Inhibition of Choroidal Neovascularization by Adenovirus-Mediated Delivery of Short Hairpin RNAs Targeting VEGF as a Potential Therapy for AMD

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PURPOSE. Choroidal neovascularization (CNV) is the leading cause of blindness in age-related macular degeneration (AMD). Several lines of evidence implicate increased levels of vascular endothelial growth factor (VEGF) in retinal pigment epithelium (RPE) from patients with AMD. Current approaches to attenuate VEGF or its receptors, including the use of small interfering (si)RNA, show significant promise, but still have limited efficacy and require repeat administrations, using procedures associated with multiple complications. The goal of this study was to develop an approach for long-term endogenous expression of short hairpin (sh)RNA that would significantly attenuate VEGF and hence act as a potential therapy for AMD.

METHODS. Several shRNAs expressed from recombinant adenovirus were developed. These shRNAs were expressed in human RPE cells in the presence of adenovirus vectors overexpressing VEGF, and the amount of VEGF attenuation was evaluated. Adenovirus vectors expressing VEGF were subsequently injected into the subretinal space of mice, and induction of CNV was measured in the presence of adenovirus vectors expressing shRNA targeting VEGF.

RESULTS. Potent shRNA sequences were identified that were able to silence VEGF in human RPE cells. When expressed from adenovirus backbones, these shRNA constructs silenced VEGF by 94% at a 1:5 molar ratio (VEGF to shRNA) and 64% at a 1:0.05 molar ratio. Adenovirus vectors expressing high levels of VEGF could induce CNV in mice within 5 days. Co-injection of VEGF-expressing viruses into mice with shRNA targeting VEGF led to a substantial (84%) reduction in CNV.

CONCLUSIONS. shRNA targeting VEGF from adenovirus vectors allows potent attenuation of VEGF and prevents CNV. This approach shows promise as a therapy for AMD. (Invest Ophthalmol Vis Sci. 2006;47:3496–3504) DOI:10.1167/iovs.05-1610

Choroidal neovascularization (CNV) is the leading cause of vision loss in age related macular degeneration (AMD). In patients with the exudative or “wet” form of the disease, choroidal blood vessels grow through Bruch’s membrane into the subretinal space, followed by leakage and accumulation of serum or blood beneath the retinal pigment epithelium (RPE), leading to insult of the retina and rapid vision loss. The advent of photodynamic therapy has shown significant promise in the treatment of a subset of neovascular lesions in patients with AMD; however, while effective in ablating established pathologic blood vessels, it does not prevent formation of new blood vessels and hence requires repeat treatments. Although the molecular basis for AMD is not well understood, several growth factors have been implicated in the disease process, including basic fibroblast growth factor (bFGF), transforming growth factor (TGF)-β, and vascular endothelial growth factor (VEGF). Several lines of evidence suggest that VEGF is a major stimulator of CNV in AMD, including an observed increase of VEGF in the RPE of maculae from patients with AMD, increase of VEGF expression in the laser model of CNV in monkeys and rats, and induction of CNV in ectopically delivered VEGF cDNAs to the RPE of rats and nonhuman primates. VEGF is a secreted peptide that has five homodimeric species formed by alternative splicing. Of these variants, the 165 amino acids (VEGF165) is the most commonly expressed isoform in the ischemic retina.

Because of cumulative evidence for the involvement of VEGF in AMD, several clinical trials targeting the VEGF pathway are under way. Strategies to modulate the effects of VEGF include the use of a VEGF-neutralizing oligonucleotide aptamer (pegaptanib), humanized anti-VEGF monoclonal antibody fragment (ranibizumab), receptor analogues (sFlt-1) or receptor-immunoglobulin fusion protein, inhibition of the tyrosine kinase signaling cascade; or degradation of VEGF mRNA by small interfering (si)RNA. For several reasons, including the limited half-lives of these molecules in vivo, repeated intraocular injections are required for therapeutic benefit. For example, in a phase III trial of pegaptanib, patients received intravitreal injections every 6 weeks for 48 weeks to achieve a 15% benefit over sham subconjunctivally injected patients. Intravitreal injections are invasive, with the potential of endophthalmitis, retinal detachment, and cataract. Although the risks are relatively low (e.g., endophthalmitis, 0.16% per dose), the cumulative effects of serial intravitreal injections over several years may be substantial. Hence, there is a continued need for therapies that are efficacious and persist for longer time frames.

