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Expression of Vascular Endothelial Growth Factor in the Human Retina and in Nonproliferative Diabetic Retinopathy

Chiara Gerhardinger,* Lawrence F. Brown,† Sayon Roy,* Masakazu Mizutani,* Charles L. Zucker,* and Mara Lorenzi*

From the Schepens Eye Research Institute and the Department of Ophthalmology and the Department of Pathology,† Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts*

Vascular endothelial growth factor (VEGF)/vascular permeability factor is a likely angiogenic mediator in proliferative diabetic retinopathy, and its role is under scrutiny in the pathogenesis of the capillary leakage characteristic of background diabetic retinopathy. To examine whether the diabetic milieu induces or increases retinal VEGF expression in humans, we examined retinas from nondiabetic eye donors and donors with 9 ± 5 years of diabetes and documented microangiopathy. To identify possible confounding effects of the postmortem period, we also studied the postmortem stability of the VEGF transcript and the expression of the VEGF protein in rat retinas. In both human and rat retina we detected by Northern analysis a 4.2-kb VEGF mRNA species and by reverse transcriptase polymerase chain reaction the transcripts encoding $VEGF_{165}$ (the most abundant), $VEGF_{121}$, and $VEGF_{189}$. By *in situ* hybridization and immunohistochemistry VEGF mRNA and protein co-localized at the ganglion cell, inner nuclear, and outer plexiform layers and in the walls of the blood vessels (where mRNA was scarce). The protein was additionally detected in photoreceptors. The abundance and distribution of VEGF mRNA and protein were not altered in the diabetic retinas, indicating that the diabetic environment is not sufficient to increase retinal VEGF expression. The demonstration that VEGF is constitutively expressed in the adult retina and is localized to discrete neural cells and their processes proposes a role for the cytokine in retinal homeostasis and/or function. ***(Am J Pathol 1998, 152:1453–1462)***

Vascular endothelial growth factor (VEGF) is a probable mediator of the unregulated angiogenesis triggered by ischemic retinal diseases, including diabetic retinopathy. Such a role is proposed and supported by the detectability of this potent endothelial mitogen[1] in the ocular fluids of patients with retinal neovascularization,[2] the temporal and spatial correlation of increased VEGF synthesis and neovascularization in monkeys with retinal vein occlusion,[3] the development of intraretinal new vessels in mice overexpressing a VEGF transgene,[4] and the at least partial prevention of retinal neovascularization in mice with ischemic retinopathy treated with inhibitors of VEGF action[5] or synthesis.[6] Rational and safe translation of such ablative strategies to clinical use requires knowledge of the biology of VEGF in the human retina, and such knowledge is currently lacking.

Because VEGF is also a potent vascular permeability factor,[7] there is a keen interest in determining whether the cytokine may additionally contribute to the early retinal microvascular abnormalities induced by diabetes and, notably, to the characteristic capillary leakage responsible for hard exudates and macular edema.[8] Intravitreal injection of VEGF in rats[9] and primates[10] induces increased retinal vascular permeability and other manifestations of diabetic microangiopathy. However, VEGF is undetectable in the ocular fluids of most diabetic patients with nonproliferative retinopathy,[2] but this finding cannot exclude subtle changes in synthesis that may be focal. Studies have reported in the retinas of patients with nonproliferative diabetic retinopathy vascular and glial VEGF immunoreactivity apparently not observed in the retinas of nondiabetic subjects[11,12] and increased VEGF immunostaining in the retinas of streptozotocin-diabetic rats when compared with control rats, especially in areas of albumin extravasation.[13] However, because VEGF is a secreted protein with diffusible as well as extracellular-matrix-bound isoforms,[14] its absence from a tissue does not mean that it is not synthesized there and secreted, just as its detection does not inform about the sites and levels of synthesis. Moreover, VEGF is a circulating molecule,[15,16] and its presence in areas of increased per-

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Address reprint requests to Dr. Mara Lorenzi, Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114. E-mail: lorenzi@vision.eri.harvard.edu.

Table 1. Characteristics of Eye Donors and Specimens

Groups	Age (years)	Sex	Diabetes duration (years)	Eyes: time to enucleation (hours)	Eyes: time to processing* (hours)
Northern hybridization study (total retina RNA)					
Nondiabetic	61 ± 7	9 M/2 F		3.0 ± 1	29 ± 6
Diabetic	63 ± 7	3 M/5 F	9 ± 5	3.0 ± 2	25 ± 9
In situ hybridization and immunohistochemistry studies (retinal sections)					
Nondiabetic	67 ± 5	4 M/5 F		3.5 ± 1	15 ± 4
Diabetic	67 ± 7	3 M/4 F	9 ± 4	3.0 ± 1	12 ± 5

M, male; F, female.
*The time elapsed from death to sample processing was longer for the eyes used in the Northern study (fresh samples) than for the eyes used in the morphological studies because the latter were fixed by the eye banks before shipment to the laboratory. Numbers are means ± 1 SD.

meability may reflect extravasation. The *in situ* hybridization studies performed in streptozotocin-diabetic rats[13] are not readily interpretable in the absence of comparative data from control rats. It is thus unknown at this time whether VEGF synthesis, secretion, or action are altered in background diabetic retinopathy, although such information would be instrumental both in reconstructing the pathogenetic process that leads to vascular leakage and identifying discrete targets for intervention.

