Vascular Endothelial Growth Factor Is Present in Glial Cells of the Retina and Optic Nerve of Human Subjects With Nonproliferative Diabetic Retinopathy

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**Purpose.** To determine whether vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which have been implicated in the development of retinal and choroidal neovascularization, are present in the retinas and optic nerves of patients with diabetes before proliferative retinopathy appears.

**Methods.** Light microscopic immunocytochemistry using antibodies to VEGF, bFGF, vimentin, glial fibrillary acidic protein (GFAP), and factor VIII on frozen sections from eyes of patients with diabetes without proliferative retinopathy, eyes of patients without diabetes and without known ocular disease, and eyes with disciform age-related macular degeneration (ARMD). Retinal vascular digest preparations to evaluate microvascular abnormalities.

**Results.** Based on morphology and on GFAP and vimentin immunopositivity, retinas from all subjects with diabetes immunostained strongly to VEGF in elongated processes that appeared to be Müller cells. Gial cells within septa surrounding axons in the anterior optic nerve also immunostained for VEGF, as did endothelial cells of some posterior retinal blood vessels and some retinal pigment epithelial cells. Retinas from eyes with disciform ARMD immunostained for VEGF, though less extensively than did those of subjects with diabetes. Retinas and optic nerves from subjects without ocular disease were VEGF negative. Basic fibroblast growth factor was expressed minimally in the inner retinal layers of subjects with and without diabetes, but it was substantial in the photoreceptor layer of all eyes. Vascular endothelial growth factor immunopositivity was present in eyes with no, or little, retinal vascular anatomic abnormality in digest preparations.

**Conclusions.** Vascular endothelial growth factor expression precedes retinal neovascularization in the retinas and the optic nerves of humans with diabetes. Its localization to gial cells of the inner retina and the anterior optic nerve suggests a relationship to neovascularization in these sites. That VEGF immunopositivity may occur when there is no anatomic evidence of retinal nonperfusion and little likelihood of retinal neovascularization suggests the possibility that ischemia may not be the sole stimulus for VEGF expression. Invest Ophthalmol Vis Sci. 1997;38:36-47.

Vascular endothelial growth factor (VEGF), a mitogenic peptide highly specific for vascular endothelial cells, has been implicated strongly in the development of retinal and iris neovascularization in proliferative diabetic retinopathy and other retinal vascular dis-
and retinal and choroidal tumors with associated retinal detachment, as well as by polymerase chain reaction in epiretinal membranes removed surgically from subjects with diabetes and proliferative retinopathy.

In immunocytochemical experiments, VEGF mRNA and protein expression recently were reported in several locations in the retinas of rats made diabetic by streptozotocin and was maintained for as little as 6 months. Sites of VEGF expression in these animals were thought to be ganglion cells, Müller cells, astrocytes, smooth muscle cells, and retinal pigment epithelial cells. These authors reported that the localization of VEGF immunoreactivity correlated with extravascular immunoreactivity for albumin, considered to indicate breakdown of the blood–retinal barrier.

Recently, Pe’er and associates presented the first description of VEGF mRNA expression in the inner and outer layers of human retinas with severe proliferative diabetic retinopathy, ischemic central retinal vein occlusion with rubeosis iridis, and retinoblastomas and choroidal melanomas with associated retinal detachment and rubeosis iridis. Although their methodology did not permit precise cellular localization of the growth factor mRNA, they suggested, on the basis of patterns of autoradiographic labeling in some specimens, that retinal astrocytes were likely to be involved.

We describe here the localization of VEGF protein by immunocytochemistry in glial cells of the retina and optic nerve and, to a lesser extent, in retinal pigment epithelial (RPE) cells and some retinal vascular endothelial cells in human subjects with diabetes who had no evidence of retinal or iris neovascularization. We could detect only to a limited degree another important mitogen, basic fibroblast growth factor (bFGF), within cells of the inner retinal layers of these eyes. These results provide evidence for glial cells as the initial cellular sites of the molecular events that may lead to human retinal and optic nerve head neovascularization. In addition, they raise questions about the role of ischemia in all instances of VEGF expression.

