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Upregulation of vascular endothelial growth factor in ischemic and non-ischemic human and experimental retinal disease

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Summary. Vascular endothelial growth factor (VEGF) is induced by hypoxia and it has been implicated in the development of iris and retinal neovascularization (NV) in ischemic retinopathies in which it has been suggested that Müller cells are responsible for increased VEGF production. VEGF, however, is also known to be a potent mediator of vascular permeability in other tissues and may perform this function in retina. Immunohistochemical staining for VEGF was performed on a variety of human and experimental ischemic and non-ischemic ocular disorders in which blood retinal barrier (BRB) breakdown is known to occur to determine if there is an upregulation of VEGF in these conditions. We found increased VEGF immunoreactivity in ganglion cells of rats with oxygen-induced ischemic retinopathy and in ganglion cells, the inner plexiform layer, and some cells in the inner nuclear layer of rats with experimental autoimmune uveoretinitis (EAU), in which there was no identifiable ischemia or NV. In rats with EAU, VEGF staining intensity increased from 8 to 11 days after immunization, coincident with BRB failure. These results were confirmed using two distinct anti-VEGF antibodies and by immunoblot and the immunohistochemical staining was eliminated by pre-incubating the antibodies with VEGF peptide.

VEGF staining was also increased in the retina and iris of patients with ischemic retinopathies, such as diabetic retinopathy and retinal vascular occlusive disease, and in patients with disorders in which retinal ischemia does not play a major role, such as aphakic/pseudophakic cystoid macular edema, retinoblastoma, ocular inflammatory disease or infection, and choroidal melanoma. VEGF was primarily localized within retinal neurons and retinal pigmented epithelial cells in these cases. In addition or in association with its role of inducing NV, VEGF may contribute to BRB breakdown in a variety of ocular disorders and blockage of VEGF signaling may help to reduce some types of macular edema.

Key words: Blood-retinal barrier, Ischemia, Vascular endothelial growth factor, Experimental autoimmune uveoretinitis, Macular edema

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Introduction

VEGF was first discovered because of its ability to increase the permeability of microvessels, primarily postcapillary venules and small veins, to circulating macromolecules and was originally named vascular permeability factor because of this property (Senger et al., 1983, 1986). It is one of the most potent vascular permeability agents known, acting at concentrations below 1 nM, which is 50,000 times more potent than histamine (Senger et al., 1990), and in some vascular beds, such as within cremaster muscle or skin, it acts within 10 minutes (Roberts and Palade, 1995). VEGF is induced under hypoxic conditions (Goldberg and Schneider, 1994; Hashimoto et al., 1994; Minchenko et al., 1994a,b; Pierce et al., 1995) and can stimulate vascular endothelial cell growth and angiogenesis associated with neoplasia (Connolly et al., 1989a,b; Leung et al., 1989; Kondo et al., 1993; Senger et al., 1993) and ischemia (Adamis et al., 1993; Aiello et al., 1994; Miller et al., 1994; Pierce et al., 1995; Youssri et al., 1995). Several recent studies have explored the possible role of VEGF in ocular NV (Adamis et al., 1994; Aiello et al., 1994; Miller et al., 1994; Murata et al., 1995; Pierce et al., 1995; Stone et al., 1995), but little attention has been given to its potential effect on retinal vascular permeability.

Recently, we have demonstrated increased immunohistochemical staining for VEGF in eyes with ocular melanoma (Vinores et al., 1995b). While staining for VEGF was sometimes found within tumor cells, it was

also found in retinal neurons, retinal pigmented epithelium (RPE), iris, and ciliary body. It was not associated with identifiable NV, but often occurred in areas of BRB breakdown as demonstrated by extravasated albumin. To our knowledge, this was the first demonstration of increased VEGF staining within retinal neurons and RPE in situ, and it raises two important questions: 1) Is the VEGF localized within retinal neurons and RPE the result of induction by tumor cells or does it also occur in other ocular disorders; 2) In addition to its role in ocular NV, does VEGF contribute to BRB breakdown? In the present study, we have sought to address these questions by assessing immunolocalization of VEGF in a variety of clinical and experimental disorders.

Materials and methods

Oxygen-induced ischemic retinopathy

Ischemic retinopathy was induced in rats by the method of Penn et al. (1993). Newborn Sprague-Dawley rats were immediately placed in a chamber and exposed to a high oxygen atmosphere alternating between 40% and 80% every 12 hrs for 14 days. The rats were then removed from the chamber and exposed to room air for 5 days. Control animals were maintained in room air for the entire 19 days. Some animals were anesthetized with a mixture of 5 mg/kg xylazine hydrochloride and 25 mg/kg ketamine hydrochloride. They were then perfused with saline followed by India ink and the retinas were whole-mounted to observe NV. Other animals were used for immunohistochemistry by fixing the eyes in 2% paraformaldehyde/5% sucrose in 0.1M phosphate buffer, pH 7.4, for 1 hr. The sucrose concentration was increased in a stepwise fashion to 20% over 2 hrs. The tissue was incubated overnight at 4 °C in 20% sucrose and frozen in a 2:1 ratio of 20% sucrose in phosphate buffer: O.C.T. compound (Miles, Elkhart, IN) using dry ice and isopentane (Barthel and Raymond, 1990). Frozen sections were cut, treated for 10 min at -20 °C with methanol containing 0.75% H_2O_2, washed with Tris-buffered saline (TBS), and used for immunohistochemistry.

Induction of S-antigen-induced experimental autoimmune uveoretinitis (EAU)

Female Lewis rats from 6-8 weeks of age (175-200 g) were immunized with 30 micrograms of S-antigen mixed 1:1 with Freund's complete adjuvant enriched with *Mycobacterium tuberculosis* strain H37Ra (DIFCO, Detroit, MI) in a final volume of 0.1 ml by injecting one hind footpad to induce EAU. At 8 or 11 days after immunization, 8 eyes at each time point from rats immunized with S-antigen or 7 eyes from normal Lewis rats were fixed in buffered formalin, paraffin-embedded, and sectioned for immunohistochemistry. The retinas were dissected from 4 other eyes from each group, frozen, and used for immunoblots.

Immunoblots

Retinas were washed, frozen, and then lysed in buffer containing 0.0625M Tris, pH 6.8, 12.5% glycerol, 1.25% sodium dodecyl sulfate (SDS), and 1.25% ß-mercaptoethanol. Cultured cells were rinsed 2 times with phosphate-buffered saline, lysed in buffer, and scraped. Cell lysates were sonicated briefly. For immunoblots, the protein concentration of the supernatant was measured and samples containing 100 μg of protein were boiled in Laemmli buffer (Laemmli, 1970) for 3 min. Proteins were resolved on 15% SDS-PAGE gels and electrophoretically transferred to nitrocellulose paper as previously described (Towbin et al., 1979). The nitrocellulose paper was blocked for 1.5 hr at room temperature with 2% nonfat dry milk (Carnation) and incubated overnight at room temperature with a 1:200 dilution of VEGF antibodies in TBS containing 2% milk. The paper was washed 3x10 min with TBS/milk and incubated for 1.5 hr with a 1:200 dilution of affinity purified rabbit anti-goat IgG conjugated to alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD). After washing, the immunoreactive bands were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Kirkegaard and Perry), as previously described (Hackett and Campochiaro, 1993).

