Human Ciliary Epithelium as a Source of Synthesis and Secretion of Vascular Endothelial Growth Factor in Neovascular Glaucoma

Kakarla V. Chalam, MD, PhD; Vikram S. Brar, MD; Ravi K. Murthy, MD

Vascular endothelial growth factor (VEGF), a secreted 46-kDa glycoprotein, is an endothelial cell–specific angiogenic and vasopermeable factor.1–6 Vascular endothelial growth factor mediates its effects through high-affinity, cell-surface transmembrane receptors, including tyrosine kinase and fetal liver kinase 1.6 Hypoxia regulates its gene expression in vitro and in vivo.7,8 Expression of VEGF messenger RNA (mRNA) is induced rapidly in a variety of cultured cells, including retinal pigmented epithelium cells, by exposure to low levels of oxygen.8,10

Neovascular glaucoma is a serious complication of a number of vascular disease processes that affect the eye. Diabetic retinopathy, retinal venous obstruction, and sickle cell disease are the leading etiologic factors for the development of neovascular glaucoma.11,12 The common features in these diseases are retinal ischemia and hypoxia, which stimulate the synthesis and secretion of VEGF in the retina13–15; subsequent diffusion of VEGF into the vitreous and aqueous humor causes intraocular neovascularization.16

Application of panretinal photocoagulation (PRP) to an ischemic retina may induce regression of iris neovascularization in most patients.17 In several refractory cases, contact Nd:YAG laser cyclophotocoagulation resulted in complete regression of iris neovascularization.18 These observations prompted us to consider the involvement of nonretinal tissue in the synthesis and secretion of VEGF. Vascular endothelial growth factor was noted in the iris and ciliary body in primary uveal melanoma.19 To search for other sites of VEGF production, we investigated the ciliary epithelium as a possible source of this growth factor with immunohistochemical analysis and in situ hybridization.

Methods

Tissue Preparation

Sixteen human enucleated eyes were investigated for the presence of VEGF with immunohistochemical analysis (VEGF protein) and in situ hybridization (VEGF messenger RNA). Eight human
eyes with neovascular glaucoma were obtained from the Georgiana Dvorak Theobald Laboratory at the University of Illinois at Chicago. The work was performed under the policies regulated by the eye banks from whom the enucleated eyes were procured. No local institutional review board approval was involved in the process. These eyes had no light perception and were enucleated because of uncontrolled neovascular glaucoma that had developed secondary to other ocular disease and failed to respond to standard care interventions, such as PRP. Baseline demographic characteristics of donor eyes were not noted. However, 8 age-matched human eyes without intraocular neovascularization (controls) were obtained from the South Carolina Lions Eye Bank for comparative analysis. All eyes were fixed in 10% formalin and processed for embedding in paraffin wax. Tissue sections 6 μm thick were cut using an RNase-free method and mounted on 3-aminopropyltriethoxysilane-coated glass slides. Tissue sections were kept at 4°C until used. The localization of VEGF protein by immunohistochemical analysis and VEGF mRNA by in situ hybridization was performed on deparaffinized and rehydrated sections.

**Immunohistochemical Analysis**

The tissue sections were treated with 3% hydrogen peroxide to eliminate endogenous peroxidase activity and 0.1% Triton X-100 to increase permeability. They were then blocked with 0.5% bovine serum albumin and incubated overnight in a 1:500 dilution of anti-VEGF receptor 2 antibody (Calbiochem Corp) at 4°C. The sections were subsequently incubated with biotinylated secondary antibody for 1 hour and with avidin-biotinylated peroxidase complex reagent (Vector Laboratories, Inc) for 5 minutes. The reaction product was visualized by using 3-amin-9-ethylcarbozole. Substitution of anti-VEGF receptor 2 antibody with normal nonimmune serum served as the negative control. The slides were washed, mounted in aqueous mounting medium, and evaluated under a light microscope.