MATERIALS AND METHODS

Donor Eyes

We obtained human donor eyes through the National Disease Research Interchange (Philadelphia, PA). There were eight eyes from subjects with diabetes—three men and five women 58 to 81 years of age. Clinical histories of the donors were not detailed. No subject had a history of diabetic retinopathy, but one had undergone bilateral cataract surgery with intracocular lens implantation and bilateral laser trabeculoplasty for chronic open-angle glaucoma. We also obtained five eyes from persons with no history of diabetes or of ocular disease as determined by gross pathologic examination. These eyes were from two men and three women 58 to 81 years of age. In addition, we obtained three eyes that, on examination under the dissecting microscope, showed the disciform lesions of ARMD. These were from three women 70 to 92 years of age. This information is summarized in Table 1. All eyes were enucleated, packed on ice, and shipped to us by express mail. However, the time of arrival varied from 20 to 51 hours after each subject's death.

Tissue Preparation

Immediately after the specimens arrived in the laboratory, a superior calotte was removed, and the interior of the globe was examined carefully under the dissecting microscope for evidence of diabetic retinopathy or other vascular disease. This was followed by gentle removal of the lens and vitreous to preserve any vascular formations anterior to the inner limiting membrane. Each specimen was placed in a cryomold and was frozen rapidly in OCT medium (Miles Laboratories, Elkhart, IN) using isopentane. Sections 18 μm thick were taken serially, air dried for 2 hours, and fixed in 2% buffered (pH 7.4, 50 mM phosphate) paraformaldehyde solution for 10 minutes at 25°C. The sections were washed in 50 mM Tris-buffered saline (TBS, pH 7.4) for 15 minutes, air dried, and stored at −70°C until use.

Immunocytochemistry

Polyclonal antibodies prepared in goats to recombinant human VEGF and bFGF were obtained from R & D Systems (Minneapolis, MN). They were diluted 1:100 before use. Rabbit antihuman factor VIII and antibovine glial fibrillary acidic protein (GFAP) were obtained from Dako (Carpinteria, CA) and diluted 1:200 and 1:500, respectively, before use. Goat antihuman vimentin was obtained from Sigma Chemical (St. Louis, MO) and diluted 1:200 before use.

Before sections were immunostained by 5-bromo-4-chloro-3-indoloyl phosphate–nitro blue tetrazolium (BCIP–NBT), endogenous peroxidase activity was quenched with a 3% hydrogen peroxide–70% methanol solution. This was not necessary when we performed fluorescence immunocytochemistry. Sections immunostained by either of the methods described below were incubated in a blocking solution containing 1% bovine serum albumin and 5% normal rabbit or normal goat serum (depending on the source of the secondary antibody) in TBS for 30 minutes at 25°C and then in diluted primary antibody for 3 hours at 25°C in a humidified chamber. After this, they were washed three times in TBS. Sections to be stained using the BCIP–NBT method were incubated in a 1:100 dilution of biotinylated secondary antibody (rabbit–antigoat immunoglobulin G or goat–antirabbit immunoglobulin G; Vector Laboratories, Burlingame, CA) at 25°C for 1 hour. Sections were washed in TBS three times for 5 minutes each and were incu-
TABLE 1. Subject Characteristics

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Gender</th>
<th>Race</th>
<th>Laser</th>
<th>Other Eye Disease</th>
<th>Cause of Death</th>
<th>Time From Death to Processing of Eye (hours)</th>
</tr>
</thead>
</table>
| Subjects with diabetes
| 70        | F      | B    | Yes; for glaucoma | Open-angle glaucoma; bilateral intraocular lenses | Cerebrovascular accident | 33                                          |
| 60         | F      | W    | No   | No                | Bowel obstruction, necrosis, multisystem failure | 30                                          |
| 63         | M      | W    | No   | No                | Cardiorespiratory arrest; possible sepsis         | 36                                          |
| 57         | F      | W    | No   | No                | Cerebrovascular accident                          | 23                                          |
| 72         | M      | W    | No   | No                | Pneumonia, congestive heart failure               | 45                                          |
| 75         | M      | W    | No   | No                | Ventricular fibrillation                          | 45                                          |
| 64         | F      | W    | No   | No                | Myocardial infarction                             | 30                                          |
| 83         | F      | W    | No   | No                | Intracranial hemorrhage                           | 20                                          |

