domain 2 of Flt1 and Ig domain 3 of Flk1 or Flt4, and a

multimerizing component, wherein the fusion polypeptide comprises the amino acid sequence of SEQ ID NO:23.

7. The therapeutic method of claim **6**, wherein the eye disorder is age-related macular degeneration or diabetic ret-inopathy.

8. The therapeutic method of claim 6, wherein administration is selected from one of eye drops, subconjunctival injection, subconjunctival implant, intravitreal injection, intravitreal implant, sub-Tenon's injection, and sub-Tenon's implant.

9. A method for the treatment of a human subject diagnosed with age-related macular degeneration, comprising administering an effective amount of a vascular endothelial growth factor (VEGF) fusion polypeptide to the human subject, wherein the fusion polypeptide consists essentially of an immunoglobulin-like (Ig) domain 2 of a first VEGF receptor Flt1 and Ig domain 3 of a second VEGF receptor Flt1 or Flt4; and a multimerizing component having between 1 and 200 amino acids in length having at least one cysteine residue, the method comprising:

- (a) administering to the subject an initial dose of at least approximately 25-4000 ug VEGF inhibitor protein per eye; and
- (b) administering to the subject a plurality of subsequent doses of the VEGF fusion polypeptide in an amount that is approximately the same or less than the initial dose, wherein the subsequent doses are separated in time from each other by at least two weeks.

10. The method of claim **9**, wherein the initial dose is at least approximately 50 ug of VEGF fusion polypeptide.

Mylan Exhibit 1016 Mylan v. Regeneron, IPR2021-00880 Page 154 Joining Petitioner: Apotex **11**. The method of claim **10**, wherein the initial dose is at least approximately 100 ug of VEGF fusion polypeptide.

12. The method of claim **11**, wherein the initial dose is at least approximately 1000 ug of VEGF fusion polypeptide.

13. The method of claim 12, wherein the subsequent doses are separated in time from each other by at least four weeks.

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14. The method of claim 13, wherein the subsequent doses are separated in time from each other by at least 3 to 6 months.15. The method of claim 9, wherein the initial dose and at least one subsequent dose is administered by intravitreal 5 injection.

* * * * *

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