Intravitreous Injections of Vascular Endothelial Growth Factor Produce Retinal Ischemia and Microangiopathy in an Adult Primate

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Purpose: The purpose of the study is to determine the effect of exogenous vascular endothelial growth factor (VEGF) on the primate retina and its vasculature.

Methods: Ten eyes of five animals were studied. Physiologically relevant amounts of the 165 amino acid isoform of human recombinant VEGF were injected into the vitreous of six healthy cynomolgus monkey eyes. Inactivated human recombinant VEGF or vehicle was injected into four contralateral control subject eyes. Eyes were assessed by slit-lamp biomicroscopy, tonometry, fundus color photography, fundus fluorescein angiography, light microscopy, and immunostaining with antibodies against proliferating cell nuclear antigen and factor VIII antigen.

Results: All six bioactive VEGF-injected eyes developed dilated, tortuous retinal vessels that leaked fluorescein. Eyes receiving multiple injections of VEGF developed progressively dilated and tortuous vessels, venous beading, edema, microaneurysms, intraretinal hemorrhages and capillary closure with ischemia. The severity of the retinopathy correlated with the number of VEGF injections. None of the four control eyes exhibited any abnormal retinal vascular changes. The endothelial cells of retinal blood vessels were proliferating cell nuclear antigen positive only in the bioactive VEGF-injected eyes.

Conclusion: Vascular endothelial growth factor is sufficient to produce many of the vascular abnormalities common to diabetic retinopathy and other ischemic retinopathies, such as hemorrhage, edema, venous beading, capillary occlusion with ischemia, microaneurysm formation, and intraretinal vascular proliferation.

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Retinal neovascularization is a major cause of vision loss in diseases such as diabetic retinopathy and retinal vein occlusion. Since 1948, published clinical and experimental data have supported the existence of a *vasoformative* factor, synthesized and released by ischemic retina, that is causative for neovascularization of the retina, optic nerve, and iris.[1–4] Recent evidence strongly supports the identification of vascular endothelial growth factor (VEGF) as the mediator of retinal ischemia-associated ocular neovascularization.[5–15]

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Vascular endothelial growth factor refers to a family of polypeptides produced through the alternative splicing of messenger ribonucleic acid (mRNA) that potently induces vascular permeability and angiogenesis.[16–20] The VEGF gene expression is regulated by oxygen levels,[21,22] and VEGF is the primary bioactive vascular endothelial cell mitogen made and secreted by hypoxic retinal cells in vitro.[9,23] Enhanced retinal VEGF gene expression is correlated with normal vessel development,[12] and high intraocular VEGF protein levels are associated temporally and spatially with pathologic neovascularization in a primate model.[5,24] In humans with retinal ischemia-associated neovascularization, elevated vitreous and aqueous VEGF levels correlate with neovascularization of the retina, optic nerve, and iris.[6–8] Vascular endothelial growth factor mRNA levels are increased in the ischemic retina of nonhuman primate eyes[5] and in human eyes with proliferative diabetic retinopathy.[11] A causal role in neovascularization was shown through the inhibition of intraocular VEGF. Iris neovascularization was prevented completely in a nonhuman primate model,[13] and retinal neovascularization was reduced in a mouse model of retinopathy of prematurity.[14] Additionally, intravitreous injections of VEGF have been shown to directly stimulate iris neovascularization and neovascular glaucoma in nonhuman primate eyes.[15] These data indicate that VEGF is both necessary and sufficient for adult retinal ischemia-associated iris neovascularization. However, the direct effect of VEGF on the adult primate retinal vasculature is not known.

In this study, human recombinant VEGF was injected into the vitreous of healthy adult primate eyes. We now show that VEGF is capable of producing a retinopathy with many features common to diabetic retinopathy and the other ischemic retinopathies, such as hemorrhage, edema, venous beading, capillary occlusion with ischemia, microaneurysm formation, and intraretinal vascular proliferation.

Materials and Methods

All animals were cared for in accordance with the Association for Research in Vision and Ophthalmology Resolution on the use of animals for research and guidelines established for animal care at the Massachusetts Eye and Ear Infirmary. Ten eyes of 5 cynomolgus monkeys (*Macacca fascicularis*) were studied. Four animals received bioactive VEGF in one eye and either inactive VEGF (animals 1 and 3) or vehicle (animals 4 and 5) in the contralateral eye. The VEGF was inactivated either by reduction to its monomer form or by coinjecting bioactive dimer VEGF with a 50:1 molar excess of a previously characterized VEGF neutralizing monoclonal antibody (mAb 4.6.1).[25,26] One animal (animal 2) received bilateral injections of bioactive VEGF of differing doses.

