Cloning and mRNA expression of vascular endothelial growth factor in ischemic retinas of Macaca fascicularis.

Cloning and mRNA Expression of Vascular Endothelial Growth Factor in Ischemic Retinas of *Macaca fascicularis*

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Purpose. To identify and isolate cDNAs for the alternatively spliced vascular endothelial growth factor (VEGF) mRNAs present in retina and to compare the relative levels of the splice variants and localization of VEGF mRNA in nonischemic and ischemic adult simian retinas.

Methods. Retinas of cynomolgous monkeys were made ischemic by laser occlusion of the main branch retinal veins. Reverse transcription–polymerase chain reaction was used to amplify the VEGF coding region of RNA from ischemic and control retinas, and amplification products were analyzed by agarose gel electrophoresis, Southern blot, and nucleotide sequencing. Analysis of VEGF mRNA expression was accomplished by in *situ* hybridization.

Results. Control and ischemic retinas produce mRNAs that correspond to the 121 and 165 amino acid diffusible isoforms of VEGF, and relatively low levels of $VEGF_{189}$, the heparin-binding isoform. There was no significant difference in the levels of the alternatively spliced VEGF transcripts between control and ischemic retinas. Cloning and sequencing revealed that simVEGF cDNAs are 99% identical to human VEGFs and are predicted to encode proteins identical to their respective human homologues. In situ hybridization of nonischemic retinas revealed a low level of VEGF mRNA in retinal ganglion cells and in the inner nuclear layer. VEGF mRNA levels were elevated in ischemic retinas as early as 1 day after laser vein occlusion, when there was a small but reproducible increase in signal. The expression peaked at approximately 13 days, coincident with maximal iris neovascularization, and was significantly reduced by 28 days, when the iris vessels largely regressed.

Conclusions. The elevation of $simVEGF_{121}$ and $VEGF_{165}$ in ischemic retinas is consistent with a role for diffusible, retina-derived angiogenic factors in the development of ocular neovascularization. Invest Ophthalmol Vis Sci. 1996;37:1334–1340.

Vascular endothelial growth factor (VEGF) is an endothelial-specific mitogen that has potent angiogenic and permeability-enhancing properties in vivo. The expression of VEGF and its receptors has been correlated with embryonic, physiological, and pathologic angiogenesis.[1] We have hypothesized that VEGF plays a role in ischemia-associated ocular neovascularization, and we have demonstrated[2] that increased VEGF protein levels in the aqueous are correlated spatially and temporally with experimental iris neovascularization in adult primates. In the primate neovascularization model, the retina is rendered ischemic by laser occlusion of the main branch retinal veins. Within 5 to 7 days, new iris blood vessels are reproducibly observed and can be quantified by their angiographic appearance. Retinas that receive laser injury without producing ischemia do not develop neovascularization.

It has been postulated that the induction of angiogenic factors is a direct response to the decreased oxygen levels in the ischemic retina.[3] In support of this hypothesis, VEGF mRNA has been shown to be elevated in the presumed hypoxic regions of tumors,[4] and retinal cells have been shown to increase their levels of VEGF mRNA, protein, and bioactivity in re-

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sponse to hypoxia.[5] Moreover, elevated levels of VEGF protein have been demonstrated in the vitreous from eyes with proliferative diabetic retinopathy, a neovascular disease clinically associated with decreased retinal vascular perfusion.[6-8]

VEGF meets many of the criteria necessary for consideration as an ischemia-induced retinal angiogenic factor. First, VEGF is a well-documented angiogenic factor and is present in the retina.[9] Second, the expression of VEGF in the retina is elevated in response to ischemia.[2] Third, the level of VEGF protein is correlated with the timing and degree of neovascularization.[2] Yet, in spite of these observations, little is known of how VEGF is regulated in the retina. An investigation of the biosynthesis and localization of VEGF mRNA, in the normal and ischemic adult primate eye, is critical to understanding how changes in local retinal metabolism trigger neovascular pathologies.