To begin learning about the biology of VEGF in the human retina and possible changes induced by diabetes, we examined in the retinas of control individuals and age-matched diabetic patients with background retinopathy the extent and sites of VEGF synthesis, the splicing pattern of the transcript, and the distribution of VEGF mRNA and protein in the same retina. To identify possible confounding effects of the postmortem period we also studied in rat retinas the postmortem stability of the VEGF transcript and the expression of the VEGF protein.

Materials and Methods

Human Subjects and Specimens

Human eyes were obtained from certified eye banks through the National Disease Research Interchange. Criteria for inclusion in the study were age less than 75 years, absence of retinal or hematological diseases or uremia, absent administration of chemotherapy or life support measures, diabetes duration of less than 15 years to address mostly background retinopathy,[17] and the fewest possible chronic pathologies other than diabetes. Eyes destined for isolation of total retinal RNA were obtained from 8 diabetic and 11 nondiabetic subjects; their characteristics are reported in Table 1. The eyes were kept at 4°C by the eye banks and shipped to the laboratory on ice; the two retinas from each donor were immediately dissected, separated from the retinal pigment epithelium, pooled, and processed for RNA isolation.[18] The eyes used for *in situ* hybridization and immunohistochemical studies were obtained from 7 additional diabetic and 9 nondiabetic donors (Table 1). These two groups included all 7 diabetic donors and 7 of the 8 nondiabetic donors whose retinal trypsin digests had been previously studied for the occurrence of microvascular cell apoptosis and vascular pathology.[19] The eyes were fixed in 10% buffered formalin by the eye banks as soon as possible after death (Table 1); on arrival to the laboratory they were cryopreserved and embedded in optimal cutter temperature compound as described.[19]

Animals and Specimens

Eyes were obtained from Sprague-Dawley male rats aged 3 to 4 months and weighing 400 to 500 g. To study VEGF mRNA stability, RNA was extracted from retinas of rat eyes 1) enucleated and processed immediately after sacrifice, 2) enucleated immediately after sacrifice and kept at 4°C for 30 hours, and 3) enucleated 5 hours after sacrifice and kept at 4°C for 25 hours. These intervals were chosen to match the timing of events reported in Table 1 for the human specimens. To study the expression and localization of retinal VEGF protein, rat eyes were enucleated immediately after sacrifice, fixed in 10% buffered formalin, and processed as described for the human eyes.

Northern Analysis

Northern analysis[20] was used to study the levels of VEGF mRNA in human retinas and the stability of the transcript in the postmortem period in rat retinas. The blots of human retinal RNA were hybridized to a ^{32}P-labeled 225-bp human VEGF cDNA obtained by polymerase chain reaction (PCR) as described below, which recognizes all VEGF transcripts,[21] and to a ^{32}P-labeled 457-bp human β-actin cDNA.[18] The blots of rat retinal RNA were hybridized to the human VEGF cDNA and to a chicken β-actin cDNA (Oncor, Gaithersburg, MD). Blots were exposed to a PhosphorImager screen and analyzed with a computing densitometer (Molecular Dynamics, Sunnyvale, CA); data are expressed as VEGF/actin mRNA ratios. After quantitation, blots were exposed to Kodak AR film, and the autoradiographs were used for photography.

In Situ *Hybridization*

In situ hybridization was performed in retinas of nondiabetic and diabetic donors to identify sites of VEGF synthesis and possible focal changes in the diabetic retinas. Radial retinal sections prepared from formalin-fixed and cryopreserved human eyes were hybridized as previously described to an antisense single-stranded ^{35}S-

labeled RNA probe specific for VEGF and its sense control.[22] The antisense VEGF probe hybridizes specifically with a region of VEGF mRNA common to all known VEGF splicing variants.[22] Because the silver grain distribution was quite uniform along the length of each individual section in both the diabetic and nondiabetic retinas, the signal was quantitated by counting the grains in four randomly selected microscopic fields in each retina. Each field was inclusive of all retinal layers. The counts were performed by an observer masked to the identity of the donors, with the aid of an image analysis system (Biological Detection Systems, Pittsburgh, PA). The results are expressed as number of grains per unit tissue area. To further ensure that such results reflected the integrated grain density throughout the length of the sections, two masked observers scored grain density on a semiquantitative scale of 0 to 4, with zero attributed to the sense control and 4 to the slide with the most abundant grains.

Reverse Transcriptase (RT)-PCR

RT-PCR was used to investigate the splicing pattern of VEGF mRNA in human and rat retina. The VEGF primers[21] used in both the human and the rat studies are derived from external exons, shared by all differently spliced VEGF mRNA species.[23] RT was performed as described.[18] Reaction mixtures containing 0.5, 1, 2, and 4 μl of each RT reaction in 50 μl of 10 mmol/L Tris/HCl, pH 8.3, 50 mmol/L KCl, 2 mmol/L $MgCl_2$, 0.2 mmol/L each dNTP, and 0.5 μmol/L each VEGF primer were covered with mineral oil, heated at 95°C for 2 minutes, and cooled at 72°C before addition of 0.125 U of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN). PCR amplification was carried out in an OmniGene DNA thermal cycler (Hybaid, Teddington, UK) at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes for 14 cycles, with a 5-minute extension at 72°C at the end of the reaction. Ten microliters of each PCR reaction was electrophoresed on a 1% agarose gel, and Southern blots were hybridized to the 225-bp VEGF cDNA. Quantification of signals was obtained with the PhosphorImager computing densitometer system.