Paraffin-embedded tissues

In addition to the eyes from Lewis rats developing EAU, formalin-fixed postmortem and surgically-removed eyes from humans with a variety of ischemic or non-ischemic ocular disorders (n=164) or without any ocular disorders (n=31) were also used for VEGF immunostaining. Accompanying pathology reports were also obtained. Paraffin sections of a rat intracerebral anaplastic glioma, a human glioblastoma in the spinal cord, and two human specimens from areas of cerebral infarction were used for comparison.

Immunohistochemistry

Affinity-purified rabbit polyclonal IgG directed against the 20 amino terminal residues of human VEGF and the control peptide against which they were directed were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). These antibodies block VEGF activity (Sioussat et al., 1993) and specifically react with native and denatured VEGF in immunoblots (Santa Cruz Biotechnology, Inc., 1994). Another anti-VEGF antibody directed against the 16 amino terminal residues of human VEGF was raised in rabbits. It was demonstrated to specifically immunoprecipitate native VEGF.

Fixed-frozen or deparaffinized sections were blocked with 10% normal goat serum (NGS) in 0.05M TBS for 30 min at room temperature to prevent

nonspecific binding of antibodies. One percent NGS in TBS was used for washing and diluting antibodies. Excess serum was removed and, without washing, the tissue was covered with a 1:20-1:40 dilution of VEGF antibodies and incubated overnight at 4 °C in a humidified chamber. After warming to room temperature, the sections were washed. The immunoreaction was developed using HistoMark Red (Kirkegaard and Perry) according to the manufacturer's instructions, which yielded a brilliant red reaction product that was easily distinguished from melanin, allowing positively-stained RPE and ciliary epithelial cells to be recognized. Frozen sections of eyes from rats with ischemic retinopathy and controls were incubated with one of the primary anti-VEGF antibodies as described above. The sections were then warmed to room temperature and incubated for 30 min with a 1:40 dilution of goat anti-rabbit immunoglobulins (Arnel, Brooklyn, NY), washed, and incubated 30 min with a 1:100 dilution of a rabbit peroxidase-anti-peroxidase complex (Arnel). After thorough washing with 0.05M Tris buffer, pH 7.6, immunoreactivity was visualized with freshly made 3,3'-diaminobenzidine-4 HCl in Tris buffer containing 0.0185% H_2O_2. The slides were mounted with Aqua Poly/Mount (Polysciences, Warrington, PA) and photographed on a Zeiss Axioskop (Carl Zeiss, Thorwood, NY) using Nomarski optics and Kodak 64T film. For controls, each anti-VEGF antibody was incubated for 2 hr at room temperature with a tenfold express of VEGF peptide (Santa Cruz Biotechnology) prior to applying it to the sections or non-immune serum was substituted for primary antibodies.

Specimens were evaluated without knowledge of the pathology and were graded according to localization and intensity of stain. VEGF immunostaining in the retina, RPE, ciliary epithelium, and iris were graded as intense (+3), moderate (+2), weak (+1), or negative (0). Statistical significance of staining at each site for the various ocular disorders compared to normal eyes was calculated using the Mann-Whitney U-test.

Color slides were scanned with an AGFA (Mortsel, Belgium) Arcus Plus flat bed scanner. Each image was treated in exactly the same manner without alteration of color and composites were labeled and printed on photographic paper with a Kodak XL 7700 Digital Continuous Tone printer.

Results

Experimental ischemic retinopathy

In the rat model of ischemic retinopathy, areas of nonperfusion (NP) and NV were visualized on retinal whole mounts following perfusion with India ink (Fig. 1), demonstrating its usefulness as a model for ischemic retinopathy. Immunostaining for VEGF with the affinity-purified Santa Cruz antibody shows minimal background staining in control rats, while the rats with ischemic retinopathy showed prominent staining in the ganglion cell layer (Fig. 2A, arrow). Incubation of the primary antibody with VEGF peptide almost completely eliminates the VEGF staining (Fig. 2B, arrow). Figure 2C shows similar staining of ganglion cells using the second VEGF antibody, which is also eliminated by preincubation with the VEGF peptide (Fig. 2D). The essentially identical results with two VEGF antibodies and the blocking with VEGF peptide indicate that the ganglion cell staining is specific for VEGF. We examined retinas from 28 rats with ischemic retinopathy and all showed prominent staining for VEGF in ganglion cells, whereas this was not seen in any of 10 control rats examined.

Experimental autoimmune uveoretinitis

Rats with EAU demonstrate prominent staining for VEGF in the inner retina 8 days after immunization with S-antigen (Fig. 3A). The chromogen, Histomark Red, gives a bright red reaction product localized in ganglion cells, astrocytes, Müller cells, and some cells in the inner nuclear layer in both the posterior and peripheral retina. Preincubation of the antibody with VEGF peptide completely eliminates the staining (Fig. 3B). Eleven days after immunization with S-antigen, the staining for VEGF is even more intense (Fig. 3C), while in a control Lewis rat there is only faint background staining (Fig. 3D).

Retinal homogenates from normal Lewis rats and Lewis rats developing EAU were used under reducing conditions for immunoblots. Using the affinity-purified Santa Cruz anti-VEGF antibody, a 21 kD cross-reacting band was detected in homogenates containing 100 μg of total protein from rats developing EAU 11 days after immunization (Fig. 4, lanes 3 and 4), but only a very weak band was seen in homogenates containing 100 μg of protein from unimmunized rats (Fig. 4, lanes 1 and 2). Fig. 4, lane 5 shows human recombinant VEGF.

Human pathologic specimens

In normal human eyes, VEGF was demonstrated in the retinas of only 13% (Fig. 5A) and the ciliary epithelium of 26%, and was not detected in the iris (Table 1). VEGF immunoreactivity in the retina and iris was significantly increased in a variety of ischemic retinopathies (Table 1, $p=0.0043$) including diabetic retinopathy ($p=0.00034$). The staining was never as intense as that seen in rats with EAU (Figs. 3A,C), but as in EAU and oxygen-induced ischemic retinopathy in rats, VEGF staining in human ischemic retinopathies was often associated with retinal neurons (Fig. 5B) and the RPE (Fig. 5C), rather than with vessels. VEGF was frequently demonstrated in the ganglion cells, the inner and outer plexiform layers, and the nerve fiber layer with occasional positivity in the inner and outer nuclear layers, the RPE, and the photoreceptor inner segments. In occasional cases of diabetic retinopathy or retinal

vascular occlusions, more intense VEGF staining was evident in the ciliary epithelium than in normal eyes, but the overall incidence was not statistically significant. VEGF-positivity was also demonstrated in the iris of 5 of 14 specimens with vascular occlusions and 1 of 26 cases of diabetic retinopathy (Table 1).

VEGF was demonstrated in the retinas of 37% of eyes with ocular inflammatory disease or infection, which was also significantly increased compared to normals ($p=0.0152$). Striking staining was often demonstrated in particular cell populations within the retina in specific cases of uveitis, rubeosis with proliferative vitreoretinopathy, cytomegalovirus retinopathy (Fig. 5D), and endogenous aspergillis endophthalmitis (Fig. 5E). VEGF immunoreactivity was not significantly increased in the ciliary epithelium of eyes with inflammatory disease or infection, but occasional cases of uveitis, Bechet's disease, rubeosis with proliferative vitreoretinopathy, cytomegalovirus retinopathy and Herpesvirus retinopathy showed VEGF positivity in the iris.