Vascular endothelial growth factor is a family of protein isoforms that exhibits different biochemical properties and tissue-specific gene expression. At least three isoforms are known, each derived from alternative splicing of a single VEGF gene.[10] The different isoforms are active in experimental models of angiogenesis and permeability in vitro and in vivo. $VEGF_{121}$ (numbers refer to the amino acids present in the mature protein) is a secreted, diffusible protein found in the conditioned media of tissue culture cells. $VEGF_{165}$ has moderate heparin affinity and is detected both in conditioned media and attached to the cell surface and extracellular matrix. $VEGF_{189}$ contains an additional heparin-binding domain that tethers the protein heparan sulfate on the cell surface and in the basement membrane. Bioactive $VEGF_{189}$ can be liberated from these sites by partial plasmin proteolysis or by disruption of its interaction with heparan sulfate proteoglycans. The functional significance of the different VEGF isoforms is not understood, though it has been hypothesized that the 165 and 189 amino acid isoforms bound to the extracellular matrix may act as a biologic reservoir for angiogenic or permeability-enhancing activity.[11]

We have characterized the structure and expression of VEGF mRNAs in ischemic retinas from eyes in which iris neovascularization has developed. The alternatively spliced forms of VEGF in control and ischemic retina were identified by reverse transcription–polymerase chain reaction (RT–PCR), Southern blot analysis and molecular cloning of simian VEGF (simVEGF) cDNAs. These cDNAs were used as molecular probes for in situ hybridization analysis of VEGF mRNA expression in normal and ischemic monkey retinas.

METHODS

All animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with guidelines established for animal care at the Massachusetts Eye and Ear Infirmary. Cynomolgous monkeys (*Macaca fascicularis*) were anesthetized for all procedures with intramuscular injection of a mixture of 20 mg/kg ketamine, 1 mg/kg diazepam or 0.25 mg/kg acepromazine maleate, and 0.125 mg/kg atropine sulfate. Supplemental anesthesia of 5 to 6 mg/kg of ketamine hydrochloride was administered as needed. Proparacaine hydrochloride (0.5% topical anesthetic drops) was administered before placement of lid speculae and for pneumotonometry. Pupils were dilated as needed with 2.5% phenylephrine and 0.8% tropicamide drops. Animals were placed in a comfortable restraint device to allow head positioning for photography and angiography. Deeply anesthetized animals were killed with an intravenous injection of Somnlethal (JA Webster, Sterling, MA), a phentobarbital-based euthanasia solution approved by the American Veterinary Medical Association.

Retinal ischemia and the resultant iris neovascularization were induced by laser occlusion of the branch retinal veins, as previously described.[2] For nonischemic controls (referred to as sham-lasered eyes), laser photocoagulation was directed adjacent to the retinal vessels to produce retinal injury without retinal ischemia. Iris neovascularization was followed by serial fluorescein angiography and was graded using standardized angiograms and photographs. In one series, the animals were anesthetized and the eyes obtained on days 4, 13, and 28 after laser occlusion. In a second series, untreated eyes were obtained, as were eyes 24 hours after occlusion.

Reverse Transcription–Polymerase Chain Reaction and cDNA analysis

Animals were anesthetized, eyes were enucleated and bisected at the equator, and retinas were dissected with forceps. Retinal tissue from day 13 ischemic retinas and nonischemic controls was flash frozen in liquid nitrogen until RNA extraction. CsCl-purified total RNA (100 ng) was reverse transcribed using MMLV reverse transcriptase (Superscript II; Gibco BRL, Grand Island, NY). Approximately 1% of RT product was used for PCR amplification with primers based on the human VEGF translational start and stop codons (5′-ACCATGAACTTTCTGCTGTC-3′ and 5′-TCACCGCCTCGGCTTGTCAC-3′). Amplification was performed using standard GeneAmp PCR buffer with 200 μM dNTP, 50 pmol primer, and 2.5 U Amplitaq (Perkin Elmer, Norwalk, CT). The parameters for amplification were: 3-minute initial denaturation at 94°C; 30 cycles of 94°C, 45 seconds each; 1-minute denaturation at 55°C; 1-minute denaturation at 72°C; and 3-minute final extension at 72°C. Products separated in agarose gels and visualized by ethidium bromide

