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Overexpression of Vascular Endothelial Growth Factor (VEGF) in the Retinal Pigment Epithelium Leads to the Development of Choroidal Neovascularization

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Vascular endothelial growth factor (VEGF) has been strongly implicated in the development of choroidal neovascularization found in age-related macular degeneration. Normally expressed in low levels, this study investigates whether the overexpression of VEGF in the retinal pigment epithelium is sufficient to cause choroidal neovascularization in the rat retina. A recombinant adenovirus vector expressing the rat $VEGF_{164}$ cDNA (AdCMV.VEGF) was constructed and injected into the subretinal space. The development of neovascularization was followed by fluorescein angiography, which indicates microvascular hyperpermeability of existing and/or newly forming blood vessels, and histology. VEGF mRNA was found to be overexpressed by retinal pigment epithelial cells and resulted in leaky blood vessels at 10 days postinjection, which was maintained for up to 31 days postinjection. By 80 days postinjection, new blood vessels had originated from the choriocapillaris, grown through the Bruch's membrane to the subretinal space, and disrupted the retinal pigment epithelium. This ultimately led to the formation of choroidal neovascular membranes and the death of overlying photoreceptor cells. By controlling the amount of virus delivered to the subretinal space, we were able to influence the severity and extent of the resulting choroidal neovascularization. These results show that even temporary overexpression of VEGF in retinal pigment epithelial cells is sufficient to induce choroidal neovascularization in the rat eye. ***(Am J Pathol 2000, 157:135–144)***

Age-related macular degeneration is a significant cause of central vision loss in aging populations. The more severe form of age-related macular degeneration is characterized by choroidal neovascularization (CNV), in which new blood vessels grow from the choroid, through the Bruch's membrane into the subretinal space. This ultimately leads to the formation of choroidal neovascular membranes (CNVMs), from which blood and serum may leak, causing vision loss.[1] The exact cause of CNV is not clear; however, it is often associated with a buildup of abnormal extracellular deposits in the form of soft drusen between the aging retinal pigment epithelium (RPE) and Bruch's membrane.[2] This, in turn, could result in localized areas of ischemia, triggering angiogenesis.

Growth factors and cell adhesion molecules that have been implicated in CNV include ICAM-1, E-selectin, CD44,[3] basic and acidic fibroblast growth factor (aFGF and bFGF),[4] and vascular endothelial growth factor (VEGF). Although evidence does support a role for other growth factors in CNV,[5–7] the potency and specificity of VEGF for vascular endothelial cells and the fact that it can be secreted would suggest it has a primary role in CNV development. VEGF, a homodimer of approximately 45 kd, is a very potent vascular endothelial cell mitogen.[8,9] Six different isoforms of human VEGF have been identified to date,[8,10–12] and all have different heparin binding capabilities, show varying tissue distribution, are up-regulated under hypoxic conditions,[13] and are potent vasopermeability factors.[9]

Over the last decade it has been well established that VEGF is crucial for normal angiogenesis and that it also plays an important role in pathological angiogenesis. However, it remains to be established whether VEGF is the sole causal angiogenic factor in the development of CNV. VEGF possesses many attributes for such a role. It is strongly and preferentially induced by hypoxia in RPE cells,[14] it is invariably associated with human CNVMs and in laser CNV models in animals,[3,5,15–19] it is strongly secreted from the basal side of the RPE toward the choroid, and high levels of VEGF receptors KDR and flt-4 are found on the choriocapillaris endothelium facing the RPE layer.[20]

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However, the role of VEGF as the only causal agent in CNV has been questioned by evidence showing that VEGF is prominently expressed by RPE cells in epiretinal membranes in which there are no blood vessels[21] and rats implanted suprachoroidally with slow release VEGF pellets show no leakage or development of CNV.[22] In addition to VEGF, transforming growth factor-β (TGF-β), aFGF, and bFGF have also been localized to human CNVMs.[4] CNV has been shown to develop in the minipig model when bFGF was perfused into the suprachoroidal space, although the neovascularization did not penetrate the Bruch's membrane.[7] However, mice with a targeted disruption of the bFGF gene are able to develop CNV after laser photocoagulation, suggesting it is not an absolute requirement for new blood vessel growth.[23]

To investigate the role of VEGF in the development of CNV, we have adopted a recombinant adenovirus gene delivery strategy previously shown to specifically target the rat RPE.[24–27] A recombinant adenovirus vector containing the rat $VEGF_{164}$ cDNA (AdCMV.VEGF) was used to determine whether short-term *in vivo* overexpression of VEGF in RPE cells was sufficient to cause CNV in the rat.

Materials and Methods

Construction of AdCMV.VEGF

The rat $VEGF_{164}$ amino acid splice form cDNA was cloned from RCS/rdy rat RPE cells grown under hypoxic conditions (2% O_2). Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using the primers AGCGGGATCCTCGCAGTCCGAGCCGGA and CTCCGGATCCCAAAGTGCTCCTCGAAG. These primers generated a PCR product of 804 bp containing the coding region of the rat $VEGF_{164}$ splice variant, flanked by 61 bp of 5′ UTR and 170 bp of 3′ UTR. The PCR product was cloned into the *Bam*HI site of pGEM11 (Promega, Madison, WI). After DNA sequence verification, the rat VEGF cDNA was subcloned into the adenovirus shuttle vector, pCA13 (Microbix Biosystems Inc., Toronto, ON) behind the human cytomegalovirus (CMV) immediate early promoter/enhancer (from −299 to +72) and before SV40 polyadenylation signals. pCA13 is used to construct replication-incompetent Ad5 vectors with inserts in the early region 1 (E1). The 293-cell line,[28] transformed with Ad5, supplies the necessary E1 function in *trans*. The resultant plasmid, pCA13.VEGF, was co-transfected, using the Ca_2PO_4 precipitation method, into 293 cells along with *Cla*I-restricted AdRSV.βgal DNA as the viral backbone. The resultant AdCMV.VEGF virus (E1/partial E3 deletion) underwent several rounds of cloning by limiting dilution on 293 cells. All viruses used in this study were expanded and purified on a two-step CsCl gradient.[29] Titer of the viral stocks was determined by limiting dilution on 293 cells. AdRSV.βgal,[30] AdCMV.βgal,[31] and AdCMV.GFP[32] have been described previously and were used as controls.