A number of other ocular disorders in which there is BRB breakdown and usually no retinal NV also had increased immunoreactivity for VEGF, primarily in retinal neurons. Intraretinal VEGF positivity was significantly increased in aphakic/pseudophakic cystoid macular edema ($p=0.045$, Fig. 5F), retinoblastoma ($p=0.0234$), and choroidal melanoma ($p=0.0084$, Fig. 5G). Several cases of aphakic/pseudophakic cystoid macular edema, keratoplasty, choroidal melanoma, or glaucoma also had conspicuous staining in the ciliary epithelium or the iris (Fig. 5H).

The paraffin sections of the rat intracerebral anaplastic glioma, the human glioblastoma, and the human brain infarcts were comparatively stained much weaker for VEGF than most positively-stained ocular specimens. The rat tumor showed only focal VEGF-positivity in some peripheral regions of the tumor, bordering on normal brain tissue. The human glioblastoma showed VEGF in tumor cells, including giant cells, particularly in areas of necrosis, and in neuronal cell bodies. In one of the cerebral infarcts, VEGF

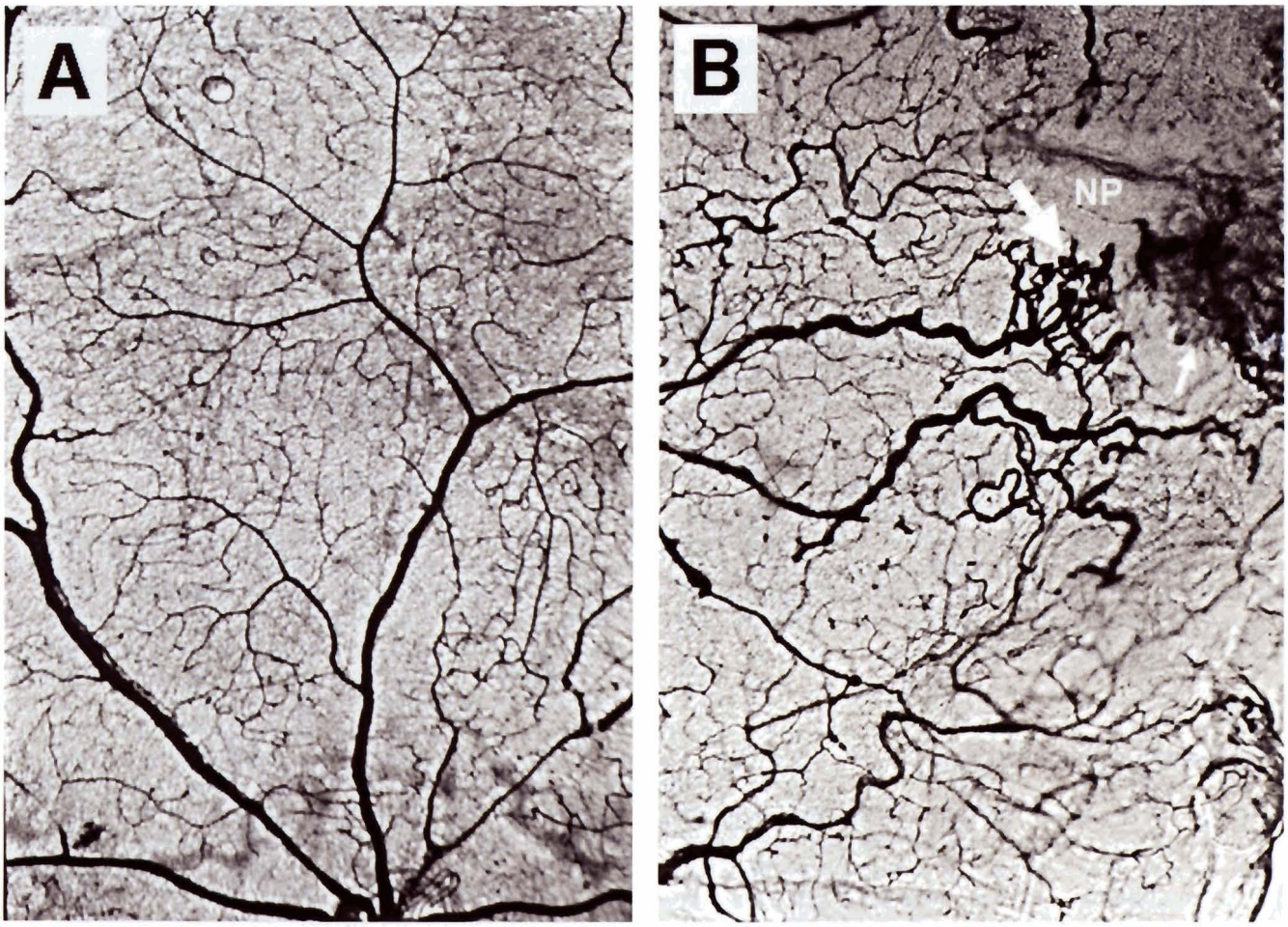

Fig. 1. Whole-mounted, India-ink-perfused retinas from a control rat **(A)** and a rat with oxygen-induced ischemic retinopathy **(B)** demonstrating areas of nonperfusion (NP) and neovascularization (arrows) in the latter.

staining was localized to vessel walls and in the other it was found within neurons in areas of infarction.

Discussion

The present study has confirmed previous reports demonstrating that VEGF immunoreactivity is increased in human and experimental ischemic retinopathies (Adamis et al., 1993, 1994; Aiello et al., 1994; Malecaze et al., 1994; Miller et al., 1994; Dastgheib et al., 1995; Pierce et al., 1995; Shima et al., 1995; Youssri et al., 1995). These data and preliminary studies administering or inhibiting VEGF (Aiello et al., 1995; Smith et al., 1995; Tolentino et al., 1995) support a possible role for VEGF as a mediator of ocular NV in ischemic retinopathies. However, we also found increased VEGF immunoreactivity in the retina in a number of ocular disorders that are not associated with NV and, although metabolic ischemia cannot always be ruled out, these cases do not present overt pathological evidence of ischemia. Since cytokines such as interleukin 1ß (Li et al., 1995) and prostaglandins E_1 and E_2 (Harada et al., 1994) have been demonstrated to increase expression of VEGF, production of cytokines by inflammatory cells (which are present in each of these disorders) or in some cases by tumor cells may be responsible for the increased VEGF seen in these conditions. Increased levels of cytokines have been demonstrated in EAU (Caspi, 1989; Mahalak et al., 1991; DeKozak et al., 1994), which is the condition that shows the most intense VEGF staining, but shows no ischemia or NV at the time points evaluated (McMenamin et al., 1993). Therefore, one important finding of our study is that VEGF immunoreactivity can be increased in the retina and RPE by stimuli other than hypoxia, and elevated cytokine levels provide a reasonable alternative. Another important finding is that high levels of VEGF immunoreactivity can occur in retina without associated NV. The stimulus for increased production of VEGF may be important in this regard. Hypoxia has been demonstrated to increase the expression of VEGF receptors (Plate et al., 1993; Thieme et al., 1995), while this has not been demonstrated for other cytokines. Perhaps simultaneous increased expression of VEGF and its receptors is required for the development of NV.