staining either were excised from gels, electroeluted, and cloned into the pCRII plasmid using a T/A cloning kit (Invitrogen, San Diego, CA), or they were transferred to nylon for Southern blot analysis with a 520-bp NcoI/BglII fragment of the human VEGF cDNA. Nucleotide sequence information was determined for more than 10 independently amplified cDNAs using dideoxy-terminated sequencing. Nucleotide and protein sequence comparisons were performed using MacDNAsis (Hitachi Software, San Bruno, CA) sequence analysis software.

In Situ Hybridization

Retinas, removed as described above, were immersed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4°C for 4 hours and then transferred to buffer and embedded in paraffin. Untreated and 24-hour ischemic retinas were from the right and left eye, respectively, of two animals. Days 4, 14, and 28 ischemic retinas were from two eyes of the same animal. One sham-lasered retina was examined. Six-micrometer sections were deparaffinized, rehydrated, and fixed in 4% paraformaldehyde–PBS for 1 hour. After washing in PBS, the sections were treated with 20 μg/ml of proteinase K (Boehringer Mannheim, Indianapolis, IN) for 15 minutes at room temperature, washed in PBS, fixed in 4% paraformaldehyde–PBS for 45 minutes, and acetylated in 0.1 M triethanolamine, pH 8, containing 0.15 M NaCl and 0.13% acetic anhydride. After dehydration, sections were prehybridized in 50% formamide, 2 × SSC, 1 × Denhardt's, 10% dextran sulfate, and 0.5% salmon sperm DNA for 2 hours at 37°C. Digoxigenin-labeled VEGF antisense and sense riboprobes were transcribed from linearized plasmids containing full-length simian $VEGF_{121}$ cDNA, using a Boehringer Mannheim Genius kit. Prehybridization fluid was removed by a brief rinse in 2 × SSC and 40 μl of probe diluted to 1 μg/ml in prehybridization solution was applied to each section, after which the tissue coverslipped to prevent evaporation. Tissue RNA and riboprobe were denatured in situ by incubation of slides at 95°C for 20 minutes in a humidified chamber, and hybridization was then allowed to proceed for 16 hours at 42°C. Slides were agitated gently in 2 × SSC to remove coverslips, washed twice in 2 × SSC for 15 minutes each, and incubated for 30 minutes at 37°C in 1 mg/ml RNase A (Boehringer Mannheim) diluted in 2 × SSC. Sections were washed twice in 0.1 × SSC for 20 minutes each at 42°C, then once for 10 minutes at room temperature, and rinsed in Genius Buffer 1 (100 mM Tris–HCl, pH 8, 150 mM NaCl). Immunohistochemical detection of specific hybridization was carried out by incubation for 2 hours with an anti-digoxigenin alkaline phosphatase conjugate (1:500; Boehringer Mannheim) and visualization of the complex using the Boehringer Mannheim nucleic acid detection kit. All experiments were repeated at least twice (two independent in situ hybridization runs) with duplicate slides for both sense and anti-sense hybridizations. Sections were visualized using Nomarski optics on a Zeiss Axiophot (Carl Zeiss, Oberkochen, Germany).

RESULTS

Identification and Molecular Cloning of Retina-Derived simVEGF

Previously, we determined by Northern blot analysis that VEGF mRNA levels are elevated dramatically in retinas rendered ischemic.[2] To examine the production of VEGF mRNA splice variants in ischemic and nonischemic (sham-lasered) retinas, the relative abundance of the various VEGF transcripts was determined by RT–PCR analysis of total RNA. In control and ischemic retinas, two molecular species of approximately 580 and 450 bp were identified. These correspond in size with VEGF cDNAs encoding the 121- and 165-amino acid forms of the protein (Fig. 1a). Southern blot analysis of PCR products (cycles 20 and 25), using a human cDNA probe, corroborated the results of the RT–PCR (Fig. 2B). Whereas the product corresponding to $VEGF_{121}$ appears to predominate in nonischemic retina, the products corresponding to $VEGF_{121}$ and $VEGF_{165}$ appear equivalent in hypoxic retinal tissue (Fig. 1b). In addition, with this more sensitive method of detection, a third product corresponding in size to the $VEGF_{189}$ transcript was detected (Fig. 1b). Identical results have been obtained with samples from different experimental and control retinas. For each experimental animal, angiography demonstrated that the retina was ischemic, and it documented iris neovascularization (data not shown).

