Constitutive Expression of VEGF, VEGFR-1, and VEGFR-2 in Normal Eyes

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PURPOSE. The expression of vascular endothelial growth factor (VEGF) and its high-affinity receptors VEGFR-1 and VEGFR-2 was investigated in normal eyes.

METHODS. Monkey and rat eyes were surgically removed in animals under deep anesthesia and immediately prepared for study. Ocular VEGF, VEGFR-1, and VEGFR-2 expression was studied using a combination of in situ hybridization, northern blot analysis, immunohistochemistry, immunohistochemistry, immunohistochemistry, and reverse transcription-polymerase chain reaction.

RESULTS. Steady state VEGF mRNA levels were detected in the normal vascularized ocular tissues of the monkey: the conjunctiva, iris, retina, and the choroid-retinal pigment epithelial complex. VEGF121 and VEGF165 were the major isoforms identified. VEGF protein was detected in the conjunctiva, retina, and the choroid-retinal pigment epithelial complex. Retinal VEGF mRNA localized to the ganglion, inner nuclear, and retinal pigment epithelial cell layers. VEGF protein localized to these same layers and to the cones of monkey retina. VEGF-1 and VEGF-2 mRNAs were detected in normal monkey iris, retina, and the choroid-retinal pigment epithelial complex. In both monkey and rat eyes, VEGF-1 and VEGF-2 mRNAs were localized to the inner nuclear layer of the retina.

CONCLUSIONS. VEGF, VEGFR-1, and VEGFR-2 are constitutively expressed in the vascularized tissues of normal eyes. (Invest Ophthalmol Vis Sci. 1999;40:2115–2121)

Vascular endothelial growth factor (VEGF) refers to a family of peptide growth factors that act primarily on blood vessels.1 The human VEGF gene produces up to five alternatively spliced mRNAs that code for proteins of 121, 145, 165, 189, and 206 amino acids.2–4 The mouse gene codes for proteins of 120, 164, and 188 amino acids.5 The different VEGF isoforms have varying affinities for heparin. The larger isoforms bind heparin avidly, whereas the smaller ones do not.6 As a result, the three smaller isoforms are detected in conditioned media and biologic fluids, and by comparison, the larger isoforms are found bound to heparin-like molecules in extracellular matrix and on cell membranes. Unlike acidic and basic fibroblast growth factor, all five VEGF isoforms contain a leader secretory peptide sequence for their efficient secretion.2,5

Two VEGF high-affinity receptor tyrosine kinases have been cloned and sequenced: VEGF-R1 (Flt-1; fms-like tyrosine kinase)7 and VEGF-R2 (KDR; kinase domain region or Flk-1 in the rodent).8 In addition, an alternatively spliced soluble variant of the membrane-bound VEGF-R1 mRNA has been described.9 VEGF-R1 and VEGF-R2 are structurally related to each other, containing seven extracellular immunoglobulin-like domains and a conserved intracellular tyrosine kinase domain.7 125I-VEGF autoradiography of rat tissues has demonstrated that VEGF binding is largely restricted to blood vessels and is associated with both fenestrated and nonfenestrated endothelium.10

VEGF, VEGF-R1, and VEGF-R2 are each essential for normal blood vessel development. Heterozygous deletion of the VEGF gene results in embryo death between days 8.5 and 9.5. The embryos are characterized by impaired angiogenesis and blood island formation.11,12 Homozygous deletion of VEGF-R2 leads to death of mice between embryonic days 8.5 and 9.5, with the embryos having no yolk–sac blood islands and endothelial cells.13 Homozygous VEGF-R1 deletion permits endothelial cell differentiation, but the vascular channels that form are grossly abnormal, and the animals die in utero.14

VEGF is a major mediator of retinal ischemia–associated ocular neovascularization.15,16 It is also the primary bioactive vascular endothelial cell mitogen synthesized and secreted by hypoxic retinal cells in vitro.17 When monkey retina is made ischemic, VEGF mRNA and protein levels increase in proportion to the amount of iris neovascularization that subsequently develops.18 The freely diffusible VEGF121 and VEGF165 are the major isoforms produced in ischemic retina.19 In humans with retinal ischemia, VEGF levels in the retina, vitreous, and aque-
ous temporally correlate with neovascularization of the retina, optic nerve, and iris. 20–22