Before injection or photography, the animals were anesthetized with 20 mg/kg of ketamine hydrochloride (Ketalar), 0.005 mg/kg of acepromazine, and 0.03 mg/kg of atropine sulfate given intramuscularly. Before pneumotonometry, or lid speculum placement, topical 0.5% proparacaine hydrochloride was administered. Pupils were dilated with 2.5% phenylephrine and 0.8% tropicamide for fundus photography. For enucleation, the animals were anesthetized deeply with 7 mg/kg intravenous Nembutal sodium. Animals were killed with 0.33 mg/kg intravenous pentobarbital. Photography and fluorescein angiography were performed as described previously.[27,28]

Human recombinant $VEGF_{165}$ was purified from the conditioned medium of transfected Chinese hamster ovary cells as described previously.[29] Previous experiments showed that simian $VEGF_{165}$ is a major isoform produced in ischemic simian retina and in cultured hypoxic human retinal cells.[9,13] Vascular endothelial growth factor endotoxin levels were assayed before injection using the E-toxate Limulus amoebocyte lysate assay[30] (Sigma, St Louis, MO) and were undetectable (i.e., less than 0.03 EU/ml). Inactive VEGF monomer was formed by reducing VEGF as described previously.[15] Briefly, dimer VEGF was reduced with 10 mM dithiothreitol (Sigma) for 30 minutes at 65° C then acetylated with 200 mM iodoacetamide (Sigma) for 30 minutes at 37° C. Equal amounts (2.5 μg) of dimer and monomer VEGF were centrifuged for 6 hours at 12,000 rpm in separate two-chamber centrifuge tubes with 3000 kd cutoff filters (Amicon, Inc, Beverly, MA) to remove the dithiothreitol and iodoacetamide. The samples were washed with phosphate buffered saline (PBS) (Sigma) three times and respun in sterile PBS at 6200 × g for 1 hour at 25° C to a final volume of 50 μl. Monomer and dimer were prepared the day before injection. Monomer production was confirmed using sodium dodecyl sulfate protein electrophoresis and silver staining to confirm reduction and single-band purity (data not shown).[31]

Previous reverse transcription and complementary DNA sequencing of the simian retina $VEGF_{165}$ isoform showed a sequence that coded for protein identical to human $VEGF_{165}$.[13] As a control, 50.0 μg of the VEGF neutralizing monoclonal Ab A4.6.1 was mixed with 1.25 μg of VEGF. An identical amount of a previously characterized monoclonal antibody (anti-gpl20) of the same isotype (IgG_1) was mixed with VEGF for injection into the contralateral eye. This antibody has not shown any effect on neovascularization in another system.[26] The antibodies and VEGF were mixed and vortexed 1 hour before injection. Before and immediately after vitreous injections, baseline intraocular pressures (IOPs) are taken with a pneumotonometer (Digilab Model 30R) and the anterior segment examined with a slit lamp.

All reagents were diluted in sterile PBS to a total volume of 100 μl. The mixtures were vortexed gently for 30 seconds and centrifuged for 1 minute at 5000 G. The reagent was drawn up into a sterile tuberculin syringe and injected using a 30-gauge needle. Injections were through the pars plana into the center of the vitreous as viewed through an operating microscope. A drop of topical gentamicin sulfate (Iolab, Claremont, CA) was applied and pneumotonometry was performed within 10 minutes after injection to confirm an IOP of less than 40 mmHg.

After enucleation, eyes were bisected at the equator and immersed in Carnoy fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 4 hours followed

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by storage in 100% ethanol until paraffin embedding for light microscopy. For light microscopy, 6 μm sections were cut with a microtome, placed on superfrost slides, heated to 60° C for 30 minutes, deparaffinized with xylene, and rehydrated in decreasing concentrations of ethanol. To stain for proliferating cell nuclear antigen (PCNA), sections were treated with 5% hydrogen peroxide and incubated in tissue unmasking factor (Signet, Dedham, MA) to increase antigen retrieval. The PCNA monoclonal antibody (Signet) diluted in 2% horse serum and 0.3% Triton-X 100 in PBS was used, and sections were incubated overnight at 25° C. Tissue was washed with PBS × 3 for 10 minutes. Using the ABC Elite Vectastain System (Vector Labs, Burlingame, CA), sections were incubated in secondary antibody and avidin-biotin complex, each for 45 minutes, followed by three 10-minute PBS washes between steps. The complex was developed using the blue Vector-SG chromagen.

