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Invited Review

Intraocular neovascularization

A. Yoshida, S. Yoshida, T. Ishibashi and H. Inomata

Department of Ophthalmology, Faculty of Medicine, Kyushu University, Fukuoka, Japan

Summary. An important character of the eye is transparency, so intraocular neovascularization, which is fragile and likely to result in hemorrhage, would cause a functional disorder of the eye and contribute to loss of vision associated with such diseases as retinopathy of prematurity, diabetic retinopathy, retinal vein occlusion, and age-related macular degeneration. Recently interest in the mechanisms of intraocular neovascularization has increased, and the mechanisms have been gradually elucidated using several in vitro and in vivo angiogenesis models. Blood vessels in the eye are composed of, and surrounded by, various types of cells that produce multiple factors. Neovascularization is regulated by complex interactions among these angiogenic factors, angiostatic factors, and adhesion molecules, and some of these angiogenesis-related molecules have also been suggested as new targets for novel therapeutic agents of intraocular neo-vascularization. This review focuses on in vivo representative angiogenesis models of the corneal pocket model and the model of oxygen-induced retinopathy, and discusses the role of some angiogenesis-related factors and adhesion molecules in intraocular neovascularization.

Key words: Neovascularization, vascular endothelial growth factor/vascular permeability factor, Interleukin-8, NF-κB, Adhesion molecule

Introduction

Angiogenesis, the formation of new capillary blood vessels, is essential for development, tissue regeneration, and remodeling. Angiogenesis also accompanies pathological conditions, including tumor growth, rheumatoid arthritis, psoriasis, and diabetic retinopathy (Folkman, 1995). Intraocular neovascularization is a major cause of blindness associated with such diseases as retinopathy of prematurity, diabetic retinopathy, retinal vein occlusion, and age-related macular degeneration (Diabetic Retinopathy Study Research Group, 1981; Brown et al., 1987; Prost, 1988; Moss et al., 1994; Thylefors et al., 1995). Retinopathy of prematurity occurs in premature neonates. In the premature baby the retina remains incompletely vascularized at the time of birth. Abnormal new proliferating vessels develop at the junction of vascularized and avascular retinas. Diabetic retinopathy is the most frequent cause of blindness in individuals of working age. In patients with diabetes mellitus, retinal capillary occlusions develop, creating areas of ischemic retina followed by neovascular proliferations from pre-existing retinal venules. Occlusion of the central or branch retinal vein induces hemorrhage, edema, exudates, and nonperfused retina followed by neovascularization mainly in adulthood. Age-related macular degeneration is a major cause of visual loss in persons over 65 years old. In age-related macular degeneration, neovascularization develops from choroidal vasculature, and extends into the subretinal space in the macula, the area of retina responsible for central vision. The neovascularization associated with these disorders commonly involves increased permeability of the retinal vasculature resulting in transduction of serum components into the retina and visual loss from macular edema. In addition, the neovascularization itself can lead to visual loss due to the fragility of the new vessels resulting in vitreous hemorrhage or due to progressive fibrovascular proliferation and contraction that may lead to macular traction, traction retinal detachment or rhegmatogenous retinal detachment (Patz, 1980; Aiello, 1997).

Recently the interest in the mechanisms of intraocular neovascularization has increased. No single growth factor acts alone to cause intraocular neovascularization. Neovascularization is induced by complex interactions among multiple angiogenic factors, angiostatic factors, and adhesion molecules. In this review, we focus on the mechanisms of intraocular neovascularization in which a variety of factors interact.

In vivo angiogenesis model

To understand the precise mechanisms of intraocular neovascularization developing in vivo and in vitro angiogenesis model systems is important, and so far several angiogenesis model systems have been developed. Among them, the corneal pocket model and

Offprint requests to: Ayako Yoshida, MD, Department of Ophthalmology, University of Michigan, Kellogg Eye Center, 1000 Wall Street, Ann Arbor, MI 48105, USA

the model of oxygen-induced retinopathy are representative models of angiogenesis in vivo and we mainly used those models to investigate the angiogenic properties of angiogenic factors.