Cell Culture

The 293-cell line was obtained from Microbix Biosystems Inc., human umbilical cord endothelial cells (HUVEC) from the American Type Culture Collection (Manassas, VA), the Long Evans and RCS/rdy rat primary RPE cells were from M. Hall, Jules Stein Eye Institute of the University of California Los Angeles (Los Angeles, CA), and the human RPE 51 cells were isolated from the retina of a 51-year-old donor as previously described.[33] All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. HUVEC cell medium was supplemented with 100 μg/ml of heparin and 37 μg/ml of endothelial cell growth supplement (ECGS; Sigma-Aldrich, St. Louis, MO).

VEGF mRNA and Protein Analysis

VEGF mRNA and protein analysis was carried out basically as described.[34] For analysis of mRNA production from AdCMV.VEGF, 1×10^5 human RPE 51 cells were transduced at an multiplicity of infection (MOI) of 50. Total RNA was isolated from cell monolayers at different time points using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Northern blots (10 μg of RNA) were hybridized using random primed rat VEGF cDNA. For analysis of the VEGF protein produced by AdCMV.VEGF, 1.5×10^6 human RPE 51 cells (in 1.5 ml medium) were transduced at MOIs of 50 and 200. Conditioned media was removed (10 μl) from the cells at different time points and subject to Western blot analysis. Endogenous VEGF secreted by RPE 51 cells was also subject to Western blot analysis following concentration of the conditioned medium (1.5 ml) on heparin sepharose CL-6B (Amersham Pharmacia Biotech, Uppsala, Sweden) as previously described.[35] The enhanced chemiluminescence system (Amersham Pharmacia Biotech) was used to detect rat VEGF protein expressed from AdCMV.VEGF in conjunction with a rabbit polyclonal antibody raised against 1–191 amino acid of the human VEGF protein (Santa Cruz Biotechnology, Santa Cruz, CA).

HUVEC Proliferation Assay

Long Evans rat RPE cells were seeded in six-well plates at 10^6 cells/well. Recombinant adenovirus, AdCMV.βgal, and AdCMV.VEGF were added at a MOI of 10 and left for 18 hours. The media was then replaced and the cells left for an additional 48 hours. The Long Evans rat RPE cell conditioned media were collected, diluted 1:10, 1:100, or 1:1000 and then added to 24-hour ECGS-starved HUVEC cells plated in 96-well plates at 5×10^2 cells/well. Heparin was added at 100 μg/ml to potentiate VEGF and VEGF receptor interactions.[36] Seven days later, proliferation of the HUVEC cells was measured by incubating at 37°C in 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) 0.7 mg/ml for 4 hours. The cells were lysed in 20% sodium dodecyl sulfate in 50% dimethylformamide, pH 4.7, for several hours and the OD read at 570 nm.

Subretinal Injections

Pigmented and nonpigmented RCS/rdy and DA rats were used for subretinal injections. The animals were anesthetized by a mixture of ketamine (40 mg/kg) and xylazine (6 mg/kg) delivered intramuscularly. The eyes were further treated with topical amethocaine drops and the pupils dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride drops. The conjunctiva was cut close to the limbus to expose the sclera. A 30-gauge needle was used to make a shelving puncture of the sclera. A 32-gauge needle was then passed through this hole in a tangential direction under an operating microscope. For histology and fluorescein angiogram studies, 2 μl of AdCMV.VEGF (4×10^5, 4×10^6, 4×10^7, or 4×10^8 pfu/eye), AdRSV.βgal (8×10^8 or 2×10^8 pfu/eye), or AdCMV.GFP (4×10^7 pfu/eye) was delivered to the subretinal space. Immediately after the subretinal injection a circular bleb was usually observed under the operating microscope. The success of each subretinal injection was further confirmed by the observation of a partial retina detachment as seen by indirect ophthalmoscopy. The needle was kept in the subretinal space for 1 minute, withdrawn gently, and antibiotic ointment applied to the wound site.

Fluorescein Angiography

Fluorescein angiography was performed as previously described with an intraperitoneal injection of 10% sodium fluorescein (0.2 ml) and a modified Canon CF-60ZA retinal camera (Kawasaki, Kawagawa, Japan).[37] All animal procedures adhered to the Animal Use guidelines of the Association for Research in Vision and Ophthalmology.

