AAV–Mediated Expression of Vascular Endothelial Growth Factor Induces Choroidal Neovascularization in Rat

Fei Wang, Katherine G. Rendahl, William C. Manning, Dulce Quiroz, Mazie Coyne, and Sheldon S. Miller

PURPOSE. To develop a small-animal model of choroidal neovascularization (CNV) by injecting adeno-associated virus (AAV)-VEGF into the subretinal space (SRS) of rats.

METHODS. An adeno-associated viral vector encoding human VEGF165 was injected into the subretinal space (SRS) of Sprague-Dawley or Long Evans rats. Expression of VEGF was identified by RT-PCR and immunohistochemistry. Physiological and pathologic changes in the retina and choroid were evaluated by electroretinography, fluorescein angiography, light microscopy, and three-dimensional reconstruction of serial sections.

RESULTS. Green fluorescent protein (GFP) and VEGF were expressed for at least 20 months in the retina and retinal pigment epithelium (RPE). Histologic sections showed extensive subretinal neovascularization, degenerating photoreceptors, and proliferating RPE at 5 weeks to 20 months after injection of AAV-VEGF. At 2 to 12 months after injection, leaking blood vessels were detected by fluorescein angiography. Electroretinogram a- and b-wave amplitudes were significantly decreased during this time. Three-dimensional reconstruction of serial sections demonstrated that choroidal blood vessels penetrated Bruch’s membrane, one of them splitting into three branches in the SRS. In the current model, CNV was produced in 95% of the animals tested (19/20). It persisted for more than 20 months, a necessary requirement for modeling the development of CNV in age-related macular degeneration (AMD).

CONCLUSIONS. In this study, a highly reproducible animal model of long-lasting CNV was developed. This model is being used to test antiangiogenic molecules to reduce or inhibit CNV and could be extended to primates. (Invest Ophtalmol Vis Sci. 2003;44:781–790) DOI:10.1167/iovs.02-0281

A ge-related macular degeneration (AMD) is a leading cause of vision loss that affects more than 11 million people in the United States. Over 1.6 million of these people, age 50 and older, experience significant vision loss in late AMD. In the exudative form of AMD, choroidal blood vessels grow through Bruch’s membrane into the sub-RPE space and extend into the subretinal space (SRS). This choroidal neovascularization (CNV) may lead to the accumulation of serum or blood beneath the RPE or retina, the detachment of RPE or retina, and the formation of disciform scars.

The pathogenesis of CNV is poorly understood, but several growth factors have been identified in choroidal neovascular membranes surgically excised from patients with AMD, including basic fibroblast growth factor (bFGF), transforming growth factor(TGF)-β, and vascular endothelial growth factor (VEGF). In RPE of macaque with AMD, expression of VEGF is significantly increased, suggesting a major role for RPE and VEGF in AMD. In the laser model of CNV in monkeys and rats, VEGF expression correlated with the development of CNV. In addition, inhibition of VEGF signaling by kinase inhibitors dramatically prevented CNV in a laser-induced murine model. These data suggest that VEGF is a major stimulator of CNV.

VEGF is an endothelial cell–specific mitogen that was originally identified as vascular permeability factor (VPF). VPF and VEGF are splice variants from the same gene (165 amino acids [aa] for VEGF and 189 aa for VPF; the other variants are 121, 145, and 209 aa). VEGF mRNA is found in cultured human RPE cells. Protein is present in cell lysate and conditioned medium. In human fetal RPE native tissue, we have confirmed the presence of VEGF and its three receptors (Flt1, KDR, and neuropilin-1). There are data to suggest that receptor activation by VEGF causes RPE cell proliferation and migration. In this process, RPE tight junctional integrity and physiology are probably also altered, suggesting that VEGF could facilitate penetration of RPE by choroidal blood vessels.

Adeno-associated virus (AAV) has been a valuable vector for gene transfer. A major attraction of AAV is that it mediates long-term expression in nondoning cells. There was little or no evidence of cytotoxic or host immune response in a phase I clinical trial of AAV vectors. Over the past several years, recombinant AAV vectors have been extensively used in retina and other systems to deliver a variety of transgenes with little or no toxicity and inflammation. It has been shown in mice and rats that AAV can mediate efficient and long-term delivery of LacZ, green fluorescent protein (GFP), or other transgenes to the RPE.