Unlike NV, breakdown of the BRB occurs in each of

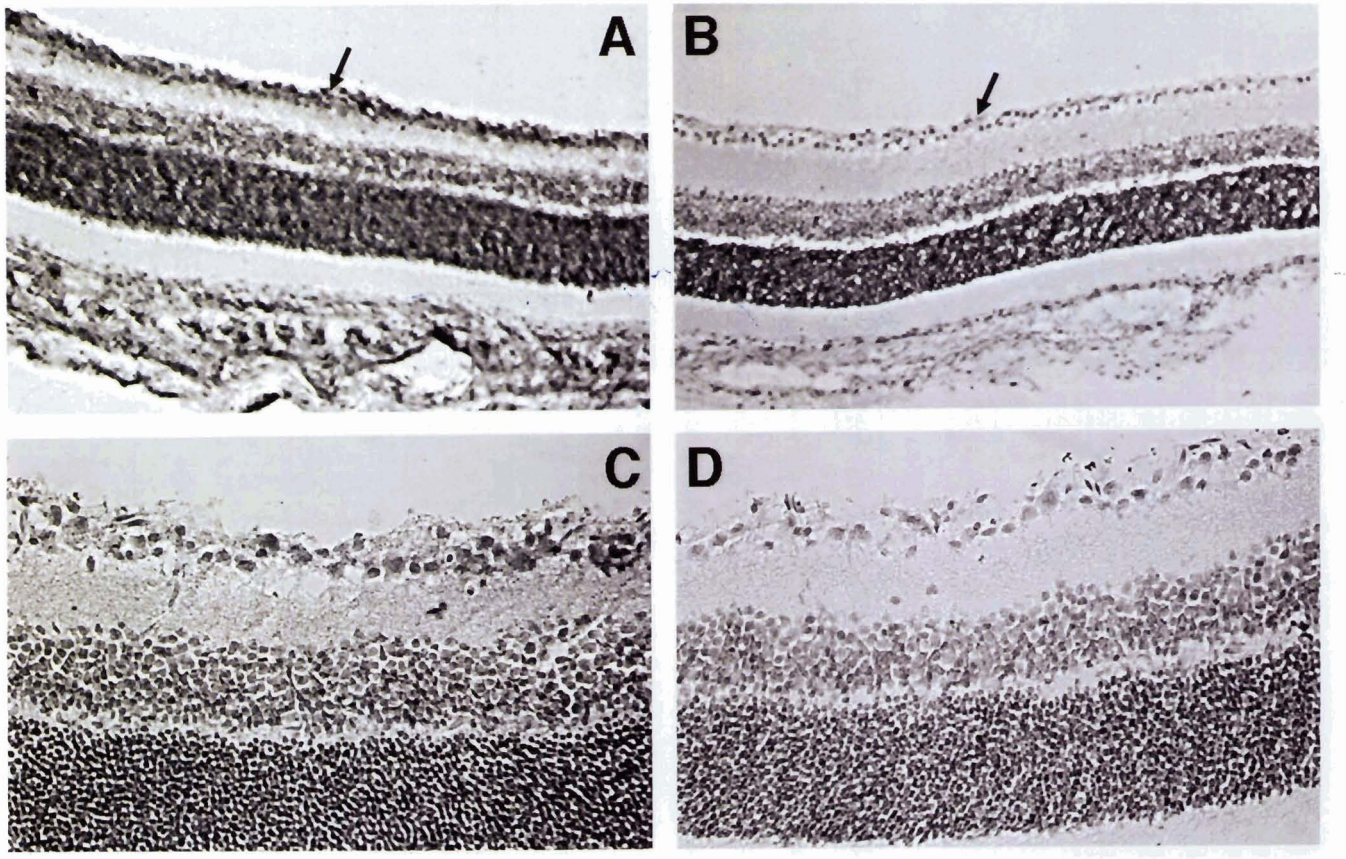

Fig. 2. Oxygen-induced ischemic retinopathy in rats. **A.** Immunohistochemical staining with the affinity-purified anti-VEGF antibody shows prominent staining of the ganglion cells (arrow). x 175. **B.** Incubation of the affinity purified antibody with VEGF peptide eliminated ganglion cell staining (arrow). x 175. **C.** The second VEGF antibody also showed positive staining of ganglion cells which was unaffected by incubation with vehicle. x 350. **D.** Incubation of the second antibody with VEGF peptide also eliminated the immunostaining of ganglion cells. x 350

the conditions in which increased VEGF immunoreactivity was demonstrated in the retina (Vinores et al., 1990a, 1994, 1995a,b; Derevjanik et al., 1995; Vinores, 1995). VEGF increases vascular permeability in other tissues (Senger et al., 1983, 1986, 1990; Connolly et al., 1989b; McClure et al., 1994; Roberts and Palade, 1995) and may also contribute to vascular leakage in the retina. VEGF increases microvascular permeability by enhancing vesicular transport through a recently described organelle, the vesiculo-vacuolar organelle (Qu-Hong et al., 1995). Increased vesicles have been demonstrated in retinal vascular endothelial cells in diabetic rats (Vinores et al., 1990a,b, 1993b), humans with diabetes (Vinores et al., 1993a), and rats with EAU (Derevjanik et al., 1995). Therefore, VEGF may play a role in modulation of the BRB.

Previous studies have suggested that VEGF is primarily localized to endothelial cells (Dvorak et al., 1991; Senger et al., 1993) or Müller cells (Miller et al., 1994; Pierce et al., 1995; Stone et al., 1995). In human and rat central nervous system tumors, VEGF is primarily localized in tumor vessels and in areas of necrosis (Plate et al., 1992, 1993; Weindel et al., 1994); however, it has also been demonstrated in some tumor cells, in neurons of a human spinal cord containing a glioblastoma, and in areas of brain infarction. VEGF mRNA has also been reported in normal brain neurons, particularly the granule cells of the cerebellum (Monacci et al., 1993), and in cultured RPE cells (Adamis et al., 1993; Kuroki et al., 1995; Kvanta, 1995). In the present study, we have demonstrated VEGF immunoreactivity in retinal neurons and RPE in situ. While VEGF is present in neurons only under pathologic conditions, the possibility arises that VEGF may have some as yet unrecognized neuron-related function. An analogous situation involves PDGF, which for many years was thought to act almost exclusively in wound repair (Pierce et al., 1991). However, PDGF is expressed in retinal