The corneal pocket model (Fig. 1) is one of the most common and useful model systems of angiogenesis, because the cornea is a transparent avascular substratum in which vessels originate from a distance, the corneoscleral limbus, thus permitting monitoring and measurement of the progress of new blood vessel growth. In this model, angiogenic factors are incorporated into pellets (ethylene-vinyl-acetate or hydron pellets) and are implanted into the pockets produced in the cornea of mice, rats, or rabbits. After initial looping of limbal capillaries, enlargement and extension of the loops into the cornea occurs. Subsequently, vascular sprouts appear at the apices of the loops, a vascular network develops with the differentiation of arterial and venous branches, and vessels continue to grow toward the pellets. In mice and rats, after 5-7 days, we can measure the neovascularization from the limbus to the pellets induced by those factors (Yoshida et al., 1997, 1998a).

The model of oxygen-induced retinopathy (Fig. 2) has come into wide use as a model of retinal angiogenesis recently. In this model, neonatal animals are exposed to hyperoxia, resulting in obliteration of the posterior retinal vessels. The animals are then returned

Fig. 1. Biomicroscopic photographs of rat corneas 6 days after implantation of EVA pellets containing CINC (50 ng) (A), bFGF (50 ng) (B), or control buffer (C). **A and B.** Cornea demonstrates growth of vessels from the corneoscleral limbus toward the pellet. **C.** No neovascularization is apparent. Bar: 500 μm. Reproduced, with permission, from Yoshida et al.: The role of NF-κB in retinal neovascularization in the rat: possible involvement of cytokine-induced neutrophil chemoattractant (CINC), a member of the interleukin-8 family. J. Histochem. Cytochem. 46, 429-436, 1998.

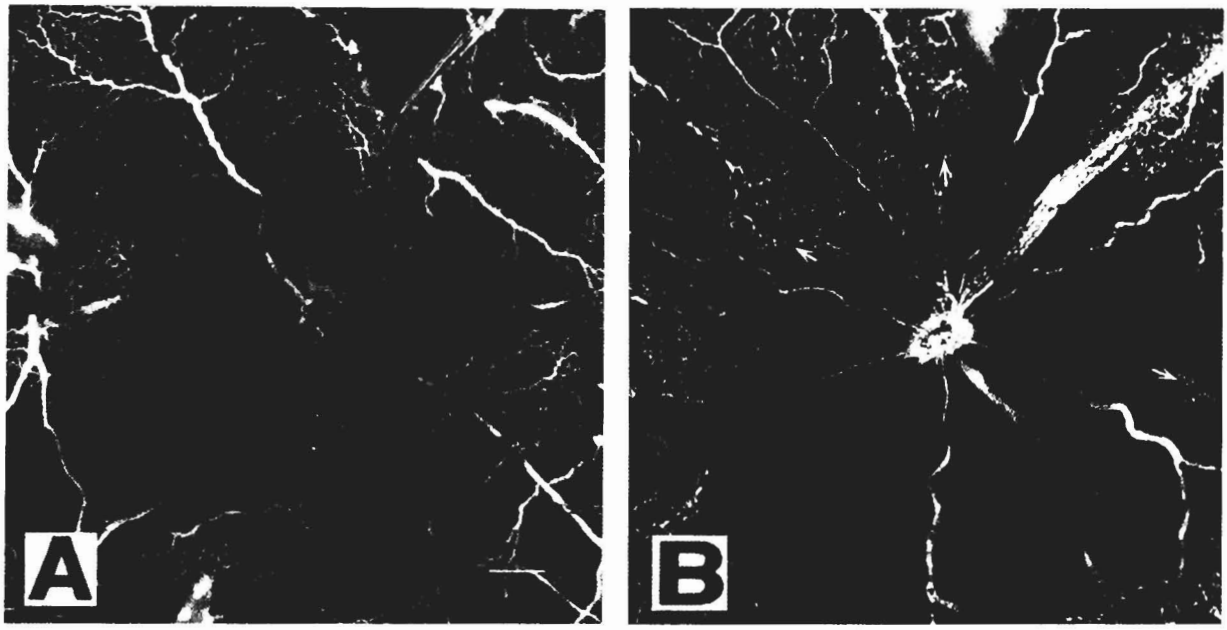

Fig. 2. Fluorescein-dextran-perfused, flat-mount retinas from animals exposed to room air or hyperoxia. **A.** The retina from postnatal day 17 mouse maintained continuously under normoxic conditions. **B.** The retina from the mouse exposed to 75% oxygen for 5 days (from postnatal day 7 to 12) and then maintained in room air for 5 days. Neovascular responses are apparent at the junction between the perfused and the nonperfused region of the retina (arrows). Bar: 200 μm.