In vivo, neovascularization can be induced by overexpressing VEGF121 or VEGF165, using a replication-deficient recombinant adenovirus vector or an AAV vector. These experiments suggested that a relevant animal model for CNV could be produced by sustained overexpression of VEGF in the RPE. The lack of such a model hampers our understanding of the pathogenesis of such diseases as AMD, and consequently we sought to develop an animal model of CNV by using AAV to overexpress VEGF in the RPE over a long period.
The titers were: AAV-VEGF, 1.0 × 10^13 particles/mL; AAV-GFP, 1.0 × 10^13 particles/mL. AAV was purified by precipitation with polyethylene glycol. The rAAV vector was concentrated in 50 mM NaCl and 50 mM HEPES (pH 7.4). The resultant construct (pD10-VEGF165) contains the CMV immediate-early promoter plus enhancer, CMV intron A, VEGF coding region, bovine amebocyte lysate (Associates of Cape Cod, Inc., Woods Hole, MA) and mouse moloney murine leukemia virus long terminal repeat sequences. Human VEGF 165 cDNAs were cloned from the pCR Blunt II TOPO vector into the pD10-CMV rAAV vector as an EcoRI fragment. The resultant construct (pD10-VEGF165) contains the CMV immediate-early promoter plus enhancer, CMV intron A, VEGF coding region, bovine growth hormone polya A site, and AAV inverted terminal repeat (ITR) sequences.

**Construction of rAAV Vectors and rAAV Production**

Human VEGF165 cDNAs were cloned from the pCR Blunt II TOPO vector into the pD10-CMV rAAV vector as an EcoRI fragment. The resultant construct (pD10-VEGF165) contains the CMV immediate-early promoter plus enhancer, CMV intron A, VEGF coding region, bovine growth hormone polyA site, and AAV inverted terminal repeat (ITR) sequences.

rAAV vector particles were produced by a triple transfection protocol. rAAV was purified from total cell lysates by heparin column chromatography. Fractions containing virus were pooled and particles concentrated by precipitation with polyethylene glycol. The rAAV pellet was suspended in 150 mM NaCl and 50 mM HEPES (pH 7.4). Viral titers were determined by DNase-resistant physical particle assay. The titers were: AAV-VEGF, 6 × 10^13 particles/mL; AAV-GFP, 1.0 × 10^13 particles/mL. Bacterial endotoxin levels were assayed using Limulus amebocyte lysate (Associates of Cape Cod, Inc., Woods Hole, MA) and were below 5 EU/mL for all vector preparations. Assuming a mean body weight of 0.25 kg and a total of 4 μL injected AAV, the animals received only 0.08 EU/kg, far below the 5.0-EU/kg limit for human use.

**Subretinal Injection**

SD (albino) or LE (pigmented) rats were injected with AAV-VEGF at the ages of P15 to P17, as previously described. Briefly, wild-type animals were anesthetized by ketamine-xylazine injection, and a local anesthetic (proparacaine HCl) was applied topically to the cornea. The pupils were dilated with 1% atropine sulfate and 2.5% phenylephrine hydrochloride. Under the microscope, a small hole was made in the medial sclera, just behind the ora serrata, with a 28-gauge needle. The subretinal injections of 2 μL of AAV-VEGF were made by inserting a blunt 32-gauge needle (Hamilton, Reno, NV) through the sclera, vitreous, and retina and delivering the rAAV suspension into the subretinal space in the superior retina. The contralateral eye was either not injected or injected subretinally with PBS or AAV-GFP. Subretinal blebs were formed in the SRS and disappeared within 24 hours as the fluid was absorbed. No choroidal bleeding was observed during and after subretinal injection, indicating that the integrity of Bruch’s membrane was not compromised.

**Immunohistochemistry**

Rats were killed by overdose of carbon dioxide inhalation and the eyes enucleated. After the cornea and lens were removed, the posterior eyecups (retina-RPE-choroid-sclera) were fixed in 4% formaldehyde for 1 hour. After three 5-minute washes in PBS, the eyecups were cryoprotected in 30% sucrose overnight at 4°C and embedded in optimal cutting temperature (OCT) compound at −20°C. Sections (15 μm) were cut on a cryostat, placed on glass slides, and allowed to dry overnight. Sections were permeabilized with 0.3% Triton X-100 (PBS) for 10 minutes and processed for fluorescent or enzymatic methods.

For fluorescence microscopy, sections were incubated in blockerm (1% BSA, 1% FBS, and 0.3% Triton X-100 in PBS [pH 7.4]) for 30 minutes, followed by 2 hours in 10 μg/mL goat anti-human VEGF primary antibody (R&D Systems, Minneapolis, MN) diluted in blocking solution. After three washes with the blocking solution, sections were incubated with 5 μg/mL fluorescein-conjugated rabbit anti-goat secondary antibodies (Alexa Fluor 488 or 546; Molecular Probes, Eugene, OR). Sections were washed six times in PBS, covered with antifade mounting medium (ProLong; Molecular Probes, Eugene, OR) to prevent photobleaching, and mounted with a coverslip. Fluorescent images were obtained with a microscope (Axioptoph; Carl Zeiss, Thornwood, NY) equipped with a charge-coupled device (CCD) camera and processed with image-management software (Photoshop 6.0; Adobe, San Jose, CA).