Immunodetection of VEGF Protein

Peroxidase immunohistochemistry and immunofluorescence were performed on human retinal sections consecutive to those studied by *in situ* hybridization to compare the topography of VEGF protein and mRNA and to compare VEGF immunoreactivity in diabetic and nondiabetic retinas. Peroxidase immunohistochemistry was also used to test the expression of VEGF in normal rat retina. Human retinal sections were rehydrated in PBS, blocked with 2% bovine serum albumin in PBS, and incubated overnight at 4°C in a moist chamber with an affinity-purified polyclonal rabbit VEGF antibody directed against the amino-terminal portion of human VEGF (A-20; Santa Cruz Biotechnology, Santa Cruz, CA), which reacts with all three VEGF isoforms known to be synthesized in the human retina. The antibody was diluted 1:6000 in PBS containing 2% bovine serum albumin and 0.3% Triton X-100. After three PBS washes, the sections were incubated for 30 minutes at room temperature with a biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), and endogenous peroxidase was inactivated by 30 minutes of incubation in 3% H_2O_2 in glass-distilled water. Sections were then treated with the avidin-biotin-peroxidase complex, and the peroxidase reaction product was developed with diaminobenzidine for 4 minutes. The slides were washed in water for 5 minutes and mounted with an aqueous mounting medium. No counterstaining was performed. In additional studies, the human retinal sections were also processed for immunofluorescence using the primary antibody diluted 1:3000 and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) diluted 1:50. The staining pattern observed with the Santa Cruz antibodies was confirmed using chicken anti-human VEGF antibodies previously described[24] and used at a dilution of 1:1000. Negative controls were obtained by substituting the primary antibody with an equivalent concentration of normal immunoglobulins of the appropriate species or by incubating sections with the primary antibody preabsorbed with a 10-fold excess of the peptide antigen. The VEGF-specific staining was scored on a scale of 0 to 4 by two independent observers masked to the identity of the subjects. A score of 0 was assigned to the negative control and 4 to the slide showing the greatest prevalence and intensity of specific staining.

The rat retinal sections were tested with the A-20 antibody diluted 1:4000, the chicken antibody diluted 1:750, and, additionally, a rabbit polyclonal antibody directed against 147 amino acids of the VEGF of human origin (147, Santa Cruz) diluted 1:250.

Statistical Analysis

The data are summarized with the mean ± 1 SD. Statistical analysis was performed with the unpaired *t*-test.

Results

Northern analysis detected in the human retina a 4.2-kb VEGF mRNA species (Figure 1). The size corresponds to the larger of the two VEGF mRNAs reported in mouse retina[25] and to one of the three mRNA species reported in fetal human vascular smooth muscle cells.[23] As noted in previous studies,[18,26] the β- actin signal was similar in the retinas of diabetic (85,270 ± 45,038 arbitrary densitometric units) and nondiabetic (97, 154 ± 52,651) subjects, and it was thus used as internal standard. In individual subjects the VEGF/actin mRNA ratio was highly reproducible ($r = 0.98$ for ratios determined for 12 samples in two different Northern blots), and was not affected by age, sex, or time elapsed from death to RNA isolation. There was a trend toward lower VEGF/actin ratios with increasing duration of diabetes ($r = -0.5$), but the inverse correlation was not statistically significant in the limited number of individuals studied. The VEGF/actin

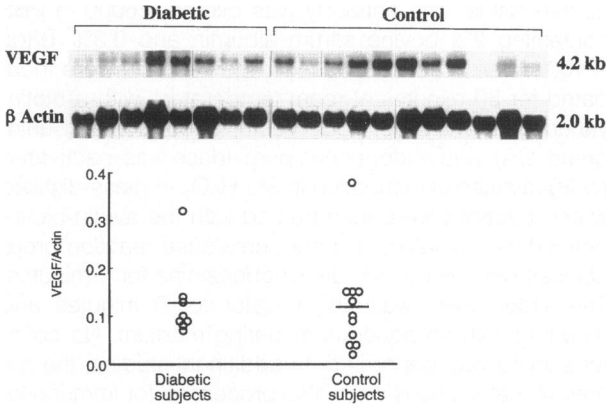

Figure 1. VEGF and β-actin mRNA levels in the retina of diabetic and nondiabetic donors. The Northern blots (**upper panel**, 8 μg of total RNA per lane) were hybridized and quantitated by phosphorimager as described in Materials and Methods. The 28 S ribosomal RNA migrated 4 mm above the detected VEGF transcript. The actin signal was similar in diabetic and control subjects, and it was used to compute for each subject a VEGF/actin ratio (**lower panel**). The elevated ratios pertain to two donors with a diagnosis of cardiomyopathy; the horizontal lines represent the mean ratio for the diabetic and control groups.

ratio was similar in the diabetic (0.11 ± 0.08) and nondiabetic (0.12 ± 0.10) group (Figure 1). One subject in each group showed a very high ratio (Figure 1); for both individuals, a 63-year-old diabetic female and a 62-year-old nondiabetic male, the listed cause of death was cardiomyopathy, a diagnosis not reported for any of the other donors.

The stability of the VEGF transcript in the postmortem period was verified in experiments with rat retinas. The VEGF/actin ratio was 1.0 irrespective of whether the rat retinas were processed for RNA isolation immediately after sacrifice or after the ~30-hour delay inherent in the processing of human eyes (Figure 2).

The primary human VEGF transcript is known to undergo alternative splicing and generate up to four species of VEGF mRNA encoding polypeptides of 206, 189, 165, and 121 amino acids, respectively.[1,23] $VEGF_{206}$ has been found only in the human fetal liver,[1] whereas most tissues express the other three forms in varying ratios.