simVEGF amplification products from independent PCR reactions were cloned, and their nucleotide sequences were determined (Fig. 2A). The predicted VEGF proteins encoded by these cDNAs are similar in organization to the previously described VEGFs, with the $simVEGF_{165}$ protein consisting of a 44-amino acid insertion into the C-terminal portion of the $VEGF_{121}$ protein backbone (Fig. 2B). At the nucleotide level, simVEGFs were nearly identical (99%) to the respective human homologues (Fig. 2C; shown is the sequence for $VEGF_{165}$). Three conservative nucleotide changes in the simVEGF sequence (asterisks) encode a protein product with a predicted sequence identical to that of human VEGF.

In Situ Hybridization Analysis of Vascular Endothelial Growth Factor mRNA Expression

In situ hybridization was used to identify the cells synthesizing VEGF mRNA in ischemic retinas obtained

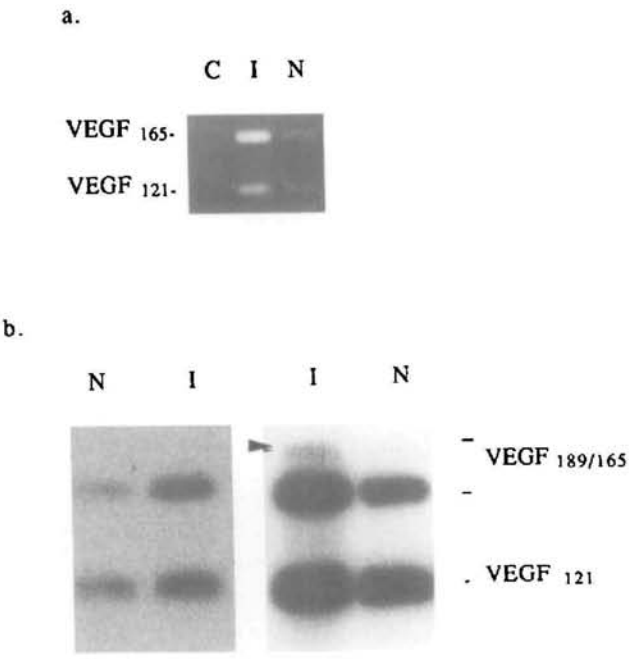

FIGURE 1. Vascular endothelial growth factor (VEGF) isoforms in control and ischemic monkey retinas. (a) Reverse transcription–polymerase chain reaction analysis of total RNA from nonischemic (N) and ischemic (I) monkey retinas. (C) Polymerase chain reaction (PCR) control containing no cDNA. Using agarose gel electrophoresis, two major amplification products are observed in both nonischemic and ischemic conditions, corresponding in size to the $VEGF_{121}$ and $VEGF_{165}$ alternatively spliced mRNAs. (b) Southern blot analysis of amplification products (*left panel*, PCR cycle 20; *right panel*< PCR cycle 25) from control and ischemic retinas using a human VEGF cDNA probe. Products corresponding to mRNAs encoding the 121-, 165-, and 189-amino acid VEGF isoforms are present in ischemic retina. With a longer autoradiographic exposures, a product corresponding to $VEGF_{189}$ is also detectable in the control retina samples but at drastically lower levels than $VEGF_{121}$ and $VEGF_{165}$ products (data not shown).