For enzymatic detection, sections were processed with a customized kit (Histostain-SP, Zymed, S. San Francisco, CA), according to the instructions. Briefly, sections were treated with a peroxidase blocking agent (PerOxo-Block; Zymed) to eliminate endogenous peroxidase activity and blocked with 10% nonimmune rabbit serum to reduce nonspecific binding. After three washes with 0.3% Triton X-100 in PBS, sections were incubated with the same primary antibody as earlier for 1 hour at room temperature. After three washes, sections were incubated with biotinylated rabbit anti-goat secondary antibody for 10 minutes, followed by incubation with a streptavidin-peroxidase conjugate. Sections were incubated with substrate-chromogen solution (hydrogen peroxide-diaminobenzidine [DAB]), counterstained with hematoxylin, dehydrated with a graded series of alcohol, cleared in xylene, and mounted (Histomount; Zymed).

**Fluorescein Angiography and GFP Expression**

**In Vivo**

Rats were anesthetized, the eyes were dilated as earlier and positioned in a special imaging setup, and fundus images were obtained with a charge-coupled device (CCD) camera coupled to a dissection microscope. Sodium fluorescein (0.1 mL 10%) was injected intraperitoneal-ly and fluorescein angiograms were taken at 5-second intervals. GFP expression in vivo was imaged with a similar procedure.

**Electroretinograms**

Rats were dark adapted overnight and anesthetized, and the eyes were dilated as described earlier. Silver chloride, wire-loop electrodes were placed on the cornea of both eyes, and reference electrodes were placed subcutaneously under each eye and on the tail. Full-field scotopic ERGs were elicited with 10-μs flashes of white light, and responses were recorded with a visual electrodagnostic system.
Stimuli were presented at intensities of 0.173, 1.896, and 3.886 log cd sec/m² at 15-second, 30-second, and 1-minute intervals. The a-wave amplitudes were measured from the baseline to the peak in the corneal-negative direction, and the b-wave amplitudes were measured from the corneal-negative peak to the major corneal-positive peak. Four responses at each intensity were averaged to reduce noise and data were computer analyzed (Excel; Microsoft, Redmond, WA).

**Histology**

As previously described,⁵⁰ rats were killed by overdose of carbon dioxide inhalation and immediately perfused intracardially with 2% formaldehyde and 2.5% glutaraldehyde in PBS (pH 7.4). Heads were removed and immersed in the same fixative overnight at 4°C. Eyes were then enucleated and bisected along the vertical meridian through the optic nerve head. Eye hemispheres were osmicated, dehydrated,
and embedded in Epon-Araldite resin. Serial sections (1.5 μm) were cut along the vertical meridian of the eye and stained with 1% toluidine blue in Tris-buffered saline (TBS). Images were obtained with the same microscope described earlier.

**Three-Dimensional Reconstruction of CNV**

Contours of structures (blood vessels, RPE, Bruch’s membrane) on each of the 32 serial sections were digitized with a motorized microscope equipped with a miniature monitor for high-resolution images (Lucivid; MicroBrightField, Colchester, VT) and were stacked by computer (Neurolucida software; MicroBrightField), according to the instruction manual. Three-dimensional (3-D) images were created from stacks of contours from selected sections using the 3-D solid rendering module of a software program (NeuroExplorer; MicroBrightField).

**Statistics**

Results are expressed as the mean ± SEM unless otherwise indicated. Statistical significance was calculated using the Student’s t-test. Given the relatively small sample size, we also used the nonparametric, Mann-Whitney test for statistical comparisons of unpaired data. P < 0.05 is regarded as significant in all comparisons.

**RESULTS**

**AAV-Mediated VEGF Expression In Vivo**

We injected 2 μL (1.2 × 10¹¹ particles) of AAV-VEGF into the subretinal space of SD rats to test the hypothesis that VEGF overexpression in RPE could generate CNV. AAV-GFP or PBS was injected into the contralateral eye as a control. Figure 1A shows a fundus image of an eye injected 15 months earlier with AAV-GFP. Figure 1B shows that the GFP expression in this eye extended over approximately one third of the distal retina. Similar results were obtained in six other animals at 2 to 17 months after injection (see Table 1). Two of these rats were examined at both 12 and 15 months. In addition, expression of GFP was measured in vitro in AAV-GFP injected eyes in two SD rats at 12 months (Table 1).