Endothelial cells and blood vessels were identified by staining for factor VIII (Dako Polyclonal, Daka, Santa Barbara, CA) with use of a standard immunoperoxidase technique, as described previously.[32,33] Each slide was covered with Crystal Mount (Biomedia Corp, Foster City, CA) and photographed using a Zeiss axiophot light microscope.

Results

All animals were documented to have healthy retinas with biomicroscopy, fundus photography, and fluorescein angiography before injections. Animals 1 through 4 had their iris neovascularization described in a previous study.[15] Careful first-hand serial observations after injections of bioactive VEGF confirmed that the animals retained significant useful vision and did not suffer undue distress.

Animal 1 received a single intravitreous injection of 1.25 μg VEGF mixed with the non-neutralizing control monoclonal antibody (anti-gpl20) in the experimental eye. This produced vessel dilation, tortuosity, and leakage (Table 1, Fig 1A). The control eye injected with VEGF mixed with VEGF-neutralizing antibody was normal in appearance. Both eyes were without signs of inflammation.

Animal 2 received a single intravitreous injection of either 0.25 (left eye) or 2.5 (right eye) μg of bioactive VEGF resulting in vessel tortuosity, dilation, and leakage that was more pronounced in the eye receiving the higher dose (Table 1). Microaneurysms appeared in the eye that received 2.5 μg VEGF (Fig 1B). Both eyes were without signs of inflammation.

Animal 3 received 1.25 μg of bioactive dimer VEGF in the experimental eye and 1.25 μg of inactive monomer VEGF in the contralateral control eye every other day for 8 days. The retinal vessels in the experimental eye became progressively tortuous, dilated, and leaky. After three injections, microaneurysm-like structures were visible (Table 1). Identical amounts of reduced monomer VEGF had no effect on the contralateral control eye. Both eyes were without biomicroscopic or histopathologic signs of inflammation. The PCNA immunostaining identified positive staining foci of proliferating cells[34] comprising the lumenal walls of dilated retinal vessels in the experimental eye (Fig 1C).[32,33] No PCNA positive cells were present in any sections of the control eye injected with inactive monomer VEGF, consistent with the very low proliferative rate for normal endothelium.[35]

Animal 4 received 1.25 μg intravitreous injections of VEGF in the experimental eye and vehicle alone (PBS) in the contralateral control eye every 3 days for 27 days and was enucleated on day 30. Initially, only vessel tortuosity, dilation, and leakiness developed after 3 days in the experimental eye (Figs 2A and 2B). The vascular changes were greatest in the second- and third-order branches of the retinal venules. After six injections, the retinal venules became very dilated and tortuous and microaneurysm-like structures appeared (Fig 2C). Venous beading was visible as were areas of nonperfusion in the midperiphery. The IOPs remained below 20 mmHg. The contralateral control eye injected with PBS alone remained unchanged in appearance throughout the course of the experiment. Neovascular glaucoma was noted in the experimental eye by day 19 (IOP > 20 mmHg), and pressure readings reached 47 mmHg on day 30 and the animal was killed. Anterior chamber paracentesis was performed immediately before the last two injections (days 24 and 27) to lower the pressure before the intravitreous injections. Histopathologic examination results showed grossly dilated vessels, intraretinal hemorrhage and edema, and foci of proliferating endothelial cells in the experimental eye (Figs 3A–3F).

Animal 5 received 1.25 μg injections of VEGF in the experimental eye or vehicle alone (PBS) in the contralateral eye every 3 days for 78 days followed by enucleation on day 81. Vessel tortuosity, dilation, leakage, intraretinal hemorrhage, venous beading, and microaneurysm formation developed in the experimental eye after three injections and became more pronounced over time (Figs 4A–4D). After four injections, large areas of capillary closure appeared (Fig 4E). The IOPs remained below 20 mmHg. As with animal 4, the most pronounced changes were in the second- and third-order branches of the retinal venules. Vitreous fibrin developed after 10 injections without an accompanying cellular reaction. Fibrin completely obstructed any view of the posterior pole after 14 injections. B-scan ultrasonography showed an attached retina. The anterior chamber showed only a mild cellular reaction, and serial paracentesis cultures were negative. A vitrectomy was performed on day 80, and cultures of the vitreous were negative. Examination results of the fundus at the time of vitrectomy showed an apparent fibrovascular stalk emanating from the disc with extensive areas of avascular retina temporally and dilated vascular loops in the superior retina. No areas of extraretinal neovascularization anterior to the internal limiting membrane were seen in serial retinal sections. Histopathologic and immunohistochemical studies showed findings similar to those of animal 4. No abnormal changes were seen in the contralateral control eye.