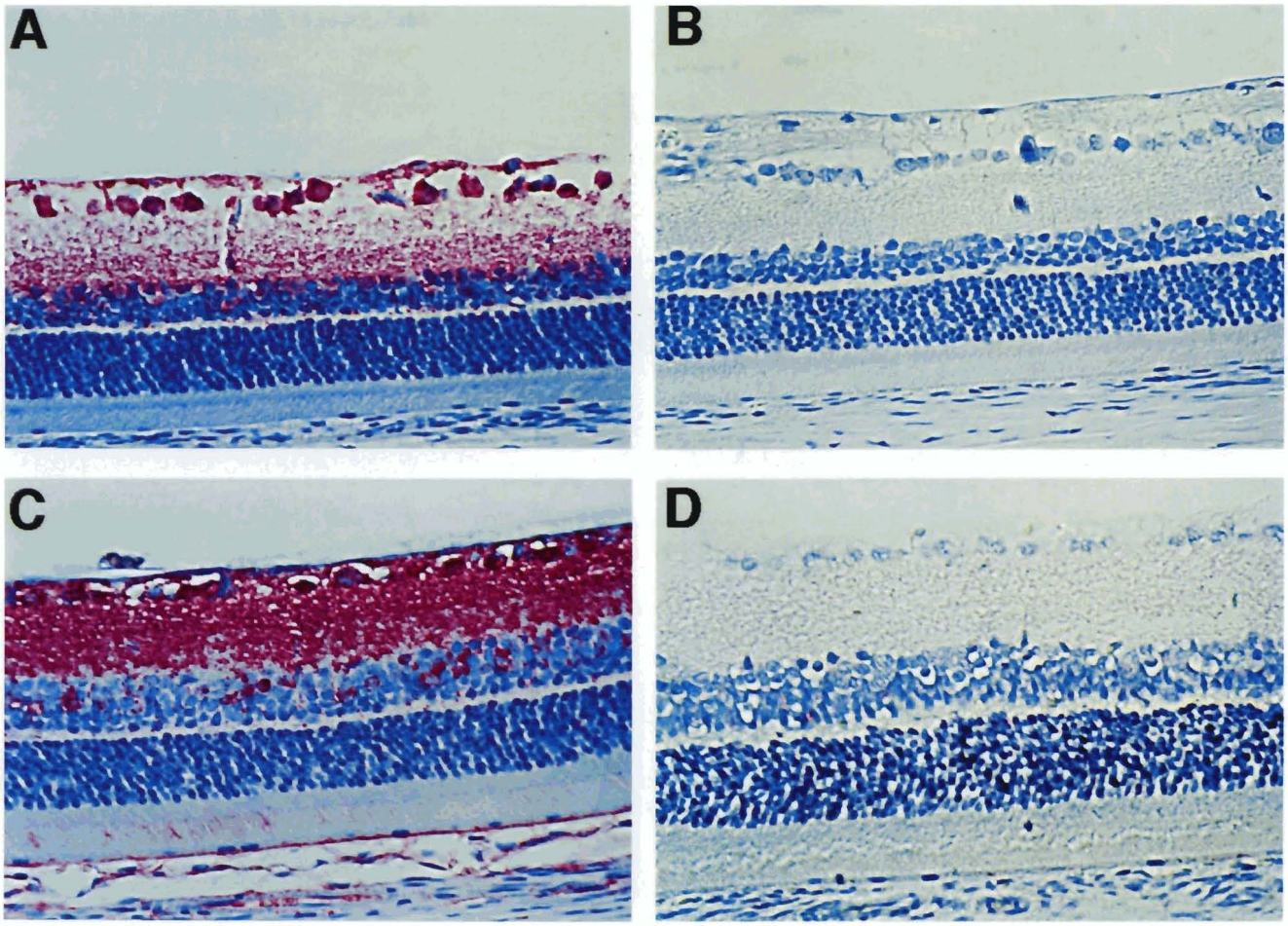

Fig. 3. Immunohistochemical staining for VEGF in rats developing EAU. **A.** Prominent immunohistochemical staining for VEGF (red) is demonstrated in the ganglion cells, nerve fiber layer, inner plexiform layer, and in some cells in the inner nuclear layer of a Lewis rat 8 days after immunization with S-antigen. **B.** Preincubation of the antibody with VEGF peptide completely eliminates the immunostaining. **C.** Eleven days after immunization with S-antigen, the staining is even more intense. **D.** In a normal unimmunized Lewis rat, there is only faint background staining for VEGF. x 350

Table 1. VEGF immunoreactivity in human ocular disorders.

DIAGNOSIS	RETINA					CILIARY EPITHELIUM				IRIS			
	N	Moderate	Weak	% positive	Significance relative to normal	N	Moderate	Weak	% positive	N	Moderate	Weak	% positive
Normal eyes	31	1	3	13	-	19	0	5	26	19	0	0	0
Ischemic retinopathies													
Diabetes	38	9	11	53	p=0.0034	26	2	0	8	26	0	1	4
Central or branch retinal vein oclusion	11	1	4	46	-	11	2	3	46	11	3	1	36
Central retinal artery occlusion	3	0	1	33	-	3	1	0	33	3	0	1	33
Retinopathy of prematurity	5	0	2	40	-	5	0	0	0	5	0	0	0
TOTAL	88	11	21	38	p=0.0043	64	5	8	20	64	3	3	9
Inflammation/infection													
Uveitis	7	1	1	29	-	6	1	0	17	6	1	0	17
Pars planitis	5	0	0	0	-	3	0	0	0	3	0	0	0
Bechet's disease	2	2	0	100	-	2	1	1	100	2	2	0	100
Rubeosis with PVR	7	1	0	14	-	7	0	1	14	7	1	0	14
Cytomegalovirus retinopathy	7	2	1	43	-	7	2	1	43	7	0	1	14
Herpesvirus retinopathy	1	1	0	100	-	1	1	0	100	1	1	0	100
Endogenous aspergillis ophthalmitis	1	1	0	100	-	-	-	-	-	-	-	-	-
TOTAL	30	8	2	33	p=0.0152	26	5	3	31	26	5	1	23
Other ocular diseases													
Aphakic/pseudophakic cystoid macular edema	17	3	5	47	p=0.045	17	2	3	29	17	3	0	18
Keratoplasty without ocular complications	6	0	1	17	-	6	0	2	33	6	0	0	0
Keratoplasty with ocular complications	8	3	5	100	-	8	3	0	37	8	1	0	12
Retinoblastoma	6	3	1	67	p=0.0234	6	0	0	0	6	0	0	0
Choroidal melanoma	37	4	12	46	p=0.0084	38	18	2	53	38	6	2	21
Glaucoma	2	1	1	100	-	2	0	0	0	2	1	0	50
Sarcoid	1	0	0	0	-	1	0	0	0	-	-	-	-

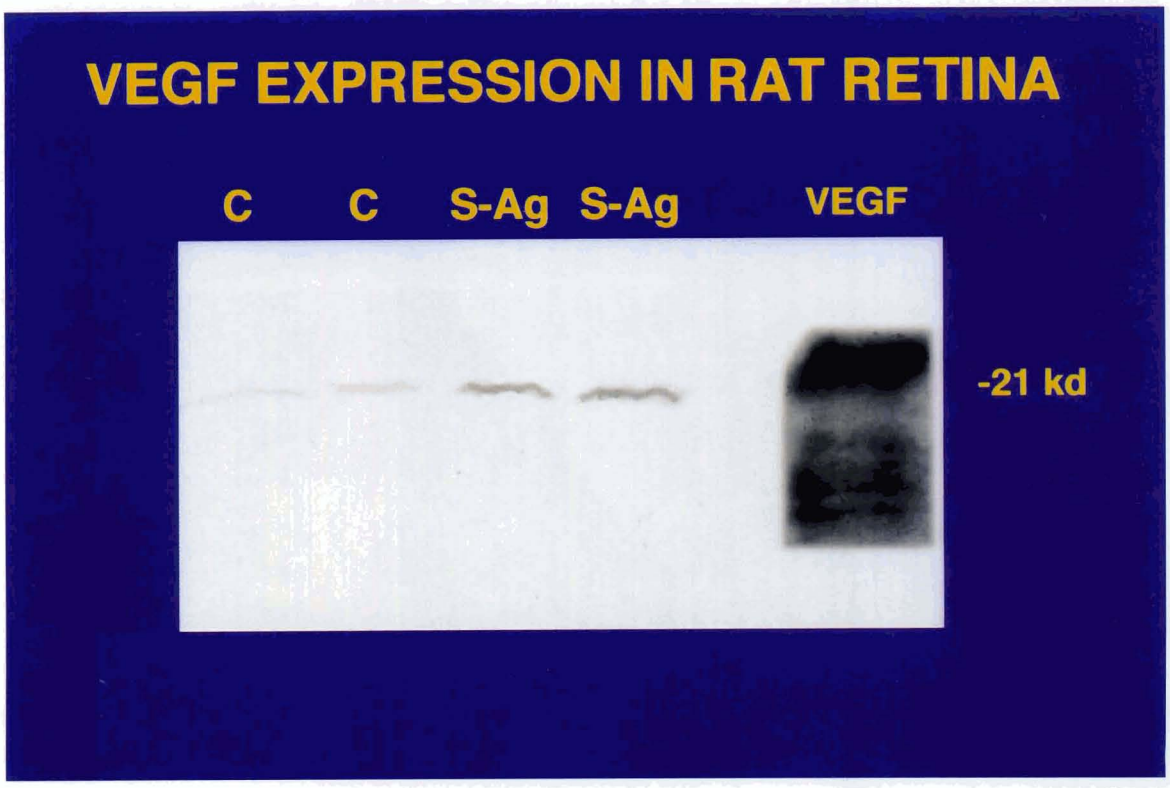

Fig. 4. Immunoblot for VEGF using the affinity-purified antibody shows only a faint band at 21 kD in homogenates of normal Lewis rat retinas (lanes 1 and 2). This band was markedly increased in retinal homogenates 11 days after immunization with S-antigen (lanes 3 and 4). Each lane was loaded with 100 µg of total protein. Recombinant human VEGF is shown in lane 5.

