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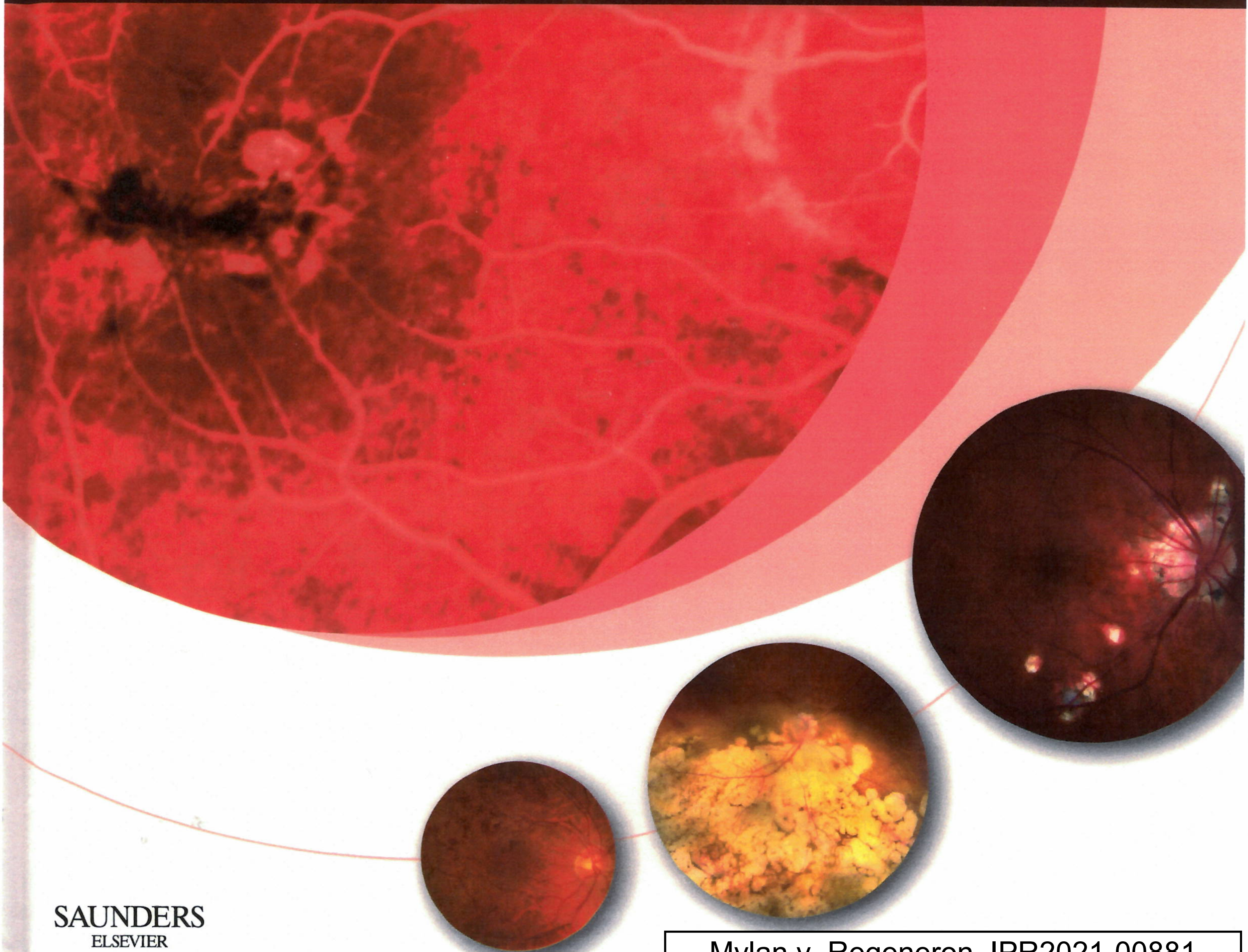
Retinal Pharmacotherapy



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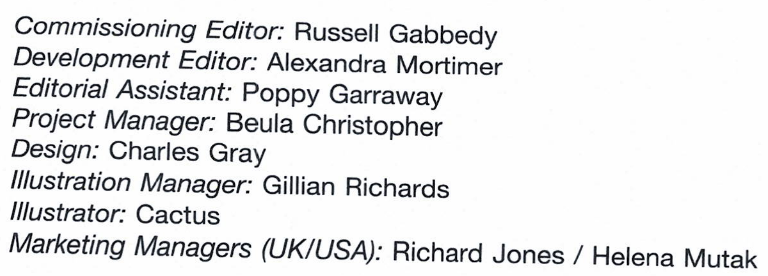

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Retinal Pharmacotherapy

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CHAPTER

4 Ocular angiogenesis: vascular endothelial growth factor and other factors

Anthony P. Adamis, MD

INTRODUCTION

The original visionary proposal by Dr. Judah Folkman[1] that antiangiogenic therapy could offer an approach to the treatment of many cancers ultimately led to a major research effort into the mechanisms which control both physiological and pathological angiogenesis. His work also contemplated the use of antiangiogenic drugs in ophthalmology. A principal focus of this research effort has been the identification of specific molecules involved in the promotion and inhibition of angiogenesis, an effort that has already led to the development of targeted therapies against vascular endothelial growth factor (VEGF). In addition, many other factors have been identified that act as promoters or inhibitors of angiogenesis (Table 4.1). This chapter will focus on those molecules whose roles have been best validated to date, and which possess particular relevance to ocular neovascularization.

PROMOTERS OF ANGIOGENESIS

VASCULAR ENDOTHELIAL GROWTH FACTOR

VEGF in physiologic and pathologic angiogenesis

VEGF (also known as VEGF-A) is a 45-kDa homodimeric glycoprotein belonging to a family that also includes VEGF-B through VEGF-E, platelet-derived growth factor (PDGF), and placental growth factor.[2] Initially isolated as a vascular permeability factor, VEGF was subsequently cloned and found to be a potent proangiogenic factor, acting as a master regulator of angiogenesis (reviewed by Ferrara and Davis-Smyth[3] and Ferrara[2]). VEGF has subsequently been found to act in a wide variety of other physiological contexts,[4] some of which, such as neuroprotection, are completely independent of its role in angiogenesis.

Alternative splicing of the human VEGF gene yields six principal isoforms of 121, 145, 165, 183, 189, and 206 amino acids.[5] The corresponding rodent isoforms are one amino acid shorter.[3] Many studies have focused on characterizing the functions of $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$. $VEGF_{121}$ is freely diffusible, while $VEGF_{189}$ and larger isoforms are found sequestered in the extracellular matrix; $VEGF_{165}$ exists in both diffusible and matrix-bound forms.[2] VEGF acts as a ligand for VEGF receptor 1 (VEGFR1) and VEGFR2; these receptor tyrosine kinases in turn activate downstream signaling cascades.

VEGF acts in many capacities in angiogenesis, including as an endothelial cell mitogen[6] and survival factor,[7] and as a chemoattractant for bone marrow-derived endothelial progenitor cells.[8] In addition, VEGF induces the upregulation of extracellular matrix-degrading enzymes, such as matrix metalloproteinases (MMPs)[9] and plasminogen activator,[10] as well as nitric oxide,[11] a downstream mediator of VEGF signaling.[12] Moreover, VEGF has two additional properties which are of direct relevance for the pathophysiology of ocular neovascular diseases. First, it is the most potent known inducer of vascular permeability,[13] an action related to the edema which often accompanies ocular neovascularization. Secondly, the retinal expression of VEGF, which is produced by a wide variety of retinal cell types,[14–16] is upregulated by hypoxia,[15,17] a response that is believed to be important in maintaining the health of both retinal neurons[18] and the choriocapillaris[17] while also creating a proangiogenic environment.

Reflecting the original focus of Dr. Folkman's proposal on the importance of angiogenesis in cancer growth and metastasis,[1] initial investigations of the role of VEGF in pathological angiogenesis demonstrated that interference with VEGF signaling inhibited tumor growth.[19] Over the course of a decade, a role for VEGF in ocular neovascular disease also was established based on three main lines of evidence: (1) correlations of VEGF elevation with the presence of ocular neovascular disease in the eyes of patients; (2) preclinical studies demonstrating that experimental elevation of VEGF levels in the eye led to neovascularization; and (3) the converse experiment, in which inhibition of VEGF signaling decreased neovascularization.