Discussion

Direct causal proof that VEGF is necessary and sufficient for retinal ischemia-associated iris neovascularization has

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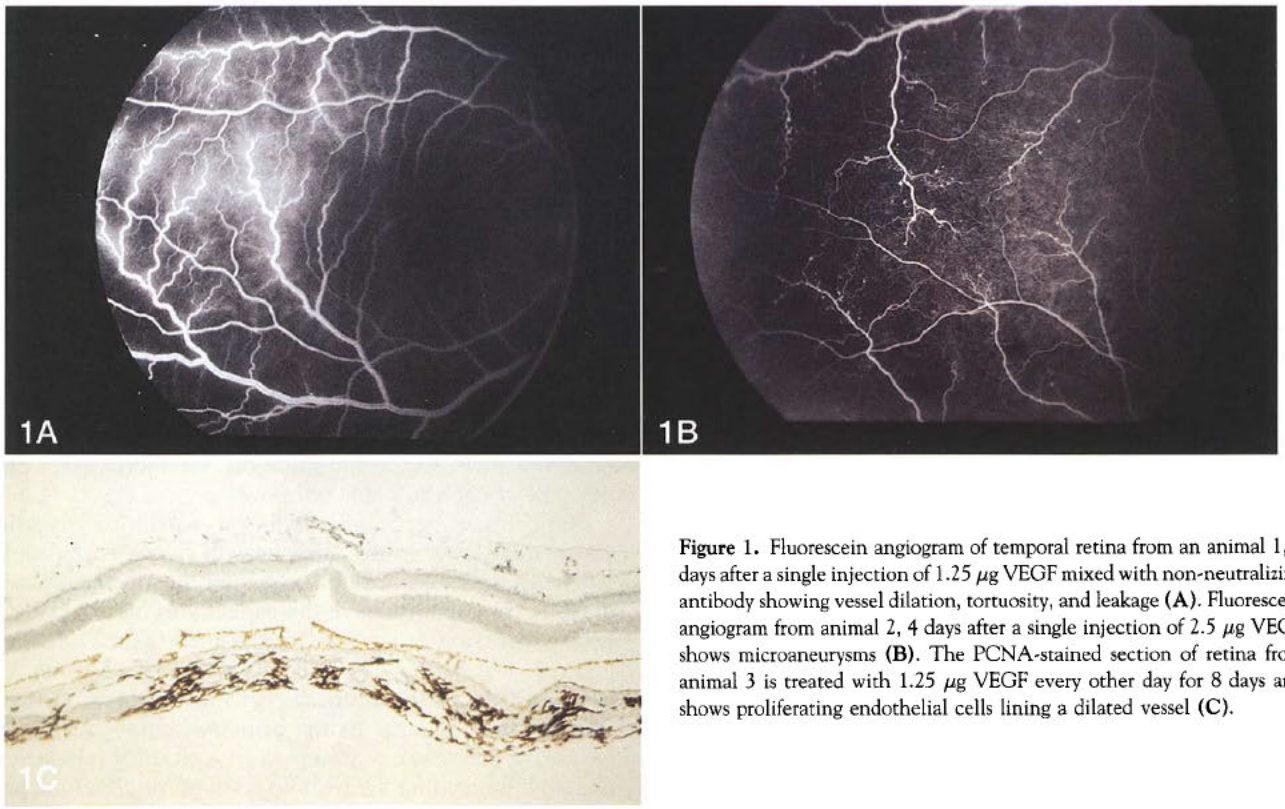

Figure 1. Fluorescein angiogram of temporal retina from an animal 1, 4 days after a single injection of 1.25 μg VEGF mixed with non-neutralizing antibody showing vessel dilation, tortuosity, and leakage **(A)**. Fluorescein angiogram from animal 2, 4 days after a single injection of 2.5 μg VEGF shows microaneurysms **(B)**. The PCNA-stained section of retina from animal 3 is treated with 1.25 μg VEGF every other day for 8 days and shows proliferating endothelial cells lining a dilated vessel **(C)**.