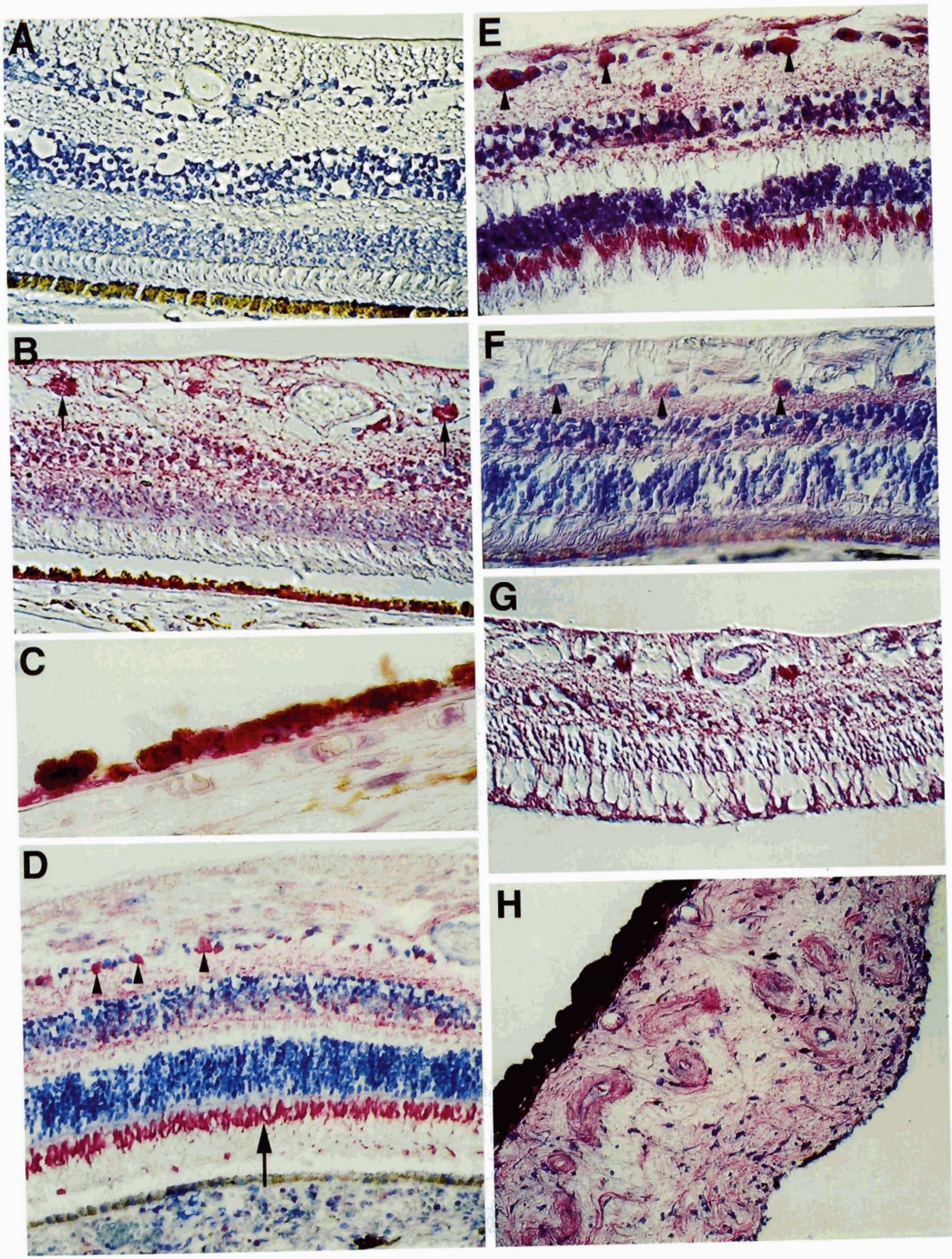
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Fig. 5. A. Normal human retina shows an absence of VEGF staining. x 260. **B.** The retina from a patient with diabetic retinopathy shows VEGF positivity (red) throughout the inner retina, particularly in ganglion cells (arrows) and in the RPE (bottom). x 260. **C.** Higher magnification clearly shows red immunoreaction product for VEGF in the RPE from a patient with proliferative diabetic retinopathy. x 710. **D.** The retina from a patient with cytomegalovirus retinopathy demonstrates conspicuous VEGF staining in the inner segments of the photoreceptors (arrow) and the ganglion cells (arrowheads) and weaker staining in the nerve fiber layer (top), inner plexiform layer, and outer plexiform layer. x 155. **E.** The retina from a patient with endogenous aspergillis endophthalmitis also shows intense red staining for VEGF in the inner segments of the photoreceptors (bottom) and the ganglion cells (arrowheads) with weaker staining of the nerve fiber layer, inner plexiform layer, and outer plexiform layer. x 260. **F.** A patient with aphakic cystoid macular edema demonstrates VEGF positivity in retinal ganglion cells (arrowheads) with weaker staining in the nerve fiber layer, the inner and outer plexiform layers, and the RPE (bottom). x 260. **G.** Diffuse immunostaining (red) for VEGF is seen throughout the retina from an eye with a choroidal melanoma. x 130. **H.** Positive staining for VEGF (red) is demonstrated in the iris, particularly around vessels, from an aphakic patient with glaucoma. x 170

ganglion cells and many other cells throughout the nervous system and may mediate interactions between neurons and glia (Richardson et al., 1988; Sasahara et al., 1991; Yeh et al., 1991; Mudhar et al., 1993). In like manner, VEGF production by neurons may mediate neuronal-vascular interactions. Also, while the VEGF receptors flt and flt-1 have thus been demonstrated only on vascular endothelial cells (DeVries et al., 1992; Terman et al., 1992; Quinn et al., 1993; Thieme et al., 1995), it is possible that other cells also express them and VEGF may mediate as yet unrecognized functions in these cells. Additional work is needed to explore this possibility and the potential role of VEGF in modulating the BRB.

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References

Adamis A.P., Shima D.T., Yeo K.-T., Yeo T.-K., Brown L.F., Berse B., D'Amore P.A. and Folkman J. (1993). Synthesis and secretion of vascular permeability factor/vascular endothelial growth factor by human retinal pigment epithelial cells. Biochem. Biophys. Res. Commun. 193, 631-638.

Adamis A.P., Miller J.W., Bernal M.-T., D'Amico D.J., Folkman J., Yeo T.-K. and Yeo K.-T. (1994). Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. Am. J. Ophthalmol. 118, 445-450.