to room air (or relatively low concentration of oxygen), which is presumed to cause relative hypoxia of the nonperfused retina, producing a neovascular response. In the past, many investigators have attempted to develop an animal model of oxygen-induced retinopathy with variable success (Patz et al., 1953; Michaelson et al., 1954; Patz, 1954; Ashton, 1968). As the interest in neovascular ocular diseases has increased in general recently, interest in animal models of proliferative retinopathy has received much attention again. Penn et al. (1993) demonstrated that retinal neovascularization is induced in neonatal rats exposed to periodically varying oxygen concentrations. Smith et al. (1994) reported an improved method to induce retinal neovascularization in neonatal mice. They allowed their newborn mice to live in room air for the first 7 days of life to permit the hyaloid vascular system to regress. Then, on day 7, the newborn mice were exposed to 75% oxygen for 5 days followed by room air. They found that 100% of the neonatal mice treated in this way developed preretinal neovascularization .

Factors affecting angiogenesis

Michaelson (1948) and Ashton et al. (1954) postulated that retinal neovascularization was caused by release of a vasoformative factor from the retina in response to hypoxia. Because of these initial hypotheses, it has become widely accepted that retinal hypoxia induces the release of factors that influence new blood vessel growth. Recently, development of new blood vessels is thought to depend upon a balance of angiogenic factors and angiostatic factors. Angiogenic factors, including vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), tumor necrosis factor α (TNF-α), angiotensin II and platelet-derived growth factor (PDGF) have been implicated in the development of intraocular neovascularization (Schultz and Grant, 1991; Robbins et al., 1994; Berka et al., 1995; Pierce et al., 1995; Spranger et al., 1995). In addition, we demonstrated that interleukin-8 (IL-8), a member of the CXC chemokine family having potent angiogenic properties, may have an important role in retinal neovascularization (Yoshida et al., 1998b).

Transforming growth factor-β (TGF-β) is known to have an angiostatic property in the eye (Pfeiffer et al., 1997). Other factors, including angiostatin, endostatin, and thrombospondin-1, may have potential angiostatic properties in the eye (Folkman, 1995; O'Reilly et al., 1997). In addition, endothelial cells interact with and respond to changes in the extracellular matrix through cell surface receptors called adhesion molecules.

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF)

VEGF/VPF is an endothelial cell-specific mitogen that was identified and cloned in several studies (Senger et al., 1983; Ferrara and Henzel, 1989; Keck et al., 1989; Leung et al., 1989). It has two main activities: increased vasopermeability and induction of angiogenesis (Senger et al., 1983; Keck et al., 1989). VEGF/VPF is a highly conserved homodimeric glycoprotein existing as four isoforms in the human as a result of alternative RNA splicing ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$). At least two of these isoforms are freely diffusable molecules within the eye. The novel VEGF family, VEGF-B and VEGF-C, also have the ability to promote proliferation of vascular endothelial cells, and are located in the retina (Takagi et al., 1998). VEGF/VPF is produced by a number of ocular cells, including retinal vascular endothelial cells, pericytes, glial cells, ganglion cells, retinal pigment epithelial cells, and invasive leukocytes (Adamis et al., 1993; Simorre-Pinatel et al., 1994; Aiello et al., 1995a; Nomura et al., 1995; Lutty et al., 1996 . Hypoxia upregulates VEGF/VPF, owing both to increased transcription mediated by hypoxia-inducible factor -1 and an increase in VEGF/VPF mRNA stability dependent on the 3' region of the mRNA (Semenza et al., 1998).

Fms-like tyrosine kinase (Flt-1), kinase insert domain containing receptor/fatal liver kinase-1 (KDR/Flk-1), fms-like tyrosine kinase-4 (Flt-4), and a soluble type of Flt-1 are receptors for the VEGF family. The expression of Flt-1 is predominant in bovine retinal pericytes and KDR/Flk-1 is expressed predominantly in retinal endothelial cells in vitro (Takagi et al., 1996). KDR/Flk-1 promoter activity is modulated by Sp1 and Sp3 (Hata et al., 1998). In vivo, increased KDR/Flk-1 and Flt-1 mRNA levels are found in ganglion cells and the inner and outer nuclear layers in diabetic animals (Hammes et al., 1998).