RT-PCR with human VEGF primers was used to distinguish between endogenous rat VEGF and human VEGF₁₆₅ that was overexpressed in rat ocular tissue. These experiments used retina and RPE-choroid from the rat eyes that had been injected 15 months earlier with AAV-VEGF or PBS. In Figure 1C, lanes 1 and 2 contain bands amplified from rat retina (Ret) and RPE-choroid (RPE) mRNA obtained from the AAV-VEGF-injected eye. In both lanes, the 655-bp band corresponds to human VEGF₁₆₅ and was confirmed by cloning and sequencing the PCR product. No band was detected in the retina (Fig. 1C, lane 3) and RPE-choroid (Fig. 1C, lane 4) from a PBS-injected eye. The 523-bp band corresponds to VEGF₁₂₁ (confirmed by cloning and sequencing). Similar results were obtained from two other animals at 17 months after injection (see Table 1).

Using two different protocols, Figure 1D–G shows VEGF expression in SD rat eyes injected 15 months earlier with AAV-VEGF or PBS control. Some VEGF expression was present in the ganglion cell layer of the PBS control eye (GCL; Fig. 1D) and in the choroid, but not in the RPE (Fig. 1D). In contrast, extensive staining was observed in the RPE and choroid and some staining in the GCL, as well (Fig. 1E; AAV-VEGF). Subretinal newvascularization was also present (Fig. 1E, arrows) and indicates that the section was from the AAV-VEGF injected area. A fluorescence-detection protocol showed that VEGF was also expressed in the RPE of an AAV-VEGF-injected eye (Fig. 1G), but not in the PBS control (Fig. 1F). VEGF staining in the choroid of both the control and AAV-VEGF–injected eyes is consistent with endogenous VEGF expression by endothelial cells, because the anti-human VEGF antibody cross-reacts with rat VEGF. Practically identical results were obtained in three other animals at 12 months after injection (see Table 1).

An inverse correlation between pigmentation and incidence of AMD has been reported.⁵¹⁵² Although this conclusion is controversial,⁵³ we also examined expression of VEGF in pigmented LE rats. Figure 2 illustrates a similar pattern of expression of VEGF in a LE (pigmented) rat 20 months after co-injection of AAV-VEGF and AAV-GFP in one eye and PBS injection in the control eye. VEGF expression was observed in AAV-VEGF–injected eye using enzymatic (Fig. 2B) and fluorescence (Fig. 2D) protocols, but not in the PBS-injected eye (Fig. 2A). The arrows in Fig 2B show proliferating RPE that expressed VEGF. Figure 2C (same eye as in Figs. 2B, 2D) shows expression of GFP in the AAV-VEGF and AAV-GFP co-injected eye. GFP expression in photoreceptors was also observed (not shown).

**VEGF-Induced Physiological Changes**

ERGs were used to evaluate physiological changes in the eye after AAV-VEGF or after control injections of PBS or AAV-GFP. Figure 3A summarizes ERG data from an animal previously injected eye using enzymatic (Fig. 2B) and fluorescence-detection protocol showed that VEGF was also expressed in the RPE of an AAV-VEGF–injected eye (Fig. 1G), but not in the PBS control (Fig. 1F). VEGF staining in the choroid of both the control and AAV-VEGF–injected eyes is consistent with endogenous VEGF expression by endothelial cells, because the anti-human VEGF antibody cross-reacts with rat VEGF. Practically identical results were obtained in three other animals at 12 months after injection (see Table 1).

An inverse correlation between pigmentation and incidence of AMD has been reported.⁵¹⁵² Although this conclusion is controversial,⁵³ we also examined expression of VEGF in pigmented LE rats. Figure 2 illustrates a similar pattern of expression of VEGF in a LE (pigmented) rat 20 months after co-injection of AAV-VEGF and AAV-GFP in one eye and PBS injection in the control eye. VEGF expression was observed in AAV-VEGF–injected eye using enzymatic (Fig. 2B) and fluorescence (Fig. 2D) protocols, but not in the PBS-injected eye (Fig. 2A). The arrows in Fig 2B show proliferating RPE that expressed VEGF. Figure 2C (same eye as in Figs. 2B, 2D) shows expression of GFP in the AAV-VEGF and AAV-GFP co-injected eye. GFP expression in photoreceptors was also observed (not shown).