Aiello L.P., Avery R.L., Arrigg P.G., Keyt B.A., Jampel H.D., Shah S.T., Pasquale L.R., Thieme H., Iwamoto M.A., Park J.E., Nguyen H.V., Aiello L.M., Ferrara N. and King G.L. (1994). Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N. Engl. J. Med. 331, 1480-1487.

Aiello L.P., Pierce E.A., Foley E.D., Sullivan R., Chen H., Ferrara N., King G.L. and Smith L.E.H. (1995). Inhibition of vascular endothelial growth factor (VEGF) reduces retinal neovascularization in the mouse. Invest. Opthalmol. Vis. Sci. 36, S401.

Barthel L.K. and Raymond P.A. (1990). Improved method for obtaining 3-µm cryosections for immunocytochemistry. J. Histochem. Cytochem. 38, 1383-1388.

Caspi R.R. (1989). Basic mechanisms in immune-mediated uveitic disease. In: Immunology of eye diseases. Lightman S. (ed). Kluwer Academic Publishers. Dordrecht. The Netherlands. pp 66-68.

Connolly D.T., Heuvelman D.M., Nelson R., Olander J.V., Eppley B.L., Delfino J.J., Siegel N.R., Leimgruber R.M. and Feder J. (1989a). Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J. Clin. Invest. 84, 1470-1478.

Connolly D.T., Olander J.V., Huevelman D., Nelson R., Monsell R., Siegel N., Haymore B.L., Leimgruber R. and Feder J. (1989b). Human vascular permeability factor. J. Biol. Chem. 264, 20017-20024.

Dastgheib K., Li Q., Chan C.-C., Roberge F.G., Csaky K. and Green W.R. (1995). Vascular endothelial growth factor (VEGF) in neovascular age-related macular degeneration. Invest. Opthalmol. Vis. Sci. 36, S102.

DeKozak Y., Naud M.-C., Bellot J., Faure J.-P. and Hicks D. (1994). Differential tumor necrosis factor expression by resident retinal cells from experimental uveitis-susceptible and -resistant rat strains. J. Neuroimmunol. 55, 1-9.

Derevjanik N.L., Vinores S.A., Peng B., Mahlow J., Chiu C., Campochiaro P.A. and Chan C.-C. (1995). Effects of cyclosporine A (CsA), dexamethasone, and thromboxane synthetase inhibitor (CGS-13080) on blood-retinal barrier breakdown in experimental autoimmune uveoretinitis: an ultrastructural study. Invest. Opathlamol. Vis. Sci. 36, S534.

DeVries C., Esobedo J.A., Ueno H., Houck K., Ferrara N. and Williams L.T. (1992). The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255, 989-991.

Dvorak H.S., Sioussat T.M., Brown L.F., Berse B., Nagy J.A., Sotrel A., Manseau E.J., Van de Water L. and Senger D.R. (1991). Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. J. Exp. Med. 174, 1275-1278.

Goldberg M.A. and Schneider T.J. (1994). Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. J. Biol. Chem. 269, 4355-4359.

Hackett S.F. and Campochiaro P.A. (1993). Modulation of plasminogen activator inhibitor-1 and urokinase in retinal pigmented epithelial cells. Invest. Ophthalmol. Vis. Sci. 34, 2055-2061.

Harada S., Nagy J.A., Sullivan K.A., Thomas K.A., Endo N., Rodan G.A. and Rodan S.B. (1994). Induction of vascular endothelial growth factor expression by prostaglandin E_2 and E_1 in osteoblasts. J. Clin. Invest. 93, 2490-2496.

Hashimoto E., Kage K., Ogita T., Nakaoka T., Matsuoka R. and Kira Y. (1994). Adenosine as an endogenous mediator of hypoxia for induction of vascular endothelial growth factor mRNA In U-937 cells. Biochem. Biophys. Res. Comm. 204, 318-324.

Kondo S., Asano M. and Suzuki H. (1993). Significance of vascular endothelial growth factor/vascular permeability factor for solid tumor growth, and its inhibition by the antibody. Biochem. Biophys. Res. Commun. 194, 1234-1241.

Kuroki M., Beerepoot L.V., Voest E.E. and Adamis A.P. (1995). Regulation of VEGF mRNA expression by reactive oxygen

intermediates. Invest. Opthalmol. Vis. Sci. 36, S895.

Kvanta A. (1995). Expression of vascular endothelial growth factor (VEGF) in choroidal fibroblasts and retinal pigment epithelial cells. Invest. Opthalmol. Vis. Sci. 36, S1046.

Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277, 680-685.

Leung D.W., Cachianes G., Kuang W.-J., Goeddel D.V. and Ferrara N. (1989). Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246, 1306-1309.

Li J., Perrella M.A., Tsai J.-C., Yet S.-F., Hsieh C.-M., Yoshizumi M., Patterson C., Endege W.O., Zhou F. and Lee M.-E. (1995). Induction of vascular endothelial growth factor gene expression by interleukin-1ß in rat aortic smooth muscle cells. J. Biol. Chem. 279, 308-312.

Mahalak S.M., Lin W.-L., Essner E. and Shichi H. (1991). Increased immunoreactivity of collagen types I, III and V, fibronectin and TGF-ß in retinal vessels of rats with experimental autoimmune uveo-retinitis. Curr. Eye Res. 10, 1059-1063.

Malecaze F., Clamens S., Simorre-Pinatel V., Mathis A., Chollet P., Favard C., Bayard F. and Plouet J. (1994). Detection of vascular endothelial growth factor messenger RNA and vascular endothelialg growth factor-like activity in proliferative diabetic retinopathy. Arch. Ophthalmol. 112, 1476-1482.

McClure N., Healy D.L., Rogers P.A.W., Sullivan J., Beaton L., Haning R.V., Connolly D.T. and Robertson D.M. (1994). Vascular endothelial growth factor as capillary permeability agent in ovarian hyperstimulation syndrome. Lancet 344, 235-236.

McMenamin P.G., Forrester J.V., Steptoe R. and Dua H.S. (1993). Ultrastructural pathology of experimental autoimmune uveitis in the rat. Autoimmunity 16, 83-93.

Miller J.W., Adamis A.P., Shima D.T., D'Amore P.A., Moulton R.S., O'Reilly M.S., Folkman J., Dvorak H.F., Brown L.F., Berse B., Yeo T.-K. and Yeo K.-T. (1994). Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am. J. Pathol. 145, 574-584.

Minchenko A., Bauer T., Salceda S. and Caro J. (1994a). Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. Lab. Invest. 71, 374-379.

Minchenko A., Salceda S., Bauer T. and Caro J. (1994b). Hypoxia regulatory elements of the human vascular endothelial growth factor gene. Cell. Mol. Biol. Res. 40, 35-39.

Monacci W.T., Merrill M.J. and Oldfield E.H. (1993). Expression of vascular permeability factor/vascular endothelial growth factor in normal rat tissues. Am. J. Physiol. 264, C995-1002.

Mudhar H.S., Pollock R.A., Wang C., Stiles C. and Richardson W.D. (1993). PDGF and its receptors in the developing rodent retina and optic nerve. Development 118, 539-552.

Murata T., Nakagawa K., Ishibashi T., Ohnishi Y., Inomata H. and Sueishi K. (1995). Temporal and spatial correlation between VEGF expression and retinal angiogenesis in neonatal rats. Invest. Opthalmol. Vis. Sci. 36, S895.

Penn J.S., Tolman B.L. and Lowery L.A. (1993). Variable oxygen exposure causes preretinal neovascularization in the newborn rat. Invest. Opthalmol. Vis. Sci. 34, 576-585.

