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Mechanisms of Retinal and Choroidal Neovascularization

Patricia A. D’Amore

Angiogenesis, or the formation of new blood vessels, is a major component of a number of retinal and choroidal diseases, including proliferative diabetic retinopathy and age-related macular degeneration. For more than 50 years, researchers and clinicians have hypothesized about the pathogenesis of vessel proliferation. The establishment of techniques for culturing cells of the vasculature and neural retina, the identification and characterization of distinct angiogenic factors, and the development of the techniques of molecular biology have allowed significant insight into the cellular and molecular basis of choroidal and retinal microangiopathy.

In general, it is likely that growth of the microvasculature is regulated by a balance between local stimulators and inhibitors (Fig. 1). These growth-stimulating molecules include polypeptide growth regulators, such as vascular endothelial growth factor—vascular permeability factor (VEGF–VPF, hereafter referred to as VEGF), acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), interleukin-8, insulin-like growth factor, and hepatocyte-growth factor (also known as scatter factor). These factors may exert their action in a soluble form, but some (for example, aFGF, bFGF, and VEGF) have been reported to be bound to heparan sulfate on the cell surface and in the extracellular matrix. Potential growth inhibitors include not only soluble factors such as transforming growth factor beta (TGF-β), but also extracellular matrix constituents such as thrombospondin, which is reported to have antiangiogenic activity.

Although the factors involved in the control of angiogenesis are limited, there are numerous levels of control. For example, the synthesis of VEGF is regulated by the oxygen level in the microenvironment of the cells. bFGF may be controlled at a number of levels, including release and sequestration by heparan sulfate. On the other hand, the control of TGF-β appears to be not at the level of synthesis but, rather, through its activation. Thus, as would be expected, the critical process of angiogenesis appears to have multiple sites of regulation (Fig. 1).

A study of the clinical course of proliferative retinopathy has provided many clues into its possible etiology. One of the earliest changes observed in background retinopathy is the loss of pericytes. The temporal association between pericyte dropout and neovascularization led to the speculation that pericytes might exert an inhibitory influence on capillary growth. Studies using tissue culture cells have provided experimental evidence to support this hypothesis. Experiments with capillary endothelial cells and pericytes indicate that the coculture of two cell types inhibits endothelial cell proliferation, and that the inhibition is mediated by TGF-β, which is activated on contact between the cells. The loss of pericytes is probably not sufficient to induce the growth of new vessels. Instead, the absence of the pericyte (and the resultant lack of the inhibitory factor TGF-β) may render the microvasculature more vulnerable to changes in local concentrations of angiogenic stimuli (Fig. 1).

Ischemia inarguably precedes the development of neovascularization in the retina as well as in other tissues. In his survey of proliferative retinopathies, Wise noted that there were characteristics common to all diseases. Among these was the fact that viable tissue is necessary for "retinitis proliferans." Accordingly, Wise postulated that after retinal capillary or vein obstruction, tissues became hypoxic and produced a vasoproliferative stimulus, leading to the development of neovascularization. In their discussion of this hypothesis, Wise and colleagues comment that "[a]lthough the actual vasostimulating factor is unknown, it is closely related to cellular hypoxia."

Background diabetic retinopathy is characterized by areas of capillary closure, ranging in size from 100 μm² in early stages of the disease to as much as 5 mm² in severe diabetic retinopathy. Numerous explanations have been put forward to explain the capillary nonperfusion, including alterations in the coagulation system. More recently, abnormal leukocyte rheology has been postulated to play a central role in capillary occlusion (reviewed by Hatchell and Sinclair). Observations in a rat model of alloxan-induced diabetes showed many capillaries occluded by macrophages. Further, analysis of circulating monocytes and granulocytes revealed a significant increase in the percentage...
of activated cells. Similarly, using video microscopy, leukocyte trapping in muscle capillaries of streptozotocin-induced diabetic rats was found to lead to a 15% increase in microvascular resistance. Studies in which experimental branch vein occlusion was created in miniature pigs by laser photocoagulation have conclusively linked ischemic retinal tissue with the development of preretinal neovascularization. Whereas the Po2 of the intervascular zones of a normal retina was 27.3 ± 2.9 mm Hg, the average Po2 of retinas in which blood flow in veins was interrupted by photocoagulation with lasers (creating ischemia) was 13.7 ± 1.1 mm Hg. These results are consistent with the hypothesis that ischemic tissues produce and release stimulators of new blood vessel formation. Furthermore, that photocoagulation of the ischemic retinas restored Po2 to the preischemic value (28.0 ± 3.0 mm Hg) supports the supposition that clinical panretinal photocoagulation inhibits and reverses vasoobliteration associated with ischemic retinopathies by eliminating hypoxia. Alternatively, the efficacy of photocoagulation may be due to the destruction of the cells responsible for production and release of the angiogenic stimulus. Of particular interest was the observation that photocoagulation of the ischemic retina did not lead to the inhibition of neovascularization in this model. The authors speculated that the lack of effect may be due to the fact that the photocoagulation was directed to only a portion of the ischemic retina. Indeed, the investigators noted that “new vessels continued to grow after this treatment toward the adjacent nonphotocoagulated territory which remained hypoxic.”