**Table 1. VEGF (or GFP) Expression Induced by AAV-VEGF (or AAV-GFP)**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time (n)</th>
<th>Eye</th>
<th>Injection</th>
<th>Results (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR for VEGF</td>
<td>15 mo</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td>17 mo</td>
<td>L</td>
<td>AAV-GFP or PBS</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Immunohistochemical staining for VEGF</td>
<td>12 mo</td>
<td>L</td>
<td>Uninjected or AAV-GFP</td>
<td>GFP (2)</td>
</tr>
<tr>
<td></td>
<td>15 mo</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>IH (2), IF (3)</td>
</tr>
<tr>
<td></td>
<td>20 mo</td>
<td>L</td>
<td>AAV-VEGF + AAV-GFP</td>
<td>IH, IF, GFP</td>
</tr>
<tr>
<td>GFP expression in vivo</td>
<td>2 mo</td>
<td>L</td>
<td>AAV-GFP</td>
<td>GFP</td>
</tr>
<tr>
<td></td>
<td>12 mo</td>
<td>L</td>
<td>Uninjected or AAV-GFP</td>
<td>GFP (5)</td>
</tr>
<tr>
<td></td>
<td>15 mo</td>
<td>L</td>
<td>Uninjected or AAV-GFP</td>
<td>GFP (2)</td>
</tr>
<tr>
<td></td>
<td>17 mo</td>
<td>L</td>
<td>AAV-GFP</td>
<td>GFP</td>
</tr>
</tbody>
</table>

The first column lists three experimental protocols that were used to assay VEGF (or GFP) expression. At various times after injection (second column), expression was examined with RT-PCR, immunohistochemistry, or in vivo imaging of GFP. IH, immunohistochemical staining for VEGF with enzymatic protocol; IF, immunofluorescent staining for VEGF. Number of animals is shown in parenthesis (n1). Third and fourth columns: eyes that were injected with PBS, AAV-GFP, AAV-VEGF, AAV-VEGF + AAV-GFP, or un.injected, as indicated. When more than one rat was examined at the same time point, the injected agent was alternated between left and right eyes. Fifth column: protocol(s) used to examine eyes. Each test eye was positive for one or more of the three protocols and (n2) indicates the number of animals that tested positive. For the control eyes, a blank space indicates that the eyes were negative for all tested protocols. In row one, for example, the left eye was injected with AAV-VEGF 15 months before sacrifice and VEGF expression was detected using RT-PCR (Fig. 1C). In row two, the right eye of the same rat was injected with PBS, and there was no VEGF expression by RT-PCR.
VEGF-Induced CNV

Blood vessel proliferation was examined in 1.5-μm sections of the whole eyecup at different times after injection of AAV-VEGF. Sections from four SD rat eyes injected with AAV-VEGF at 5 weeks, 7 weeks, 12 months, and 15 months before death are shown in Figure 4. All sections showed extensive subretinal neovascularization and retinal degeneration. As summarized in Table 2, we observed subretinal neovascularization in 19 (95%) of 20 of the animals studied, at 12 time points, between 5 weeks and 20 months. A similar result was obtained by using pigmented LE rat eyes (n = 2; see Table 2). Figure 5A is a histologic section from the PBS injected eye (20 months) showing normal retinal morphology. In contrast, the AAV-VEGF-injected eye shows RPE proliferation (inverted white arrowhead) above Bruch’s membrane. In addition, proliferating blood vessels are shown in the subretinal space (black arrows).

VEGF-induced proliferation of blood vessels was limited to areas approximate to the injection site and analyzed in 1.5-μm sections of the whole eyecup. In Figure 6A, the left half of the eyecup indicates the area of morphologically normal-looking photoreceptors and blood vessels. Part of this area is illustrated at higher magnification in Figure 6B. In the right half of the eyecup (Fig. 6A) between the two black arrows was the area of AAV-VEGF injection, also shown at higher magnification in Figure 6C. In Figure 6C, the black arrows indicate new blood vessels in the SRS that are enveloped by proliferating RPE cells (white arrowheads). The white arrow shows a rosette of disorganized photoreceptors. The areas denoted by asterisks in Figures 6C and 6D show a consequence of retinal degeneration in which the RPE proliferates and forms a self-contained lake with the apical membrane facing inward, as evidenced by apical processes that rim the interior of the lake. This structure is seen much more clearly in electron microscopic (EM) sections (Matthew LaVail, personal communication, July 2001).

Electroretinograms (A, ERG) and fluorescein angiogram (B, C) of AAV-VEGF and PBS injected eyes. (A) ERG a- and b-wave amplitudes in the AAV-VEGF-injected eye (thick line) were approximately 50% smaller than in the PBS-injected eye (thin line). (B) Fundus image of a rat eye at 5 months after injection of AAV-VEGF and before it was killed for serial sectioning (see Fig. 6). (C) Two minutes after fluorescein injection leakage of fluorescein was observed at the AAV-VEGF injection site (arrow).
TABLE 2. Choroidal Neovascularization Induced by AAV-VEGF