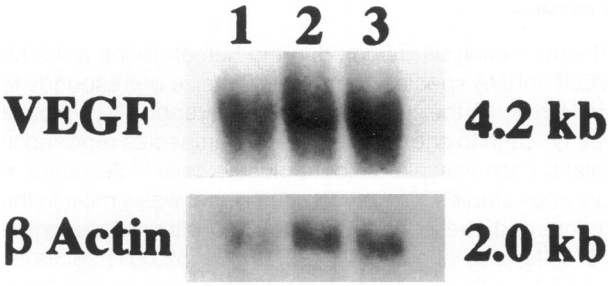

Figure 2. Northern blot of rat retinal VEGF and β-actin mRNAs. Retinas were isolated and processed immediately after sacrifice (**lane 1**), isolated from eyes enucleated immediately after sacrifice and kept at 4°C for 30 hours (**lane 2**), and isolated from eyes enucleated 5 hours after sacrifice and kept at 4°C for 25 hours to match the timing of events reported in Table 1 for the human specimens (**lane 3**). The two retinas isolated from each rat were assigned to different protocols to minimize the effect of inter-individual variations. Twelve micrograms of total RNA was electrophoresed in each lane, and the Northern blot was hybridized and quantitated by phosphorimager as described in Materials and Methods. The 28 S ribosomal RNA migrated 5 mm above the VEGF transcript.

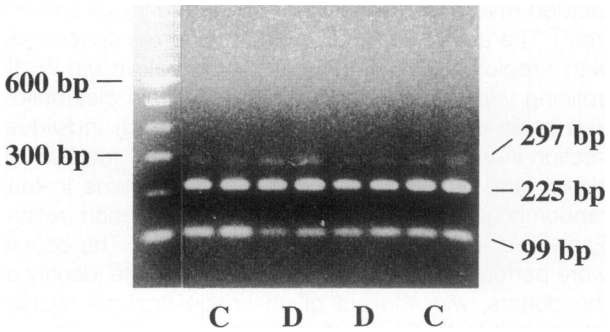

Figure 3. VEGF mRNA species in human retina. RT-PCR was performed as described in Materials and Methods; for the purpose of enhancing visualization of all products, the reverse-transcribed cDNAs were subjected to 30 cycles of amplification. Twenty microliters of the PCR reaction was loaded in each lane and resolved on a 1% agarose gel stained with ethidium bromide. The lane at the extreme left carries molecular weight markers. For each control (C) and diabetic (D) sample, 2 and 4 μl of the RT reaction were amplified.

Because the isoforms have different properties and may subserve different functions,[1] and were not discretely identified by Northern analysis, RT-PCR was used to investigate the pattern of VEGF splicing in the human retina and whether it is affected by diabetes. Fragments of 297, 225, and 99 bp, corresponding to the mRNAs encoding $VEGF_{189}$, $VEGF_{165}$, and $VEGF_{121}$, respectively, were detected in both the nondiabetic and diabetic retinas (Figure 3). The relative expression of the three VEGF mRNAs, quantitated by Southern analysis of PCR products generated in the exponential phase of the PCR reaction, was similar in diabetic and control subjects with the 225-bp species being the most abundant in both groups. Also in the rat retina RT-PCR showed a VEGF band of 225 bp as the major product, indicating that the mRNA encoding $VEGF_{165}$ ($VEGF_{164}$ in the rat retina[27]) is the predominant species constitutively expressed in the retina.

In situ hybridization of human retinas (Figure 4) showed expression of VEGF mRNA mostly in the cells of the inner nuclear and ganglion cell layers and in the region of the outer plexiform layer, whereas the frequency of grains overlaying the outer nuclear layer was not obviously different from that observed in the sense controls (Figure 4, A and A′). In both nondiabetic and diabetic retinas there was some degree of clustering in the grain distribution, but the pattern was consistent along the length of each section. The abundance of VEGF mRNA was highly variable among individuals (Figure 4, B and C *versus* D and E), and the prevalence of retinas with intense labeling was similar in the nondiabetic and diabetic group. Of note, VEGF expression was never prominent around large blood vessels, even in the diabetic specimens (Figure 4, B and C). The quantitation of grains/unit area yielded a value of 31 ± 16 for the nondiabetic group and 37 ± 17 for the diabetic group ($P = 0.4$). The semiquantitative scores assigned by masked observers were 2.3 ± 0.9 and 2.9 ± 1.0, respectively, and showed in individual specimens excellent correlation with the grain counts ($r = 0.81$, $P < 0.001$). Neither the grain counts nor the scores correlated with age, time to enucleation or fixation, length of fixation, or diabetes duration.