The impetus for the present study was to take a step toward the development of a strategy that would allow potent and long-term suppression of the overactive VEGF pathway found in AMD. In contrast to relatively short-lived siRNA used in previous studies, we describe use of endogenously expressed short hairpin (sh)RNAs expressed from DNA templates on adenovirus backbones that potentially will allow long-term suppression of overexpressed VEGF due to the innate longevity of expression from integrated or episomally stable DNA vectors relative to ectopically administered RNA, siRNA, and oligonucleotides. Adenovirus vectors have been shown to allow transgene expression for years in nonhuman primates.
and “lifetime” persistence and transgene expression in rodents in vivo.

In ocular tissues, adenovirus has been shown to persist in the RPE for at least 6 months in vivo, the latest period examined. Although most preclinical ocular gene therapy studies in animal models have used adeno-associated virus (AAV) or lentiviruses as the gene transfer vector, adenovirus has been used in the only two human ocular gene therapy trials performed to date. In these trials, no adverse events or dose limiting toxicities were recorded up to the highest dose of virus tested: 10^{11} virion particles. However, mild inflammation that could be corrected was observed in some patients at these high doses.

In this study, we have used a model of CNV previously well characterized by several investigators—that of CNV caused by adenovirus or AAV-mediated overexpression of VEGF in the RPE. These investigators have demonstrated that virally delivered VEGF cDNAs to the RPE replicate some of the hallmarks of CNV caused by adenovirus or AAV-mediated overexpression of VEGF in the RPE. These investigators have demonstrated that virally delivered VEGF cDNAs to the RPE replicate some of the hallmarks that found in patients with wet AMD, including but not limited to fragmentation of Bruch’s membrane followed by invasion of choroidal blood vessels into the subretinal space: proliferation of RPE cells and the presence of pericytes and endothelial cells in the subretinal space; and, obviously, increased levels of VEGF in the RPE, as is found in patients with AMD.

Adenovirus serotype 5 vectors have almost exclusive increased levels of VEGF in the RPE, as is found in patients with endothelial cells in the subretinal space; and, obviously, marks found in patients with wet AMD, including but not delivered VEGF cDNAs to the RPE replicate some of the hallmarks that found in patients with wet AMD, including but not limited to fragmentation of Bruch’s membrane followed by invasion of choroidal blood vessels into the subretinal space: proliferation of RPE cells and the presence of pericytes and endothelial cells in the subretinal space; and, obviously, increased levels of VEGF in the RPE, as is found in patients with wet AMD.

### MATERIALS AND METHODS

#### shRNA Constructs

A human VEGF cDNA was PCR amplified using the primers pSV-F (forward) and pSV-R (reverse) as previously described. To construct the shRNA constructs, a 266 bp fragment from the human VEGF gene was amplified with the primer combination pShBFPH1VEGFi7F and pShBFPH1VEGFi7R; VEGFi8F, VEGFi8R; and VEGFiNSF, VEGFiNSR, and ligating into HindIII/ClaI digested pShVP22GFPRFP. The VEGF RNAi sequences were cloned into pShBFPH1 by annealing the oligonucleotide pairs VEGFi4F, VEGFi4R, VEGFi5F, VEGFi5R, VEGFi7F, VEGFi7R, VEGFi8F, VEGFi8R, and VEGFiNSF, VEGFiNSR, and ligating into HindIII/BgIII-digested pShBPH1 to create pShBFPH1VEGF Fi4, pShBFPH1VEGF Fi5, pShBFPH1VEGF Fi7, pShBFPH1VEGF Fi8, and pShBFPH1VEGF FiNS, respectively. Oligonucleotide sequences were used to design primers for qRT-PCR.

### Transfections and Adenovirus Infections in Cell Culture

The human embryonic retinoblast cell line 911 was maintained in DMEM with 10% FBS and the human retinal pigment epithelial cell line ARPE-19 (ATCC, Manassas, VA) in DMEM/F-12, 1:1 with 10% FBS. 911 cells (1.2 × 10^7) were transduced (Lipofectamine 2000; Invitrogen, Carlsbad, CA) with 6 μg VEGF to VEGF DNA in a 1:1.2 molar ratio. ARPE-19 cells (1.2 × 10^7) were infected with VEGF and VEGFi viruses at 1:0.05, 1:0.1, 1:0.25, 1:0.5, 1:2, or 1:5 infectious unit ratio using 2.24 × 10^7 particles of AdVEGFRFP. Cells were harvested for total RNA extraction 60 hours after transfection or infection. To confirm VEGF expression from AdVEGFRFP, 1.2 × 10^6 911 cells were infected with 2 × 10^6 particles of either AdCMVGFPRII (a control virus expressing GFP instead of VEGF) or AdVEGFRFP and harvested 30 hours later for Western blot analysis.