<table>
<thead>
<tr>
<th>Time (mo)</th>
<th>Eye</th>
<th>Injection</th>
<th>Results (n2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 wk (1)</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>H</td>
</tr>
<tr>
<td>6 wk (2)</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>ERG (2)</td>
</tr>
<tr>
<td>7 wk (1)</td>
<td>R</td>
<td>PBS</td>
<td>H</td>
</tr>
<tr>
<td>8 wk (2)</td>
<td>L</td>
<td>PBS or AAV-GFP</td>
<td>H (1), FA (1)</td>
</tr>
<tr>
<td>3 mo (5)</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>H (5), FA (2), ERG (5)</td>
</tr>
<tr>
<td>6 mo (1)</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>H</td>
</tr>
<tr>
<td>7 mo (2)</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>ERG (2)</td>
</tr>
<tr>
<td>8 mo (2)</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>H (1), ERG (1)</td>
</tr>
<tr>
<td>12 mo (2)</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>H (1), FA (1), ERG (2)</td>
</tr>
<tr>
<td>12 mo (3)</td>
<td>L</td>
<td>AAV-GFP</td>
<td>R Uninjected</td>
</tr>
<tr>
<td>15 mo (2)</td>
<td>L</td>
<td>AAV-VEGF or (AAV-VEGF + AAV-GFP)</td>
<td>H (2)</td>
</tr>
<tr>
<td>16 mo (2)</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>H (2)</td>
</tr>
<tr>
<td>17 mo (2)</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>H (2)</td>
</tr>
<tr>
<td>18 mo (1)</td>
<td>L</td>
<td>AAV-VEGF</td>
<td>R PBS</td>
</tr>
<tr>
<td>20 mo (1)</td>
<td>L</td>
<td>PBS</td>
<td>R AAV-VEGF</td>
</tr>
</tbody>
</table>

First column: the time after injection and the numbers of animals used (n1). Second and third columns: eyes that were injected with PBS, AAV-GFP, AAV-VEGF, AAV-VEGF+AAV-GFP, or uninjected, as indicated. When more than one rat was examined at the same time point, the injected agent was alternated between left and right eyes. Fourth column: protocols used to examine eyes. H, histology; FA, fluorescein angiography; or ERG, electroretinogram; as indicated. Each test eye was positive for one or more of the four protocols, and (n2) indicates the number of animals that tested positive. A blank space indicates control eyes that tested negative in one or more protocols. For example, at 3 months five animals were studied. In all five animals, the eyes injected with AAV-VEGF showed extensive neovascularization by histology. The eyes injected with AAV-VEGF showed extensive leakage on FA in two animals, and the eyes injected with AAV-VEGF showed a reduction in ERG amplitude in three animals. None of these changes were observed in the control eyes (hence, the blank space in the fourth column). In these eyes, multiple procedures were performed, for example, in two of the five rats the ERG was measured first, then the FA was obtained. Animals were then killed for histology.

✱ There was no histological evidence for neovascularization in this AAV-VEGF injected eye.

and in Figure 7. The two black arrowheads in the center of Figure 6D point to a break in Bruch’s membrane penetrated by a choroidal blood vessel.

Serial sections were cut to demonstrate that the penetrating blood vessels in Figure 6D originated in the choroid. (A set of 32 sections clearly showed the choroidal origin of the blood vessels that penetrated Bruch’s membrane and can be viewed at http://www.millercnvmodel.com.) Six of these sections are shown in Figures 7, which focuses on three blood vessels, labeled 1, 2, and 3. Bruch’s membrane is denoted by the black arrows that point toward the choroid. In each section, Bruch’s membrane was easily identified in regions of normal morphology and then traced to the neovascularized area. Figure 7A shows blood vessel 2 lying just below Bruch’s membrane in the choroid. In Figure 7B, it is shown after breaking through Bruch’s membrane, consistent with CNV. In Figures 7C–F, blood vessels 1 and 3 coalesced and then separated, and blood vessel 2 resided on the retinal side. We have frequently seen this pattern of anastomosing capillaries in our lectin-BrdU stained retinas (not shown).

The 3-D structure of the blood vessels that penetrated Bruch’s membrane in Figure 7 was reconstructed from the full set of 32 serial sections using the image management software (Neurolucida; MicroBrightField). The results of this reconstruction analysis are shown in Figure 8. Both images contain two invariant reference structures, on each side of Bruch’s membrane, one on the retinal side and the other in the choroid. In the retina, the reference structure appears as a white RPE lake (Figs. 4 through 7, asterisks) and in the choroid, it appears as a yellow blood vessel (Fig. 8). In Figure 8A, a blood vessel (green) broke through Bruch’s membrane (gray) and entered the SRS. In Figure 8B, all three choroidal blood vessels (red, green, or blue) penetrated Bruch’s membrane and extended into the SRS. After it penetrated Bruch’s membrane, a blood vessel (3, red) split into three branches in the SRS. The choroidal origin of these three arterial blood vessels is indicated by the branching pattern and the relatively smaller sizes of the three SRS blood vessels (red) compared with the choroidal