The ocular role of VEGF/VPF has been investigated in a variety of animal models. Tolentino et al. (1996) demonstrated that intravitreal injection of VEGF/VPF induced retinal hemorrhage, edema, venous beading, capillary occlusion with ischemia, microaneurysm formation, and intraretinal vascular proliferation that are common to diabetic retinopathy and other ischemic retinopathies. Over-expression of VEGF in the retina is sufficient to cause intraretinal and subretinal neovascularization (Okamoto et al., 1997). In oxygen-induced retinopathy, VEGF/VPF mRNA and protein production increases rapidly following the induction of hypoxia (Pierce et al., 1995, Vinores et al., 1997). The use of antisense phosphorothioate oligodeoxynucleotides against VEGF/VPF and human Flt-1 or murine KDR/Flk-1 chimeric protein inhibits retinal neovascularization and VEGF/VPF synthesis in the oxygen-induced retinopathy model (Aiello et al., 1995b; Robinson et al., 1996).

Intraocular VEGF/VPF concentrations increase in patients with active intraocular neovascularization arising from proliferative diabetic retinopathy, central retinal vein occlusion, retinopathy of prematurity, and subretinal neovascularization (Aiello et al., 1994; Wells

et al., 1996). In these studies, patients who had no neovascular disorder, no active neovascularization within the eye or who once had active neovascularization that was now quiescent all had low concentrations of intraocular VEGF/VPF. In the early stage of diabetic retinopathy, VEGF/VPF production also increases (Amin et al., 1997). Because we cannot find a non-perfused area in this stage, we believe VEGF/VPF is also induced by factors other than hypoxia. VEGF/VPF may be induced by other cytokines such as TNF-α and IL-1β produced by invasive leukocytes (Yoshida et al., 1997). Another candidate is advanced glycated end product (AGE), which induces VEGF/VPF production in retinal pigment epithelial cells, vascular endothelial cells, and glial cells (Murata et al., 1997). Vinores et al. (1997) showed that VEGF/VPF was also increased in human and experimental ischemic and non-ischemic disorders in which blood retinal barrier breakdown is known to occur, suggesting that VEGF/VPF may also contribute to blood retinal barrier breakdown in addition or in association with its role of inducing neovascularization. Furthermore, Gerhardinger et al. (1998) demonstrated that in the retina of individuals without diabetes mellitus and retinal diseases several types of cells constitutively synthesize VEGF/VPF, suggesting that VEGF/VPF may have actions affecting the modulation of neuronal function or homeostasis in the retina.

The choroidal neovascular membrane removed from human patients with age-related macular degeneration shows evidence of VEGF/VPF expression (Lopez et al., 1996). In choroidal neovascularization in animal models, retinal pigment epithelial cells, Müller cells, ganglion cells, and invasive macrophages show VEGF expression (Ishibashi et al., 1997).

Therefore, VEGF/VPF may have an important role in the development of intraocular neovascularization, and it may provide a target for therapeutic intervention in retinal neovascularization.

Fibroblast growth factor (FGF)

In the FGF family, bFGF and acidic FGF (aFGF), single-chain proteins of approximately 140 amino acids, are well known for angiogenic factors. FGF is produced by many cells, including retinal pigment epithelial cells, vascular endothelial cells and glial cells. Both bFGF and aFGF have no secretory signaling sequence typical of proteins secreted by the endoplasmic reticulum-Golgi apparatus (Mignatti et al., 1992). Basic FGF influences vascular endothelial cells by both paracrine and autocrine control. In vitreous fluids from patients with proliferative diabetic retinopathy, bFGF concentration increases compared to controls (Sivalingam et al., 1990; Boulton et al., 1997). Basic FGF is expressed in choroidal neovascular membrane from patients with age-related macular degeneration (Frank et al., 1996; Kitaoka et al., 1997).

However, when bFGF-deficient mice were compared with wild-type mice in an oxygen-induced retinopathy model, bFGF-deficient mice developed the same amount of retinal neovascularization as wild-type mice (Ozaki et al., 1998). In addition, mice with bFGF overexpression in retinal photoreceptors showed no significant difference in the amount of retinal neovascularization compared with wild-type mice in the same model. In ocular neovascularization, the importance of bFGF may be less than previously assumed.