Subjects without diabetes, no eye disease

| 79         | M      | W    | No   | No                | Ventricular fibrillation                          | 34                                          |
| 58         | F      | W    | No   | No                | Cerebrovascular accident                          | 36                                          |
| 83         | F      | W    | No   | No                | Metastatic carcinoma                              | 29                                          |
| 81         | F      | W    | No   | No                | Respiratory arrest                                | 35                                          |
| 72         | M      | W    | No   | No                | Subdural hematoma                                 | 33                                          |

Subjects with age-related macular degeneration

| 84         | F      | W    | No   | No                | Respiratory arrest                                | 30                                          |
| 70         | F      | W    | No   | No                | Congestive heart failure                          | 39                                          |
| 92         | F      | W    | No   | No                | Acute renal failure                               | 51                                          |

bated in an avidin-conjugated alkaline phosphatase solution (Sigma) for 30 minutes at 25°C, washed again three times in TBS, and incubated in a BCIP–NBT liquid substrate system (Sigma) as directed by the supplier. Some sections were counterstained with eosin. Alternatively, instead of a biotinylated secondary antibody, we used a secondary antibody that had been conjugated to fluorescein isothiocyanate (FITC; Vector Laboratories) at a dilution of 1:400 at 25°C for 1 hour. Sections prepared with either secondary antibody were then washed in TBS, coverslipped, and examined microscopically. For FITC-conjugated antibody staining, an appropriate light source and filters for fluorescence microscopy were used.

Control slides of the same tissues were incubated concurrently with test slides. In some controls, nonimmune goat or rabbit serum (Sigma) was substituted for the primary antibody. In other controls, specificity of the antigrowth factor antibodies was tested by adding a concentration of 10–6 M of either recombinant human bFGF or recombinant human VEGF (each obtained from R & D Systems) to the solution containing the primary antibody.

Results described in this article were derived from examination of at least three slides of the entire posterior ocular segment per eye immunostained with each antibody. Although the descriptions that follow are not expressed on a quantitative or a semiquantitative basis, a description of strong or intense staining means that many cells stained intensely throughout the extent of the retina or optic nerve on all the sections examined for an eye or a group of eyes.

Retinal Vascular Digests

These were performed by a combination of the methods of Kuwabara and Cogan12 and Laver et al13 which we found gives optimal results. Superior ocular calottes were fixed for 24 hours in 4% paraformaldehyde in 50 mM phosphate-buffered saline, pH 7.4. Pieces of retina up to 1 cm² in area were excised and washed overnight with several changes of distilled water. These were transferred to a petri dish and were incubated in a water bath at 37°C with a solution of 3% trypsin (Gibco, Grand Island, NY) in 0.15 M Tris-HCl buffer, pH 7.8, until the retinal pieces appeared soft and pliable. This usually required 1½ to 2 hours. The pieces were washed thoroughly with distilled water and incubated in 0.1 M Tris–HCl buffer, pH 8.5, with 50 U/ml elastase (type 1, 2X crystallized; Sigma) for 15 to 20 minutes. Digestion was observed under a dissecting microscope, and occasionally it had to be repeated two to three times until nonvascular elements could be removed. The digest was floated onto a slide, and care was taken to have it spread flat, air dried,
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RESULTS

Figure 1A shows a section from the midperipheral retina of a 70-year-old woman with diabetes, and Figure 1B shows a section from the posterior retina, taken within 0.35 mm of the optic nerve of the same person. The sections were immunostained for VEGF using a biotinylated secondary antibody and the BCIP-NBT reaction system, and they were counterstained with eosin. Numerous processes within the inner retinal layers stain. Some of these have elongated (Fig. 1A, arrowheads) and even bulbous (Fig. 1B, arrowhead) profiles and are located within the inner plexiform and inner nuclear layers; occasionally they extend through the entire width of these layers. We observed few VEGF-positive processes in the outer plexiform or outer nuclear layer using this immunostaining method. A minimal amount of patchy stain was seen in the basal portions of some RPE cells (Fig. 1B, open arrows), and there was positive VEGF immunostaining of the endothelial lining of several large retinal vessels; in some, the endothelial cells immunostained for VEGF (Fig. 1B, filled arrows). There was some lighter but more diffuse staining in the ganglion cell and nerve fiber layers of this section.