Correlations between elevations in ocular levels of VEGF and ocular neovascular disease have been reported and include conditions such as iris neovascularization, retinal vein occlusion, diabetic retinopathy (DR), diabetic macular edema (DME), neovascular glaucoma, and retinopathy of prematurity (reviewed by Starita et al.[4]). Elevated expression of VEGF also has been detected in surgically removed maculae[20] and choroidal neovascularization (CNV) membranes of eyes with age-related macular degeneration (AMD).[21]

A variety of approaches have been employed to demonstrate that elevated ocular levels of VEGF are sufficient to induce ocular neovascularization. These have included direct intravitreal injection of VEGF[22] and retinal vein photocoagulation[23] in monkeys; in rodent models, studies have included intravitreal injection of VEGF-expressing vectors,[24] and the use of transgenic mice engineered to overexpress VEGF in the retinal pigment epithelium (RPE).[25]

The experiments demonstrating that VEGF elevations are necessary for the development of ocular neovascularization have also employed various techniques. Agents used to block the actions of VEGF have included VEGFR fusion proteins,[26–28] anti-VEGF antibodies,[29,30] an anti-VEGF monoclonal antibody antigen-binding fragment (Figure 4.1),[31] an aptamer directed against $VEGF_{165}$,[28] and $VEGF_{165}b$, a VEGF variant which binds VEGFR2 but cannot activate it.[32] Agents used to block the ocular production of VEGF or VEGFR1 at the transcriptional or translational level have included small interfering RNAs (siRNAs) specific for VEGF[33] or VEGFR1,[34] and antisense oligonucleotides specific for VEGF.[35] Blocking the actions of VEGF in the eye by various means inhibited neovascularization of the iris,[29] cornea,[30] retina,[26,28,32,34,35] and choroid.[27,31,33,34]

Further detailed investigations into the mechanisms underlying VEGF's importance have revealed that the isoform $VEGF_{165}$ is especially pathogenic. In a murine model of ischemia-associated ocular neovascularization, retinal expression of $VEGF_{165}$ was found to be dramatically elevated compared to other isoforms; moreover, intravitreal injection of a $VEGF_{165}$-specific RNA aptamer was as efficient at inhibiting the pathological neovascularization as injection of a VEGFR-Fc fusion protein that inactivated all VEGF isoforms (Figure 4.2).[28] In addition, $VEGF_{165}$ acts as an especially potent inflammatory cytokine, a property of direct relevance given the importance of inflammation in

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Table 4.1 Proangiogenic and antiangiogenic factors

Proangiogenic factors	Antiangiogenic factors
Angiogenin	Angioarrestin
Angiopoietin-1	Angiostatin (plasminogen fragment)
Complement factors C3 and C5	Antiangiogenic antithrombin III
Cryptic collagen IV fragment	Cartilage-derived inhibitor (CDI)
Developmentally regulated endothelial locus 1 (Del-1)	CD59 complement fragment
Fibroblast growth factors: acidic (aFGF) and basic (bFGF)	Endostatin (collagen XVIII fragment)
Follistatin	Fibronectin fragment
Granulocyte colony-stimulating factor (G-CSF)	Growth-related oncogene (Gro-β)
Hepatocyte growth factor (HGF)/scatter factor (SF)	Heparinases
Interleukin-8 (IL-8)	Heparin hexasaccharide fragment
α5 integrins	Human chorionic gonadotropin (hCG)
Leptin	Interferon α/β/γ
Midkine	Interferon-inducible protein (IP-10)
Pigment epithelium-derived growth factor	Interleukin-12
Placental growth factor	Kringle 5 (plasminogen fragment)
Platelet-derived endothelial cell growth factor (PDECGF)	Metalloproteinase inhibitors (TIMPs)
Platelet-derived growth factor-BB (PDGF-BB)	2-Methoxyestradiol
Pleiotrophin (PTN)	Pigment epithelium-derived growth factor
Progranulin	Placental ribonuclease inhibitor
Proliferin	Plasminogen activator inhibitor
Transforming growth factor-α (TGF-α)	Platelet factor-4 (PF4)
Transforming growth factor-β (TGF-β)	Prolactin 16-kDa fragment
Tumor necrosis factor-α (TNF-α)	Proliferin-related protein (PRP)
Vascular endothelial growth factor (VEGF)	Retinoids
	Soluble VEGFR-1
	Tryptophanyl-tRNA synthase fragment
	$VEGF_{xxx}b$
	Tetrahydrocortisol-S
	Thrombospondin-1 (TSP-1)
	Transforming growth factor-β (TGF-β)
	Vasculostatin
	Vasostatin (calreticulin fragment)

Adapted from: Angiogenesis Foundation. Understanding angiogenesis. List of known angiogenic growth factors. Available online at: http://www.angio.org/understanding/content_understanding.html.

pathological neovascularization. Laser injury has been shown to upregulate retinal expression of intercellular cell adhesion molecule-1 (ICAM1), thereby promoting leukocyte adhesion to the vascular endothelium through CD18, the leukocyte ligand for ICAM1.[36] Genetic ablation of either molecule significantly reduced the formation of laser-induced CNV (Figure 4.3).[36] In this context, it is noteworthy that $VEGF_{165}$ was found to be significantly more potent at upregulating ICAM1 expression on endothelial cells than $VEGF_{121}$.[37] In addition, depletion of macrophages has been found to inhibit the development of pathological neovascularization in a rat model of retinopathy of prematurity (Figure 4.4)[28] and in laser-induced CNV.[38] $VEGF_{165}$ was more potent at chemotaxis of monocyte/macrophages than $VEGF_{121}$.[37] Since macrophages produce VEGF,[39] their infiltration serves as an amplification mechanism in further promoting angiogenesis.

Investigational approaches to VEGF inhibition in ocular neovascularization

The extensive research effort into elucidating VEGF's role in ocular neovascularization has provided a sound foundation for the development of anti-VEGF therapies. Three agents, pegaptanib,[40] ranibizumab,[41] and bevacizumab,[42] are already in widespread use, and are discussed in dedicated chapters of this text. A brief account of other approaches currently under evaluation in clinical trials follows.

RNA interference

RNA interference abrogates gene expression through a cellular defense mechanism mediated by double-stranded RNA sequences of at least 21 nucleotides long, resulting in targeted destruction of specific mRNA species.[43] RNA interference has been used to target VEGF mRNA in animal models, leading to suppression of corneal neovascularization[44] as well as CNV induced either by laser[33] or by overexpression of VEGF from a transgene.[45] Sirna-027, an agent targeting the expression of VEGFR1, also has been shown to suppress both retinal and CNV in murine models.[34]

Currently there are two siRNA agents undergoing evaluation in clinical trials for treatment of neovascular AMD. Bevasiranib (Ophi Health), directed against VEGF, has successfully completed a phase II trial and is currently recruiting patients for the phase III COBALT trial in which it will be combined with ranibizumab.[46] In addition, a phase I trial of the anti-VEGFR1 agent AGN211745 (Allergan; previously Sirna-027) has been completed,[47] and enrollment for a phase II trial is ongoing.[48] Recent evidence suggests that antiangiogenic siRNAs work nonspecifically and through a nonclassical siRNA mechanism in suppressing CNV.[49]

Soluble VEGFR fusion protein: VEGF-Trap

Work demonstrating the potential of soluble VEGFR fusion proteins to suppress retinal neovascularization[26] provided a basis for the development of VEGF-Trap, a fusion protein combining components of both VEGFR1 and VEGFR2.[50] VEGF-Trap, which was engineered with a view to optimizing pharmacokinetic properties as well as efficacy, binds to all isoforms of VEGF as well as placental growth factor.[50] Intravitreal injection of VEGF-Trap inhibited laser-induced CNV in mice, as well as preventing VEGF-induced blood–retinal barrier breakdown.[27] It is now being evaluated in a phase III study.[51]

Anecortave acetate

Anecortave acetate is a member of a group of corticosteroids, first isolated in Dr. Folkman's laboratory,[52] that have angiostatic properties but lack conventional anti-inflammatory activity.[53] In a rat retinopathy of prematurity model, anecortave significantly reduced pathologic retinal neovascularization without affecting normal retinal angiogenesis.[54] In other studies with this model, anecortave was found to reduce retinal expression of VEGF,[55] and of insulin growth-factor-1 and its receptor.[56] Anecortave also inhibited VEGFR2 expression in a murine model of retinoblastoma.[57] These findings suggest that the angiostatic effects of anecortave may at least in part be mediated through VEGF signaling pathways.[52]

Anecortave acetate has shown some promise as a treatment for neovascular AMD, administered as a juxtascleral depot either alone[58] or in combination with photodynamic therapy.[59] Although anecortave acetate did not meet its efficacy endpoint in a phase III noninferiority trial comparing it to photodynamic therapy with verteporfin,[58] it remains under study as a prophylactic treatment to slow the progression of neovascular AMD.[60]

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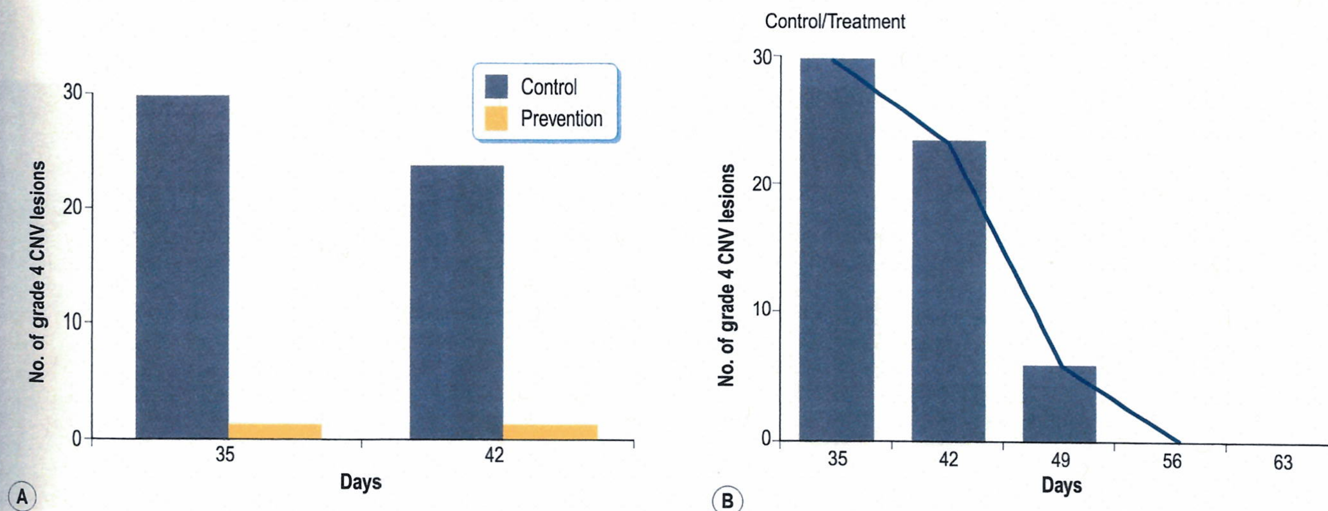