![Figure 4](image-url) AAV-VEGF induced CNV in SD rats. In 1.5-µm sections from separate eyes at 5 (A) and 7 (B) weeks and 12 (C) and 15 (D) months after AAV-VEGF injection showing new blood vessels in the SRS (black arrows), disorganized photoreceptors (white arrows), RPE proliferation (white arrowheads), RPE lakes (•), and Bruch’s membrane (black arrowheads).
blood vessel (red). As supplementary data, we include a Web site that provides a video containing a 3-D reconstruction of the 32 serial sections (http://www.iovs.org/cgi/content/full/44/2/781/DC1).

**DISCUSSION**

Long-lasting CNV (up to 20 months) was induced in 95% of the animals tested by injecting AAV-VEGF into the subretinal space of the rat eye. Characteristics of CNV that have been described in human disease (and some animal models) include: endothelial cell and pericyte proliferation, the proliferation of RPE along newly formed blood vessels, and the disruption of Bruch’s membrane.5,40 Proliferating blood vessels in the SRS, the first of these characteristics, were observed by using two different sectioning techniques (Figs. 1E, 4, 6C, 6D, black arrows) and fluorescein angiography (Fig. 3C). RPE proliferation was commonly observed in the area of neovascularization and also formed a barrier that prevented the spread of neovascularization from the SRS into the retina (Figs. 4, 6C, 6D; white arrowheads). In principle, pigmentation could affect the development of CNV,51,52 although the inverse correlation between melanin content of the RPE and AMD is still controversial.53 Histologic sections from pigmented LE rats injected with AAV-VEGF showed extensive blood vessel and RPE proliferation in the SRS (Figs. 2B, 5B), very similar to those observed in non-pigmented SD rats (Figs. 1E, 4, 6C, 6D) and patients with AMD (see Fig. 6 in Green5). Finally, we were able to demonstrate the penetration of Bruch’s membrane by choroidal blood vessels, using serial sections (Figs. 6C, 7) and a 3-D reconstruction of these sections (Fig. 8). These serial sections are reminiscent of those obtained from patients with AMD (see Fig. 8 from Green5). These data, taken together, provide a basis for a model of CNV that has many of the hallmarks present in patients with AMD.

Overexpression of VEGF in the outer retina or SRS is not sufficient to cause CNV, perhaps because VEGF cannot diffuse through the RPE to reach the choroidal blood supply although there is evidence in primate to suggest that vitreous injection of rVEGF leads to proliferation of endothelial cells in the choroid.55 However RPE65 promoter-driven overexpression of VEGF in the RPE also failed to induce subretinal neovascularization.56 In both sets of experiments, VEGF may not have reached the critical concentration that would induce CNV.57 For example, one of the transgenic mouse lines (rhodopsin promoter), with a very high VEGF copy number, developed CNV but only during the early stages of photoreceptor degeneration.7,54 This suggests that the development of CNV may have been rate limited by secretion of VEGF from degenerating photoreceptors. This is not a limiting factor in our model, because VEGF was mainly secreted by the RPE into the choroid.

However, high VEGF levels per se may not be sufficient to induce CNV. It may require other growth factors or injury or breakdown of Bruch’s membrane. In one CNV model using Ad-VEGF,39 subretinal injection was made from the choroidal side, and the integrity of Bruch’s membrane was probably...
compromised, perhaps facilitating the development of CNV. Rupture of Bruch’s membrane with laser has been a reliable way to generate CNV in a variety of animals, including monkey,13 rat,15 and mouse47 but also has disadvantages.40,43,56 In our experiments, all injections were performed under the microscope, and, although we took precautions to avoid damaging Bruch’s membrane, this possibility cannot be completely ruled out. However, the difference between bleeding that originates from retinal versus that from choroidal blood vessels can be observed. In retinal bleeding, blood leaks into the vitreous, whereas bleeding from the choroid remains in the subretinal or sub-RPE space and does not leak into vitreous. Most important, no choroidal bleeding was observed during and after subretinal injection. The size and shape of the break in Bruch’s membrane (Figs. 6D, 7) indicates that the break was not caused by the injection needle, but by blood vessel penetration, because the needle would cause a much larger break.