Interleukin-8 (IL-8)

IL-8, a chemotactic cytokine for T lymphocytes and neutrophils (Eckmann et al., 1993; Jung et al., 1995; Rasmussen et al., 1997) is the most well known CXC chemokine and is induced by various stimuli, including TNF-α, IL-1β, and hypoxia (Kasahara et al., 1991; Karakurum et al., 1994; Yoshida et al., 1997). IL-8 has been shown to be able to mediate both in vitro endothelial cell chemotactic and proliferative activity (Koch et al., 1992), as well as in vivo angiogenesis (Strieter et al., 1992), and it also contributes to such angiogenesis-dependent disorders as rheumatoid arthritis, psoriasis, wound repair, malignant melanoma, bronchogenic carcinoma, and diabetic retinopathy (Koch et al., 1992; Endo et al., 1994; Nickoloff et al., 1994; Smith et al., 1994; Elner et al., 1995; Singh et al., 1995; Strieter et al., 1995). We showed that the IL-8 concentration in vitreous fluids from patients with such active retinal neovascularization as proliferative diabetic retinopathy and retinal vein occlusion is significantly higher than that for individuals with quiescent neovascularization or without a neovascular disorder (Yoshida, et al., 1998b). The predominant risk factor for the development of intraocular neovascularization is the extent and duration of retinal ischemia (ischemic drive) (Shimizu et al., 1981; Magargal et al., 1982). In patients with proliferative diabetic retinopathy, retinal ischemia generally begins in the midperipheral retina, and retinopathy progresses with a relatively low ischemic drive (Magargal et al., 1982). In contrast, the avascular region in patients with retinal vein occlusion tends to appear rapidly and to spread extensively. In our study, the IL-8 concentration in vitreous fluids from patients with retinal vein occlusion was higher than that for patients with active proliferative diabetic retinopathy (Yoshida et al., 1998b. The consistency of the clinical observations with the IL-8 concentration implicates IL-8 in ischemia-associated retinal neovascular diseases, such as proliferative diabetic retinopathy and retinal vein occlusion. The greater the ischemic drive in the retina, presumably the greater the extent of hypoxia experienced by retinal cells and the greater the amount of IL-8 produced. Furthermore, vitreous fluids containing a high concentration of endogenous IL-8 induced angiogenesis in vitro. We detected IL-8 expression immunohistochemically in vascular endothelial cells and in glial cells in the retinas of

patients with neovascularization. Thus, retinal glial cells and endothelial cells are likely sources of IL-8 in the ocular fluid of patients with ischemic retinal diseases.

The promoter of the IL-8 gene contains potential binding sites for the transcription factor NF-κB (Matsusaka et al., 1993; Mukaida et al., 1994; Oliveira et al., 1994). NF-κB, which was originally identified as a heterodimeric complex of 50- and 65-kDa (p65) subunits (Baeuerle, 1991), is central to the regulation of numerous inflammatory and proliferative response genes (Lenardo and Baltimore, 1989; Grilli et al., 1993; Liao et al., 1993, 1994). NF-κB may regulate the initiation of angiogenesis in vitro (Shono et al., 1996; Stoltz et al., 1996). IL-8 produced through NF-κB activation contributes to TNF-α-induced angiogenesis (Yoshida et al., 1997). In addition, activated NF-κB and cytokine-induced neutrophil chemoattractant (CINC), a rat homolog of IL-8 or Gro, are both expressed in retinal vascular endothelial cells and glial cells in the oxygen-induced retinopathy (Yoshida et al., 1998a). We also demonstrated that CINC has an angiogenic property using the corneal micropocket assay. In bovine retinal glial cells in vitro, hypoxia induced NF-κB activation and IL-8 gene expression, and both these effects were inhibited by pyrrolidine dithiocarbamate, a specific inhibitor of NF-κB activation (Yoshida et al., 1998b). Thus, hypoxia-induced expression of IL-8, mediated by activation of NF-κB, in retinal glial cells and vascular endothelial cells may be important in the pathogenesis of retinal neovascularization. In addition to the IL-8 gene, NF-κB regulates many other angiogenesis-related genes, including those encoding TNF-α, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) (Baeuerle and Henkel, 1994). Therefore, NF-κB, in addition to IL-8 and VEGF, may also provide a target for therapeutic intervention in retinal neovascularization.