Figure 4.1 Inactivation of all vascular endothelial growth factor (VEGF) isoforms potently inhibits laser-induced choroidal neovascularization (CNV) in the nonhuman primate. (A) Cynomolgus monkeys (n = 10) received 500 µg of recombinant humanized monoclonal anti-VEGF antibody (rhuFab VEGF) in one eye and vehicle in the other, every 2 weeks. On day 21, CNV was induced by laser wounding. The bar graph shows the total number of grade 4 CNV lesions in the eyes receiving rhuFab VEGF (gold bar) compared to those in control eyes that received vehicle (blue bar); assessments were made 2 weeks after laser induction (day 35), and 3 weeks after the laser induction (day 42). Adapted from Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 2002;120:338–346.

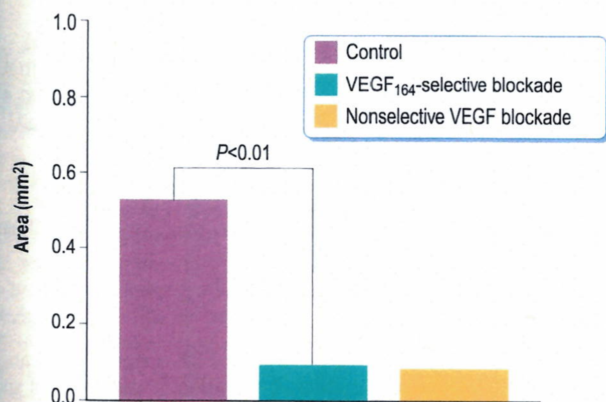

Figure 4.2 Vascular endothelial growth factor ($VEGF_{164/165}$) is especially potent in promoting pathological neovascularization. In a rat model of ischemia-induced retinal neovascularization, intravitreal injection of an aptamer specific for $VEGF_{164/165}$ was as effective in inhibiting pathological neovascularization as a VEGFR1-Fc fusion protein which binds all VEGF isoforms. Adapted from Ishida S, Usui T, Yamashiro K, et al. VEGF164-mediated inflammation is required for pathological, but not physiological, ischemia-induced retinal neovascularization. J Exp Med 2003;198:483–489.

PLATELET-DERIVED GROWTH FACTOR

The PDGF family consists of four related dimeric polypeptides (PDGF-A through PDGF-D)[61] that are structurally related to VEGF.[2] In general they occur as homodimers, although the PDGF-AB heterodimer has also been identified.[61] PDGFs are ligands for two receptor tyrosine kinases, PDGFR-α and PDGFR-β, of which PDGFR-β is principally responsible for signal transduction on cells associated with the vascular system, including endothelial cells, pericytes, and smooth-muscle cells.[62] Similarly, PDGF also has a widespread distribution among these same cell types.[62] In addition to its central role in vascular system development, PDGF signaling is important for processes such as wound healing and central nervous system development.[62]

Studies have revealed a central role for the PDGF-B homodimer in vascular development, as it was found to stimulate the proliferation,[63] and induce capillary tube formation[64] of endothelial cells. PDGF-B is especially critical for the recruitment of mural cells (pericytes and smooth-muscle cells) to the developing vasculature.[65] Genetic ablation of PDGF-B leads to perinatal death from hemorrhages and vascular system abnormalities[66] while ablation of the PDGFR-β gene results in a similar phenotype.[67] Proliferation of mural cells was significantly reduced in mice lacking either PDGF-B or PDGFR-β.[65] Also, administration of an aptamer specific for PDGF-B led first to pericyte loss and then to regression of tumor vessels in a murine tumor model.[68] These findings indicate that PDGF-B produced by endothelial cells is essential for the proliferation, migration, and recruitment of mural cells to the developing capillaries (Figure 4.5).[65]

Studies of ocular neovascularization in mice have provided further evidence in support of this model. Inhibition of PDGF-B signaling, whether by genetic ablation in endothelial cells[69] or PDGFR kinase inhibitors,[70] led to deficient pericyte recruitment in models of retinal[69] and corneal[70] neovascularization.

Studies using three different models of ocular neovascularization, in which PDGF-B and VEGF signaling were blocked by administration of an antibody to PDGFR-β or pegaptanib, respectively, have further delineated the respective roles of these molecules.[71] Physiological retinal angiogenesis was inhibited on postnatal day 3 by blocking PDGF-B, but not by blocking $VEGF_{164}$; however, combined blockade provided greater inhibition. Conversely, VEGF blockade alone inhibited the development of laser-induced CNV, whereas blocking PDGF-B signaling was ineffective on its own; again, greater inhibition occurred if both pathways were blocked. Finally, in a corneal model of neovascularization, PDGF-B blockade between days 10 and 20 postinjury led to detachment of mural cells from corneal neovessels; in contrast, VEGF blockade reduced neovascularization when applied immediately after wounding, but it did not induce regression of vessels after they were established. However, vessel regression was enhanced if both inhibitors were given (Figure 4.6).[71] These experiments suggest that a combination strategy targeting both VEGF and PDGF-B may be more effective, both in treating established neovascularization and in preventing new vessel growth.

FIBROBLAST GROWTH FACTOR 2 (FGF2)

FGF2 (also known as basic FGF) is a heparin-binding growth factor that occurs in several isoforms. FGF2 signals through four receptor tyrosine kinases (FGF receptor 1 through FGF receptor 4) and acts in a variety of developmental processes, including angiogenesis.[72]

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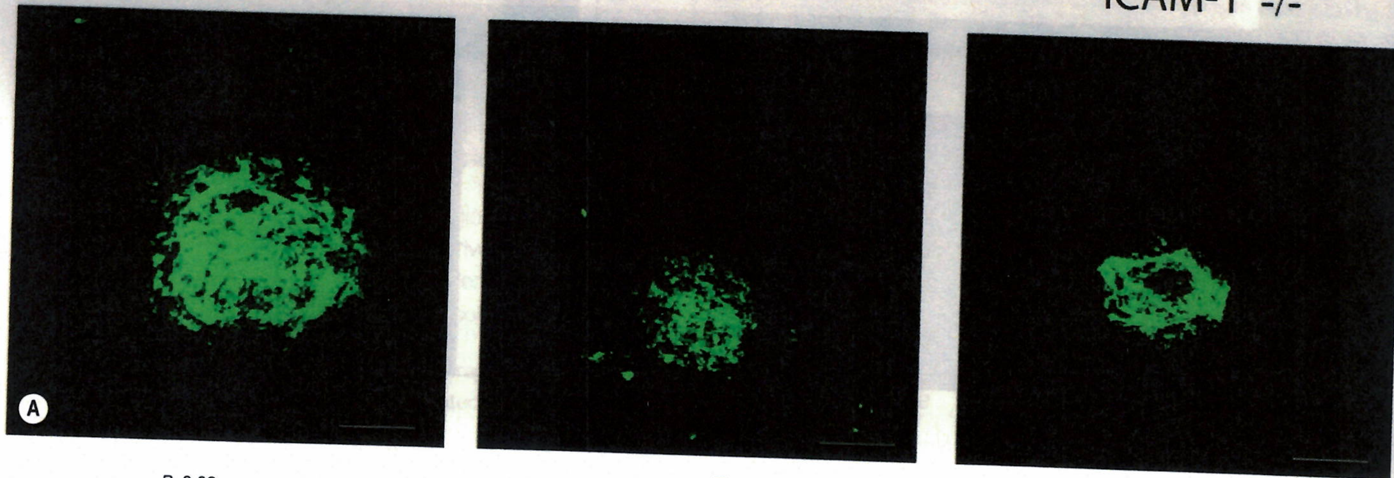

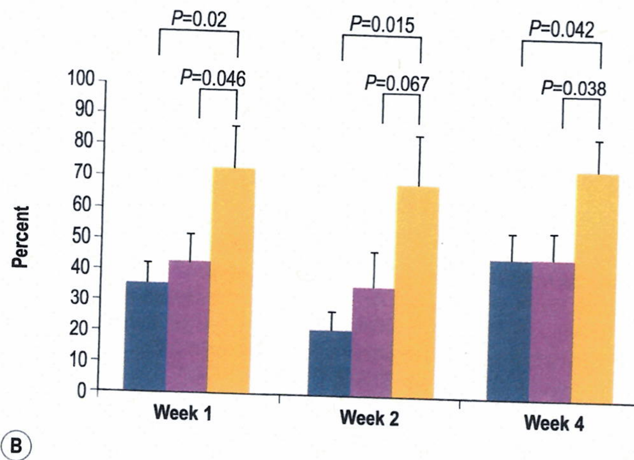

Figure 4.3 Evidence of the role of inflammation in the model of laser-induced choroidal neovascularization (CNV). (A) Genetic ablation of either CD18 or intercellular cell adhesion molecule-1 (ICAM1) led to marked diminution of the size of laser-induced CNV. Two weeks following laser injury, stacked confocal images were taken of fluorescein *Griffonia simplicifolia* lectin I-labeled tissue within the laser scars. CNV membranes were significantly reduced in both mutant strains, compared to wild-type mice. Scale bar, 100 μm. (B) Loss of either CD18 or ICAM1 resulted in fewer lesions of pathological significance. Fluorescein angiography performed at 1, 2, and 4 weeks after laser photocoagulation demonstrated that ablation of either CD18 (blue bar) or ICAM1 (purple bar) resulted in significantly fewer grade 2B lesions (those showing pathologically significant leakage) than were seen in wild-type mice (gold bar) (mean ± SEM; $n = 5$ for all groups). Adapted from Sakurai E, Taguchi H, Anand A, et al. Targeted disruption of the CD18 or ICAM-1 gene inhibits choroidal neovascularization. Invest Ophthalmol Vis Sci 2003;44:2743–2749.