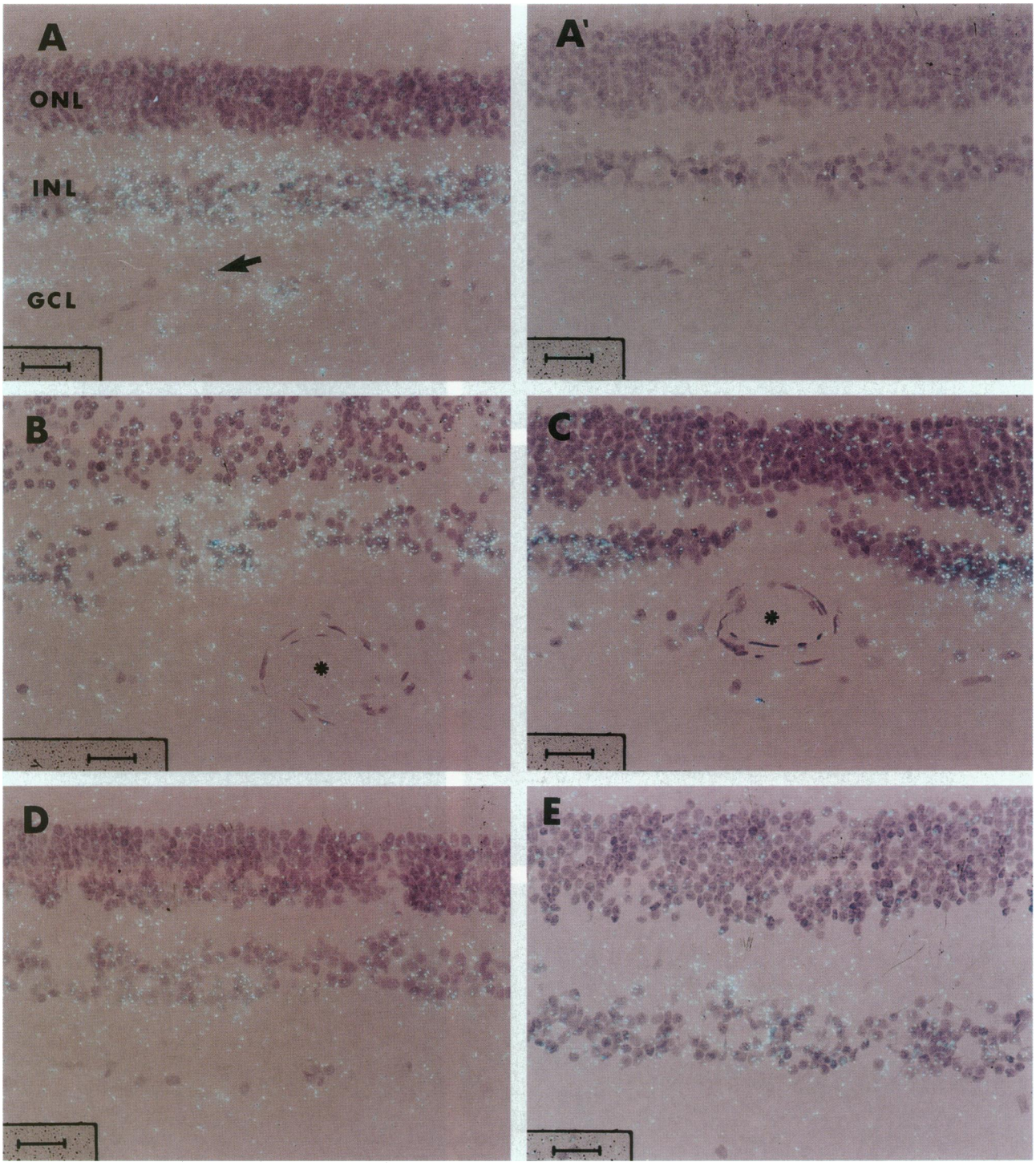

Figure 4. Localization of VEGF mRNA by *in situ* hybridization in nondiabetic and diabetic retinas. In retina sections hybridized with the antisense probe (**A**) intense labeling is present over the inner nuclear layer (INL) and the ganglion cell layer (GCL) but not over the outer nuclear layer (ONL). Hybridization of the same retina with the control sense probe (**A'**) shows minimal nonspecific labeling. The signal is abundant in some retinas (**B** and **C**, showing, respectively, the retina of a 72-year-old nondiabetic female and the retina of a 66-year-old male with a 15-year history of diabetes) and less prominent in others (**D** and **E**, showing, respectively, the retina of a 56-year-old nondiabetic male and the retina of a 57-year-old male with a 12-year history of diabetes). The **arrow** points to ganglion cells; **asterisks** indicate large vessels. The photographs were taken under combined polarized fluorescence and bright-field microscopy. Bars, 25 μm.

There was a correlation in individual specimens between the silver grain counts and the intensity of immunohistochemical staining for VEGF protein (r = 0.5, P = 0.04), but the protein distribution was not identical to that of the mRNA. Specific VEGF staining (Figure 5) was present in the vascular walls, in ganglion cells and their dendritic processes and fibers, in cells of the inner nuclear layer (especially the distal portion), in the synaptic terminals of the photoreceptors (cone pedicles and rod spherules) in the outer plexiform layer, and in compo-