been obtained in a nonhuman primate model.[13,15] However, the evidence that VEGF is involved in adult retinal neovascularization is indirect. Vascular endothelial growth factor receptors are present on cultured retinal capillary endothelial cells,[36] and hypoxic retina cell-derived VEGF is the major mitogen stimulating the proliferation of endothelial cells in vitro.[9] Intraocular VEGF levels are correlated spatially and temporally with retina, optic nerve, and iris neovascularization[5–8]; and neovascularization of the iris, retina, and optic nerve frequently are associated in the ischemic retinopathies. This observation supports a similar pathophysiologic mechanism for all three types of neovascularization. Finally, retinal neovascularization is suppressed when VEGF is inhibited in the mouse model of retinopathy of prematurity.[14]

In this study, we show that intravitreous VEGF, in amounts similar to those measured in human eyes with neovascularization, is sufficient to produce intraretinal vascular proliferation in the adult nonhuman primate. Additionally, VEGF can produce much of the pathologic

Table 1. Vascular Endothelial Growth Factor Effects on Retinal Vasculature

	PBS (animals 4 + 5)	VEGF Monomer 1.25 μg Every 2 Days for 8 Days (animal 3)	VEGF Single Dose 1.25 μg + Anti-VEGF mAb (animal 1)	VEGF Single Dose 0.25 μg VEGF (animal 2)	VEGF Single Dose 2.5 μg VEGF (animal 2)	VEGF Single Dose 1.25 μg + Anti-gp120 mAb (animal 1)	VEGF 1.25 μg Every 2 Days for 8 Days (animal 3)	VEGF 1.25 μg Every 3 Days for 27 Days (animal 4)	VEGF 1.25 μg Every 3 Days for 78 Days (animal 5)
Dilation	0	0	0	+	++	+	++	+++	+++
Tortuosity	0	0	0	+	++	+	++	+++	+++
Leakage	0	0	0	+	++	+	++	+++	+++
Microaneurysms	0	0	0	0	+	0	++	+++	+++
Venous beading	0	0	0	0	0	0	0	+++	+++
Capillary nonperfusion	0	0	0	0	0	0	0	+++	+++
Extraretinal neovascularization	0	0	0	0	0	0	0	0	0

PBS = phosphate buffered saline; VEGF = vascular endothelial growth factor; mAb = monoclonal antibody; + = mild; ++ = moderate; +++ = severe.

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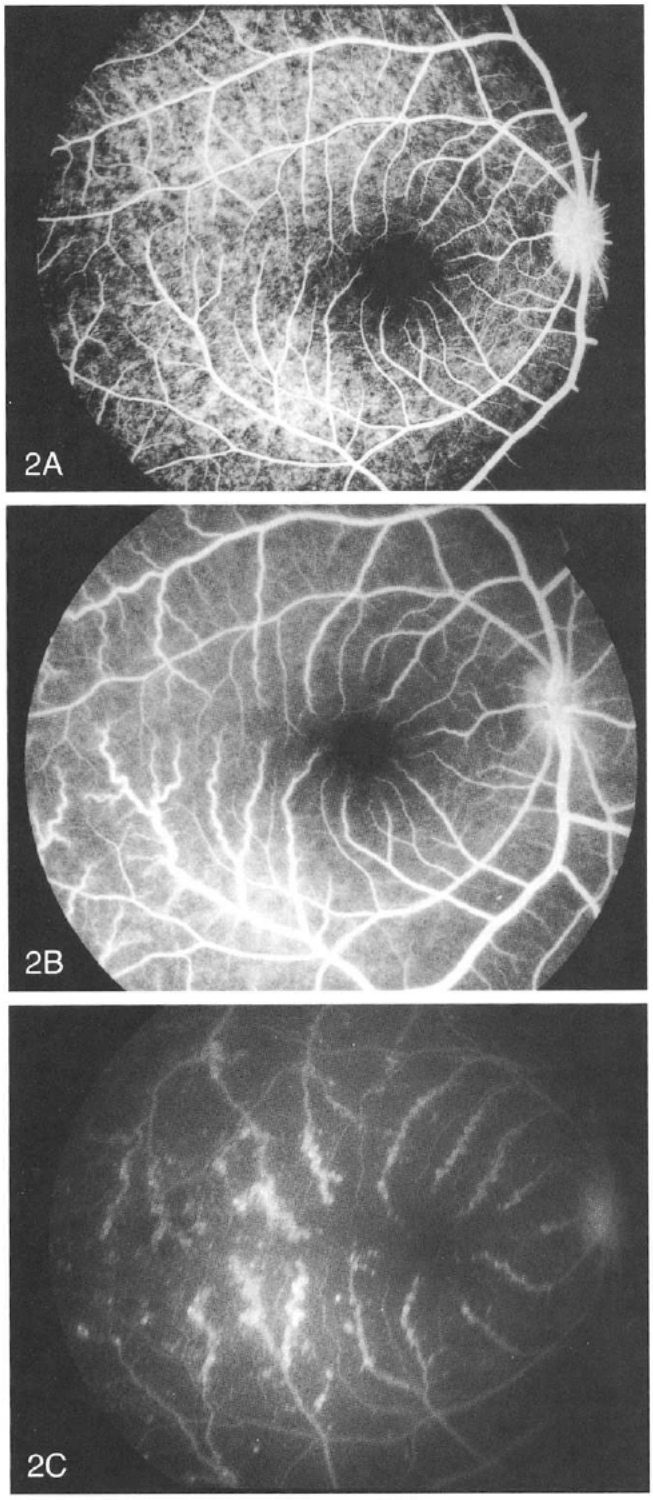