Inflammatory cytokines

Monocytes/macrophages are known to produce a variety of cytokines, such as TNF-α, IL-1β and bFGF, upon activation and are important in angiogenesis. Macrophage-derived TNF-α may trigger angiogenesis by inducing IL-8, VEGF, or bFGF in an autocrine or paracrine manner (Yoshida et al., 1997). In addition, tumor-associated monocyte/macrophage infiltration correlates with tumor angiogenesis in individuals with invasive breast cancer (Leek et al., 1996), and the accumulation of monocytes/macrophages that produce TNF-α and bFGF is associated with angiogenesis after femoral artery occlusion in the rabbit (Arras et al., 1998). Monocytes/macrophages also participate in neovascular disorders, such as diabetic retinopathy. Furthermore concentration of monocyte chemoattractant protein-1 (MCP-1) and TNF-α also increase in vitreous fluids from patients with proliferative diabetic retinopathy (Elner et al., 1995; Spranger et al., 1995). MCP-1, a CC chemokine, attracts monocytes, T cells, mast cells, and basophils, and it may also play an important role in intraocular angiogenesis.

Adhesion molecules and extracellular matrix

Adhesion molecules are important for intraocular neovascularization. Some adhesion molecules, such as VCAM-1, ICAM-1, and E-selectin are involved in diabetic retinopathy. Recently, integrins have also been demonstrated to play an important role in intraocular angiogenesis (Brooks, 1996). They are composed of noncovalently associated α and β chains, and can bind to an array of extracellular matrix (ECM) components, including laminin, collagen, fibronectin, thrombospondin, fibrinogen, and vitronectin. The binding of an integrin to an ECM component sends an intracellular signal that initiates a variety of endothelial responses, such as adhesion, migration, proliferation, and apoptosis. Basic FGF-induced angiogenesis depends on integrin $\alpha_v\beta_3$ using rabbit corneal assay and chick chorioallantoic membrane assay. In contrast, VEGF/VPF-induced angiogenesis depends on integrin $\alpha_v\beta_5$ (Friedlander et al., 1995). In oxygen-induced retinopathy, the α_v-integrin antagonist peptide reduced retinal neovascularization (Hammes et al., 1996). In this model the laminin receptor is present (Stitt et al., 1998). Furthermore, integrin $\alpha_v\beta_3$ is expressed in choroidal neovascular membranes from patients with age-related macular degeneration and ocular histoplasmosis syndrome (Friedlander et al., 1996) and both integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are on fibrovascular membranes from patients with proliferative diabetic retinopathy.

A fragment of matrix metalloproteinase 2 (MMP-2), comprising the C-terminal hemopexin-like domain, termed PEX, prevents MMP-2 binding to $\alpha_v\beta_3$ and blocks cell surface collagenolytic activity. Brooks et al. (1998) demonstrated that a naturally occurring form of PEX can be detected in vivo in conjunction with $\alpha_v\beta_3$ expression during developmental retinal neovascularization, suggesting that it would interact with endothelial cell $\alpha_v\beta_3$ where it would serve as a natural inhibitor of MMP-2 activity, thereby regulating the invasive behavior of new blood vessels.

Conclusions

Recent work has elucidated the molecules and mechanisms that induce the development of intraocular neovascularization. Many studies have demonstrated the involvement of multiple factors found in association with tumor-related angiogenesis, such as Tie-1 and Tie-2 in intraocular neovascularization. Because the eye is a highly differentiated and unique sense organ in which transparency is very important, intraocular neovascularization may be involved in the factors characteristic of the eye. However, the mechanisms are not fully understood and an effective treatment for retinal neovascularization remains elusive. Current clinical treatment for active intraocular neo-

vascularization involves destroying the peripheral retina by either laser photocoagulation or cryotherapy. Although often effective, this procedure induces substantial changes in capillary hemodynamics and in the quality of vision because of its inherently destructive property. In patients with very severe neovascularization, laser alone may not prevent severe visual loss. Pharmacological therapy for retinal neovascularization would be a major benefit to patients with intraocular angiogenic diseases. An improved understanding of neovascularization may suggest new approaches to therapy.

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