A causal role for VEGF in retinal ischemia-associated neovascularization has been demonstrated in two animal models. VEGF-neutralizing antibodies completely prevented iris neovascularization in a primate model, 15 and soluble VEGF receptors suppressed retinal neovascularization in a mouse model. 16 In gain-of-function experiments, exogenous VEGF has been shown to trigger iris and retinal neovascularization in monkeys, 24,25 and retinal neovascularization develops in transgenic mice that overexpress VEGF in the retina. 20

VEGF also appears to play an important role in other types of ocular neovascularization. Endogenous VEGF is required for corneal neovascularization in a rat model, 27 and VEGF, VEGFR-1, and VEGFR-2 have been temporally and spatially associated with choroidal neovascularization in humans and monkeys. 28,29

Several lines of evidence suggest that VEGF is also operative in the survival of newly grown vessels. The regression of experimental iris neovascularization is preceded by a decrease in aqueous VEGF levels in monkeys, 10 and a similar decrease is observed after panretinal photocoagulation in humans. 21 In the developing mouse eye, declining retinal VEGF levels have been correlated with the regression of newly formed vessels. 30 Moreover, intravitreal injections of VEGF have been shown to rescue these new vessels from regression. It is not known whether VEGF is a survival factor for long-standing normal adult vessels, or whether it serves other nonvascular functions in the eye. To begin to investigate these questions, the expression and distribution of VEGF and its high-affinity receptors was studied in normal adult eyes.

METHODS

Anesthesia and Enucleation

Animals were cared for in accordance with the ARVO Resolution for the Use of Animals in Ophthalmic and Vision Research and guidelines established by the Massachusetts Eye and Ear Infirmary, Children’s Hospital, and the Genentech Animal Care Committees. General anesthesia for monkeys was performed as previously described. 15 Briefly, cynomolgus monkeys (Macaca fascicularis) and Rhesus monkeys were anesthetized for all procedures with an intramuscular injection of ketamine (20 mg/kg; Parke-Davis, Morris Plains, NJ), acepromazine (0.15 mg/kg; Ayerst Laboratories, Rouses Point, NY), and atropine sulfate (0.125 mg/kg; Gensia Laboratories, Irvine CA). Male Sprague-Dawley rats (weight, 200–250 g) were anesthetized with intramuscular ketamine hydrochloride (25 mg/kg) and xylazine (10 mg/kg).

Seven monkey and four rat eyes were used. Different eyes were used for the polymerase chain reaction (PCR), northern blot analysis, immunohistochemistry, and in situ hybridization studies. Eyes were enucleated with animals under deep anesthesia. The globes were bisected at the equator, and the vitreous was removed. The bisected globes were then immediately placed in fixative as described later, or individual tissues were isolated using McPherson forceps and Vannas scissors. The retina, iris, lens, conjunctiva, and cornea were separately isolated, placed in a 50-mL conical plastic tube, and snap frozen in liquid nitrogen. Homogeneous samples of choroid and retinal pigment epithelium (RPE) could not be prepared rapidly, and they were therefore studied as a whole (choroid-RPE). The time from enucleation to isolation of all the tissues was less than 5 minutes. The animals were then killed with an overdose of sodium pentobarbital.

In Situ Hybridization

The in situ hybridization protocol and the preparation of 32P-labeled riboprobes have been previously described. 31 Briefly, species-specific PCR primers were designed from available human and murine VEGF sequences to amplify a 0.9-kb (human) or 0.65-kb (rat) cDNA fragment. The antisense probes hybridized to regions of VEGF mRNA coding sequence common to all known splice variants of VEGF. The human VEGF probe was used on monkey tissues, and the rat VEGF probe was used on rat tissues. Human VEGFR-1, human VEGFR-2, and murine VEGFR-2 probes were designed from the transmembrane region of each sequence. The 0.3-kb human VEGFR-2 probe had less than 5% homology with VEGFR-1 and was used on monkey tissues. The 0.5-kb human VEGFR-1 probe had more than 95% homology among human, rat, and mouse and was used on both monkey and rat tissues. The 1.2-kb murine VEGFR-2 probe was used on rat tissues and had 51.1% homology with VEGFR-1. Appropriate sense and antisense riboprobes were generated by in vitro transcription in the presence of 32P-uridine triphosphate. Adult monkey and nonpigmented rat eyes were fixed in 4% paraformaldehyde, paraffin embedded, and hybridized as previously described. 31 Slides were examined after 5 weeks’ exposure.