#### Subretinal Injections

The use of animals in this work was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57Bl/6j mice were purchased from Jackson Laboratories, bred, and maintained in a 12-hour light-dark cycle and cared for in accordance with federal, state and local regulations. Subretinal injections were performed as previously described with a 5-μl glass syringe (Hamilton, Reno, NV). Particles of AdVEGFRFP or AdCMVGFPRII (8.6 × 10^6) were injected alone or with 4.3 × 10^6 AdVEGFRFP with either 4.3 × 10^6 particles of AdBPH1VEGF Fi7 or pShBFPH1VEGF FiNS.

### Fluorescence Microscopy

Cells and frozen sections were visualized with a microscope (Eclipse TS100; Nikon, Tokyo, Japan) with a 120-W metal halide lamp and appropriate emission and excitation filters for RFP, BFP, and FITC. Images were captured with a camera and associated software (Cool- Snap; Photometrics).

### Quantitative Real-Time PCR In Vitro Studies

For qRT-PCR, 1 μg of DNA-treated RNA was reverse transcribed using oligo (dT)_{16} and Taq polymerase (TaqMan; Applied Biosystems, Inc. [ABI], Foster City, CA) reverse transcription kit, according to the manufacturer’s instructions. Single PCR reactions were performed on cDNA (TaqMan Universal PCR Master Mix; ABI) and analyzed (Prism 7900HT Sequence Detection System; ABI). Human VEGF mRNA was detected (Assays-on-Demand ID Hs00173626_m1; ABI). RFP was detected with the primer-probe combination RFP-forward primer (5'-GGAGGGGCGGTATGAGC-3'), RFP-probe primer (5'-GGGATCTCCATTTTGCA-3'), and RFP-probe (5'-CACGACGGGTTCTCCT-3'), an assay custom designed by ABI. All probes contained the covalently linked reporter FAM dye at their 5’ ends and a TAMRA quencher dye at their 3’ ends.

### Northern Blot Analysis

Northern blot analysis was performed by using standard procedures on 15 μg of total RNA probed with a 32P-labeled 665-bp SacI/HindIII fragment of pShVEGFRFP (VEGF) and 748-bp BglII/XhoI fragment of pDSred2-N1 (RFP).
Western Blot Analysis
Cells were lysed in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, and 1.0% Triton-X-100 containing leupeptin (10 μg/ml), aprotinin (10 μg/ml), and phenylmethylsulfonyl fluoride (PMSF; 0.1 mM). Lysates were loaded onto a 12% Tris-glycine polyacrylamide gel (BMA, Inc., Lowell, MA). VEGF was detected using anti-human VEGF antibody (R&D Systems, Minneapolis, MN) and RFP using a DsRed monoclonal antibody (BD Biosciences, Franklin Lakes, NJ). Secondary detection was achieved using HRP-conjugated anti-goat and anti-mouse antibodies respectively (Jackson ImmunoResearch, West Grove, PA).

Histology and Lectin Staining
Five days after injection, mice were euthanized by CO2 inhalation. For lectin staining, eyes were enucleated, fixed for 30 minutes in 4% paraformaldehyde, embedded in OCT, and sectioned to 14 μm thickness. Sections were incubated for 10 minutes at 37°C with 500 μg/ml BSA in phosphate-buffered saline followed by staining for 1 hour at 37°C with 100 μg/ml fluorescein-conjugated GSL I-isolectin B4 (Vector Laboratories, Burlingame, CA).

Fluorescein Angiography and CNV Measurement
Fluorescein angiography was performed as described previously, with FITC-dextran MW 2 × 10⁶ (Sigma-Aldrich, St. Louis, MO). Measurement of CNV size was performed on images captured as described earlier (ImagePro software; Media Cybernetics, Silver Spring, MD). The size of CNV was determined by calibration of images against a stage micrometer with a resolution of 0.01 mm.