**In Situ Hybridization**

The complementary DNA (cDNA) for VEGF (human VEGF-121) was provided by Anthony Adams, MD (Harvard Medical School). Plasmid cDNAs were produced and isolated by an isolation kit (High Pure Plasmid Isolation Kit, Roche). The VEGF cDNA was cut with EcoRI (Promega Corporation) and transcribed in vitro (Genius System, Roche) with T7 polymerase or cut with BamIII and transcribed with T3 polymerase for generation of antisense and sense (control) probes, respectively. The product size was confirmed by electrophoresis using 1% agarose gels, and the probes were labeled with digoxigenin. The yield and quantity of the probes to be used were determined by dot hybridization.

The tissue slides were treated with 0.2N hydrochloride for 20 minutes at room temperature, heated for 15 minutes at 70°C in 2× saline–sodium citrate (SSC [150mM sodium chloride and 15mM sodium citrate]), and digested with proteinase K solution at 37°C for 30 minutes. Glycine solution was used to halt digestion, and postfixation was performed using freshly prepared 4% paraformaldehyde for 5 minutes.

The tissues were prehybridized with 50% formamide and 2× SSC for 2 hours at 37°C and hybridized overnight at 50°C with the probes diluted 1:100 in hybridization buffer (10mM Tris hydrochloride, pH 7.5, 12.5% Denhardt solution that contained 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 2× SSC, 50% formamide, 0.5% sodium dodecyl sulfate, 250 μg/mL of salmon sperm DNA, and 5 μg/mL of sodium pyrophosphate). After hybridization, the sections were washed twice in 2× SSC, incubated with 100 μg/mL of RNase A for 30 minutes to digest any unbound probes, and washed with 0.1× SSC solution and digoxigenin buffer (100mM Tris hydrochloride, pH 7.5, and 150mM sodium chloride). For detection of the probe, the sections were blocked in buffer that contained 2% normal serum for 30 minutes, washed in buffer, and incubated with alkaline-phosphate–conjugated digoxigenin antibody (Roche) diluted 1:1000 for 4 hours. After washing, the slides were incubated in a substrate solution of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche) for up to 30 minutes while the color reaction was monitored. The chromogen reaction was halted by rinsing the slide in 20mM Tris hydrochloride, pH 7.5, and 5mM EDTA. The tissue sections were dehydrated and mounted in glycerogel for examination by light microscopy.

**Results**

**Immunohistochemical Analysis**

Figure 1 shows the ciliary tissue from a control eye without iris neovascularization. A minimal amount of VEGF immunostaining was seen. In eyes with neovascular glaucoma, strong immunoreaction products were observed in the nonpigmented epithelial cells of the ciliary processes and in the retina (Figure 2 and Figure 3). Negative controls in which nonimmune serum substituted for anti-VEGF receptor 2 antibody revealed minimal staining (eFigure 1 in the Supplement).

**In Situ Hybridization**

At least 2 sections of each eye were examined, and within each group of controls and neovascular glaucoma eyes, consistent
results were obtained from all the cases. In controls, in which there is no intraocular neovascularization, minimal or no staining of VEGF mRNA in ocular structures was observed (eFigure 2 in the Supplement). In contrast, eyes with neovascular glaucoma had strong staining for VEGF mRNA in the pigmented epithelial cells (Figure 4). Expression of VEGF was also observed in the ganglion cell layer, internal nuclear layer, and outer nuclear layer of eyes with rubeosis iridis (Figure 5). Adjacent sections hybridized with VEGF sense probe revealed only minimal background staining (eFigure 3 in the Supplement).

**Discussion**

Panretinal photocoagulation is used clinically to treat iris and retinal neovascularization that occurs secondary to diabetic retinopathy and retinal vein occlusion. In this treatment, large areas of the retina are ablated using laser photocoagulation, and distant neovascularization on the optic nerve or iris subsequently regresses. Possible explanations for the efficacy of this treatment include destruction of cells producing an angiogenic factor, attenuation of the metabolic demand, induction of an angiogenic inhibitor, and reduction of hypoxia by increasing diffusion from the choroid. By reducing hypoxia, PRP may downregulate VEGF, leading to regression of neovascularization.