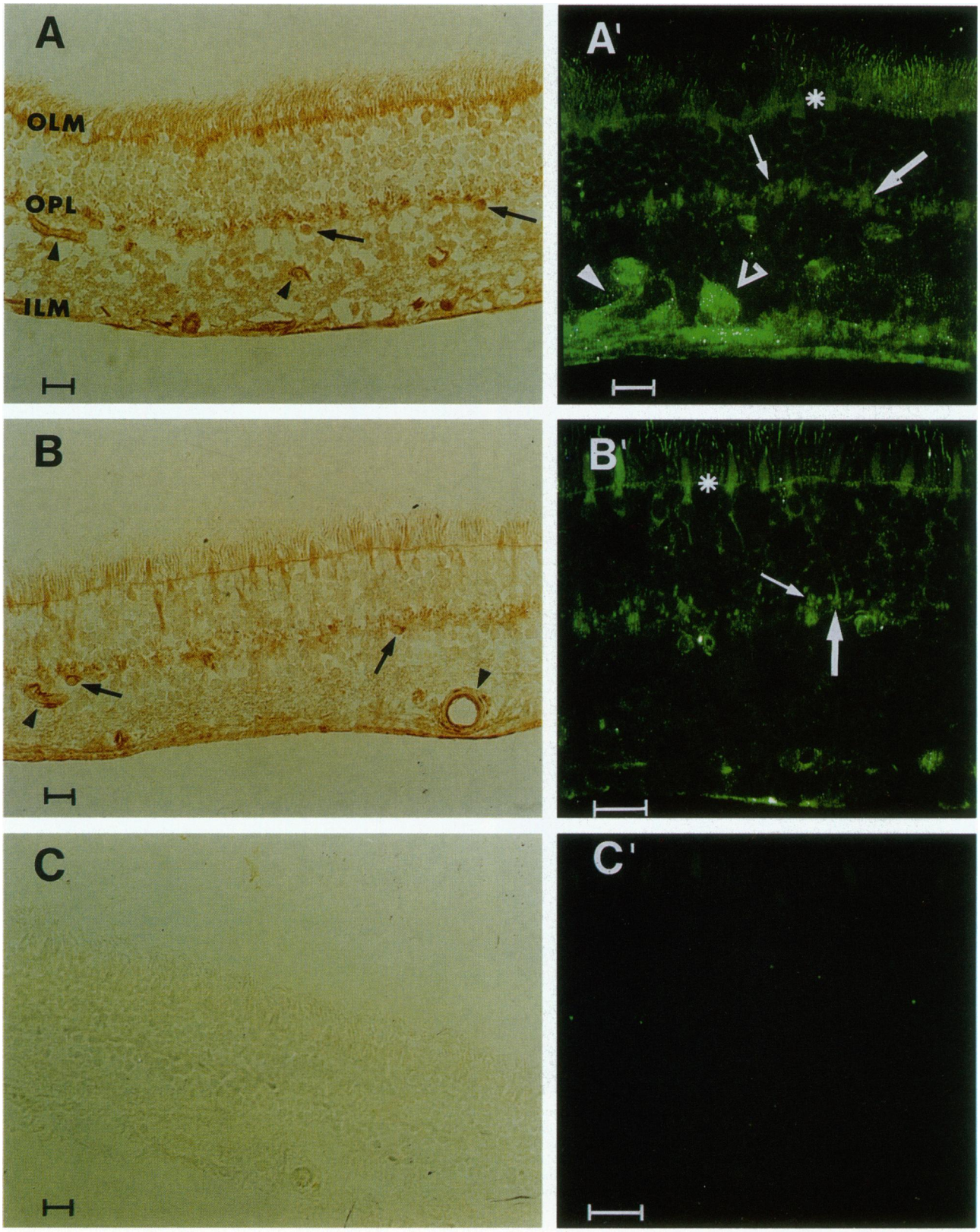

Figure 5. Localization of VEGF protein by peroxidase immunohistochemistry (**left panels**) and immunofluorescence (**right panels**) in nondiabetic and diabetic retinas. In the retina of a 72-year-old nondiabetic female (**A**) and of a 74-year-old female with a 10-year history of diabetes (**B**), intense immunostaining is present along the outer limiting membrane (OLM), the outer plexiform layer (OPL), cells in the outer portion of the inner nuclear layer (**small arrows**), the vascular walls (**arrowheads**), and the internal limiting membrane (ILM). Immunofluorescence of the same retinas viewed in confocal microscopy (**A′** and **B′**) highlights the immunoreaction in rod and cone photoreceptors (**asterisks**), their synaptic terminals in the OPL (rod spherules, **small arrows**; cone pedicles, **large arrows**) and in ganglion cells (**open arrowhead**) and their fibers (**arrowhead**). The negative control shown for peroxidase immunohistochemistry (**C**) was obtained by preabsorbing the primary antibody with a 10-fold excess of the peptide antigen; in the negative control for immunofluorescence (**C′**), the primary antibody was substituted with nonimmune rabbit immunoglobulin. Bars, 25 μm.

nents of the photoreceptors distal to the outer limiting membrane. The distribution of VEGF immunoreactivity was similar in control and diabetic retinas, as were the semiquantitative scores for staining intensity (1.6 ± 0.7 and 2.1 ± 0.5, respectively; $P = 0.1$). Of note, we observed an inverse correlation ($r = 0.64$, $P = 0.01$) between duration of fixation and intensity of VEGF immunoreaction. This did not affect our results because the length of fixation for the diabetic samples (54 ± 37 hours) was similar or slightly shorter than that for the nondiabetic sample (65 ± 43 hours).

To obtain observations in retinas that had not borne agonal events or postmortem processes, the presence and localization of the VEGF protein were also examined in rat retinas. The pattern of staining observed with three different antibodies (Figure 6) was internally consistent and similar to that noted in the human retina. The only qualitative difference among the three antibodies was that the staining of the photoreceptors with antibody 147 extended to the outer segment, and the only qualitative difference between the staining pattern in rat and human retina was the intense staining in the rat retina of cells in the proximal portion of the inner nuclear layer (probably amacrine cells).

Discussion

This study demonstrates that in the normal human retina several types of cells constitutively synthesize VEGF and that the localization of the protein is compatible with both autocrine and paracrine actions of VEGF, which may include the modulation of both neuronal and non-neuronal cell types in the retina. The study also documents that background diabetic retinopathy is not accompanied by changes in the steady-state levels, splicing pattern, or sites of synthesis of the retinal VEGF transcript or by changes in the distribution and abundance of the VEGF protein.