RESULTS

shRNA-Mediated Silencing of VEGF165 in Transfected Human Embryonic Retinoblasts

To develop constructs that are effective at silencing human VEGF165, we targeted three different regions of VEGF165, using design criteria similar to those used previously for shRNA-mediated silencing of rhodopsin.⁵⁷ These VEGF shRNAs are henceforth referred to as VEGF15, VEGF17, and VEGF18, targeting human VEGF165 codons 100-107, 181-188, and 44-51, respectively (Fig. 1). In addition, we adapted a previously published siRNA sequence targeting VEGF165, which we refer to as VEGFiNS (Fig. 1). This sequence was selected based on its lack of complete homology to any nonspecific shRNA—a construct referred to as VEGFiNS (Fig. 1)—selected based on its lack of complete homology to any sequence in the National Center for Biotechnology Information (NCBI; Bethesda, MD) nucleotide database. An expression cassette for red fluorescent protein (RFP) was included in the VEGF-expressing plasmid (pShVEGF-RFP) in cis to allow for accurate normalization of transfection efficiency in vitro and the convenient identification of cells containing the VEGF-targeting shRNA in vivo. The ability of each of the shRNA constructs to silence VEGF165 was measured relative to silencing of VEGF165 by a nonspecific shRNA—constructed according to methods established in this study (Fig. 1)—selected based on its lack of complete homology to any sequence in the National Center for Biotechnology Information (NCBI; Bethesda, MD) nucleotide database. An expression cassette for red fluorescent protein (RFP) was included in the VEGF-expressing plasmid (pShVEGF-RFP) in cis to allow for accurate normalization of transfection efficiency in vitro and the indirect identification of cells expressing VEGF in vivo. pShVEGF-RFP was cotransfected into human embryonic retinoblasts with each of the shRNA constructs and the level of silencing achieved measured qualitatively by Northern blot analysis and quantitatively by real-time RT-PCR. Northern blot analysis clearly revealed knockdown of VEGF165 at the level of mRNA (Fig. 2A) and real-time RT-PCR revealed (Fig. 2B) silencing of VEGF165 mRNA by VEGF17, VEGF15, and VEGF18 to be 28.94% ± 5.26%, respectively (n = 8, each experiment; Fig. 2B). Given that these experiments were performed in a plasmid molar ratio of 1:1:2 (VEGF:shRNA), the knockdown observed is considerable, especially since VEGF165 was expressed from a strong viral (CMV) promoter relative to the shRNA, which is expressed from a cellular H1 promoter. Not surprisingly, while we observed significant overlap between RFP- and BFP-positive cells, some cells were RFP positive only, as determined by fluorescent microscopy (data not shown), which may explain in part the lack of complete silencing of VEGF. Whereas each of VEGF17, VEGF15, and VEGF18 were designed to target human VEGF165, VEGF15 also targets murine VEGF at codons 180-187 by virtue of sequence homology between murine and human VEGF cDNAs. Based on these data, VEGF17 and VEGF15 were selected for further study.

shRNA-Mediated Silencing of VEGF165 in Adenovirus-Infected Human RPE Cells
To deliver shRNA expression cassettes to RPE in vivo, we rescued recombinant adenovirus constructs containing BFP with one of VEGF17, VEGF15, or VEGF18—these adenovirus clones are henceforth generally referred to as AdBFPH1VEGF1.
We also rescued a clone concomitantly expressing VEGF165 and RFP (AdVEGFRFP). The adenovirus clones have a deletion in region E1, rendering them replication deficient outside of the packaging cells (Fig. 3A). Cell lysates prepared from 911 cells infected with purified viruses were used to confirm expression of VEGF165 and RFP by Western blot analysis (Fig. 3B). To predict the optimal ratios of AdVEGFRFP to AdBFPH1VEGFi viruses for effective silencing of VEGF165 in the context of Ad in RPE cells, we performed a dose–response experiment in the human RPE cell line ARPE-19. Specifically, we measured the amount of silencing of VEGF165 achieved with increasing ratios of AdBFPH1VEGFi virus. Given that silencing of overexpressed VEGF165 down to normal levels (and not beyond) would probably be optimal for clinical efficacy, these data would reveal whether regulated levels of VEGF165 silencing may be achieved by modifying viral dose. We tested ratios ranging from 1:0.05 to 1:5; AdVEGFRFP to AdBFPH1VEGFi. We found that at the highest doses tested (VEGF to VEGFi of 1:5), VEGF165 mRNA was silenced by 93.55% ± 0.07% and 88.25% ± 0.21% by VEGFi7 and VEGFi5, respectively (Fig. 4). The potency of VEGFi7 was more obvious at lower doses where as much as a 100-fold dilution of VEGFi7 without reduction in VEGFi165 levels (a ratio of 1:0.05) led to silencing of VEGFi165 by 64.30% ± 1.98%, a reduction in potency of only 29.25% ± 2.53%. In contrast, use of 50-fold less VEGFi5 led to silencing of VEGFi165 by 23% ± 3.39%, a reduction in potency of 65.25% ± 3.6% (n = 3–6, each experiment). Based on its substantial potency, we selected VEGFi7 for in vivo studies.