Immunostained sections from the anterior portion of the optic nerve from the same person shown in Figure 1 are shown in Figure 2. Elongated profiles—either within the septa between the optic nerve fibers or branching off from the septa—show intense VEGF stained with periodic acid–Schiff reagent and hematoxylin, mounted, and coverslipped.

Figure 1. (A) Photomicrograph of a retina from the temporal periphery of a 70-year-old woman with diabetes that was immunostained for vascular endothelial growth factor (VEGF) and counterstained with eosin. Arrowheads indicate elongated processes, one of which includes branches, that extend from the nerve fiber layer to the outer plexiform layer. The inner limiting membrane has been disrupted artefactually. (B) Photomicrograph of a section, from the macular retina of the subject shown in A, that was immunostained for VEGF and counterstained with eosin. Although the outer nuclear layer is present in a portion of the section, the photoreceptor layer has been artefactually disrupted. The arrowhead indicates a bulbous, immunopositive process at the junction of the outer plexiform and inner nuclear layers. Open arrows at the bottom of the micrograph demonstrate regions of immunopositivity at the bases of the retinal pigment epithelial cells, and the filled arrows demonstrate immunopositive endothelial cells lining large retinal vessels. Magnification, ×100.

Figure 2. (A) Photomicrograph of a portion of the anterior optic nerve from the subject shown in Figure 1, immunostained for vascular endothelial growth factor and counterstained with eosin. (B) Photomicrograph of another anterior optic nerve than the portion of the subject shown in A, immunostained for glial fibrillary acidic protein and counterstained with eosin. Magnification, ×250.
FIGURE 3. (A) Vascular endothelial growth factor (VEGF) immunostain using fluorescein isothiocyanate (FITC)-labeled secondary antibody. A retinal section from a 60-year-old woman with diabetes is labeled. Note the elongated processes that immunostain and extend through all retinal layers except the photoreceptor layer. (arrowhead) Positive immunostain in the endothelial layer of a large retinal arteriole. The neurosensory retina has been detached artefactually. (B) Gial fibrillary acidic protein immunostain using FITC-labeled secondary antibody of the second serial retinal section from the same subject shown in A. (C) Vimentin immunostain using FITC-labeled secondary antibody of the third serial retinal section from the subject shown in A and B. (D) A control section from the same eye, stained using anti-VEGF antibodies incubated with $10^{-5}$ M of the recombinant human growth factor. No specific immunostain can be seen, even though the photograph was overexposed considerably. However, in this section, the retinal pigment epithelium was not fully detached artefactually, and its considerable autofluorescence is evident. Magnification, $\times 450$ (A to D). (E) Factor VIII immunostain using FITC-labeled secondary antibody of a nonserial section of retina from the same subject. Multiple vascular profiles label, but the pattern is different from those shown in A to C. Magnification, $\times 200$. 
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with diabetes that was stained for VEGF. Labeled pro-

tivity. Similar VEGF immunostaining of the posterior

cells of the retinal vessels, some of which were immu-

prominently in the inner retina where the Müller cell

to demonstrate using fluorescent secondary antibodies be-

immunofluorescent preparations labeled fine pro-

for bFGF in the photoreceptor layer (Fig. 4A) in all

EFAP (Fig. 3B) and for vimentin (Fig. 3C); the latter

fluorescence was demonstrated. Immunostains for fac-

immunostaining of the RPE could not be demon-

VEGF immunostaining was more intense and diffuse within

moderate amount of staining that had a cellular distri-

magnification in Figure 7D, and they include exten-

deoxyribonuclease I digestion (Fig. 7B) of the region enclosed by the box in

DISCUSSION

This study has several major conclusions. The first is

bFGF, whether we substituted nonimmune immunoglo-

together with an excess of the appro-

in eyes without diabetes, with or without disciform submacu-

avascular zone surrounding the larger arterioles. Addi-

These abnormalities are even more evident at higher

None of the five eyes from subjects without diabetes;

DISCUSSION

This study has several major conclusions. The first is

occasional patchy staining appeared in the basal por-

None of the five eyes from subjects without diabetes;

bFGF immunostaining of the structures within the inner retinal layers of these

not label elongated processes extending into the outer

immunostaining for either VEGF or for bFGF, whether we substituted nonimmune immuno-

in eyes from older human subjects.