The role of FGF2 in ocular neovascular disease is not well defined. Elevated expression of FGF2 has been detected in CNV membranes from patients with AMD[73] and in epiretinal membranes from patients with proliferative DR.[73] However, exogenous administration of FGF2 produced only subretinal neovascularization that did not penetrate Bruch's membrane in an experimental model of CNV.[74] Other studies found that transgenic mice with elevated retinal FGF2 expression developed CNV following low-intensity laser (sufficient to disrupt photoreceptors but not Bruch's membrane) while wild-type mice did not.[75] Taken together with studies demonstrating that genetic ablation of the FGF2 gene did not inhibit the formation of laser-induced CNV,[76] these findings suggest that FGF2 is in itself not sufficient to provoke CNV in the absence of an additional stimulus and that FGF2 may also not be required to induce CNV.

TUMOR NECROSIS FACTOR-α (TNF-α)

TNF-α is the prototypic member of a superfamily of cytokines that mediate a variety of biological functions, signaling through a correspondingly large family of receptors.[77] Several studies have examined the role of TNF-α as a mediator of angiogenesis, but a unified picture is not yet apparent.

TNF-α has been found to stimulate angiogenesis in the corneas of rats[78] and rabbits.[79] It is not clear if these represent direct or indirect effects since TNF-α has been demonstrated to induce expression of VEGF[80] and VEGFR2[81] potently in cultured endothelial cells. TNF-α also upregulates the synthesis of other factors associated with angiogenesis, including angiopoietin 1 and angiopoietin 2[80] as well as MMP2 and MMP9.[82]

Several studies have assessed the role of TNF-α signaling in angiogenesis. In ischemic-induced neovascularization in the limbs of mice, TNF-α was essential for the mobilization and survival of bone marrow-derived endothelial progenitor cells, induction of VEGF expression and collateral vessel development.[83] In another report, administration of infliximab (a monoclonal antibody to TNF-α)[84] or etanercept (a soluble TNF receptor fusion protein) both inhibited the size of laser-induced CNV in mice.[84] Gene knockout studies, however, have been inconsistent; some studies found a dependence of retinal neovascularization on TNF-α function[85] whereas others did not.[86]

In clinical studies, elevated levels of TNF-α have been found in fibrovascular membranes of patients with proliferative DR[87] and in surgically excised CNV membranes.[88] Intriguingly, intravenous administration of infliximab for treatment of rheumatoid arthritis caused regression of CNV in patients with AMD[89]; moreover, intravenous infliximab also led to reductions in macular edema in patients with DME.[90] It is not clear if these effects of TNF-α are independent of its upregulation of VEGF; if separate pathways are involved, TNF-α inhibition alone or in combination with VEGF inhibition could provide an additional therapeutic option.

EPHS AND EPHRINS

Ephs comprise a large family of receptor tyrosine kinases that are activated upon binding with their cognate membrane-bound ligands, the ephrins.[91,92] EphrinAs are attached to the cell membrane by a glycosylphosphatidyl anchor while the ephrinBs have transmembrane and cytoplasmic signaling domains (Figure 4.7).[93] The Ephs also fall into two

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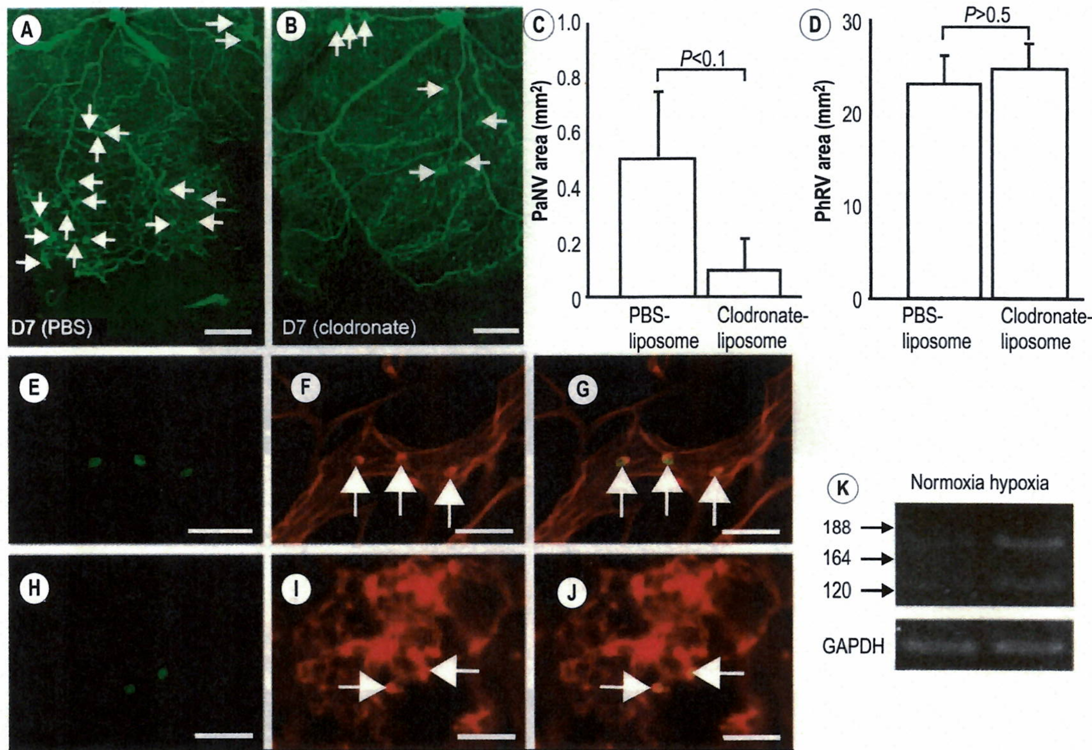

Figure 4.4 Monocytes contribute to pathological retinal neovascularization. In a retinopathy of prematurity model, postnatal day zero (P0) rats were maintained for 10 days in 80% oxygen, interrupted daily by 30 minutes in room air, followed by a progressive return to 80% oxygen. This treatment led to an avascular retina. On P10, corresponding to study day 0 (D0), retinal revascularization was induced by maintaining the rats in room air for an additional 7 days (D7). (A–C) At D7, pathological neovascularization (PaNV; arrows in A and B) was significantly inhibited by treatment with clodronate liposomes compared to control liposomes ($n = 8$ for both treatments; means ± standard deviation). (D) Physiological neovascular area (PhRV) was not significantly affected by treatment with clodronate liposomes ($P > 0.05$). (E–J) Influx of monocytes was observed just before and during pathological neovascularization. (H–J) Monocytes were labeled with a fluorescein conjugated antibody to CD13 (E and H), while rhodamine-conjugated *Concanavalin* A was used to label the retinal vasculature and adherent leukocytes (F and I). As shown by superposition of these figures (panels G and J), the concanavalin A and CD13 staining co-localized, indicating that the adherent leukocytes were monocytes. (K) In cultured peripheral blood monocytes obtained from retinopathologic rats at D7, exposure to hypoxia (1% oxygen) led to marked increase in expression of vascular endothelial growth factor mRNA compared to exposure to normoxia (21% oxygen). PBS, phosphate-buffered saline. Scale bars: (A and B) 0.5 mm and (E–J) 50 μm. Reproduced from Ishida S, Usui T, Yamashiro K, et al. VEGF164-mediated inflammation is required for pathological, but not physiological, ischemia-induced retinal neovascularization. J Exp Med 2003;198:483–489.