It is possible that CNV was produced in part by inflammatory responses to the needle injection, the viral vector, or VEGF. Subretinal neovascularization in a laser-induced primate model was inhibited by steroids,58 suggesting that the inflammatory response may play an important role in the breakdown of Bruch’s membrane59 and in the development of experimental CNV. Unlike adenovirus,60 which may contribute to the development of CNV in the models involving Ad-VEGF,39,40 AAV causes little or no inflammatory response61 and probably did not contribute to CNV in the present study. VEGF itself, however, could recruit macrophages62 and may cause inflammatory responses that contribute to the development of CNV.7 Retinal degeneration (Figs. 1, 2, 4, 5, 6) may have been secondary to retinal detachment caused by CNV.63 We speculate that it also may have been due to secretion of VEGF by the RPE. Either possibility could explain the reduction in ERG a- and b-wave amplitudes (Fig. 3A). It has been shown that VEGF alters RPE physiology that could indirectly alter photoreceptor function.21,64 Retinal degeneration has been observed in two Ad-VEGF–induced CNV models39,40 and in transgenic mice with high VEGF copy numbers.54 In these three studies, retinal degeneration was probably caused by retina detachment and overexpression of VEGF. An understanding of the cause of retinal degeneration in this animal model may shed light on the mechanism of retinal degeneration in AMD.

In vivo, adenovirus-mediated expression of protein has been reported as early as 48 hours,65 whereas AAV-delivered genes reach maximum expression in 2 weeks.66 In models with Ad-VEGF40 or laser,13 CNV was induced within 1 or 3 weeks, respectively. In the present experiments, CNV was present at 5 but not at 4 weeks (n = 1) after injection, perhaps because there was insufficient time for expression and the development of neovascularization. In Ad-VEGF–induced CNV models, CNV regression was observed approximately 3 weeks.

**FIGURE 7.** Choroidal blood vessels penetrate Bruch’s membrane. (A–F) From an AAV-VEGF–injected eye showing penetration (black arrowheads, B–F) of Bruch’s membrane by blood vessels (2, 3). Bruch’s membrane denoted by black arrows pointing toward choroidal blood supply. (A, B, C) Separated by 7.5 μm; (D, E) separated by 3 μm; (E, F) separated by 1.5 μm (*). RPE lakes. The full set of 32 sections can be viewed at http://www.iovs.org/cgi/content/full/44/2/781/DC1.

**FIGURE 8.** Three-dimensional reconstruction of choroidal neovascularization from 14 (A) and 32 (B) serial sections. The illustrations contain two invariant fiducial marks: a white area of RPE proliferation (A, *) on the retinal side (Ret) of Bruch’s membrane (gray, Br) and a yellow blood vessel in the choroid (Ch). Three choroidal blood vessels (green, blue, and red) penetrated Bruch’s membrane and remained in the SRS. As supplementary data, a Web site has been provided with video containing a 3-D reconstruction of the 32 serial sections: http://www.iovs.org/cgi/content/full/44/2/781/DC1.
after injection,40 whereas laser-induced CNV in the macula lasted for approximately 13 weeks.53 These relatively transient effects may not appropriately model retinal degenerative diseases that progress slowly over the course of years. By comparison, AAV has been shown to mediate long-term gene expression55. In the present experiments, expression of GFP and VEGF was maintained for at least 20 months after injection (Fig. 2), and CNV was observed for at least 20 months.

The success rate of producing CNV is another criterion by which these models may be compared. For example, in the Ad-VEGF-induced CNV model,40 the success rate was 50% at 4 weeks (n = 4). In the present experiments, the success rate was considerably higher. There was one failure at 4 weeks, as noted earlier and another at 16 months (Table 2, asterisk). The reason(s) for the second failure is not clear, but it may have been caused by a faulty injection, because CNV developed in the contralateral eye of this animal. CNV was observed in histology sections from 95% of the animals (19/20 rats; 23/25 eyes, Table 2). In addition, VEGF message or protein was detected in all eight animals injected with AAV-VEGF, and GFP expression was detected in all seven animals injected with AAV-GFP (Table 1).

The present work has established a small animal model of CNV by AAV-induced VEGF overexpression in RPE where vector dose can be titrated to control transgene expression and the development of CNV. The strategy for generating this model may also be applied to primates.53 The relatively long period of AAV-VEGF-induced CNV and a very high induction rate should enable us to study the pathogenesis of CNV and retinal degeneration associated with AMD and to test anti-angiogenic molecules that may be therapeutic against CNV.

Acknowledgments

The authors thank John Flannery for the use of laboratory space and equipment; Dana Lau and Eric Green for initial technical advice; Jeffery Winer and Peter Ohara for advice on the use of NeuroLucida; Rita Mukhtar and Anahita Jafari for technical assistance; Matthew LaVail for consultation throughout the project; and James Handa, David Saperstein, John Flannery, and Ward Peterson for critical comments on an earlier version of the manuscript.

References