Our observation moderately intense immunostaining

or oval profiles with central lumina, but did not

immunostaining in glial cells in the optic nerves in three

to demonstrate them using the BCIP–NBT method. Figure 3D

mild, patchy bFGF immunostaining of structures within the inner retinal layers of these

immunostaining of the posterior retinas and optic nerves was observed in all eight eyes

retina. Immunostaining of the RPE could not be demon-

than that for VEGF, and immunostaining was more intense and diffuse within

budation similar to what we observed in the subjects with
diabetes, in the three eyes we examined with disciform macular scars, presumably from ARMD. These eyes

had no observable retinal vascular abnormalities, nor did the donors have a history of diabetes. The intensity

of the VEGF immunostain in the neural retinas of these eyes was, in all cases, substantially less than in

the eyes we examined from subjects with diabetes, although immunostaining of the RPE usually was con-

siderably more (Fig. 5B). Immunostain for GFAP of retinal and optic nerve sections from eyes of subjects

without diabetes, with or without disciform submu-

DISCUSSION

This study has several major conclusions. The first is

none of diabetes before proliferative retinopathy has de-

including those with disciform ARMD, showed none

immunostaining for VEGF (Fig. 3A, arrowhead). All these

immunofluorescent staining using FITC-labeled

immunostain for either VEGF or for

Figure 7A shows highly cellular capillaries with infre-

intensity was in all cases, substantially less than in

This study has several major conclusions. The first is

bFGF expression occurs in the retinas of subjects

and also in the retinas of subjects without
diabetes with choroidal, but not retinal, neovasculari-

This extends the findings described in the recent report of Pe’er et al,17 who examined enucleated
human eyes that had advanced neovascular disease. We, of course, can only speculate as to whether, and when, retinal neovascularization would have developed in the eyes we studied had those persons lived longer. It seems highly unlikely that the eyes from the subjects with diabetes, which we received consecutively, came from persons who, at the time of death, were all on the verge of having proliferative retinopathy. Additionally, VEGF was expressed in the neural retinas of eyes from three subjects without diabetes.
but with choroidal neovascularization underlying their macular retinas. Almost surely, new vessels arising from the retinal circulation would never have developed in these persons. Furthermore, in the eyes from subjects without diabetes but with choroidal neovascularization (who also showed positive retinal immunostaining for VEGF), and in the eyes from subjects with diabetes with minimal retinopathy (which we could demonstrate by examination of the open globe through the dissecting microscope and by examination of vascular digest preparations of the superior calottes), immunostaining was positive throughout the entire retina, ora serrata to ora serrata. We were able to see this because the sections were cut through the whole eye.

Without additional evidence, the function of VEGF in retinas without evidence of vasoproliferation is a subject only for speculation. As noted, VEGF does produce increased vascular permeability, and it has been suggested that it plays a role in the breakdown of the blood–retinal barrier, perhaps leading to diabetic macular edema. Several reports have indicated that a basal level of VEGF is produced normally in many types of cells in the retina and throughout the body. Based on the finding of VEGF expression in cultured human RPE cells, it has been suggested that low basal levels of VEGF secretion may be responsible for the trophic function of the RPE on choriocapillary maintenance. Vascular leakage and neovascularization are presumed to occur only at much higher levels of growth factor secretion after upregulation caused by pathologic influences. We have not attempted to study the integrity of the blood–retinal barrier in the current experiments and cannot, therefore, comment on this possible role of VEGF. Miller et al initially stated that VEGF mRNA was “barely detectable” in northern blots of normal, nonischemic monkey retinas and that there was “minimal” VEGF mRNA labeling on in situ hybridization of microscopic sections of such retinas. Recently they revised this conclusion using more sensitive methods, at least in a preliminary report of their results. If basal levels of VEGF are secreted by cells of the normal human retina, they are not present above the threshold of the detection techniques used in our own experiments. Although we have no evidence of its role in the retinas of subjects without diabetes, the substantial upregulation of VEGF protein expression we have observed in the eyes of subjects with diabetes but without evident retinopathy appears to be biologically important.