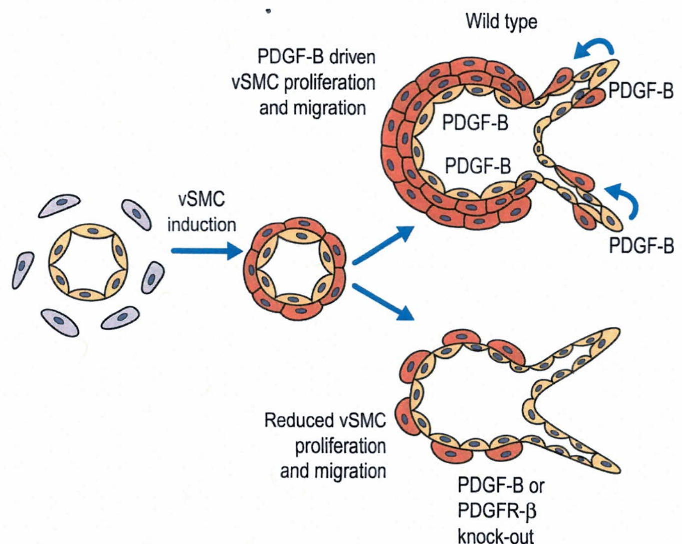

Figure 4.5 Platelet-derived growth factor (PDGF)-B regulates the development of blood vessel walls. During blood vessel development, the nascent endothelial tube (yellow) is surrounded by undifferentiated mesenchymal cells (gray) which are induced to differentiate into vascular smooth-muscle cells (vSMC), and to form a surrounding sheath (red). During further development of the vascular network, with concomitant growth and sprouting of blood vessels, PDGF-B derived from the endothelium further promotes vSMC proliferation and migration. These proliferative and migratory responses are reduced in mice in which PDGF-B or PDGFR-β have been genetically ablated, leading to defective coating of capillaries by pericytes, as well as to vSMC hypoplasia in larger vessels. Reproduced from Hellstrom M, Kalen M, Lindahl P, et al. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development 1999;126:3047–3055.

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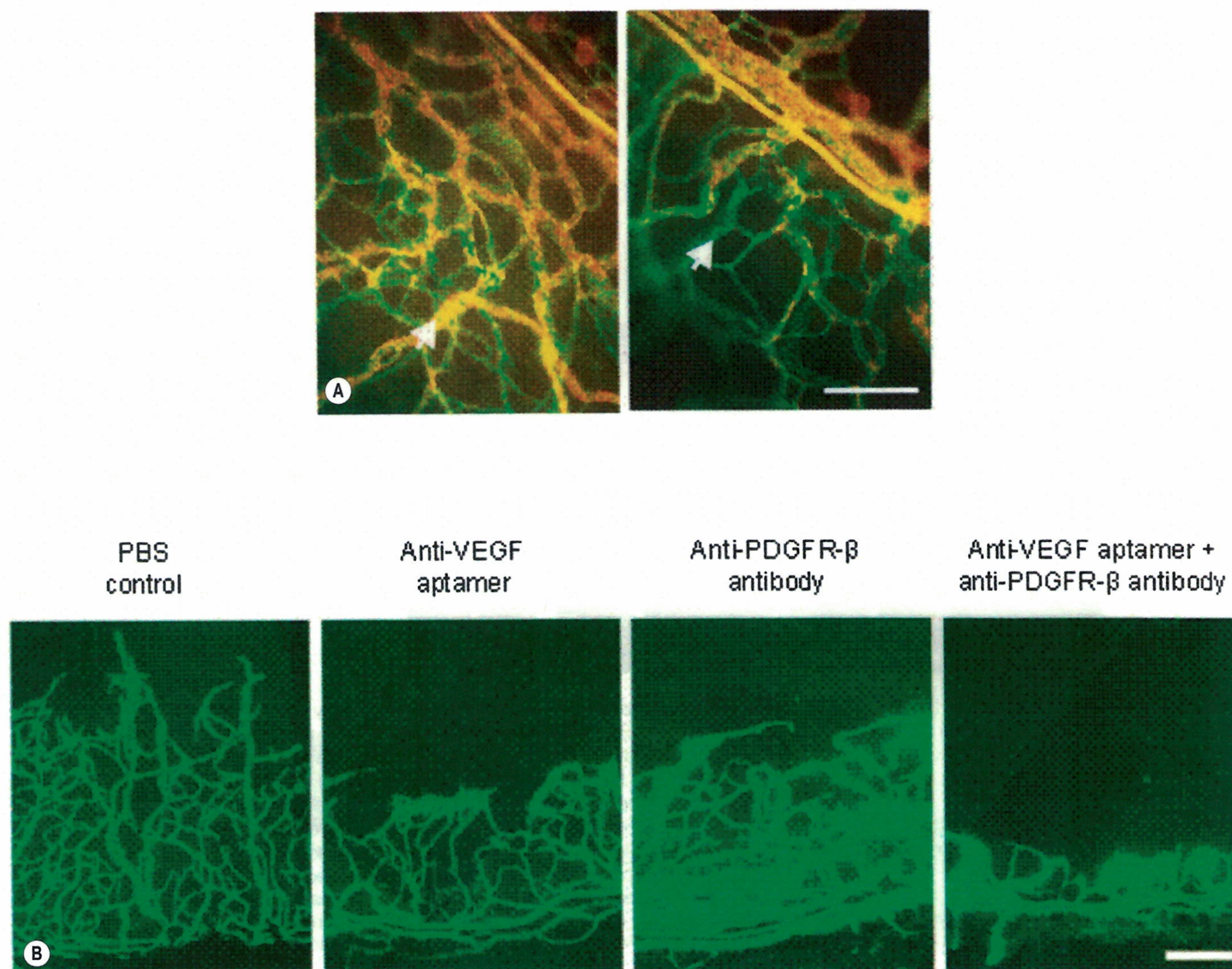

Figure 4.6 The role of platelet-derived growth factor (PDGF)-B on blood vessel growth and mural cell coverage in a corneal neovascularization model. (A) Endothelial cells were labeled by staining with lectin (green) and mural cells were stained with an antibody against smooth-muscle actin (red). Starting at 10 days following corneal injury, mice received daily intraperitoneal injections of an anti-PDGFR-β antibody or phosphate-buffered saline (PBS), and were sacrificed at 20 days postinjury. Treatment with the anti-PDGF-β antibody led to reduced mural cell coverage compared to controls (arrow). Scale bar = 20 μm. (B) Following induction of corneal injury, mice received daily intraperitoneal injections of one of the following: PBS, a polyethylene-glycolated anti-vascular endothelial growth factor (VEGF) aptamer, an anti-PDGFR-β antibody, or both the anti-VEGF aptamer and the anti-PDGFR-β antibody. Neovasculature (green) was stained by fluorescein isothiocyanate-concanavalin A. Neovascularization was significantly reduced by the anti-VEGF aptamer compared with either PBS or the anti-PEGFR-β antibody ($P < 0.01$); inhibition of both VEGF and PDGF-B signaling led to a further significant reduction ($P < 0.05$), compared to inhibition of VEGF signaling alone. Scale bar = 100 μm. Adapted from Jo N, Mailhos C, Ju M, et al. Inhibition of platelet-derived growth factor B signaling enhances the efficacy of anti-vascular endothelial growth factor therapy in multiple models of ocular neovascularization. Am J Pathol 2006;168:2036–2053.

broad groups, EphA and EphB, with the EphAs binding primarily, although not exclusively, to members of ephrinA subclass, while EphBs similarly tend to bind preferentially to ephrinB ligands.

Owing to the association of ephrins to cell membranes, ephrin-Eph signaling requires cell–cell contact. A notable feature of their interaction is that signaling can proceed not only in the forward direction, through activation of Eph kinases, but also in the reverse direction. Their interactions are critical for a wide variety of processes, including proper patterning in the development of the nervous[94] and cardiovascular[91] systems, immune cell trafficking,[95] angiogenesis,[92] and insulin secretion by pancreatic β cells.[96]

There are relatively few studies of ephrinA/EphA signaling in angiogenesis or ocular neovascularization. EphA-deficient endothelial cells were unable to migrate and form capillary tubes,[97] and the administration of soluble EphA2 receptors, which would be expected to block EphA2 signaling, was found to inhibit neovascularization in rodent corneal[98] and retinal[99] models.

There is more support for the importance of ephrinB/ephB interactions in angiogenesis, especially with respect to ephrinB2 and EphB4; ablation of either gene led to defective vascular development.[100,101] Their respective expression patterns are believed to underlie the establishment of arterial or venous identity, with ephrinB2 reported to

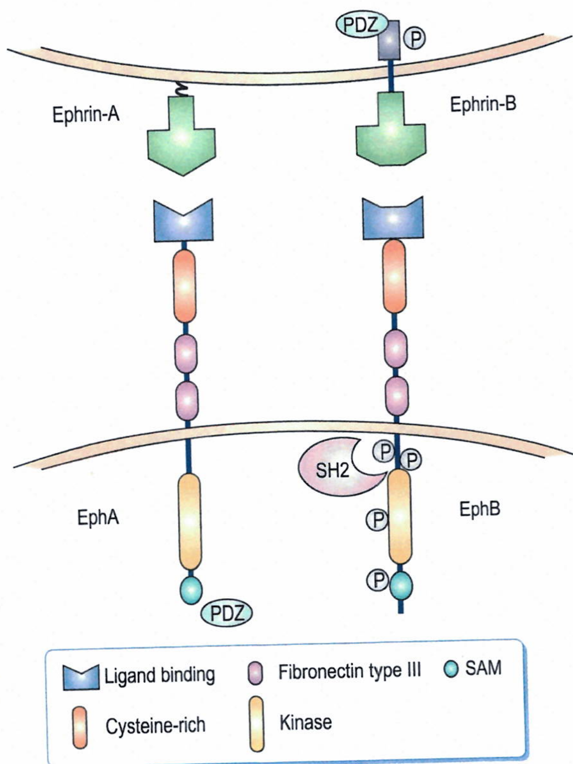

Figure 4.7 Ephrins and their Eph receptors. While both ephrins and Eph receptors are membrane-tethered proteins, ephrinBs traverse the membrane and possess a cytoplasmic signaling domain while ephrinAs do not. Ephrin-Eph binding results in receptor clustering, followed by autophosphorylation of multiple tyrosine residues and docking of downstream effectors through *src*-homology domains. The presence of a sterile alpha motif (SAM) and a PDZ domain (shown here for the carboxy-terminus of EphA, but also present in EphB), promotes ligand-induced receptor clustering. Reproduced from Dodelet VC, Pasquale EB. Eph receptors and ephrin ligands: embryogenesis to tumorigenesis. Oncogene 2000;19:5614–5619.

be expressed primarily on arteries[100] and EphB4 predominantly on veins.[100,101] EphrinB2 also has been found to be involved in recruitment of mural cells to microvessels.[102]

EphrinB2, EphB2, and EphB3 were all expressed in fibroproliferative membranes of patients with retinopathy of prematurity and proliferative DR.[103] However, experimental models have yet to resolve completely the role of EphB/ephrinB signaling in ocular neovascular disease. Angiogenesis was promoted in corneal models by ephrinB2[104] as well as by fusion proteins EphB1-Fc,[105] and ephrinB2-Fc[106]; in contrast, soluble monomeric EphB4[107,108] or ephrinB2 inhibited the development of pathological neovascularization.[108] Further investigation is required to delineate the molecular mechanisms involved in these effects.