Retinopathy of prematurity may also have a significant ischemic component. In retinopathy of prematurity, the exposure of immature retinal vessels to high levels of oxygen appears to lead to the cessation of their growth. It has been postulated that the undifferentiated state of the growing vessels, including inadequate capacity to scavenge toxic oxygen byproducts, renders them vulnerable to hyperoxia. The hyperoxia sensed by the developing vessels may be absolute (if the infant is exposed to high levels of oxygen) or relative (because Po2 in utero is 40 mm Hg, breathing room air might represent a hyperoxic insult). The retina is a metabolically active tissue and requires among the highest blood flow rates of any tissue. Neuronal retinal development continues for many weeks after the birth of a premature infant. If the need for additional vasculature to meet the oxygen requirement is not met, the result is ischemic retinal tissue. Thus, the final phase of retinopathy of prematurity may not be too dissimilar from diabetic retinopathy—both may involve ischemia leading to the induction or release of an angiogenic factor(s).

Although the data reported to date do not prove that the elaboration of angiogenic factors by ischemic tissue is the culprit in ocular neovascularization, the findings are consistent with and in strong support of this hypothesis. The list of factors that have been shown to be angiogenic has expanded rapidly (for a review, see Klagsbrun and D’Amore10). This review will deal with the two most thoroughly characterized angiogenic factors, VEGF and bFGF. The possible involvement of other factors, such as growth hormone, insulin-like growth factor-1, nerve growth factor, platelet-derived growth factor, cerebrospinal fluid, and epidermal growth factor, in pathologic ocular neovascularization has been discussed in various reviews.11,12

VASCULAR ENDOTHELIAL GROWTH FACTOR

Vascular endothelial growth factor, a 46-kd homodimer, was first identified and purified on the basis of its ability to increase microvascular permeability (for review see Ferrara et al13 and Klagsbrun and Soker14). Since that time, however, VEGF has been shown to be a potent angiogenic factor and endothelial cell-specific mitogen. Vascular endothelial growth factor refers to a family of four polypeptides of 121, 165, 189, and 206 amino acids, derived from the same gene by alternative splicing. At least three tyrosine kinase receptors for VEGF, flt-1, kdr/flk-1, and flt-4, have been identified. Although the precise mechanism of action of VEGF as an angiogenic agent has not been elucidated, its ability to stimulate endothelial cell proliferation indicates that it may induce new blood vessel formation through direct action on endothelial cells. This hypothesis is supported by observations of tumor vascularization. Investigations of glioblastoma revealed VEGF mRNA in tumor cells, whereas receptor expression was confined to the capillaries growing in...
the same vicinity.\textsuperscript{15,16} In situ hybridization during mouse development revealed \textit{flk-1} mRNA at high levels in the endothelium of proliferating endothelial cells but dramatically reduced in the quiescent endothelium of adult capillaries. The spatial and temporal correlation between VEGF and its receptor during development strongly suggest that VEGF may mediate angiogenesis during development.

Perhaps the most exciting aspect of recent progress in the study of VEGF is the observation that expression of this angiogenic agent is influenced by local oxygen concentrations. In the study of tumor vascularization referred to above, VEGF transcripts were observed in tumor cells at the periphery of necrotic areas, cells presumed to be hypoxic. The direct regulation of VEGF mRNA levels by hypoxia was demonstrated by culturing tumors cells in a hypoxic environment; a 13-fold induction in VEGF mRNA was measured in cells grown under anoxic conditions.\textsuperscript{16} Evidence for a causal role of VEGF in vascularization is provided by studies in which monoclonal antibodies against VEGF were shown to inhibit the growth of tumors transplanted into nude mice but to have no effect on the proliferation of the tumor cells in culture.\textsuperscript{17} Taken together, these recent findings provide strong evidence for a role for VEGF as a mediator of both normal and pathologic ischemia-induced angiogenesis.