1, 4, 13, and 28 days after laser-induced branch retinal vein occlusion and to compare these with the expression pattern in sham-lasered controls. In this model of neovascularization, the peak of new vessel growth in the iris occurs 10 to 14 days after laser vein occlusion, and vessel regression is observed within 25 to 35 days of occlusion. We looked to see how rapidly after vein occlusion in the retina elevated levels of mRNA might be visualized. As early as 24 hours after occlusion, there was a small but reproducible increase in VEGF mRNA levels compared to those of normal, untreated controls, particularly in the ganglion cell layer (compare Figs. 3A, 3B). Levels of VEGF mRNA, in the ischemic retina 13 days after occlusion, were significantly higher than in either the day 4 or the day 28 ischemic retina (Figs. 3C to 3E). Normal, untreated retinas investigated with an antisense probe showed very low levels of signal (Fig. 3A). The highest level of nonspecific hybridization using the sense probe was observed in the day 13 ischemic retina (Fig. 3F). A low level of VEGF mRNA was detected in day 13 sham-lasered retinas (Fig. 3G). Using Northern blot analysis, we have shown that laser-induced retinal injury in the absence of ischemia does not result in the induction of VEGF mRNA. Consistent with these findings, there was only weak VEGF mRNA signals in day 13 sham-lasered retinas in ganglion cells and scattered cells of the inner nuclear layer (data not shown).

At each time point, VEGF mRNA was localized in ganglion cell and inner nuclear layers. The most intense staining was associated with cells of the ganglion cell layer (based on their size, morphology, and distribution, we believe these to be ganglion cells). In the inner nuclear layer, there was heterogeneity in the intensity of the hybridization signal. Cells most proximal to the internal plexiform layer were as strongly positive as ganglion cells, but elsewhere in the inner nuclear layer some cells appeared completely negative. This heterogeneity was most apparent in the day 13 retina (Fig. 3D). By day 28 after laser occlusion (Fig. 3E), a time when the iris vessels have largely regressed, the VEGF mRNA level had diminished significantly.

DISCUSSION

Results of RT–PCR and molecular cloning of retina VEGFs indicate that in response to ischemia, primate retinas produce primarily the diffusible isoforms, $VEGF_{121}$ and $VEGF_{165}$, characterized by their low affinity for heparin. These data support the hypothesis that the primate retina responds to ischemia with a rapid increase in the production of diffusible angiogenic factors. Further, these findings are consistent with the belief that a retina-derived angiogenic factor(s) is responsible for neovascularization at distant sites, such as the iris. Results of in situ hybridization revealed that VEGF mRNA is elevated as early as 24 hours after the induction of retinal ischemia, an observation consistent with the detection of a significant elevation in aqueous VEGF protein at 48 hours.[2] Further, increased VEGF mRNA in the retina parallels the severity of iris neovascularization and levels of VEGF in the aqueous, which reach their maximum between 10 and 14 days after laser occlusion. After this peak of neovascularization, VEGF mRNA and aqueous VEGF protein levels decline and are followed by regression of the new iris vessels. These temporal correlations provide additional evidence that the ischemic retina itself produces the VEGF protein found in the vitreous and aqueous of these animals.[2]

Relative to the human homologue, simVEGF cDNA contains three conservative nucleotide changes,