Immunohistochemistry

Rabbit polyclonal anti-human VEGF (1:100; Oncogene Science, Cambridge MA) was used on formalin-fixed, paraffin-embedded Rhesus monkey sections after microwave antigen retrieval with citrate buffer (BioGenex, San Ramon CA). The antibodies recognize exons 1 to 5, which are common to all VEGF isoforms. Nonimmune rabbit serum was used as a negative control. The secondary antibody was detected with streptavidin-alkaline phosphatase (Kirkegaard & Perry, Gaithersburg, MD) and visualized with Fast Red TR/Naphthal Phosphatase (Research Genetics, Huntsville, AL)

RNA Isolation and Northern Blot Analysis

Total RNA was isolated by the method of Chomczynski and Sacchi 32 after tissue homogenization (Polytron). RNA (15 μg) was electrophoresed through a 1% agarose-formaldehyde gel and transferred to nylon filters (Gene Screen Plus; New England Nuclear, Boston, MA). The filters were prehybridized in buffer containing 50% deionized formamide, 5 × SSPE, 5 × Denhardt’s solution, 0.5% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and denatured salmon sperm DNA (100 μg/ml) and hybridized at 42°C in fresh buffer without salmon sperm DNA. The hybridization buffer contained either a 520-bp Neo/BglII fragment of the human VEGF cDNA (gift of Herbert Weich), a 226-bp EcoRI/BamHI fragment of human VEGF-1, or a 286-bp fragment of human VEGFR-2. 33 The blots were stripped and reprobed with a 400-bp fragment encompassing the 3′ untranslated region of the human β-actin cDNA. The cDNA probes were labeled with a random primed DNA labeling kit using [α-32P] deoxy-CTP (Boehringer Mannheim, Indianapolis, IN). Filters were washed in 2 × 0.5 × SSPE, 0.1% SDS, for varying times and at increasing temperatures. The washes
were titrated for maximum signal-to-noise ratio. The hybridized and washed filters were exposed to x-ray film (X-Omat AR; Eastman Kodak, Rochester, NY) with an intensifying screen at −70°C for 12 to 72 hours. Densitometry was performed on all blots and normalized to the corresponding actin signal for each lane using a digital imaging system (IS-1000 with ver. 1.97 software; Alpha Inotech, Torrence, CA).

Reverse Transcription–Polymerase Chain Reaction

Total RNA (1 μg) from the indicated tissues was reverse transcribed with a commercially available kit (Advantage RT-for PCR; Clontech, Palo Alto, CA) using the random hexamer primer according to the manufacturer’s protocols. Duplicate reactions without MMLV reverse transcriptase were included as negative control samples. A constant fraction (1/20) of each reverse transcription reaction was amplified with Taq DNA polymerase (Gibco, Gaithersburg, MD) using oligonucleotide primers derived from highly conserved sequences in intron 4 and the 3’ untranslated region of the VEGF gene. The 5’ primer was GCGGAATTCATYATGCGGATCAAACCYCACCA, and the 3’ primer was TACGGATCCTCCGGACCCAAAGTGCTC. These primers can detect all VEGF isoforms. Based on the sequence of human VEGF, the expected sizes of the amplified products for each VEGF isoform were 593 bp (VEGF206), 542 bp (VEGF189), 470 bp (VEGF165), 410 bp (VEGF145), and 338 bp (VEGF121). The latter four isoforms were observed after RT-PCR of RNA from monkey lung (data not shown). The identity of the monkey VEGF products was verified by cloning and sequencing.

Enzyme-Linked Immunosorbent Assay

Tissue lysates were prepared by washing the tissues with phosphate-buffered saline (PBS) three times followed by addition of 1 ml/well lysis buffer (50 mM Tris, [pH 8.0], 2 M NaCl, 0.1% CHAPS, 1% NP40, 2 mM EDTA, 1 μg/ml aprotinin, 2 mM AEBSF, and 1 μg/ml leupeptin; Sigma, St. Louis, MO). The lysate was cleared of debris by centrifugation at 14,000 rpm for 30 minutes (4°C), and the supernatant was assayed for VEGF. Total protein was determined by the method of Bradford using a commercial assay (Bio-Rad, Hercules, CA). Supernatant VEGF levels were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN) and normalized to total protein.

RESULTS

Northern blot analysis for VEGF, VEGFR-1, and VEGFR-2 identified steady state levels for each transcript in normal cynomolgus monkey choroid–RPE complex, retina, and iris (Fig. 1). VEGF was also detected in the conjunctiva; however, all three transcripts were absent in the lens. Insufficient RNA was isolated from the corneas for reliable analysis. RT-PCR identified VEGF121 and VEGF165 as the major VEGF transcripts in the tissues found to express VEGF (Fig. 2). Faint PCR products were observed occasionally in these negative control lanes, presumably because of plasmid DNA contamination (Fig. 2).