NOTCH

Notch is a 300-kDa transmembrane protein, represented in mammals by four members, Notch1 through Notch4. Notch is activated by transmembrane ligands during cell to cell contact; in mammals, these ligands are Jagged1, Jagged2, and the Delta-like family (Dll1 through Dll4, with Dll4 being the most intensively investigated). Ligand binding leads to the proteolytic cleavage of Notch, releasing an intracellular domain that is translocated to the nucleus, inducing transcription of Notch-activated genes.[109,110] Notch signaling plays a key role in pattern formation in a wide variety of tissues, and is essential for such disparate processes as somitogenesis, neurogenesis, and development of the kidney and the cardiovascular system.[109]

Studies on Notch signaling in angiogenesis have identified Dll4 as the principal Notch ligand mediating vascular development.[110] In a study of the developing retinal vasculature, Dll4 was expressed in tip cells at the end of vascular sprouts, as well as in stalk cells, capillaries, arterial endothelium, and mural cells of mature arteries.[111] In addition, inhibition of Dll4/Notch signaling caused dramatic increases in tip cell formation, endothelial cell proliferation, and filopodial extension.[112] Furthermore, heterozygous ablation of the murine *dll4* gene also led to hyperbranching of the retinal vasculature (Figure 4.8)[112] while in a tumor model, blockade of Dll4 led to increased, but poorly organized, tumor vascularity and decreased tumor growth.[113]

Taken together, these findings suggest that Dll4 acts as a negative regulator of VEGF signaling to control aberrant angiogenic sprouting and branching. It remains to be seen whether interference with Notch signaling will provide another means of controlling angiogenesis, independent of VEGF, or whether it leads to excessive sprouting without a reduction in neovascular mass.

ANGIOPOIETINS

The angiopoietins 1 through 4 (Ang1–Ang4) are secreted ligands for Tie2, a receptor tyrosine kinase that is found primarily on endothelial cells and plays an essential role in the development and remodeling of the vasculature. Ang1 and Ang2 are the most intensively investigated members of the group, with Ang1 activating Tie2 and Ang2 usually functioning as a Tie2 antagonist.[114] Genetic ablation of Tie2 was found be embryonically lethal due to vascular defects[115]; similar defects occurred with Ang1 ablation[116] or overexpression of Ang2.[117] Both Ang1 and Ang2 have been studied extensively as potential therapeutic targets for affecting angiogenesis.

Angiopoietin 1

In angiogenesis, Ang1 acts as a chemoattractant for endothelial cells[118] while also promoting endothelial cell sprouting and facilitating tissue invasion by nascent blood vessels through activation of MMPs.[119] In transgenic mice, blood vessels induced by overexpression of VEGF were leaky, while the vessels induced by overexpression of Ang1 were nonleaky; coexpression of both molecules had an additive effect on angiogenesis but the resulting vessels were nonleaky, suggesting that Ang1 may reduce the vascular permeability resulting from chronic inflammation and elevated levels of VEGF.[120] Ang1 also has been found to suppress VEGF-mediated induction of inflammatory markers such as ICAM-1,[121] vascular cell adhesion molecule-1,[121] and tissue factor.[122] These actions are consistent with the overall action of Ang1 as a stabilizer of the quiescent vasculature.[123]

In studies with rodent models, the overexpression of Ang1 was found to inhibit laser-induced CNV and ischemia-induced retinal neovascularization, while also reducing VEGF-mediated retinal vascular permeability,[124] but it had no effect on established neovascularization.[125] Together these studies suggest that intravitreal injection of Ang1 could prove a useful approach in preventing ocular neovascularization and inflammation.

Angiopoietin 2

The principal sites of Ang2 synthesis are endothelial cells,[126] and arterial smooth-muscle cells.[126] Ang2 expression is especially marked at sites of vascular remodeling,[114] and it is upregulated by hypoxia and VEGF.[127,128] Ang2 acts primarily to destabilize the vascular endothelium; it is stored in Weibel–Palade bodies of endothelial cells (Figure 4.9),[95] and is released in response to exogenous stimuli such as proinflammatory cytokines.[129]

Clinically, Ang2 has been found in association with VEGF in highly vascular areas of CNV membranes in patients with a variety of ocular conditions,[130] as well as in the vitreous of eyes of patients with DR.[131] There is evidence that Ang2 and VEGF may act cooperatively in inducing ocular neovascularization. In rodent models, Ang2 enhanced corneal neovascularization in combination with VEGF, while

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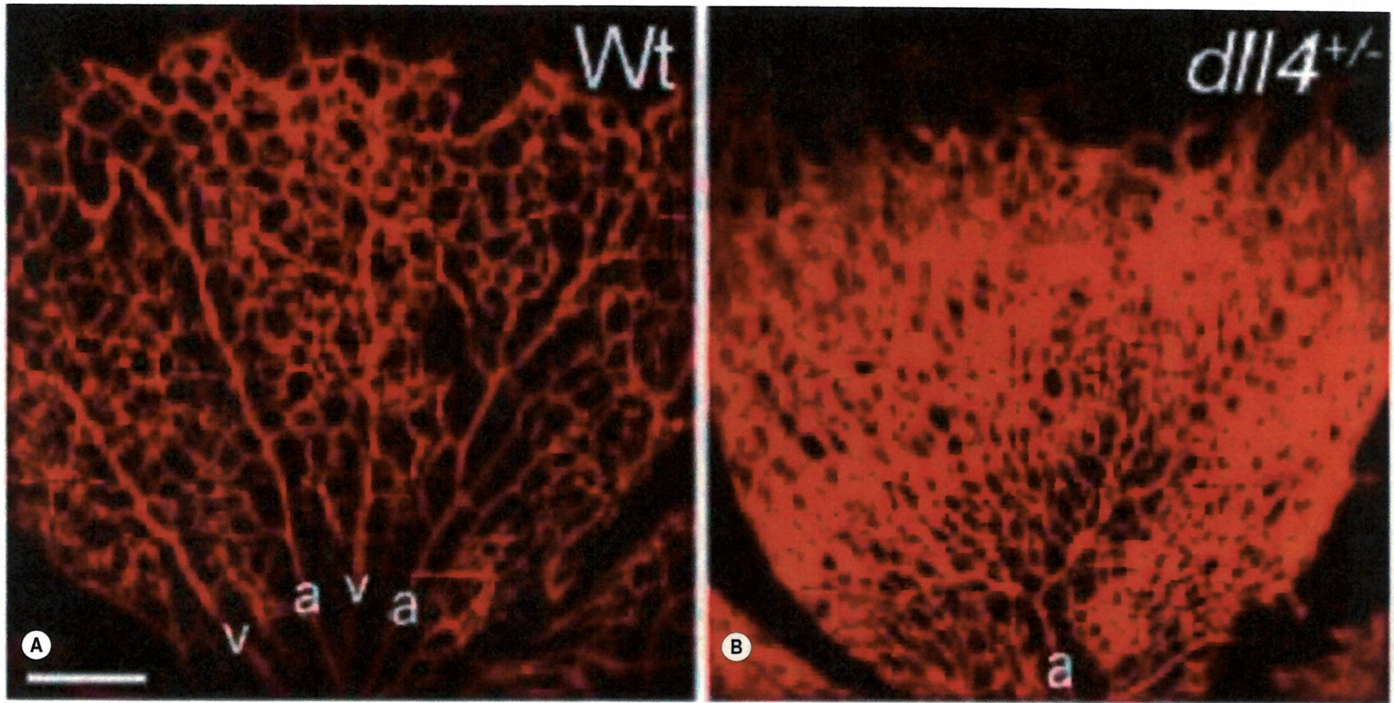

Figure 4.8 Inhibition of Delta4 signaling leads to increased vessel mass. Compared to wild-type (Wt) mice (A), retinal vessels stained at postnatal day 5 in mice for which *dll4* was heterozygously ablated (B) show hyperbranching within the vascular plexus (a, artery; v, vein). Retinal vessels were stained with isolectin B_4. Scale bar, 250 µm. Adapted from Suchting S, Freitas C, le Noble F, et al. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc Natl Acad Sci USA 2007;104:3225–3230.