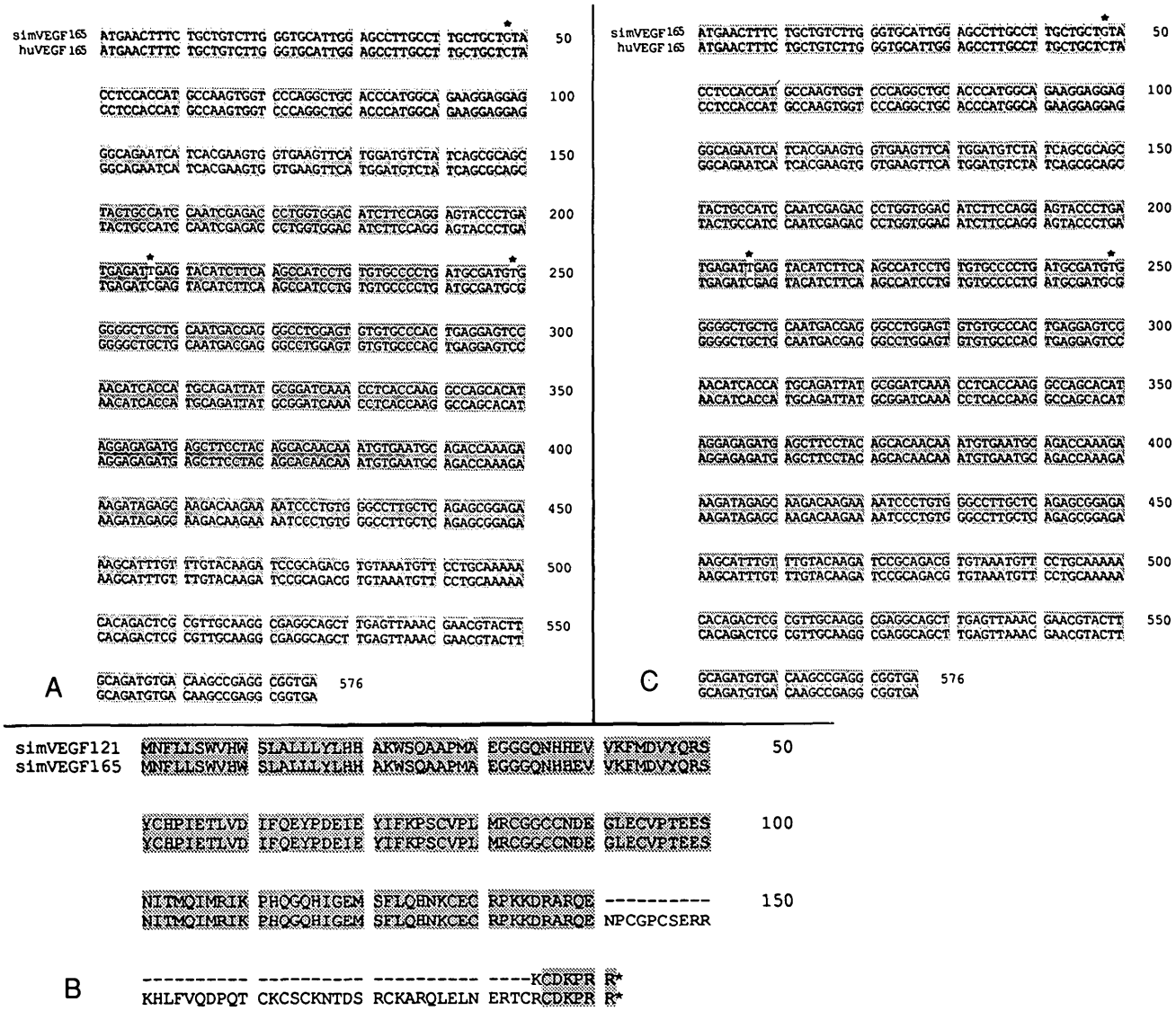

FIGURE 2. simVEGF$_{121}$ and simVEGF$_{165}$ nucleotide and predicted protein sequences. (**A**) Nucleotide sequence alignment for the simVEGF cDNAs. (**B**) Alignment of simVEGF cDNAs predicted protein products. (**C**) Alignment of the cDNA sequences for simian and human VEGF$_{165}$. Asterisks denote three conservative nucleotide changes in the simVEGF cDNA that encode a protein product identical to human VEGF. The same nucleotide substitutions are present in the SimVEGF$_{121}$ cDNA (data not shown). Shaded areas denote regions of nucleotide–amino acid sequence identity. It should be noted that the 5 N- and C-terminal amino acids are derived from primers containing human VEGF sequence and do not necessarily represent the actual simian sequence. VEGF = vascular endothelial growth factor.

resulting in an identical protein product. This degree of identity has allowed us to pursue studies in this simian model of ocular neovascularization using monoclonal antibodies raised against human VEGF, which had been demonstrated to be species specific (i.e., did not cross-react with murine or avian VEGF).[12] As a result, recently we have been able to show that intraocular injection of anti-VEGF monoclonal antibodies prevent new iris vessel growth, demonstrating that VEGF is a necessary factor for ischemia-induced ocular neovascularization.[13]