Figure 2. Fluorescein angiogram of animal 4 before VEGF injections **(A)** and 2 days after a single injection of 1.25 μg VEGF **(B)**. There is pronounced dilation and tortuosity of the second- and third-order venules. Fluorescein angiogram of the same animal after six 1.25 μg VEGF injections shows marked vessel dilation and tortuosity as well as multiple microaneurysms **(C)**.

results of diabetic retinopathy and the other ischemic retinopathies-leakage, edema, venous beading, hemorrhage, microaneurysm formation, and retinal ischemia. The latter finding is novel and unexpected. The prevailing paradigm states that retinal ischemia increases VEGF gene expression.[21,22] These data show that VEGF alone can trigger retinal ischemia through capillary closure in normotensive eyes. This activity could initiate a positive feedback loop, further increasing VEGF levels.

Experiments show the increased expression of intercellular adhesion molecule-1 by endothelial cells exposed to VEGF (V. Perez, M. Lu, and A. P. Adamis; unpublished data, 1995). Intercellular adhesion molecule-1 is known to increase the adhesion of activated monocytes and granulocytes to endothelium.[37] These data are consistent with studies showing increased intercellular adhesion molecule-1 expression in the retinal vessels of human diabetics[37] and capillary occlusion secondary to activated granulocytes and monocytes in experimental diabetic retinopathy.[38] If VEGF levels are elevated in the retina before ischemia, then the initial stimulus for increased VEGF gene expression remains unknown.

Reactive oxygen intermediates, advanced glycation endproducts, and insulin-like growth factor-1, molecules with elevated intraocular levels that are correlated with diabetic retinopathy, have the capacity to increase VEGF gene expression in vitro and in vivo (M. Kuroki, R. Punglia, M. Lu, M. J. Tolentino, I. Kim, A. P. Adamis; unpublished data). We propose that these factors, alone or in combination, serve as the primary stimuli to increase retinal VEGF gene expression in background diabetic retinopathy. Exceeding a threshold level of VEGF could promote capillary occlusion, leading to further VEGF production. This hypothesis is supported by recent immunohistochemical evidence of increased VEGF expression in the eyes of diabetic rats with early retinopathy.[39] The development of vessel tortuosity, leakage, venous beading, hemorrhages, microaneurysms, edema, and capillary nonperfusion secondary to intravitreous VEGF injections further supports such a role for VEGF in background and preproliferative retinopathy. Although elevated VEGF levels were not detected in the vitreous of diabetics with background retinopathy,[8] it is possible VEGF levels are elevated in the retina, but not detected. Only small amounts of VEGF can produce biologic effects. Vascular endothelial growth factor is the most potent permeability factor known.[16,40] On a molar basis, it is 50,000 times more powerful than histamine in the Miles assay of vascular permeability.[40]

The angiographic findings of dilation, tortuosity, and microaneurysm formation were confirmed as representing vascular endothelial cell proliferation with PCNA and factor VIII immunostaining (Figs 3A–E). These data show that the dilation and tortuosity of pre-existing vessels is more than strictly a mechanical phenomenon. Vascular endothelial growth factor-induced microaneurysms, manifesting as nests of proliferating endothelial cells, appeared much like those described in the vasculature of diabetic retinas (Figs 3D–3F).[41]

Second- and third-order venules showed the greatest degree of dilation and tortuosity (Figs 2A–2C). As with diabetes, the venous side of the vasculature was most affected.[41] This could be because of the higher concentration of VEGF in the venules as VEGF was cleared from the vitreous, or it could indicate the heterogeneity of the

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