Our second major conclusion is that VEGF expression in eyes without demonstrable retinal neovascularization at first primarily occurs in glial cells, including the Müller cells of the retina and the glial cells at least of the anterior portion of the optic nerve. Immunostaining for this growth factor was evident, but was much less prominent, in the endothelial cells of major retinal vessels and in the basal portions of RPE cells. The morphology in the human retinal sections we studied is not ideal because of the time that elapsed between subject death and tissue processing in our laboratory and because we used very thick, unfixed, frozen tissue sections through each whole globe to examine each entire retina and choroid. We do think, however, that these preparations were adequate for the studies we have described. The identification of VEGF-positive elements within the anterior optic nerve as glial cells is obvious from their morphology, their GFAP positivity, and their anatomic localization within the interaxonal septa. We identified many of the retinal elements that immunostain for VEGF as Müller cells because of their anatomic appearance—processes extending from the inner limiting membrane of the retina to the outer limiting membrane (which differentiates them from the perivascular glia of the retina)—and because of their immunopositivity for GFAP and vimentin, particularly in processes that could be identified in several serial sections, and because of morphologic characteristics using antibodies to these two substances that other authors have identified as typical of Müller cells (Figs. 3A to 3C).

An examination of Figures 3A to 3C reveals that the perivascular glia may have been VEGF positive (note the curved, immunopositive process that partially surrounds the large arteriole in these figures). Müller cells are the principal elements that stain. Although we found that some vascular endothelial cells were VEGF positive, the very different configuration of the

**FIGURE 5.** (A) Photomicrograph of midperipheral retina, from a 77-year-old man without diabetes, immunostained for vascular endothelial growth factor (VEGF) and counterstained with eosin. (B) Photomicrograph of peripheral retina, from a 70-year-old woman without diabetes, with a disciform scar underlying the macular retina. The section has been immunostained using antihuman basic fibroblast growth factor in the presence of the growth factor at a concentration of $10^{-6}$ M. The section has been counterstained with eosin. Magnifications, ×200.

**FIGURE 6.** (A) Photomicrograph of a section, from the retina of a 70-year-old woman with diabetes, incubated with antihuman vascular endothelial growth factor in the presence of the growth factor at a concentration of $10^{-6}$ M. The section has been counterstained with eosin. (B) Photomicrograph of a section, from the retina of a 60-year-old woman with diabetes, immunostained using antihuman basic fibroblast growth factor in the presence of the growth factor at a concentration of $10^{-6}$ M. The section has been counterstained with eosin. We observed similar results when we substituted nonimmune immunoglobulin G for the antibody and did not add growth factor during the incubation. Magnifications, ×200.
FIGURE 7. (A) A trypsin–elastase digest of the retinal vasculature from a region of the superior calotte of the eye of a 75-year-old man with diabetes. In several areas, nonvascular tissue remains (asterisk), but this does not interfere with the evaluation of the vascular tree. (box) This region is shown at higher power in B. Periodic acid–Schiff stain. Magnification, ×100. (B) High-power view of the region enclosed by the box in A. In general, the capillary tree is highly cellular, but there are some acellular capillaries (arrow) and pericyte “ghosts” (arrowheads), as well as atrophic strands. Magnification, ×250. (C) Low-power view of a digest preparation from the superior calotte of the eye of a 70-year-old woman with diabetes. Even at this power, the marked decrease in vascular density and cellularity is evident by comparison with A, and numerous microaneurysms can be seen. (box) The area shown at higher magnification in D. Magnification, ×100. Periodic acid–Schiff stain. (D) High-power view of the region enclosed by the box in C. Magnification, ×250.