In Situ *Hybridization*

For *in situ* hybridization, nonpigmented RCS/rdy rats were co-injected subretinally with AdCMV.VEGF (2×10^8 pfu/eye) and AdCMV.GFP (2×10^7 pfu/eye) which expresses green fluorescent protein when excited by UV light (490 nm). The purpose of using AdCMV.GFP was to help locate the injection site during sectioning. Four days later, the eyes were enucleated and snap-frozen in OCT compound (Sakura Fine Technical Co., Tokyo, Japan). Seven-micron sections were cut, fixed in 4% paraformaldehyde for 15 minutes, washed, dehydrated, then rehydrated through graded methanol/phosphate buffered saline steps and washed in phosphate buffered saline/0.1% Triton X-100. The sections were subject to Pronase E (100 μg/ml) treatment followed by DNase (20 U/ml) treatment, both for 15 minutes at 37°C. Postfixing in 4% paraformaldehyde for 10 minutes was followed by rinsing the slides in phosphate buffered saline/0.1% Triton X-100 and then 2 $\times$ SSC. The sections were acetylated in 0.1 mol/L triethanolamine with acetic anhydride for 10 minutes at room temperature. Following further rinsing in 2 $\times$ SSC, the sections were dehydrated through graded ethanol steps and air-dried. A digoxigenin RNA labeling kit (Roche Molecular Biochemicals, Mannheim, Germany) was used to generate VEGF RNA probes according to the manufacturer's instructions using a murine $VEGF_{164}$ cDNA as the template DNA. The RNA probes were incubated on the sections at a concentration of 500 ng/ml overnight at 50°C. Color detection was carried out using the digoxigenin color detection kit as according to instructions (Roche Molecular Biochemicals). Tissue sections were counterstained with methyl green and mounted.

Histology

For histology, eyes were enucleated and fixed in 4% paraformaldehyde for 4 hours and embedded in paraffin. Five-micron sections were cut and hematoxylin/eosin-stained for light microscopy.

Results

In Vitro *Characterization of AdCMV.VEGF*

AdCMV.VEGF was successfully produced by homologous recombination in 293 cells. Southern blot analysis of recombinant adenovirus DNA showed the correct restriction enzyme pattern for AdCMV.VEGF (data not shown). Northern blot analysis demonstrated that the recombinant adenovirus produced VEGF mRNA that could be detected as early as 6 hours after transduction (Figure 1A) with the expression increasing up to 72 hours. AdCMV.VEGF produced a VEGF mRNA of 804 bp, which distinguished it from the endogenously produced VEGF mRNA of approximately 3.7 kb. Endogenous VEGF mRNA could only be detected after extended autoradiograph exposure (data not shown).

AdCMV.VEGF produced large amounts of VEGF protein in human RPE 51 cells. Western blot analysis was unable to detect VEGF protein 24 hours post-transduction. However, by 48 hours a protein band of approximately 23 kd, corresponding to denatured $VEGF_{164}$, was observed, which increased in concentration up to 72 hours (Figure 1B). Endogenously produced VEGF could be demonstrated only in nontransduced RPE 51-conditioned medium after concentration of approximately 150-fold (Figure 1B).

Biological Activity of AdCMV.VEGF

To test the biological activity of VEGF produced from AdCMV.VEGF, a HUVEC proliferation assay was performed. HUVEC cells were sparsely plated in 96-well plates in the presence of 100 μg/ml of heparin but without any ECGS. The conditioned media from Long Evans rat RPE cells that had been transduced with AdCMV.VEGF and AdCMV.βgal (both MOI 10) were added to the HUVEC cells in increasing dilutions. Seven days later, the relative proliferation of HUVEC cells in the presence of exogenous and endogenous VEGF was determined. In conditioned media diluted 1:10, there was still sufficient endogenous VEGF and other growth factors produced by nontransduced and control virus AdCMV.βgal-trans-