Adenovirus-Mediated Delivery of Human VEGF165 to Murine RPE In Vivo

Adenovirus or adeno-associated virus (AAV)-mediated delivery of VEGF165 to rat retina has been described in detail by other investigators.11–13 Because our experiments are performed in a different species (mice) using a different set of constructs, we briefly examined the effects of adenovirus-mediated delivery of human VEGF165 to murine retina. In contrast to previous studies, the coexpression of RFP in the VEGF-expressing adenovirus allows accurate comparison of vascular disturbance between the site of injection (location of highest viral dose) and sites distant from the injection, which is useful given that VEGF is a secreted protein.

Six-week-old C57 mice were injected with either AdVEGF-RFP or the control AdCMVRFP virus (which contains only the RFP cassette) into the subretinal space and euthanatized 5 days after injection. We also rescued a clone concomitantly expressing VEGF165 and RFP (AdVEGFRFP). The adenovirus clones have a deletion in region E1, rendering them replication deficient outside of the packaging cells (Fig. 3A). Cell lysates prepared from 911 cells infected with purified viruses were used to confirm expression of VEGF165 and RFP by Western blot analysis (Fig. 3B). To predict the optimal ratios of AdVEGFRFP to AdBFPH1VEGFi viruses for effective silencing of VEGF165 in the context of Ad in RPE cells, we performed a dose–response experiment in the human RPE cell line ARPE-19. Specifically, we measured the amount of silencing of VEGF165 achieved with increasing ratios of AdBFPH1VEGFi virus. Given that silencing of overexpressed VEGF165 down to normal levels (and not beyond) would probably be optimal for clinical efficacy, these data would reveal whether regulated levels of VEGF165 silencing may be achieved by modifying viral dose. We tested ratios ranging from 1:0.05 to 1:5; AdVEGFRFP to AdBFPH1VEGFi. We found that at the highest doses tested (VEGF to VEGFi of 1:5), VEGF165 mRNA was silenced by 93.55% ± 0.07% and 88.25% ± 0.21% by VEGFi7 and VEGFi5, respectively (Fig. 4). The potency of VEGFi7 was more obvious at lower doses where as much as a 100-fold dilution of VEGFi7 without reduction in VEGFi165 levels (a ratio of 1:0.05) led to silencing of VEGFi165 by 64.30% ± 1.98%, a reduction in potency of only 29.25% ± 2.53%. In contrast, use of 50-fold less VEGFi5 led to silencing of VEGFi165 by 23% ± 3.39%, a reduction in potency of 65.25% ± 3.6% (n = 3–6, each experiment). Based on its substantial potency, we selected VEGFi7 for in vivo studies.

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later. Frozen sections of retina revealed RFP localization to the RPE (Figs. 5A, 5D), consistent with previous observations that Ad5 tropism is almost exclusively for the RPE. In AdVEGFRFP injected retina, GSL-1 (FITC) staining of these same sections revealed abnormalities to retinal structure including vasculature emanating from the choroid into the subretinal space, as well as disruption of Bruch’s membrane and the RPE (Fig. 5B). These pathologies are comparable to some of the hallmarks of wet AMD. Merged images indicate complete overlap between RFP-positive RPE (Figs. 5A, 5D), consistent with previous observations that Ad5 tropism is almost exclusively for the RPE. In AdVEGFRFP injected retina, GSL-1 staining in the region of viral injection (Fig. 5F). However, there was a small amount of GSL-1 staining in the RPE of this control retina in the region of viral injection, (Fig. 5F). However, there was a small amount of GSL-1 staining in the region of viral injection, perhaps caused by disruption of Bruch’s membrane during subretinal injection that was performed by the transchoroidal approach.