Figure 1. Northern blot of normal cynomolgus monkey tissues for VEGF, VEGFR-1 (flt-1), and VEGFR-2 (KDR). Numbers denote relative densitometric expression levels after normalization to β-actin signal.

In situ hybridization of cynomolgus monkey retina identified VEGF mRNA in the ganglion and inner nuclear cell layers and the RPE (Figs. 3A, 3B). To a lesser extent, positive signal was seen in the outer nuclear layer as well (Figs. 3A, 3B). A similar expression pattern was seen in the rat retina (Figs. 3C, 3D), although low levels of nonspecific labeling were seen in the photoreceptor layer (Fig. 3D). In the monkey, VEGFR-1 mRNA was present in the inner nuclear layer and individual cells subjacent to the RPE (Figs. 4A, 4B). VEGFR-1 mRNA was also weakly associated with larger vessels in the ganglion cell layer (Fig. 4A). In the rat, specific labeling for VEGFR-1 mRNA was most visible in the inner nuclear layer (Fig. 4C). VEGFR-2 mRNA was more evident than VEGFR-1 in the normal monkey retina and localized to the vessels of the ganglion cell layer. The inner half of the inner nuclear layer also labeled for VEGFR-2, and a faint signal was seen in focal areas subjacent to the RPE (Figs. 5A, 5B). Similarly, specific labeling for VEGFR-2 mRNA in the rat was most prominent in the inner nuclear layer and in association with a few isolated cells subjacent to the RPE (Fig. 5C).

Immunohistochemical staining of monkey retina and choroid sections identified VEGF protein in the same layers as the
mRNA, namely ganglion cell and inner nuclear layers, and the retinal pigment epithelium (Figs. 6A through D). However, definite staining was also seen in a subset of cone photoreceptors that did not express VEGF by in situ hybridization (Figs. 6C, 6D). This positive pattern of staining was not observed in normal rat or mouse retina (data not shown). Nonimmune serum controls showed no staining (Figs. 6C, 6D). Immunoreactive VEGF did not localize to the clearly discernible large-caliber vessels that were visible by light microscopy, and the VEGF staining was patchy and also generally not associated with visible vessels at the level of the inner nuclear layer (Figs. 6C, 6D).

**DISCUSSION**

These analyses demonstrate that some normal ocular tissues constitutively express VEGF and its cognate receptors VEGFR-1 and VEGFR-2. The expression patterns for all three genes in normal monkey and rat eyes are generally consistent. The tissues studied were surgically isolated from living animals and immediately prepared for study. The rapid preparation of the tissues limited the possibility of artifactual gene expression and permitted analyses accurately reflecting the in vivo situation.

VEGF expression was largely limited to those tissues that are vascularized in the normal state: conjunctiva, iris, retina, and choroid–RPE. Little or no VEGF was detected in the avascular lens and cornea. The lens result may have been accentuated by lower RNA loading. The cornea result confirms our previously published finding that VEGF protein is virtually absent in normal cornea. We were also unable to detect message for VEGFR-1 or VEGFR-2 in lens. The in situ hybridization studies confirm the northern blot data and further

**FIGURE 3.** Normal cynomolgus monkey retina VEGF in situ hybridization (A, B) with antisense (left) and sense (right) probes. Normal rat retina (C) and choroid–RPE layer. (D) VEGF in situ hybridization with antisense (left) and sense (right) probes. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptors; RPE, retinal pigment epithelium; C, choroid. Magnification, (A, B) ×30 Magnification, (C, D) ×300.

**FIGURE 4.** Normal cynomolgus monkey retina VEGFR-1 in situ hybridization (A, B) with antisense (left) and sense (right) probes. Rat retina VEGFR-1 in situ hybridization with antisense (left) and sense (right) probes (C). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptors; RPE, retinal pigment epithelium; C, choroid. Magnification, ×30.
demonstrate that VEGF mRNA expression is largely restricted to nonvascular cells. Further, little or no expression was seen in the circulating blood elements within vessels (data not shown). This is important because leukocytes possess the capacity to express VEGF. Finally, the ELISA and immunohistochemical data confirmed that the VEGF mRNA is translated into VEGF protein.