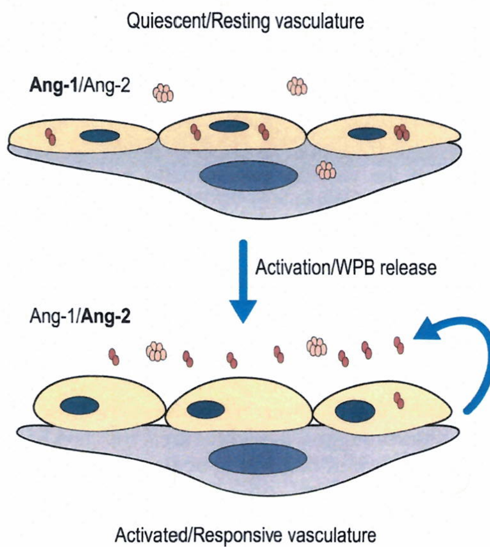

Figure 4.9 Regulation of vascular responsiveness by angiopoietins Ang1 and Ang2. Ang1 (multimeric, white) is secreted constitutively at a low level by mural (periendothelial) cells, and acts on the resting endothelium to sustain a low-level activation of Tie2, thereby helping to maintain the luminal cell surface in an antithrombotic and antiadhesive state (upper panel). Ang2 (dimeric, grey) is stored in Weibel–Palade bodies (WPB) in the endothelium, and during endothelial cell activation is released from them, along with other stored factors, leading to the Ang1/Ang2 ratio being altered more in favor of Ang2 (lower panel). As a result, the endothelial cell layer becomes destabilized and more responsive to proinflammatory stimuli. Reproduced from Pfaff D, Fiedler U, Augustin HG. Emerging roles of the angiopoietin-Tie and the ephrin-Eph systems as regulators of cell trafficking. J Leukoc Biol 2006;80:719–726.

insufficient on its own,[132] and inhibition of Ang2 prevented VEGF-induced corneal neovascularization.[133] Studies also have shown that Ang2 and VEGF can act synergistically to enhance the permeability of retinal endothelium.[134] Depending on the concomitant levels of molecules such as VEGF or Ang1, Ang2 can increase or decrease angiogenesis.[135] In transgenic mice, induction of Ang2 expression in the presence of elevated VEGF levels led to increased neovascularization, whereas induction of Ang2 when VEGF was not elevated led to its regression.[136] These interactions have led to the suggestion that the administration of Ang2, in combination with a VEGF antagonist, might provide a therapeutic approach in treating ocular neovascularization.[137]

ERYTHROPOIETIN

Erythropoietin is a 30-kDa glycoprotein, upregulated by hypoxia, and known primarily for its actions as an inducer of erythropoiesis.[138] Other functions of erythropoietin are being defined, however, including neuroprotection,[139] and promotion of angiogenesis. Erythropoietin has been shown to contribute to angiogenesis in response to ischemia through upregulation of VEGF/VEGFR and in promoting recruitment of endothelial progenitor cells.[140]

Clinical evidence supporting a role for erythropoietin in ocular neovascularization comes from studies demonstrating elevations of erythropoietin in the eyes of patients with DME,[141] and DR, especially in cases of active proliferative disease,[142] and an increased risk of retinopathy of prematurity in infants treated with erythropoietin.[143] Moreover, in a murine model of retinopathy of prematurity, neovascularization was significantly inhibited by a soluble form of the erythropoietin receptor.[142] While these preliminary findings are suggestive, additional evidence is required to establish erythropoietin as a molecular target for antiangiogenesis therapy.

MATRIX METALLOPROTEINASES

MMPs, a large group of enzymes that promote angiogenesis through their degradative action on the extracellular matrix, have been extensively investigated given their importance in tumor vascularization and

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metastasis.[144] Several studies also have examined their role in promoting ocular neovascularization. In cultured RPE cells, MMP expression was upregulated by angiogenic factors, including VEGF,[82] FGF2,[82] and TNF-α.[82] MMPs were also found to cleave matrix-bound forms of VEGF, releasing soluble fragments and altering its bioavailability.[145] Another MMP action related to angiogenesis involved exposing a cryptic epitope of collagen IV that is needed for full expression of laser-induced CNV in mice.[146]

INTEGRINS

Integrins comprise a family of heterodimeric cell surface receptors that mediate cellular responses to extracellular matrix ligands such as fibronectin and vitronectin. They have been studied intensively for their importance in cancer, where they affect tumor angiogenesis, growth, and metastasis.[147] At least two dozen combinations between different α and β subunits have been identified.

Several studies have defined a role for α_v integrins (particularly $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$) and $\alpha_5\beta_1$ in the pathogenesis of ocular neovascular disease. Expression of $\alpha_v\beta_3$ was identified in active neovascular lesions of eyes with AMD and proliferative DR, while $\alpha_v\beta_5$ was found only in eyes with proliferative DR; neither integrin was expressed on mature quiescent blood vessels.[148] Inhibition of ocular neovascularization by blocking $\alpha_v\beta_3$ integrin responses has been demonstrated in experimental models using peptide antagonists,[149] and a monoclonal antibody conjugated to mitomycin C.[150] Agents that target both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ have also shown utility in preventing experimental ocular neovascularization; these include a peptide antagonist,[148] a peptide conjugated to a proapoptotic sequence,[151] and the small-molecule antagonists, SB-267268,[152] EMD478761,[153] and JNJ-26076713.[154]

Interactions between the $\alpha_5\beta_1$ integrin and its ligand fibronectin have been found to contribute to an angiogenic pathway that is distinct from that of VEGF[155]; moreover, blocking $\alpha_5\beta_1$-mediated responses has been shown to reduce ocular neovascularization in a variety of murine models. JSM5562, a small-molecule antagonist, decreased corneal neovascularization,[156] while the related molecule JSM6427 reduced the formation of laser-induced CNV[157] and ischemia-induced retinal neovascularization.[158] JSM6427 also blocked migration and tube formation in cultured endothelial cells, suggesting a key role for $\alpha_5\beta_1$/fibronectin interactions in these processes.[158] Taken together, these investigations suggest that inhibiting integrin-mediated responses is a promising approach in the treatment of ocular neovascular disease.

COMPONENTS OF THE COMPLEMENT CASCADE

Several lines of evidence have identified a role for the complement cascade in ocular neovascular disease. Genetic studies have identified an association between specific haplotypes of factor H, a regulatory component in complement function, and an elevated risk of developing neovascular AMD.[159] Complement factors C3a and C5a have been detected in the drusen found in the eyes of patients with AMD; furthermore, subretinal deposits of C3a and C5a were generated early in the course of laser-induced CNV in mice.[160]

Studies have demonstrated that genetic ablation of C3[161] or the receptors for C3a and C5a[160] inhibited laser-induced CNV in mice. These findings also correlated with reductions in the levels of VEGF[160,161] and in leukocyte recruitment,[160] supporting the hypothesis that complement-mediated inflammation plays an active role in CNV.

INHIBITORS OF ANGIOGENESIS

Much research has focused on the factors that promote angiogenesis in ocular neovascular disease, yet several naturally occurring endogenous inhibitors also have been identified. These include pigment epithelium-derived factor (PEDF), soluble VEGFR1, the complement regulatory protein CD59, $VEGF_{xxx}b$ isoforms, and the tryptophanyl-tRNA fragment.

PIGMENT EPITHELIUM-DERIVED FACTOR

PEDF is a 50-kDa glycoprotein, highly expressed by the RPE,[162] that exhibits many properties expected of an endogenous inhibitor of angiogenesis. Specifically, PEDF inhibited endothelial cell migration[163] and induced endothelial cell apoptosis *in vitro*[164] and inhibited aberrant blood vessel growth in a murine model of ischemia-induced retinopathy.[164] Moreover, PEDF has been shown to downregulate VEGF expression in endothelial cells,[165] to inhibit VEGF-induced endothelial cell permeability,[166] and to inhibit VEGF-induced signaling through VEGFR1.[167]

Clinical studies have yielded inconsistent findings, in that vitreous PEDF levels have been reported to be lower[168] or, alternately, higher[169] in patients with proliferative DR. While retinal neovascularization in preclinical models was shown to be inhibited by PEDF administered by injection[163] or by expression from a transgene,[170] another study determined that the effects of PEDF on laser-induced CNV were dose-dependent, with inhibition occurring at low doses and promotion seen at higher doses.[171]

PEDF has undergone evaluation in a phase I trial in which a single intravitreal injection of an adenoviral vector expressing human PEDF was administered to patients with AMD; results were favorable, with most subjects experiencing either an improvement or no change in vision at 6 months postinjection.[172] However, until the inconsistencies found in animal models with regard to dose-related promotion of CNV are resolved,[171] particular caution is required in the clinical use of PEDF.

SOLUBLE VEGF RECEPTOR 1

Soluble VEGFR1 is an alternately spliced, secreted isoform that lacks the exons coding for the transmembrane and signaling domains.[173] Since soluble VEGFR1 binds to VEGF and blocks its interaction with VEGF receptors, it acts as a naturally occurring inhibitor of neovascularization and has been found to be essential for preserving corneal avascularity.[174] As previously mentioned, an engineered molecule containing the VEGF-binding domains, both VEGFR1 and VEGFR2 (VEGF-Trap), is being examined clinically as a therapeutic agent.