Pierce G.F., Mustoe T.A., Altrock B.W., Deuel T.F. and Thomas A. (1991). Role of platelet-derived growth factor in wound healing. J. Cell. Biochem. 45, 319-326.

Pierce E.A., Avery R.L., Foley E.D., Aiello L.P. and Smith L.E.H. (1995). Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. Proc. Natl. Acad. Sci. USA 92, 905-909.

Plate K.H., Breier G., Weich H.A. and Risau W. (1992). Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas in vivo. Nature 359, 845-848.

Plate K.H., Breier G., Millauer B., Ullrich A. and Risau W. (1993). Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. Cancer Res. 53, 5822-5827.

Qu-Hong, Nagy J.A., Senger D.R., Dvorak H.F. and Dvorak A.M. (1995). Ultrastructural localization of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) to the albuminal plasma membrane and vesiculo-vacuolar organelles of tumor microvascular endothelium. J. Histochem. Cytochem. 43, 381-393.

Quinn T.P., Peters K.G., DeVries C., Ferrara N. and Williams L.T. (1993). Fetal-liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in endothelium. Proc. Natl. Acad. Sci. USA 90, 7533-7537.

Richardson W.D., Pringle N., Mosley M.J., Westermark B. and Dubois-Dalcq M. (1988). A role for platelet-derived growth factor in normal gliogenesis in the central nervous system. Cell 53, 309-319.

Roberts W.G. and Palade G.E. (1995). Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. J. Cell Sci. 108, 2369-2379.

Santa Cruz Biotechnology, Inc. (1994). Research Product Catalogue. Santa Cruz, CA. p 77.

Sasahara M., Fries J.W.U., Raines E.W., Gown A.M., Westrum L.E., Frosh M.P., Bonthron D.T., Ross R. and Collins T. (1991). PDGF B-chain in neurons of the central nervous system, posterior pituitary, and in a transgenic model. Cell 64, 217-227.

Senger D.R., Galli S.J., Dvorak A.M., Perruzzi C.A., Harvey V.S. and Dvorak H.F. (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219, 983-985.

Senger D.R., Perruzzi C.A., Feder J. and Dvorak H.F. (1986). A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. Cancer Res. 46, 5629-5632.

Senger D.R., Connolly D.T., Van DeWater L., Feder J. and Dvorak H.F. (1990). Purification and NH_2-terminal amino acid sequence of guinea pig tumor secreted vascular permeability factor. Cancer Res. 50, 1774-1778.

Senger D.R., Van DeWater L., Brown L.F., Nagy J.A., Yeo K.-T., Yeo T.-K., Berse B., Jackman R.W., Dvorak A.M. and Dvorak H.F. (1993). Vascular permeability factor (VPF, VEGF) in tumor biology. Cancer Metast. Rev. 12, 303-324.

Shima D.T., Deutsch U. and D'Amore P.A. (1995). Hypoxic induction of vascular endothelial growth factor (VEGF) in human epithelial cells is mediated by increases in mRNA stability. FEBS Lett. 370, 203-208.

Sioussat T.M., Dvorak H.F., Brock T.A. and Senger D.R. (1993). Inhibition of vascular permeability factor (vascular endothelial growth factor) with antipeptide antibodies. Arch. Biochem. Biophys. 301, 15-20.

Smith L.E.H., Pierce E.A., Aiello L.P., Foley E.D., Sullivan R., Rook S.L. and Robinson G.S. (1995). Inhibition of proliferative retinopathy using antisense phosphorothioate oligonucleotides against vascular endothelial growth factor (VEGF/VPF). Invest. Ophtlamol. Vis. Sci.

36, S871.

Stone J., Itin A., Alon T., Pe'er J., Gnessin H., Chan-Ling T. and Keshet E. (1995). Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. J. Neurosci. 15, 4738-4747.

Terman B.I., Dougher-Vermazen M., Carrion M.E., Dimitrov D., Armellino D.C., Gospodarowicz D. and Bohlen P. (1992). Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem. Biophys. Res. Commun. 187, 1579-1586.

Thieme H., Aiello L.P., Takagi H., Ferrara N. and King G.L. (1995). Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. Diabetes 44, 98-103.

Tolentino M.J., Miller J.W., Gragoudas E.S., Moulton R., Chatzistefanou K., Flynn E., Ferrara N. and Adamis A.P. (1995). Vascular endothelial growth factor is sufficient to produce ocular neovascularization in a non-human primate. Invest. Opthalmol. Vis. Sci. 36, S-402.

Towbin H., Staehelin T. and Gordon J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350-4354.

Vinores S.A. (1995). Assessment of blood-retinal barrier integrity. Histol. Histopathol. 10, 141-154.

Vinores S.A., Campochiaro P.A., Lee A., McGehee R., Gadegbeku C. and Green W.R. (1990a). Localization of blood-retinal barrier breakdown in human pathologic specimens by immunohistochemical staining for albumin. Lab. Invest. 62, 742-750.

Vinores S.A., McGehee R., Lee A., Gadegbeku C. and Campochiaro P.A. (1990b). Ultrastructural localization of blood-retinal barrier breakdown sites in diabetic and galactosemic rats. J. Histochem. Cytochem. 38, 1341-1352.

Vinores S.A., VanNiel E., Swerdloff J.L. and Campochiaro P.A. (1993a). Electron microscopic immunocytochemical demonstration of blood-retinal barrier breakdown in human diabetics and its association with aldose reductase in retinal vascular endothelium and retinal pigment epithelium. J. Histochem. 25, 648-663.

Vinores S.A., VanNiel E., Swerdloff J.L. and Campochiaro P.A. (1993b). Immunohistochemical evidence for the mechanism of blood-retinal barrier breakdown in galactosemic rats and its association with aldose reductase expression and inhibition. Exp. Eye Res. 57, 723-735.

Vinores S.A., Amin A., Derevjanik N.L., Green W.R. and Campochiaro P.A. (1994). Immunohistochemical localization of blood-retinal barrier breakdown sites associated with post-surgical macular edema. Histochem. J. 26, 655-665.

Vinores S.A., Küchle M., Derevjanik N.L., Henderer J.D., Mahlow J., Green W.R. and Campochiaro P.A. (1995a). Blood-retinal barrier breakdown in retinitis pigmentosa: light and electron microscopic immunolocalization. Histol. Histopathol. 10, 913-923.

Vinores S.A., Küchle M., Mahlow J., Chiu C., Green W.R. and Campochiaro P.A. (1995b). Blood-ocular barrier breakdown in eyes with ocular melanoma: a potential role for vascular endothelial growth factor/vascular permeability factor. Am. J. Pathol. 147, 1289-1297.

Weindel K., Moringlane J.R., Marmé D. and Weich H.A. (1994). Detection and quantification of vascular endothelial growth factor/vascular permeability factor in brain tumor tissue and cyst fluid: the key to angiogenesis? Neurosurgery 35, 439-449.

Yeh H.-J., Ruit K.G., Wang Y.-X., Parks W.C., Snider W.D. and Deuel T.F. (1991). PDGF A-chain gene is expressed by mammalian neurons during development and in maturity. Cell 64, 209-216.

Youssri A.I., Luna J., Vinores S. and Campochiaro P.A. (1995). Immunohistochemical localization of vascular endothelial growth factor (VEGF) in retinas with oxygen-induced ischemic retinopathy and non-ischemic retinas. Invest. Opthalmol. Vis. Sci. 36, S401.

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