VEGF is an angiogenic factor. Because no active neovascularization occurs in normal eyes, it is possible that the VEGF observed in these studies is not bioactive. VEGF bioactivity was not tested, and it is conceivable that the VEGF that was detected was present in an inactive stored form bound to heparin. Basic fibroblast growth factor is stored in such a manner and is quickly released on injury. However, that VEGF₁₂₁ and VEGF₁₆₅ were the major isoforms identified in these studies argues against this possibility. VEGF₁₂₁ and VEGF₁₆₅ have lower heparin affinities and are less likely to bind to matrix and/or cell surfaces.

The constitutive expression of VEGF and its receptors in normal eyes is consistent with VEGF serving as a vascular survival factor. Several pieces of evidence support this hypothesis. Destruction of the RPE causes secondary degeneration of the subjacent choriocapillaris, a result consistent with the RPE’s producing a vascular survival factor. In these studies, VEGF mRNA and protein were shown to be present at relatively high levels in the RPE. Previous reports in pigmented human and mouse eyes have also shown an autoradiographic signal for VEGF in the RPE layer; however, this expression pattern may have been caused by the pigment granules in the RPE. Through the use of nonpigmented rat eyes in this study, the signal for VEGF in the RPE was confirmed. Dorey et al. have made a similar observation in the RPE of newborn albino rats. These data, together with the data showing constitutive VEGFR-1 and VEGFR-2 expression in the subjacent choriocapillaris, are consistent with VEGF acting as a choriocapillaris survival factor. The second piece of evidence comes from the rat model of retinopathy of prematurity (ROP) discussed earlier, in which VEGF withdrawal was associated with endothelial cell apoptosis and vessel regression. In that model, intravitreal injections of VEGF were able to rescue the new vessels, strongly implicating VEGF in the survival of newly grown vessels.

A third possible explanation for the constitutive expression of VEGF concerns its possible interaction with nonendothelial cells in the retina. Yang et al. recently demonstrated that VEGFR-2 is expressed in retinal progenitor cells of mouse retina. They also showed that the expression of VEGFR-2 persists throughout retinal neurogenesis and that retinal cell VEGFR-2 can be activated by VEGF in vitro. Consistent with these observations, our data show that VEGFR-1 and VEGFR-2 message is distributed throughout the inner nuclear layer in a constitutive manner.

![FIGURE 5](image5.png)  
**FIGURE 5.** Normal cynomolgus monkey retina VEGFR-2 in situ hybridization (A, B) with antisense (left) and sense (right) probes. Rat retina VEGFR-2 in situ hybridization using antisense (left) and sense (right) probes (C). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptors; RPE, retinal pigment epithelium; C, choroid. Magnification, ×30.

![FIGURE 6](image6.png)  
**FIGURE 6.** Normal Rhesus monkey retina VEGF in situ hybridization with antisense probe (A, B). Rhesus monkey retina VEGF immunohistochemistry (C, D, left) and corresponding nonimmune serum controls (C, D, right). VEGF is identified by red reaction product. Counterstained with hematoxylin and eosin. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptors; RPE, retinal pigment epithelium; C, choroid. Magnification, (C, D, left) ×30; (C, D, right) ×45.
pattern that appears to extend beyond the inner retinal vasculature. Other recently published data have also demonstrated that VEGF receptors are not exclusively expressed on endothelial cells in vivo. Brown et al.53 recently identified VEGFR-1 and VEGFR-2 receptors on uterine smooth muscle cells in vivo. When these cells were cultured in vitro, VEGFR-1 could be phosphorylated and was capable of inducing smooth muscle cell proliferation. Similarly, Soker et al.44 have shown that neuropilin-1 enhances binding of VEGF165 to VEGFR-2 on tumor cells. Thus, the biology of VEGF may be more complex than originally thought.

The expression pattern analyses shown here also suggest that oxygen may not be the sole physiological stimulus for VEGF expression in vivo. RPE cells express relatively high VEGF levels (Fig. 1), yet they are exposed to some of the highest regional pO2 levels in the body.45 Stimuli known to exist within the eye (e.g., hypoglycemia, reactive oxygen intermediates, insulin-like growth factor-1, and advanced glycation end products) are each capable of increasing VEGF expression46–49 and may serve to modulate VEGF expression in the eye.

Because VEGF, VEGFR-1, and VEGFR-2 are constitutively expressed in the eye, it is possible that the pharmacologic inhibition of VEGF bioactivity may have adverse consequences. Methods that permit the inducible silencing of specific genes in adult ocular tissues should be used to investigate this theoretical possibility.50

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