Previous studies found that PRP or panretinal cryoablation was effective in most patients with retinal ischemia-induced intraocular neovascularization. However, this mode of treatment does not result in the regression of iris neovascularization in certain patients with neovascular glaucoma. On the other hand, Nd:YAG laser cyclophotocoagulation or cyclocryotherapy provides an effective treatment in such patients. These observations indicate that, although the retina is a major source of VEGF, the ciliary epithelium represents a possible complementary source.
Our immunohistochemical analysis found that the density of VEGF immunostaining in the ciliary epithelium was greatly augmented in eyes with neovascular glaucoma compared with those without intraocular neovascularization. This finding provides evidence that the nonpigmented ciliary epithelium is another important source of VEGF in the eye, thereby contributing to the presence of this mitogen in the aqueous humor and iris neovascularization. In an animal study, Ford et al.24 found that in the ciliary body of adult mice, VEGF-A was expressed by the pigmented epithelium, whereas VEGF receptor 2 was localized primarily to the nonpigmented epithelium. However, their experiments were conducted under physiologic conditions and cannot be extrapolated to VEGF elaboration under ischemic states.

To further determine the role of ciliary epithelium in the development of neovascular glaucoma, we performed in situ hybridization to locate the expression of VEGF mRNA. Our findings indicate that the nonpigmented ciliary epithelium in patients with neovascular glaucoma exhibits a strong upregulation of VEGFA gene (OMIM 192240) expression, whereas eyes without intraocular neovascularization have minimal VEGF mRNA expression. These results correlated well with those of the immunohistochemical analysis and suggest that the nonpigmented ciliary epithelium is indeed an important site of VEGF synthesis in patients with neovascular glaucoma. Colocalization of VEGF receptor 2 in the ciliary body epithelium by immunohistochemical analysis would have added further strength to our hypothesis that VEGF produced by the ischemic ciliary body results in local neovascularization.

Quantification of VEGF levels helps to titrate PRP treatment and reduce the production of VEGF from retinal layers. Furthermore, Nd:YAG laser cyclophotocoagulation or cyclo-therapy may prevent development of neovascular glaucoma in high-risk patients through destruction of ciliary epithelium, another source of VEGF. However, we did not correlate intensity of VEGF staining with the amount of pretreatment ischemia, which would help in titration of the amount of ciliary body ablation.

The treatment of neovascular glaucoma has undergone a paradigm shift with the advent of anti-VEGF agents.25 In patients with progressive anterior segment neovascularization despite PRP, the use of anti-VEGF agents, such as bevacizumab, to induce rapid regression of neovascularization has gained widespread clinical acceptance.26-28 We found that off-label intracameral administration of bevacizumab (anti-VEGF monoclonal antibody) was effective in controlling iris neovascularization in patients with neovascular glaucoma.29 Sasamoto et al.30 found reduction in VEGF levels in the aqueous humor after intravitreal injection of bevacizumab.

In our series of patients with neovascular glaucoma, PRP failed to control the neovascularization of iris and anterior chamber angle, and intraocular hypertension could not be controlled. Subsequently, the eyes were enucleated. In such eyes, it is not surprising that we found elevated VEGF expression and production in the ciliary epithelium, which may continue to contribute to iris neovascularization despite complete ablation of the retina.

Conclusions

Our study confirms nonpigmented ciliary epithelium as a complementary source of VEGF in patients with neovascular glaucoma. In addition, our study provides an explanation for the treatment resistance to panretinal photocoagulation found in some cases of iris neovascularization secondary to ischemic retinal disorders.

REFERENCES


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**Xanthic Scotoma**

Kapil G. Kapoor, MD; Andrew J. Barkmeier, MD

This patient noted a yellow spot in her vision and optical coherence tomography revealed foveal shadowing from a pseudo-operculum. This classic case of xanthic scotoma is distinguished from a true operculum by the normal foveal contour.