In situ analysis of VEGF mRNA expression in the retina confirms the alterations in VEGF mRNA levels observed by Northern blot analysis and provides further information regarding the localization of the VEGF response to ischemia. The hybridization pattern of VEGF mRNA in the ganglion cell layer strongly suggests that ganglion cells are a major source of the growth factor. Ganglion cell production of VEGF in retinal ischemia could explain the clinical observation that proliferative diabetic retinopathy does not occur in the setting of optic atrophy or ganglion cell loss.[14] It also has been noted that successful panretinal photocoagulation, leading to regression of optic nerve,

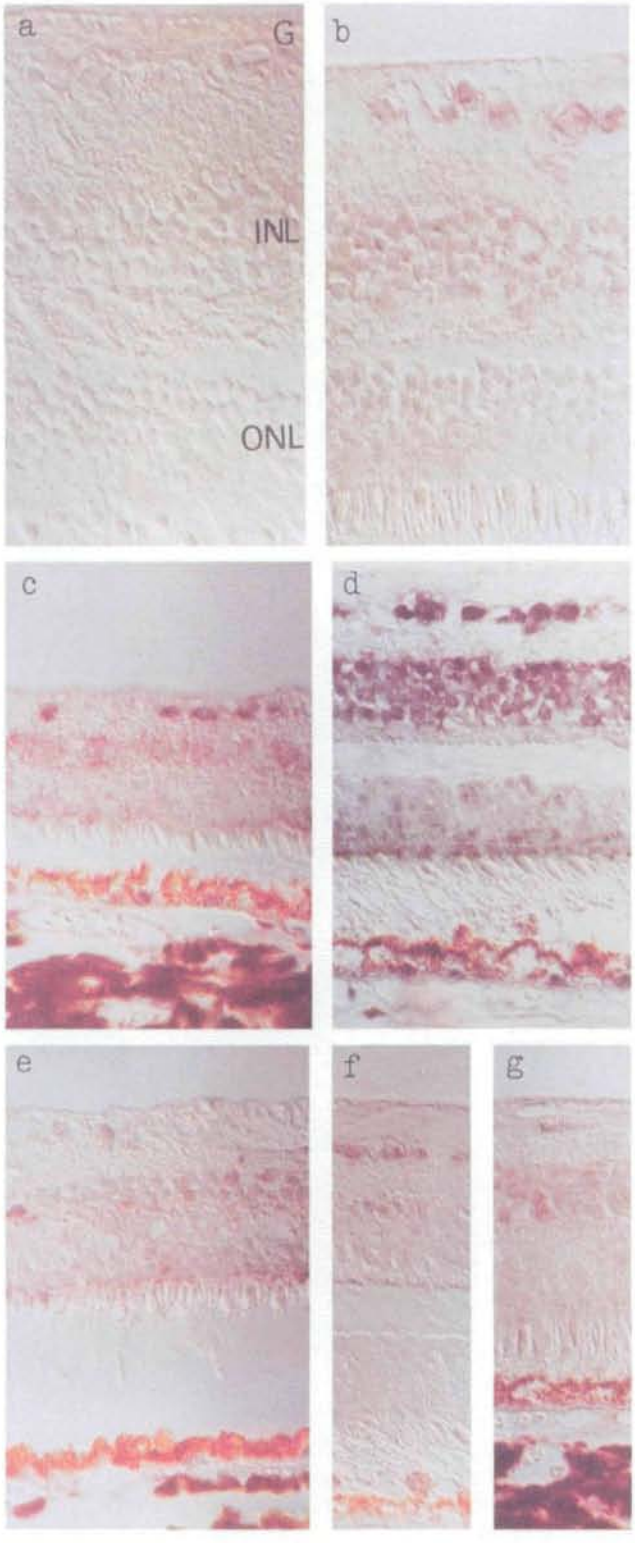

FIGURE 3. In situ localization of vascular endothelial growth factor (VEGF) mRNA in normal and ischemic retinas. Cellular localization of VEGF mRNA expression by hybridization with an antisense VEGF riboprobe in (**A**) normal untreated retina, (**B**) 24-hour ischemic retina, (**C**) 4-day ischemic retina, (**D**) 13-day ischemic retina, (**E**) 28-day ischemic retina. Sense riboprobe hybridized in (**F**) 13-day ischemic retina and (**G**) 13-day day retina. Magnification, ×330.