The interpretation of data obtained in postmortem human tissue requires analysis of possible confounding factors and integration with evidence obtained in other species. VEGF is responsive to ischemia/hypoxia,[3,28] and its presence in postmortem retina may reflect up-regulation during the period preceding or following death rather than constitutive expression. However, constitutive expression of VEGF has been noted in various adult tissues,[1,29] including the retina, with a distribution that suggests functions other than induction of angiogenesis and modulation of vascular permeability. For example, VEGF is detectable by Northern analysis in the rat brain and is especially abundant in the cerebellar cortex,[29] which is the area with the greatest density of neurons in the brain. With regard to the retina, the adult mouse,[4] rabbit,[30] and rat (this study) retinas express VEGF, and the transcript is detectable by Northern analysis, indicating that it is present in abundance at steady state. Moreover, the VEGF receptor Flk-1 is constitutively expressed in both the adult mouse[31] and rat[32] retinas, and the expression appears not to be restricted to retinal endothelial cells. The extent of retinal VEGF up-regulation is small after 24 hours of severe ischemia in the living animal,[33] and such a response may be expected to be curtailed in the postmortem period by complete lack of perfusion. Indeed, we demonstrated that the levels of VEGF mRNA measured in the rat retina 30 hours postmortem were similar to those measured immediately after sacrifice. These observations support the concept that the VEGF mRNA and protein present in the human retina reflect constitutive expression.

The combined information obtained in this study on spatial localization of VEGF mRNA and protein on the one hand, and isoforms synthesized on the other, provides some insight into the biology of retinal VEGF at steady state. The four mature homodimeric proteins that result from alternative splicing of the VEGF mRNA bind to heparin with affinity that increases in parallel with their length.[14] As VEGF message was rare around the retinal vessels, some of the immunoreactivity observed in the vascular walls is likely to reflect secretion by neighboring cells and accumulation in the basement membranes of $VEGF_{165}$, the only isoform that is both diffusible and capable of binding to heparin-containing proteoglycans of the matrix.[14] The pattern of intense vascular staining for VEGF protein in the face of absent or minimal labeling for VEGF mRNA has been reported in other tissues[34] and identifies vessels as targets of paracrine VEGF. VEGF is probably not synthesized by the photoreceptors as no mRNA signal was detected over the outer nuclear layer or the external portion of the outer plexiform layer containing most of the photoreceptors cytoplasm. Hence the reproducible staining of the photoreceptors inner segment and synaptic terminal regions suggests that photoreceptors may bind or accumulate diffusible VEGF isoforms ($VEGF_{165}$ and/or $VEGF_{121}$) synthesized and released by several cell types in the inner nuclear layer. The presence of VEGF in photoreceptors and ganglion cell fibers and the synthesis of VEGF in ganglion cells and cells of the inner nuclear layer suggest that VEGF may have in the retina actions relevant to modulation of neuronal function or homeostasis.

The diabetic retinas examined for VEGF expression and immunostaining *in situ* were obtained from donors with documented histological lesions of retinopathy and accelerated death of vascular endothelial cells and pericytes.[19] The abundance and pattern of distribution of VEGF mRNA in these specimens were not different from those recorded in nondiabetic subjects, confirming the results obtained by Northern analysis in a different group of diabetic patients and respective controls. VEGF mRNA levels are expected to be informative of both synthesis and secretion of the cytokine. VEGF synthesis appears to be regulated pretranslationally,[3,21,25] and because there is no intracellular storage form of VEGF, secretion must be through the constitutive pathway, which is linked to synthesis directly. We also did not find changes in the level and distribution of the VEGF protein in diabetic retinas. This is at variance with the observations by Lutty[11] and Amin[12] who reported increased VEGF immunoreactivity in the vessels and Müller cell processes of diabetic retinas. A puzzling aspect of the above studies is the minimal or absent VEGF staining reported in the