**VEGF IN THE OCULAR NEOVASCULARIZATION**

The regulation of VEGF by hypoxia makes it a compelling candidate for the long-sought "vasoformative factor," postulated to mediate ischemia-induced retinal neovascularization. Part of the difficulty encountered in identifying agents that might be involved in pathologic neovascularization has been the lack of reliable models of ocular angiogenesis. Recent data using a primate model of iris neovascularization indicate a spatiotemporal association of VEGF with ischemia-induced iris neovascularization.\textsuperscript{18} Laser occlusion of the branch retinal veins in a cynomologus monkey was used to create iris neovascularization. Although proliferative retinal disease is a major issue under investigation, the greater accessibility of the iris for visualization makes this a useful model. Furthermore, it is assumed that the iris neovascularization in this model results from the diffusion of angiogenic agents released from ischemic retinal tissue, simulating neovascularization in the inner retinal veins. Indeed, analysis of the aqueous fluid of eyes that had been made ischemic by laser occlusion of the retinal veins revealed elevated levels of VEGF. The levels exceeded those necessary for maximal stimulation of endothelial cells. VEGF was undetectable in the aqueous of control eyes. More important, the time course of the increased VEGF preceded the development of the iris neovascularization, and the subsequent decrease in the aqueous levels was followed by regression of the vessels. Northern analysis indicated a dramatic increase in VEGF mRNA in the ischemic retinas, and in situ hybridization revealed VEGF transcripts in cells of the inner nuclear layer. Although data are not available regarding the site of production of VEGF, Müller cells and vascular wall cells (smooth muscle and pericyte) are likely candidates. The likely relevance of this model to human disease is evidenced by analysis of VEGF levels in the vitreous of human eyes with proliferative retinopathy, which showed significant increases compared to controls.\textsuperscript{19}

In vitro analysis of human retinal pigment epithelial cells (hRPE) has revealed that these cells are also capable of making VEGF\textsuperscript{20} and that its synthesis is induced in a hypoxic environment.\textsuperscript{21} VEGF mRNA was elevated as early as 6 hours after exposure to hypoxia and continued to increase for up to 24 hours. The elevated mRNA was correlated with an increase in the synthesis and secretion of the protein. Pigment epithelium removed directly from donor eyes contained significant levels of VEGF protein. However, because these donor eyes were studied at least 24 hours after death and because upregulation of VEGF is known to be rapid (elevated VEGF mRNA was measured in myocardium after two 10-minute cycles of ischemia and 30 minutes of reperfusion\textsuperscript{22}), one cannot assume that normal pigment epithelium in situ constitutively synthesizes comparable levels.

These results indicate that pigment epithelium is capable of synthesizing substantial levels of VEGF when activated. A similar sequence of events may contribute to the choroidal neovascularization associated with age-related macular degeneration. We speculate that pigment epithelium situated on an intact Bruch’s membrane is maintained in its most differentiated and quiescent state, and that it synthesizes relatively low levels of VEGF. Activation of the pigment epithelium, as might occur with the thickening or disruption of Bruch’s membrane, or with ischemia as a result of the loss of the choroidal circulation, would then lead to upregulation of VEGF synthesis and to a directed neovascular response in the underlying choriocapillaris. Although ischemia has not been extensively documented as a factor in choroidal neovascularization, recent studies indicate angiopathic changes, including loss of the endothelium and capillary dropout in retinas from older patients,\textsuperscript{23} capillary dropout, arteriovenous anastomoses, neovascularization, and degeneration of Bruch’s membrane in choroid from patients with diabetes,\textsuperscript{24} and fibrin deposits in choroid of patients with sickle cell disease.\textsuperscript{25}
FIBROBLAST GROWTH FACTOR

Basic fibroblast growth factor, a prototypic member of a family of heparin-binding growth factors, was first purified from tumors on the basis of its ability to adhere to heparin and to stimulate the proliferation of endothelial cells. Since this original observation, a great deal of information has been gathered regarding the sources of FGF and their target cell specificity (for a review, see Klagsbrun and D’Amore). In general, virtually every tissue examined contains at least one member of the FGF family. The actions of FGF are varied: It has been implicated in the induction of the mesoderm in early embryogenesis and in the induction of differentiation of some cells (e.g., neural cells) and the suppression of differentiation in others (e.g., myoblasts). In spite of strong indications that bFGF plays important physiological roles, speculation regarding the mechanisms of FGF action have been hampered by the fact that bFGF lacks a signal sequence and appears, for the most part, not to be secreted. This fact has led to the suggestion that bFGF might be secreted by alternate pathways, including cell injury or cell death (for a review, see D’Amore).