retinal, and iris neovascularization, is accompanied by optic atrophy.[15] Panretinal photocoagulation may lead to regression of neovascularization by causing the death of ganglion cells, the source of the angiogenic stimulus, and not by adjusting retinal metabolism or transretinal oxygen gradients, as has been suggested.[16] The cells synthesizing VEGF mRNA in the inner nuclear layer have yet to be identified definitively; candidates include Müller cells, amacrine cells, and horizontal or bipolar cells.

Not surprisingly, the level of VEGF gene expression was highest in the ganglion cell and inner nuclear layers. Because the retinal circulation affected by laser vein occlusion supplies the inner retina, retinal ischemia is most severe in the inner portion of the retina, whereas the outer retina, nourished by choroidal circulation, does not experience as severe ischemia.[17] Retinal ischemia in the inner retina secondary to laser vein occlusion has been documented in a related model by Pournaras and coworkers.[18] They measured O_2 concentrations of 16.5 mm Hg in tissue of the inner retina after the disruption of retinal circulation (the normal concentration is 26 mm Hg).

Interestingly, VEGF synthesis in the ganglion cell layer appeared to be confined to the ganglion cells. This is in contrast to VEGF expression in murine retinal vascularization. During developmental angiogenesis in rat and cat retinas, the growth factor was shown to be produced by astrocytes and Müller cells in response to physiologic hypoxia.[19] Similarly, in an experimental model of retinopathy of prematurity using the mouse, VEGF mRNA was localized to Müller cells.[20] The difference in sites of expression may be the result of species differences. However, it is more likely that the degree of hypoxia is a critical variable. Chang-Ling and Stone[21] have shown that astrocytes are preferentially lost in the experimental models of retinopathy of prematurity. We suspect that the severe ischemia created in the monkey model of laser-induced ischemia leads to the loss of astrocytes and that ganglion cells upregulate VEGF in a compensatory fashion. The staining in the vicinity of the outer limiting membrane, particularly at day 13 in ischemic retinas, appears to be associated with cell processes possibly belonging to bipolar cells, photoreceptor cells, or both. Although it is possible that they belong to Müller cells, we did not see the distinct signal outlining the Müller cell processes that Stone[19] and Pierce[20] observed. Upregulation of VEGF mRNA in this area may be caused by the fact that this portion of the retina receives part of its oxygen supply from retinal vessels[18] and, hence, also would experience ischemia.

VEGF appears to be expressed at a low level in the nonischemic retina. Results from PCR analysis of VEGF from control retinas is supported by analysis of VEGF mRNA by Northern blot and in situ hybridiza-

tion, as well as by immunodetection of VEGF protein, all of which demonstrate low but detectable VEGF.[2] The increase in VEGF mRNA in ischemic retinas is not associated with changes in alternative splicing. Approximately equal amounts of $VEGF_{121}$ and $VEGF_{165}$ are detectable by PCR in control retinas, and the relative contribution of each splice variant to the total amount of VEGF mRNA remained constant after the induction of ischemia. Low levels of $VEGF_{189}$, the cell-associated isoform, are present in ischemic and non-ischemic retinas. Although our in situ analysis of normal retina does not indicate significant levels of VEGF mRNA, immunohistochemical analysis[9] and immunoassay[8] indicate a low level of constitutive protein production. In addition to the retina, numerous other adult tissues are known to have low levels of VEGF expression, suggesting that VEGF may act as a maintenance/survival factor for mature blood vessel beds.[22]

Key Words

angiogenesis, ganglion cells, hypoxia, in situ hybridization, neovascularization

Acknowledgments

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