Consistent with these frozen cross sections, choroidal flatmounts prepared from adenovirus-injected animals intracardially injected with FITC-dextran revealed large areas of CNV ranging from 534 to 1362.71 μm² and colocalizing with RFP in AdVEGFRFP injected retina (Figs. 5G, 5H). Although CNV was also observed in mice injected with the control virus AdCMVRFp (Figs. 5M, 5N), it occurred to a much smaller extent, in the range of 46.36 to 93.65 μm². In addition, we noted differences in the gross morphology of RFP-positive RPE cells in the region of CNV between AdVEGFRFP (Figs. 5I, 5J) and AdCMVRFp (Figs. 5O, 5P) injected retina. However, there was no substantial difference detectable at this resolution between the structures of retinal vessels in AdVEGFRFP (Figs. 5K, 5L) or AdCMVRFp (Figs. 5Q, 5R) retina, both of which were relatively normal, indicating that most of the neovascularization occurred in the choroid.

**Figure 4.** qRT-PCR of human VEGF expression in ARPE-19 cells infected with AdVEGFRFP and increasing doses of AdBFPH1VEGF15 or AdBFPH1VEGF17 (VEGF to VEGF: 0.05-5).

Rescue of VEGF165-induced CNV by Adenovirus-Expressed shRNA In Vivo

Having demonstrated a murine model for VEGF165-induced CNV, we attempted to inhibit the formation of CNV by coexpression of shRNA from adenovirus vectors. In these experiments AdVEGFRFP was co-injected with either AdBFPH1VEGF15 (a nonspecific shRNA-expressing virus) or AdBFPH1VEGF17—both at a ratio of 1:10, VEGF to VEGF. Experiments were performed exactly as described herein for characterization of the animal model.

Five days after injection, examination of sections from frozen retinas by GSL-1 staining in the region of RFP expression (Figs. 6A, 6C) indicated substantial amounts of CNV in retinas co-injected with AdVEGFRFP + AdBFPH1VEGF15s (Fig. 6B), including increased GSL-1 staining in the choroid and invasion of subretinal space by choroidal blood vessels. However, in animals co-injected with AdVEGFRFP + AdBFPH1VEGF17 there was relatively little or no CNV (Fig. 6D), despite the presence of RFP (Fig. 6C) in the RPE, confirming the presence of VEGF165-expressing virus. Choroidal flatmounts with intact RPE prepared from FITC-dextran-injected retinas confirmed a widespread distribution of RFP (and hence VEGF165) and BFP (and hence shRNA) in both sets of experiments (Figs. 6F, 6G, 6L, 6M). Inside the area of strong RFP and BFP fluorescence, large CNV tufts were readily observable in AdVEGFRFP- and AdBFPH1VEGF15s-injected retina (Fig. 6H). Closer examination of these tufts (Fig. 6K) revealed numerous blood vessels (Fig. 6K, arrow) emanating from the choroid. In contrast, no CNV tufts were visible within RFP- and BFP-positive areas in mice injected with AdVEGFRFP and AdBFPH1VEGF17 (Fig. 6N). However, outside the injection site (as determined by absence of significant amounts of RFP and BFP), a small number of blood vessels was visible (Fig. 6Q, arrow). Statistical analysis of choroidal flatmounts revealed that AdBFPH1VEGF17 reduced CNV by 83.82% ± 7.65% relative to the nonspecific AdBFPH1VEGF15s on co-injection with AdVEGFRFP (Fig. 6E).

Given that a small amount of CNV was caused by injection of control virus alone—possibly owing to upregulation of murine VEGF that cannot be targeted by VEGF17—the silencing of auxiliary CNV caused by overexpression of human VEGF165 could be considered almost complete. Similar experiments performed at lower doses of VEGF17 (1:5) resulted in variable inhibition of CNV between animals and were determined not to be statistically significant.