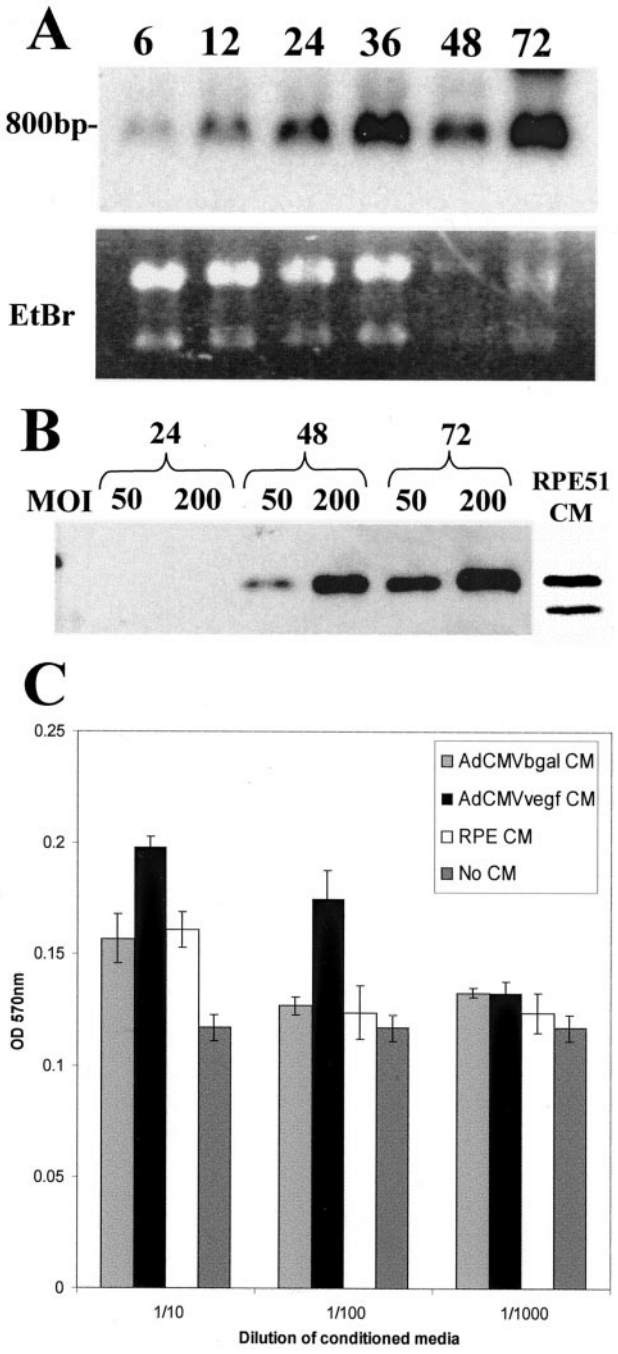

Figure 1. A: Northern blot analysis of rat $VEGF_{164}$ mRNA produced from AdCMV.VEGF-transduced human RPE 51 cells harvested at 6, 12, 24, 36, 48, and 72 hours post-transduction. AdCMV.VEGF mRNA was approximately 800 bp in size. Ethidium bromide staining showed equality and integrity of RNA loading. **B:** Western blot analysis of rat $VEGF_{164}$ produced from AdCMV-.VEGF-transduced human RPE 51 cells. Conditioned medium from human RPE 51 cells transduced at a MOI of either 50 or 200 with AdCMV.VEGF harvested at 24, 48, or 72 hours post-transduction. Ten microliters were subject to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and VEGF was visualized (23 kd) with a polyclonal anti-human VEGF antibody and detected using enhanced chemiluminescence. Endogenous VEGF produced by RPE 51 cells was also visualized after concentrating 1.5 ml of conditioned medium from nontransduced cells. The smaller endogenous VEGF molecule visible in RPE 51-conditioned medium is thought to be a nonglycosylated form of $VEGF_{164}$. **C:** HUVEC proliferation in response to conditioned media from AdCMV.βgal (MOI 10), AdCMV.VEGF (MOI 10), or non-transduced Long Evans RPE cells. "No CM" refers to fresh DMEM with heparin added. Error bars show SD from triplicate wells.

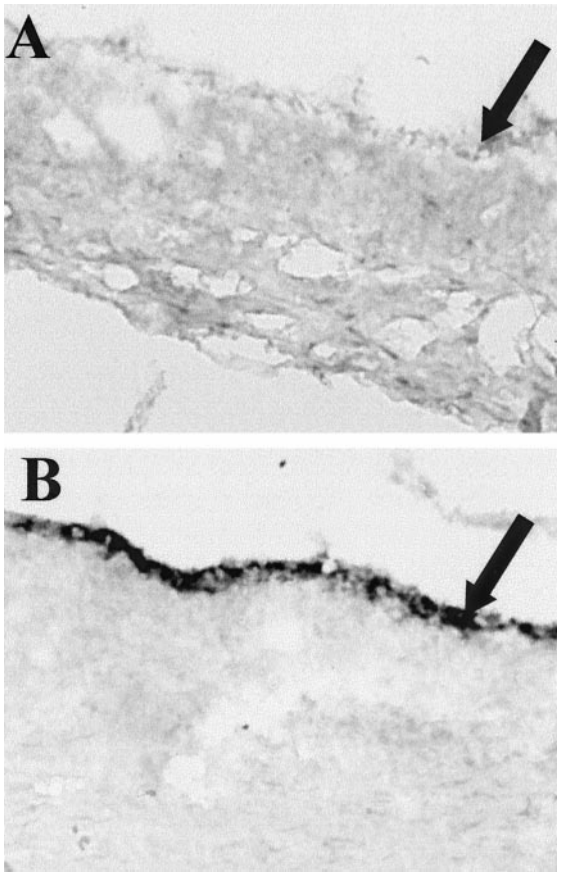

Figure 2. *In situ* hybridization of AdCMV.VEGF subretinal injection in RCS/rdy nonpigmented rat eye. Nonpigmented eyes were used to facilitate visualization of the chromogen. Area of retina away from (**A**) and close to (**B**) the injection site; original magnification, ×40. **Black arrow** shows RPE layer above the choroid and sclera.

duced Long Evans RPE cells to induce HUVEC cell proliferation compared to the nonconditioned medium control (Figure 1C). However, AdCMV.VEGF-transduced Long Evans rat RPE cells induced significantly more cell proliferation. At a 1:100 dilution, the effect of endogenous VEGF was no longer detectable, whereas AdCMV.VEGF-transduced Long Evans rat RPE cell-conditioned medium still exerted a proliferative effect (Figure 1C). However, at a dilution of 1:1000, there was no longer sufficient VEGF produced from Long Evans rat RPE cells transduced with AdCMV.VEGF at a MOI of 10 to induce HUVEC cell proliferation.

Expression of Recombinant VEGF mRNA in Vivo

$VEGF_{164}$ protein is normally secreted from the cell to exert its influence on vascular endothelial cells. To verify that it was the RPE cells that were overexpressing VEGF mRNA, *in situ* hybridization was performed 4 days postinjection of 2×10^8 pfu of AdCMV.VEGF into the eyes of nonpigmented RCS/rdy rats. VEGF mRNA was not detected in the RPE away from the injection area (Figure 2A). In contrast, very strong staining was seen in the RPE layer of AdCMV.VEGF injected eyes within the injection

Table 1. Leakage and CNV Scores of Eyes Subretinally Injected with Recombinant Adenovirus

Virus (pfu/eye)	Eye	Leakage				CNV
		Day 10	Day 17	Day 24	Day 31	Day 80
AdCMV.GFP (4×10^7)	1	0	0	−	0	0
AdRSV.βgal (2×10^8)	1	0	0	−	0*	0†
AdCMV.VEGF (4×10^5)	1	ND	ND	ND	ND	0/RD
	2	0	−	−	+	+
	3	0	−	−	0*	0
	4	0	−	−	+	0
AdCMV.VEGF (4×10^6)	1	+	−	−	+	+
	2	+	−	−	+	++
	3	+	+	+	++	+†
	4	+	++	++	++	+
AdCMV.VEGF (4×10^7)	1	ND	+	+	+	++
	2	ND	ND	ND	ND	++
	3	+	+	+	++	++
	4	+	ND	++	+++	++
AdCMV.VEGF (4×10^8)	1	++	++	+++	+++*	ND
	2	ND	ND	ND	ND	++/RD
	3	+	++	++	++	++†
	4	+	++	++	++	++