FIBROBLAST GROWTH FACTOR IN OCULAR NEOVASCULARIZATION

Although the evidence of a role for FGF in ocular angiogenesis is less compelling than that for VEGF, FGF as a mediator of neovascularization cannot be excluded. Both acidic and basic FGF have been demonstrated in the normal adult retina. bFGF has been localized to the astrocytes of the retinal ganglion cell layer, as well as to the cells of the inner nuclear layer, and reports of FGF associated with the interphotoreceptor matrix are mixed.

Total FGF-like activity was reported to be elevated in the retinas of newborn mice exposed to hypoxia. In a study in which krypton laser burns were used to induce subretinal neovascularization, uninjured RPE was observed to be negative for aFGF and bFGF, whereas after laser treatment, the cells stained positively for both acidic and basic FGF, leading the authors to suggest a role for FGF in the neovascular process. Experiments to assess the effects of hypoxia on the expression of angiogenic factors by hRPE revealed a significant increase in cell-associated bFGF with acute (24-hour) hypoxia and the release of bFGF with cell injury induced by chronic (48-hour) hypoxia.

Numerous studies have examined ocular fluids from patients with proliferative diabetic retinopathy. Some report elevated levels of FGF associated with vasoproliferation, whereas others report no significant changes in bFGF. The inconsistency in these studies may derive from differences in the sensitivity of assay systems or from improper storage of samples before analysis (bFGF is known to be denatured by exposure to proteolytic enzymes, which are likely to be present in such samples). The high affinity of FGF for heparin may help to facilitate the local action of FGF by sequestering it on the cell surface or in the basement membrane. This might explain the difficulty in detecting FGF in body fluids. Factors such as bFGF, however, most likely act locally in an autocrine or paracrine fashion. Thus, their presence or absence in a tissue fluid may not be a meaningful reflection of their role.

THE VASOFORMATIVE FACTOR

The recent demonstration of the regulation of VEGF by hypoxia makes this factor an ideal candidate as a mediator of ocular neovascularization. Thus, retinal ischemia may lead to a rapid induction of VEGF, resulting in retinal angiogenesis. Ischemic or activated pigment epithelium may upregulate VEGF, leading to choroidal neovascularization. Because at least two forms of VPF-VEGF are freely diffusible, a majority of the peptide should be exported to the surrounding tissue. By analogy to the glialoma, endothelial cells of the surrounding vessels might increase their expression of VEGF receptors, leading to their increased proliferation and the formation of new vessels.

bFGF may also contribute to the angiogenic response. Unlike VEGF, which does not appear to be constitutively synthesized at significant levels, bFGF has been shown to be present in the retina. Although hypoxia may increase bFGF levels (see above), a key to bFGF regulation may be its accessibility. For example, hypoxia may induce changes in cell permeability, leading to the release of the cytoplasmic bFGF. In support of this hypothesis, cell injury induced by prolonged hypoxia, scraping, irradiation, and stretching have all been shown to lead to the release of bFGF. Complement-mediated release of growth factors from endothelial cells has also been demonstrated. In light of the compelling evidence for a role for monocyte adhesion in microvascular obstruction (see above), this observation takes on new relevance.

In conclusion, most conditions associated with new blood vessel formation are characterized by relative ischemia—hypoxia. This is true not only for ocular neovascularization but also for angiogenesis elsewhere in the body, including wound healing and tumor vascularization. Biochemical and molecular data indict VEGF as a mediator of ocular neovascularization. Although the evidence is less compelling, bFGF may also play a role in the genesis of pathologic neovascularization, perhaps because of its passive leakage, which occurs with cell injury. Convincing data indicate that bFGF and VEGF are synergistic in their angiogenic...
effects. Because both VEGF and bFGF are constituents of the retina, it may be the combined actions of the two agents that lead to the new vessel growth. The existence of reproducible animal models of ocular neovascularization, the identification of candidate angiogenic agents, and the availability of strategies for their neutralization should permit the design of experiments that will definitively reveal the identity of the vasoformative factor(s).

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