$VEGF_{XXX}b$ ISOFORMS

$VEGF_{xxx}b$ denotes a family of VEGF isoforms, parallel to those normally considered for their impacts on angiogenesis, but which have an altered carboxy-terminus due to alternative splicing; the resulting variants can bind VEGFR-2, but since they cannot mediate downstream signaling they serve as endogenous competitive inhibitors of VEGF.[175] $VEGF_{xxx}b$ isoforms constituted 64% of the total VEGF in the vitreous of nondiabetic patients and only 12% of the total VEGF in the vitreous of diabetic patients.[176] In studies with murine models of corneal[175] and retinal[32] neovascularization, administration of $VEGF_{xxx}b$ inhibited blood vessel growth. Together, these findings suggest that $VEGF_{xxx}b$ isoforms may be a component of normal homeostasis and that their downregulation may contribute to the pathogenesis of ocular neovascular disease. The efficacy of anti-VEGF agents may thus depend on the local VEGF isoform expression pattern.

COMPLEMENTARY REGULATORY PROTEIN C59

As mentioned previously, the extent of laser-induced CNV in a mouse model was dependent on several components of the complement cascade. Further support for this mechanism has come from a recent study showing that ablation of CD59, a complement regulatory protein, promoted the development of CNV in mice, while intravitreal or intraperitoneal administration of a soluble CD59-Fc fusion protein was

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inhibitory.[177] These findings suggest that C59 serves as an endogenous inhibitor of ocular neovascularization, by downregulating the complement cascade, and it has been proposed that a soluble form of C59 could serve as a therapeutic agent.

TRYPTOPHANYL-tRNA SYNTHASE FRAGMENT

Tryptophanyl-tRNA synthase fragment (T2-TrpRS) is a 43-kDa natural cleavage product of tryptophanyl-tRNA synthase[178] that was shown to inhibit both physiological retinal angiogenesis and VEGF-induced angiogenesis in murine models.[178] In a retinopathy of prematurity model, T2-TrpRS dramatically inhibited preretinal pathological tuft formation while enhancing physiological revascularization of the obliterated retinal vasculature.[179] These actions may result from its binding to vascular endothelial cadherin, a component of the intercellular junctions between endothelial cells.[180] Recently, the combination of T2-TrpRS and an anti-VEGF aptamer strongly inhibited pathological neovascularization in a retinopathy of prematurity model.[181] This promising combination approach merits further exploration in the treatment of ocular neovascular disease.

OTHER INHIBITORS

As indicated in Table 4.1, there are numerous endogenous factors which have antiangiogenic activity. While the present chapter has focused on certain factors for which evidence supports a role in ocular neovascularization, a comprehensive discussion of endogenous inhibitors is beyond the scope of this chapter. For a comprehensive discussion of these factors, the reader is referred to the review by Zhang and Ma.[182]

SUMMARY

Systematic study of the mechanisms underlying pathological ocular neovascularization in preclinical models as well as in humans has yielded a wealth of knowledge about the numerous proangiogenic and antiangiogenic factors that modulate these processes. A major focus of research has been the role of the angiogenic promoters, the most potent of which (identified to date) is VEGF. VEGF's properties as the principal inducer of vascular permeability and its upregulation in a hypoxic environment also greatly influence the pathology associated with ocular neovascularization. PDGF-B, a molecule structurally related to VEGF, is especially crucial for the recruitment of pericytes and smooth-muscle cells to the developing vasculature and plays a key role in neovascularization of the retina and cornea. The contributions of TNF-α and erythropoietin in angiogenesis have not been as well elucidated.

Investigations involving several other ligand receptor systems have also provided evidence of their contributions to vascular development. These include the Eph kinases and their ephrin ligands, which appear to be critical for establishing arterial and venous identity, and the angiopoietins, Ang1 and Ang2. Overall, Ang1 acts to stabilize the vasculature, and inhibits VEGF-induced increases in vascular permeability, while Ang2 is primarily a destabilizing agent, which, depending on the experimental conditions, can interact with VEGF either to promote neovascularization or to induce its regression. Another signaling pathway, the Dll4-Notch system, also acts to regulate vascular patterning by inhibiting VEGF-induced angiogenic sprouting and branching.

Finally, three other important classes of angiogenic promoters have been identified: the MMPs, integrins, and components of the complement cascade. The MMPs affect VEGF signaling by releasing it from sequestered deposits in the extracellular matrix and also by exposing a cryptic collagen epitope that has been found to promote ocular neovascularization. The integrins, which are well established as mediators of interactions between the extracellular matrix and intercellular components, have recently been shown to be involved in ocular neovascularization as well. With respect to the complement cascade, genetic studies have demonstrated an increased risk of AMD for certain haplotypes of factor H, while preclinical studies have demonstrated roles for factors C3a and C5a in the development of ocular neovascularization.

Several naturally occurring factors have been identified as potential inhibitors of ocular neovascularization. PEDF acts in many assays to inhibit angiogenesis, but it may also promote angiogenesis in some contexts. Endogenous inhibitors of VEGF signaling have also been found; these include soluble VEGFR1 and a group of alternately spliced isoforms, denoted $VEGF_{xxx}b$. Finally, T2-TrpRS, a naturally occurring fragment of the enzyme tryptophanyl-tRNA synthase as well as the complement regulatory protein C59, have been found to inhibit ocular neovascularization in experimental models.

Research investigating the roles of these molecules in regulating angiogenesis has already yielded clinical benefits. Two agents targeting VEGF, pegaptanib and ranibizumab, have received clinical approval for AMD while alternative strategies for inactivating VEGF signaling, including RNA interference and a VEGF receptor fusion protein, are under active study. Infliximab, an antibody against TNF-α, has also shown promise in small-scale clinical studies, while preclinical studies suggest that PDGF-B, components of the complement cascade, and the α_5 integrins are potential molecular targets. Moreover, the endogenous inhibitors may also prove clinically useful. Thus a variety of agents, whether administered alone or as adjunctive therapy with agents targeting VEGF, offer the promise of expanding the range of treatments for ocular neovascular diseases.

Key points

- Systematic study of the mechanisms controlling angiogenesis has led to the identification of a number of proangiogenic and antiangiogenic factors active in ocular neovascularization.
- VEGF has been established as a master regulator of angiogenesis and a potent promoter of vascular permeability, making it an attractive target in treating ocular neovascularization. Two anti-VEGF agents have been approved, with others in clinical trials.
- PDGF-B plays a crucial role in the recruitment of mural cells to developing blood vessels; combination approaches targeting PDGF-B and VEGF are especially effective against ocular neovascularization in preclinical models.
- TNF-α has been shown to promote pathological angiogenesis in preclinical studies while small case series involving TNF-α inhibition have demonstrated therapeutic effects in ocular neovascular disease.
- In keeping with its inflammatory nature, components C3a and C5a of the complement cascade may contribute to the development of ocular neovascularization.
- Integrins are involved in an angiogenic pathway distinct from that of VEGF; small-molecule inhibitors of $\alpha_5\beta_1$ have been shown to reduce ocular neovascularization in preclinical models.
- MMPs promote angiogenesis by degrading the extracellular matrix to facilitate invasion by nascent blood vessels, by releasing matrix-bound growth factors, and by exposing cryptic proangiogenic epitopes.
- Several ligand receptor systems, including the angiopoietins-Tie2, ephrins-Eph kinases, and Delta4-Notch, are all essential for angiogenesis, but further work is required before this knowledge can be exploited in developing new therapies.
- Various endogenous inhibitors of angiogenesis have been identified which may prove useful as therapeutic agents. These include PEDF, complement regulatory protein C59, soluble VEGFR1, a fragment of tryptophanyl-tRNA synthase, and alternatively spliced VEGF isoforms.

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Retinal Pharmacotherapy

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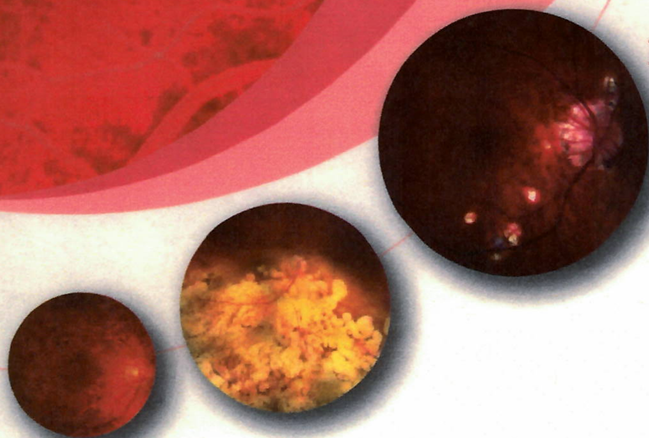

Retinal Pharmacotherapy is the first comprehensive resource devoted to pharmacologic agents and their therapeutic application in retinal and uveitic diseases. Designed for retina specialists, uveitis and ocular inflammation specialists, and comprehensive ophthalmologists, this book serves as a user-friendly, all-in-one reference to provide busy clinicians with comprehensive yet practical information about potential agents that they may consider for their patients.

- Provides a comprehensive approach to give you a complete understanding on the background and current application of each agent, including sections on: Retinal Molecular Biology; Animal Models and Routes for Retinal Drug Delivery; Retinal Diseases Amenable to Pharmacotherapy; Drugs and Mechanisms in Retinal Diseases; and, Pharmacotherapy and Surgery
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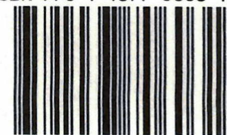

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