Retinal structures that immunostained for factor VIII (Fig. 3E) clearly indicates that retinal capillaries sectioned longitudinally were not the principal VEGF-positive structures we observed. It is interesting, however, that VEGF immunostaining was most prominent in the inner layers of the retina, where the retinal vessels are found (Figs. 1, 5B). In particular, we found that immunostaining patterns with secondary antibodies complexed to two different chromogens, FITC and BCIP–NBT, yielded labeling patterns that were consistent with one another but that had different abilities to demonstrate antigen in Müller cell processes located in the outer retinal layers. Immunostaining by the BCIP–NBT method, though generally more sensitive than that with FITC-labeled secondary antibodies, was less able to detect the antigens we studied in Müller cell processes in the outer retinal layers. The reason for this is not clear, but several studies using fluorescent-labeling immunocytochemical methods to demonstrate a number of antigens in retinal Müller cells have shown much more prominent labeling of the thick Müller cell processes in the inner retinal layers.\textsuperscript{4,21–23} Although the amplification afforded by adding the avidin–biotin-linked enzyme reaction to antigen–antibody binding should make the BCIP–NBT color reaction even more sensitive than immunofluorescence alone, perhaps some slight reduction in penetration of the reagents involved in the color reaction through cell membranes reduces the efficacy of this method for demonstrating the presence of antigens in the thin Müller cell processes visible in the outer retinal layers.
With one exception,7 all previous studies of VEGF expression in human ocular vasoproliferative diseases2,5,10 have involved sampling of ocular fluids or surgically excised epi-retinal membranes at advanced stages of disease. Two studies in nonhuman mammals using in situ hybridization and northern blot analysis9,10 showed that the rise and decline of VEGF mRNA expression in the retina coincide with the onset and regression of retinal or iris neovascularization. Additionally, Pierce et al4 performed immunocytochemistry using a fluorescent anti-VEGF antibody and confocal microscopy for cellular localization. Their findings are particularly relevant to our current results because they found VEGF in the retinal Müller cells of neonatal mice that developed retinal neovascularization after a 5-day exposure to 75% oxygen.

Müller et al2 used in situ hybridization and localized VEGF mRNA to the inner nuclear layer of the monkey retina 10 to 20 days after they produced ischemic retinal vein occlusion and iris neovascularization by laser occlusion of the major retinal veins. Their results are consistent with ours and with the study by Pierce et al4 but their cellular localization of VEGF expression is not as precise. Stone et al19 described VEGF expression by in situ hybridization, most likely in Müller cells and astrocytes, in the nerve fiber layer and the inner nuclear layer of the developing retinas of rats and cats. They related the growth factor expression to hypoxia in the metabolically highly active developing tissue. A fourth study11 reported that VEGF could be demonstrated immunocytochemically surrounding the retinal blood vessels, as well as in cells presumed to be ganglion cells and glial cells of diabetic rats in which no histologically identifiable retinal vascular lesions had developed, although the presence of extravascular albumin presumably indicated that breakdown of the blood–retinal barrier had occurred. These results are surprising to us because Murata et al11 performed immunocytochemistry on formalin-fixed, paraffin-embedded tissues, which, in our experience, lose their VEGF antigenicity. Closest to our current work is the study of Pe’er et al,7 who suggested the possible involvement of retinal astrocytes but who did not mention the expression of VEGF mRNA in the RPE or optic nerve. In our study, the morphology of the cell processes that immunostained predominately for VEGF, GFAP, and vimentin much more strongly resembled that of Müller cells than of astrocytes. In any event, this could not have been responsible for the outer retinal labeling described by Pe’er et al7 in tumors with retinal detachment because glia other than Müller cells infrequently appear in the avascular outer layers of the retina.20 In situ hybridization demonstrates the site of synthesis of a molecule, whereas immunocytochemistry demonstrates its localization within a tissue. Thus, to reconcile the finding of Pe’er et al7 that apparently substantial VEGF mRNA appears in the outer retina of eyes whose principal disease process was in the outer retina and choroid, with our observation that the most prominent VEGF immunolabeling occurred in the inner retina, we submit that perhaps VEGF synthesis is localized to any of several areas along the great length of the Müller cell, where the angiogenic stimulus is maximal. Perhaps then the newly synthesized protein is transported rapidly to the vascularized inner retina, where it can be secreted by a much shorter route to its target, the vascular endothelial cell.