Eyes injected on day 0 with viruses were followed using fluorescein angiography for 31 days. Leakage was scored as 0, no leakage; +, small area of leakage; ++, medium-sized area of leakage; and +++, large areas of leakage. Several eyes were followed for 56 days post-injection with no change in the leakage scores. −, No angiograms performed. ND, no data on these eyes at these time points due to corneal haziness or hemorrhaging. CNV scores of each eye were graded as follows: 0, small local scar around injection site with no sign of new blood vessels; +, discrete and localized CNV membrane with visible new blood vessels; ++, larger and multifocal areas of CNV corresponding to large areas of photoreceptor degeneration. RD refers to eyes that showed evidence of unrelated retinal degeneration.

*Fluorescein angiograms of these eyes shown in Figure 3.

†Histology of these eyes is shown in Figure 4.

area (Figure 2B). This is in agreement with previous work showing that adenovirus preferentially transduces rat RPE cells *in vivo* when delivered subretinally.[24] The inner nuclear layer showed very low level staining, indicative of endogenous VEGF mRNA expression (data not shown). It was also noted that the occasional cells in the neural retina, possibly Müller cells, stained strongly for VEGF mRNA, suggesting that adenovirus transduction of these cells had occurred (data not shown).

Detection of Vascular Leakage by Fluorescein Angiography

To determine whether overexpression of VEGF in the RPE had a vasopermeability effect on blood vessels, fluorescein angiograms used to detect vascular leakage. Sixteen eyes of pigmented RCS/rdy rats were subject to subretinal injection with AdCMV.VEGF. Four eyes were each injected with 4×10^5, 4×10^6, 4×10^7 or 4×10^8 pfu. Two eyes were injected with control viruses, AdRSV.βgal (2×10^8 pfu) and AdCMV.GFP (4×10^7 pfu). Fluorescein angiography was used to determine the extent of vascular leakage. All animals were observed 10 and 31 days postinjection; certain individual animals were observed more frequently, as documented in Table 1. The three AdCMV.VEGF (4×10^8 pfu)-injected eyes were followed for 56 days postinjection and the AdRSV.βgal-injected eye for 80 days postinjection; however, there was no change to their day 31 leakage scores. It was difficult to determine leakage in three eyes due to hemorrhaging or corneal haziness after the subretinal injection, and they were totally excluded from Table 1. The results summarized in Table 1 show that, in general, as the amount of AdCMV.VEGF injected increased, the size of the vascular leakage area and the number of leaky areas also increased. Inconsistencies in Table 1 were attributed to the technical difficulty of subretinal injections, resulting in some variability. A fluorescein angiogram representative of a control phosphate buffered saline-injected eye and a virus control AdRSV.βgal-injected eye is shown (Figure 3, A and B). The effect of AdCMV.VEGF at the four different dilutions, 4×10^5 (Figure 3C), 4×10^6 (Figure 3D), 4×10^7 (Figure 3E), and 4×10^8 pfu (Figure 3F), representing the four leakage scores, are also shown. Areas of intense white indicate fluorescein leakage and are shown by the arrows. The leaky area at 4×10^6 pfu (Figure 3D) was small and localized to the area surrounding the injection site, whereas the higher titer injections show leakage in the circular pattern of the bleb caused during the injection (Figure 3F).

Histological Analysis of AdCMV.VEGF-Injected Eyes

To determine whether the leakage detected by fluorescein angiography preceded new blood vessel formation and was not due only to leakage from existing blood vessels, eyes were harvested 80 days postinjection and analyzed by histology. Normal rat retina is shown in Figure 4, A and B. Eyes injected with high titers (8×10^8 pfu) of the control virus, AdRSV.βgal, showed no change except a small area around the injection site. The injection site was identified by sclera damage from the needle