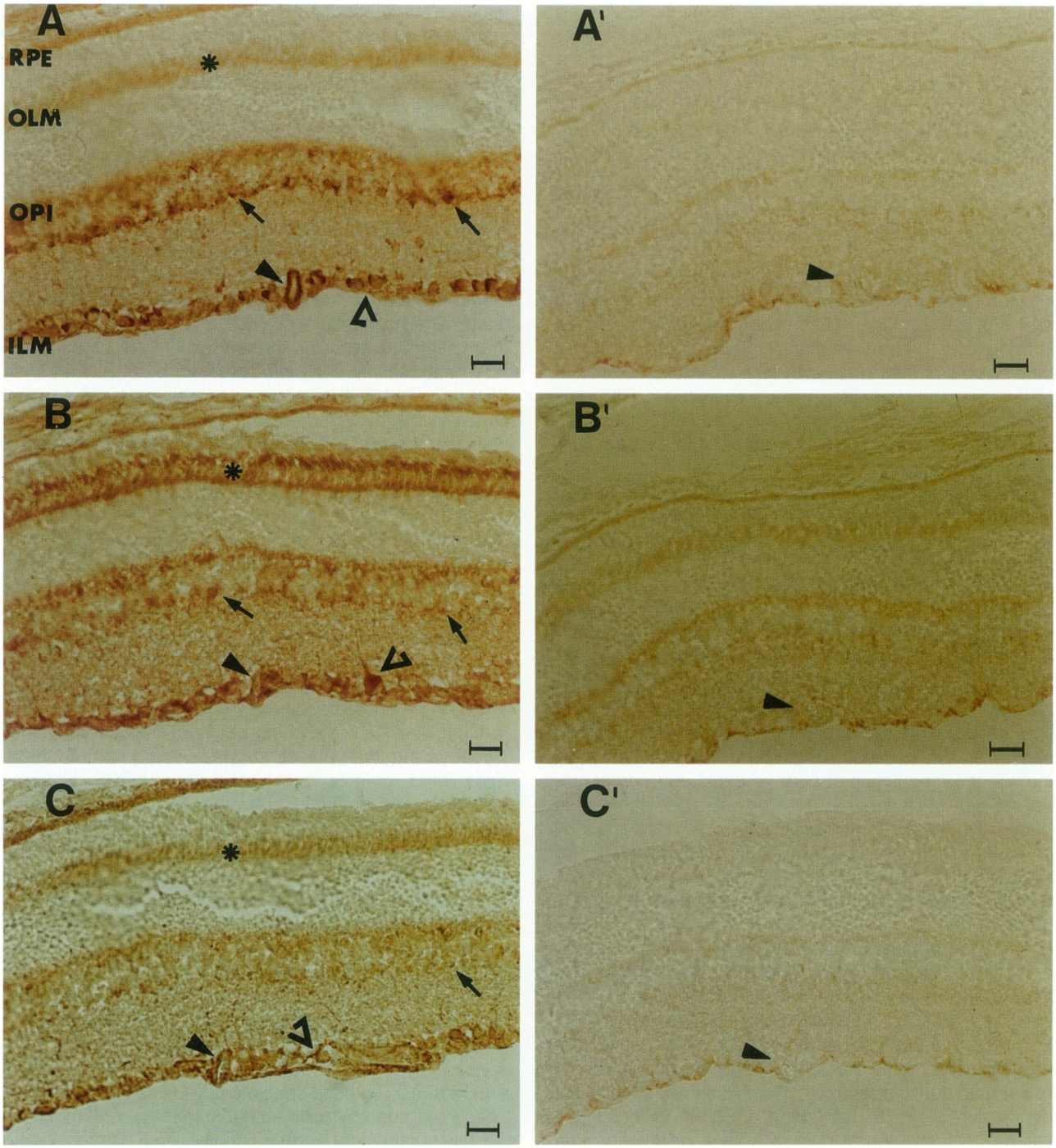

Figure 6. Localization of VEGF protein by peroxidase immunohistochemistry in rat retina using as primary antibody Santa Cruz A-20 (**A**), Santa Cruz 147 (**B**), and the chicken antibody (**C**) described in Materials and Methods. The respective negative controls (**A′**, **B′**, and **C′**) were obtained by substituting the primary antibody with nonimmune immunoglobulin of the appropriate species. On the consecutive sections, the **arrowheads** point to a landmark blood vessel, immunostained for VEGF; the **open arrowheads** point to immunostained ganglion cells (a dendritic process is clearly evident in **B**); the **arrows** point to positive cells in the inner nuclear layer; and the **asterisks** indicate immunostained photoreceptors. In addition to the retina, the sections show the retinal pigmented epithelium (RPE), which stains for VEGF. Bars, 25 μm.

retinas of nondiabetic donors whose age was similar to that of our study population. Albeit of variable intensity, we detected VEGF immunoreactivity in all retinas studied, irrespective of the primary antibody used and of whether the detection method was peroxidase histochemistry or fluorescence, and in individual retinas we found the signal for VEGF protein to be correlated with the signal for VEGF mRNA. VEGF staining of multiple types of neurons and their processes in the postmortem human retina has been reported in abstract form by other investigators.[35] A possibly important difference between our study and the studies by Lutty and Amin is that in the latter the eyes were frozen or fixed in the laboratories an average of 30 hours postmortem,[11,12] whereas the eyes used in our

study were fixed by the eye banks before shipment, 12 to 15 hours after death. The suboptimal preservation of the retinal tissue acknowledged by Amin[12] may have contributed to both the inconsistency of staining intensity among retinas and of staining pattern with different detection methods, and in fact, the authors[12] indicate that their results are not expressed on a quantitative or semiquantitative basis. In the study by Lutty, an exaggeration of the differences between nondiabetic and diabetic subjects may have been caused by the severe stages of retinopathy examined, occurring in patients with 10, 26, and 36 years of insulin-dependent diabetes, and defined as preproliferative.[11]

The observation that VEGF expression is not altered in background diabetic retinopathy is not in contrast with data indicating an effect of high glucose levels on VEGF synthesis or action. High glucose has been reported to increase VEGF synthesis *in vitro* by retinal pigmented epithelial cells[36] and aortic smooth muscle cells[37] with very different kinetics and to induce in the rat increased permeability of skin granulation tissue preventable by neutralizing VEGF antibodies.[38] None of these models incorporates the cell types (mostly neural) that synthesize VEGF in the retina, and regulation *in vivo* may be vastly more complex and controlled than *in vitro*. It appears from our study that the development of background retinopathy is not accompanied by a degree of ischemia/hypoxia that would substantially increase VEGF expression. We cannot, however, exclude that VEGF functional activity may be exaggerated or otherwise altered in the diabetic retina as a result of changes in VEGF receptors or post-receptor-signaling pathways. Other types of studies are needed to examine this possibility and to define the role(s) that constitutively expressed VEGF may have in retinal homeostasis. The latter studies will also provide perspective for the rational and safe use of strategies that interfere with VEGF synthesis or action with the goal of arresting retinal neovascularization.

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