Our third major conclusion is that although one eye from a subject with diabetes showed evidence of extensive retinal ischemia based on the substantial capillary acellularity demonstrated in the digest preparation, the others did not. We could not, of course, perform digest preparations on the entire retinal vasculature of these eyes, so there may have been areas of ischemia we missed. However, the absence of acellularity in the portions of the retina we did study, together with the extensive VEGF immunostaining through the entire retina to the far periphery in the sections we cut, suggests that ischemia related to nonperfusion of the retinal microcirculation may not explain completely the appearance of VEGF in the retinas of these subjects with diabetes. This point is made even more emphatically based on our observation of VEGF immunoreactivity in Müller cells of eyes from subjects without diabetes with choroidal neovascularization. Digest preparations from these eyes showed no evidence of capillary acellularity to suggest retinal vascular nonperfusion. This greatly reduces the possibility that focal regions of VEGF expression might be related to small regions of hypoxia caused by capillary nonperfusion in diabetic eyes or to a hypoxic stimulus arising perhaps from retinal circulatory insufficiency in the macular regions of subjects with ARMD. Decreased retinal blood flow in subjects with diabetes might account for relative hypoxia even in the absence of anatomic abnormalities of the vessels. Such decreases have been observed in subjects with diabetes without retinopathy, but their effects on retinal oxygenation and on growth factor expression within the retina remain to be determined.26,27 Although there are no studies of retinal blood flow in subjects with neovascular ARMD, it seems unlikely that retinal blood flow in them is reduced substantially in comparison with retinal blood flow in normals. Yet we found VEGF expression in the neural retinas of these subjects as well.

It might be argued that in some of these eyes without evident retinal neovascularization, our results were unphysiologic and related to metabolic processes occurring in the eyes after death.7 We do not think this is so because the eyes we examined from subjects without diabetes or choroidal neovascular disease did not express VEGF immunocytochemically, and there were no systematic differences between these eyes and the eyes without diabetes or neovascular disease.
that immunostained positively in the elapsed time between death and processing of the eyes in our laboratory.

Our fourth conclusion is that the difference between anti-VEGF and anti-bFGF labeling of these tissues is of considerable interest. In the inner retina, bFGF immunostaining was much less prominent than was VEGF immunostaining. In the photoreceptor layer, bFGF staining was very strong in all eyes, including eyes from subjects without diabetes or other ocular disease. We interpret these results with caution, however, because variations in antibody labeling may occur with differences in fixation techniques and in antibody source. This point is true in immunocytochemistry generally, but recently it has been noted with particular reference to bFGF immunostaining. As one might expect, and as we have demonstrated in our current study, maximal immunocytochemical demonstration of bFGF in the retina is found when sections are prepared from frozen, unfixed tissue.

If VEGF was present in these retinas, why did retinal neovascularization not develop in them? If most of the eyes we studied do not represent examples of imminent retinal vascular proliferation, two possibilities follow. First, VEGF alone may be insufficient to initiate retinal neovascularization. We cannot state this point with absolute certainty because our methods did not permit quantitation of biologically active VEGF in the retinas we studied. An alternative explanation for the absence of retinal neovascularization in the eyes we studied may be simply that the quantity of VEGF expressed in these eyes was too small. Second, as the hypoxia theory of the pathogenesis of retinal neovascularization implies, the initial biochemical lesions of proliferative diabetic retinopathy and other retinal neovascular diseases must develop in nonvascular cells of the retina and optic nerve before anatomic lesions appear in the vessels. If local hypoxia is necessary for the expression of VEGF, how can it be present in eyes with nonproliferative diabetic retinopathy to a sufficient degree to stimulate growth factor production? Without greater knowledge of antemortem vascular physiology (for example, from fluorescein angiographic studies) or without more extensive visualization of the vascular anatomy in the retinas we studied—which we were unable to obtain using our methods of tissue preparation—any answer to this question must be speculative. However, Williamson et al have proposed the term “hyperglycemic pseudohypoxia” to reflect those metabolic perturbations of cells in severe hyperglycemia with normal tissue PO2 that resemble the disturbances that occur in hypoxic tissues. These authors have proposed that this “pseudohypoxic” state may initiate the chain of events leading to the development of the vascular complications of diabetes. This hypothesis could explain why VEGF might be expressed in the retinas of subjects with diabetes in whom anatomic lesions of the retinal blood vessels extensive enough to produce clinically apparent nonperfusion have not yet developed. It does not suffice to explain VEGF expression in the retinas of subjects without diabetes but with choroidal neovascularization. Additional studies are required to understand this unexpected observation.

Key Words

basic fibroblast growth factor, diabetic retinopathy, glial cell, optic nerve, retina, vascular endothelial growth factor

References