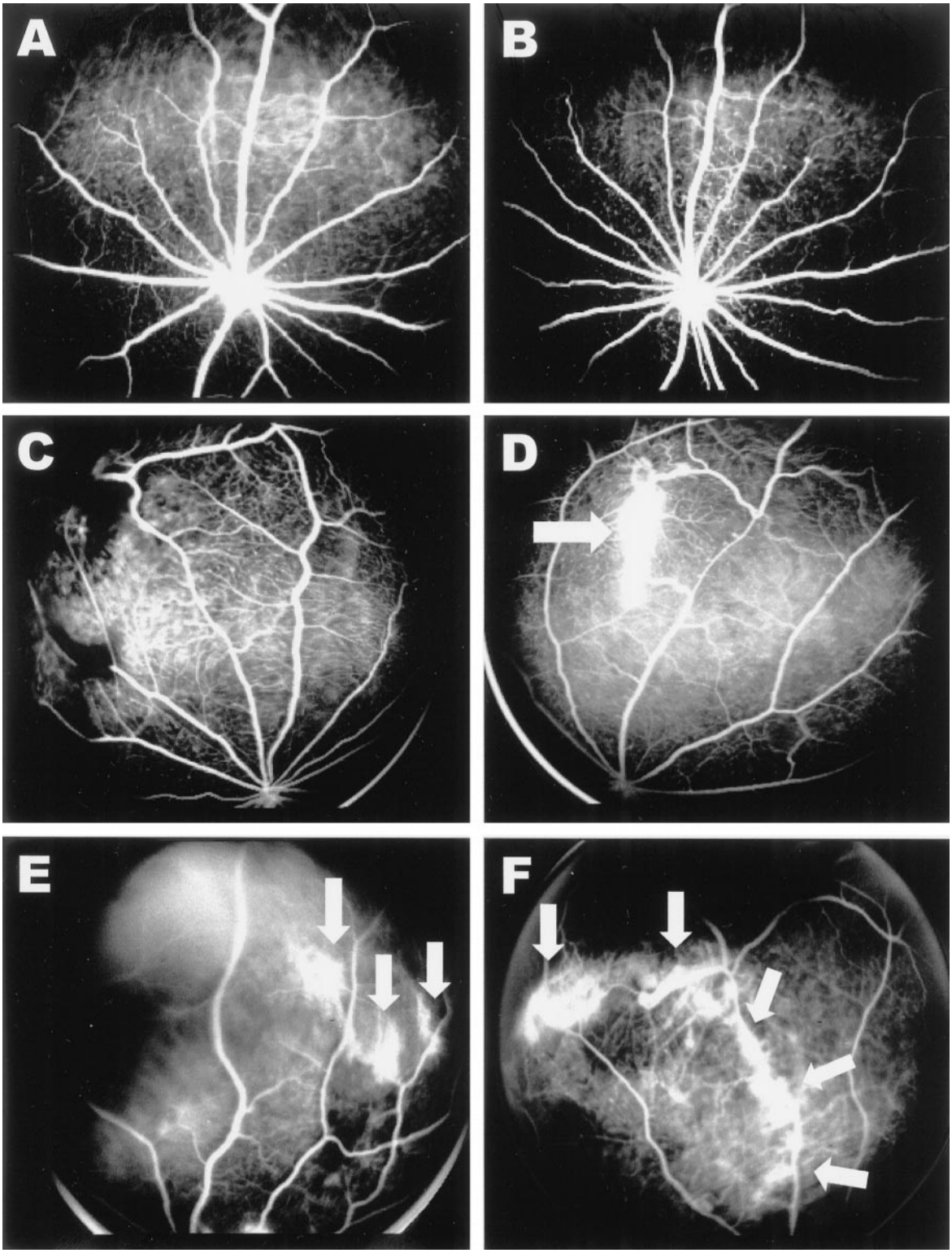

Figure 3. Fluorescein angiograms 31 days post-subretinal injection with recombinant adenovirus. **A:** Control PBS injection. **B:** AdRSV.βgal (2×10^8 pfu). **C–F:** AdCMV.VEGF injected eyes and leakage scores: 4×10^5 pfu, 0 (**C**); 4×10^6 pfu, + (**D**); 4×10^7 pfu, ++ (**E**); 4×10^8 pfu, +++ (**F**). **Arrows** indicate areas of vascular leakage.

insertion (low magnification, Figure 4C, black arrows). The detachment of neural retina from the RPE cell layer on both sides of the scar was caused during fixation and, provided all cell layers were present, was considered normal. There were no major morphological changes after the injection of AdRSV.βgal except for a small area of photoreceptor cell loss directly above the needle insertion site (Figure 4C, white arrows). Higher magnifica-

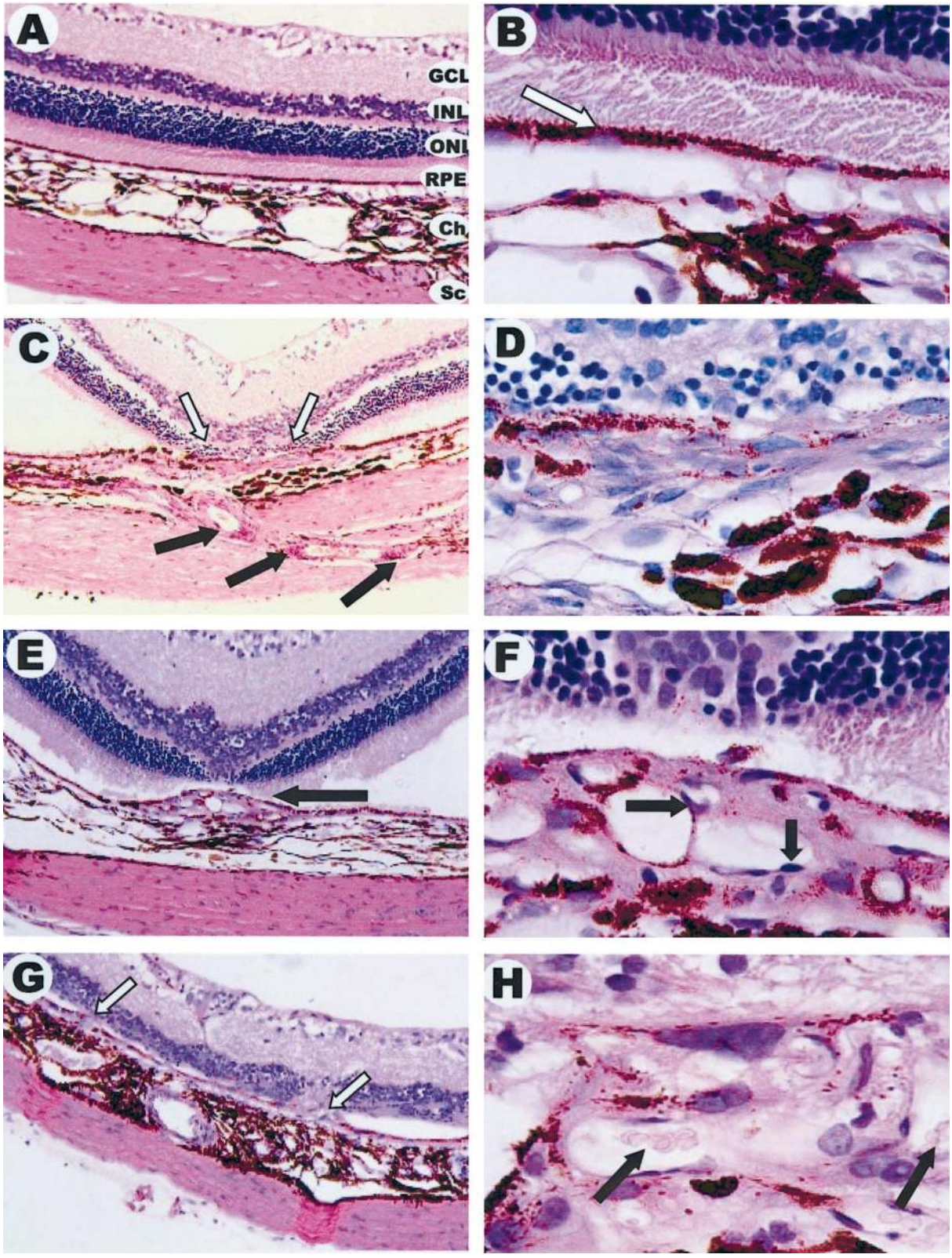

Figure 4. Histology of eyes 80 days post-subretinal injection with recombinant adenovirus. Five-micron sections of paraffin-embedded eyes were stained with hematoxylin and eosin. **A:** Normal rat retina (×20). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer (containing photoreceptor cells); RPE, retinal pigment epithelium; Ch, choroid; Sc, sclera. **B:** Normal retina (×100); **arrow** indicates pigmented RPE layer. **C** (×20) and **D** (×100): AdRSV.βgal (8×10^8 pfu). **Black arrows** indicate injection scar and **white arrows** missing photoreceptor cells: **E** (×20) and **F** (×100): AdCMV.VEGF (4×10^6 pfu). **Arrow** in **E** shows CNVM, and **arrows** in **F** shows endothelial cell nuclei. **G** (×20) and **H** (×100): AdCMV.VEGF (4×10^8 pfu); **arrows** in **G** show infiltrating choroidal blood vessels and proliferating RPE cells, and **arrow** in **H** show erythrocytes present in infiltrating blood vessel with total RPE disruption.

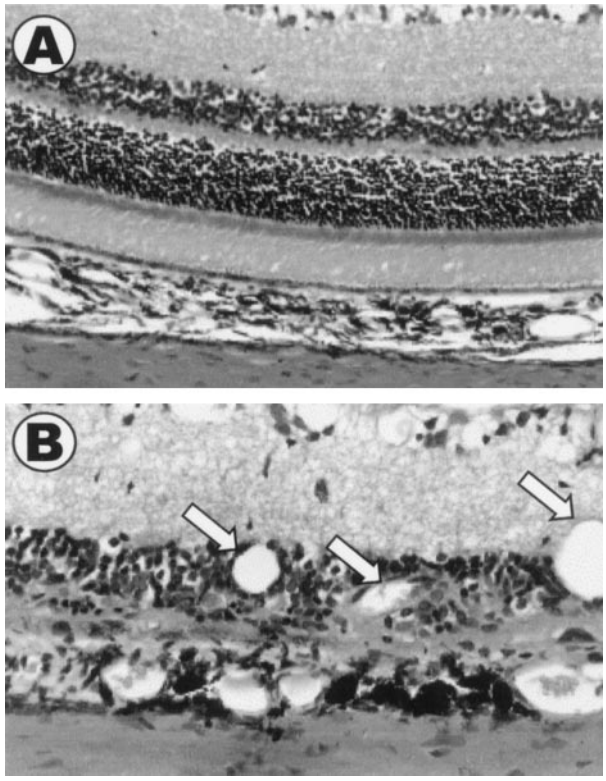

Figure 5. Histology of normal retina (**A**) and AdCMV.VEGF (4×10^7 pfu)-injected eye (**B**) 80 days postinjection showing retinal vessel dilation. Three dialted blood vessels (**white arrows**) can been seen in the inner nuclear layer. The RPE is disorganized and the outer nuclear layer has degenerated. Original magnification, ×20.

tion shows the small nonvascular scar that had formed, consisted of several layers of RPE cells with reduced pigmentation (Figure 4D). The appearance of nonvascular scar tissue observed in AdRSV.βgal-injected eyes was in strong contrast to eyes injected with AdCMV-.VEGF. Of the 15 eyes injected with AdCMV.VEGF that were examined, 12 showed some degree of CNV. Three eyes injected with the lowest concentration of AdCMV-.VEGF showed no evidence of new blood vessels. Two of the examined eyes also showed evidence of severe retinal degeneration thought to be a result of the injection trauma. A CNV score for each eye is shown in Table 1 along with the corresponding leakage score. At relatively low titers of 4×10^5 and 4×10^6 pfu, highly vascularized CNVMs were observed with overlying photoreceptor cell loss (Figure 4E, black arrow). These CNVMs were located away from the needle penetration site, as indicated by no visible scleral damage. Under higher magnification, the nuclei of vascular endothelial cells were clearly visible within the CNVM (Figure 4F, black arrows). The higher titers of AdCMV.VEGF (4×10^7 and 4×10^8 pfu/eye) caused major disruptions to the RPE and photoreceptor layers of the retina (Figure 4G and 4H; 4×10^8 pfu/eye). Many new blood vessels (Figure 4G, white arrows) and their erythrocytes (Figure 4H, 4×10^8 pfu, black arrow) can be seen extending from the choroid toward the neural retina surrounded by proliferating RPE cells. The inner nuclear layer in the more severe cases were also showing signs of degeneration. At the higher titers of AdCMV-.VEGF it was not unusual to see large blood vessels in the inner nuclear layer, suggesting that retinal vessel dilation had also occurred (Figure 5, white arrows).

Discussion

VEGF is a potent endothelial cell mitogen and angiogenic factor. In the present study, overexpression of VEGF in the RPE is shown to induce vascular leakage, new choroidal blood vessel growth, the development of CNVMs, and neural retina degeneration. These results are in contrast to experiments that used the slow release of bioactive VEGF protein from pellets implanted between the choroid and sclera.[22] The lack of CNV or vascular leakage in these experiments may mean that the development of CNV requires VEGF to be secreted from the RPE toward the inner choroid where high levels of VEGF receptors have been found.[20] This could create a chemotactic gradient for the proliferating and migrating vascular endothelial cells.[38] This may also explain why transgenic mice expressing VEGF in photoreceptor cells develop subretinal neovascularization of retinal vasculature origin and not from the choroid.[39,40] In these mice VEGF secreted from the photoreceptor cells may be unable to pass through the outer blood-retinal barrier to exert any effect on the choroid.[40]

Alternatively, the needle puncture of Bruch's membrane, which was required in this study to gain access to the subretinal space, could play a role in the development of the observed CNV. It has long been proposed that compromise of the intact barrier between the retina and choroid can lead to CNV.[41] Although it is a rare complication, choroidal blood vessels are known to grow through induced breaks in Bruch's membrane after surgery for retinal detachment.[42,43] However, the frequency and severity of CNV observed in this study was much greater than that reported in surgical cases. In addition, vascular leakage and CNV extended far beyond the initial puncture site, whereas no evidence of CNV was observed in any control virus injections. This data also show that the amount of AdCMV.VEGF injected correlates to the extent of the resulting CNV. Nonetheless, using this experimental approach, we cannot completely discount the importance of rupturing Bruch's membrane. The development of a suitable transgenic model or a less invasive injection route may help clarify this point.

In this study, recombinant adenovirus-mediated delivery of VEGF to the subretinal space resulted in the overexpression of VEGF in the RPE. This is in agreement with previous studies showing that subretinal mediated delivery of adenovirus vectors leads to transduction of the RPE.[24–27] The duration of VEGF expression in this study was likely to have been short-term, as transgene expression in the RPE from subretinally delivered adenovirus vectors in immunocompetent hosts is generally transient in nature. It has been shown previously that by 3 to 4 weeks postinjection, most transgene expression from subretinally delivered recombinant adenovirus, as determined histologically, has disappeared,[24,27,44] although individual positive cells can be detected for longer.[25]

Thus, the generation of CNV in this model required only short-term expression of VEGF in RPE cells, which was sufficient to induce permanent vascular structures at 80 days postinjection.

Previous experiments have shown that sustained intravitreal delivery of VEGF in animal models can cause widespread retinal vascular dilation.[45] Similarly, it was observed in this study that eyes subretinally injected with the higher titers of AdCMV.VEGF showed dilation of retinal blood vessels in addition to CNV. One explanation is that neural retina cells were transduced with AdCMV.VEGF, leading to retinal expression of VEGF. This is supported by the *in situ* hybridization results that showed strong VEGF mRNA expression 4 days postinjection in the occasional neural retina cell. It has been noted previously that subretinal injection can lead to transgene expression in Müller cells.[24,27] Alternatively, retinal blood vessel dilation could be the result of VEGF secreted in an apical direction from RPE cells. Apical secretion of VEGF does occur in RPE cells, albeit at a lower level than basal secretion.[20] It remains to be determined whether the RPE cells transduced with AdCMV.VEGF and producing large amounts of VEGF are able to maintain the normal apical/basal expression ratio.

Although we showed that overexpression of VEGF in RPE cells is sufficient to induce CNV in the rat, we cannot exclude a role for other growth factors in the pathogenesis of human CNV. In addition to VEGF, transforming growth factor-β (TGF-β), aFGF, and bFGF have also been localized to human CNVMs, and it has been proposed that bFGF and aFGF, which contain no signal sequence for secretion, can be released from damaged RPE cells.[4] Recent studies have suggested that VEGF and bFGF act synergistically on vascular endothelial cell proliferation,[46,47] and it has been observed that VEGF isoforms containing exon 6 can release bioactive bFGF that had been sequestered by the extracellular matrix.[48] Although VEGF may have a primary role in the development of human CNV, it likely does so in concert with bFGF and other growth factors.

Our model relies on the sudden and strong overexpression of VEGF that is unlikely to occur in the development of human CNV disease. Instead, in age-related macular degeneration patients, it is thought that the accumulated changes associated with aging gradually result in conditions suitable for CNV development. It has been suggested that chronic ischemia is one such condition, perhaps as a result of poor choroidal circulation.[49] Ischemia could also potentially result from large drusen deposits becoming confluent, which is known to increase the risk of vascular invasion.[2] Along with *in vitro* evidence showing VEGF can be up-regulated under hypoxic conditions,[13] this has led to the proposal that CNV development is the result of RPE cells up-regulating VEGF expression due to an ischemic stimulus.[50] Our results lend support to this hypothesis by showing that VEGF overexpression in the RPE is sufficient to induce CNV and, as such, is an ideal candidate for targeted anti-angiogenic gene therapy in the eye.

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