**Discussion**

Attenuation of VEGF activity has been a central theme in most therapies currently under investigation for treatment of exudative AMD. Many of these therapies show some promise but require repeated administrations that are associated with a variety of complications. Hence, therapies that are efficacious and yet administered less frequently are needed. Ectopic administration of anti-VEGF therapies also suffer from the drawback of leakage of active agent from the ocular environment into systemic circulation. Given that VEGF is required systematically for wound healing, bone growth, cyclic endometrial development, and placental vascularization, it is preferable to sequester the VEGF-inhibiting agent in the ocular environment and, ideally, in the cells that are overproducing VEGF. Breakdown of the blood–ocular barrier is a common occurrence during CNV, exacerbating the need for ocular sequestration of anti-VEGF molecules. The approach described in this study addresses some of these concerns by virtue of the fact that adenovirus injected into the subretinal space almost exclusively infects the RPE and hence the anti-VEGF molecule can only be synthesized in the RPE cells and not escape readily.
without breakdown of the RPE cell membrane. Relative to small molecules like siRNA, adenovirus vectors injected into the subretinal space may have lower probabilities of crossing intact and disrupted blood ocular barriers, given their significantly larger size.

Although intravitreal injections of anti-VEGF therapies are easier to administer than subretinal injections, in the former case, the active agent has direct access to the ganglion cells and hence may be transported to the brain through anterograde transport.39 VEGF has important roles in neuronal function including memory and learning40 and its receptors are widely expressed in the brain. Indeed, mice deficient in VEGF have a neurodegenerative phenotype analogous to amyotrophic lateral sclerosis.41 Hence, subretinal injections targeting the RPE and perhaps photoreceptor cells might be a safer and more practical route for delivering anti-VEGF therapies in the long term if single treatments were to last for years. Because adenovirus serotype 5 has very high tropism for the RPE, non-RPE associated silencing of VEGF is unlikely. This limited tropism does not necessarily limit the use of adenovirus as a vector, given that it is the RPE from the macula of patients with AMD that typically exhibits high levels of VEGF.8 Additional cell types in the retina, such as photoreceptors, may also be targeted if necessary through pseudotyping of adenovirus vectors.42,43 Although not investigated in this study, regulation of shRNA expression may allow greater control in knockdown of VEGF levels through the use of inducible and tissue-specific promoters,44 although significant further development is still necessary for tissue-specific regulated expression of shRNA.

The central findings of the present study were that shRNA expressed from adenovirus vectors can efficiently silence high levels of VEGF.8 Additional cell types in the retina, such as photoreceptors, may also be targeted if necessary through pseudotyping of adenovirus vectors.42,43 Although not investigated in this study, regulation of shRNA expression may allow greater control in knockdown of VEGF levels through the use of inducible and tissue-specific promoters,44 although significant further development is still necessary for tissue-specific regulated expression of shRNA.

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significant toxicity, with efficacy lasting for more than 38 months.\textsuperscript{27} Mild inflammation that can be managed is observed, however, in approximately 25% of patients. More recently,\textsuperscript{26} adenovirus has been used to deliver pigment epithelium-derived factor safely to ocular tissues of AMD-affected eyes at doses of $10^{9.5}$ particle units. These combined studies bode well for the future of adenovirus as an ocular gene therapy vector.

We confirmed in the present study that CNV is primarily caused by exogenous human VEGF165 and not exclusively by disruption of Bruch’s membrane and/or an immune response to the adenovirus. This was shown by silencing of exogenous VEGF165, that led to an approximately 84% reduction in the size of CNV. The remaining CNV may have been caused by subretinal injection of virus, as we observed increased FITC-dextran staining at the site of injection of the control virus (Fig. 5N). Closer histologic examination, however, indicated that this material did not invade the subretinal space (Fig. 5E). Scar tissue and CNVs have been observed by previous investigators at the site of injection of control virus.\textsuperscript{12,45}

Although the results of our study reveal that shRNA expressed from adenovirus vectors for silencing VEGF165 may be a promising avenue for the treatment of wet AMD, there are

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\caption{Frozen retinal sections from (A, B) AdVEGFRFP+AdBFP1VEGF17 and (C, D) AdVEGFRFP+AdBFP1VEGF17-injected mice. (E) Analyses of total CNV area (mean ± SD) of mice injected with either AdVEGFRFP+AdBFP1VEGF17 (VEGF17) or AdVEGFRFP+AdBFP1VEGF17 (VEGF17). P = 0.0031. Choroidal flatmounts from (F–K) AdVEGFRFP+AdBFP1VEGF17-injected mice or (L–Q) AdVEGFRFP+AdBFP1VEGF17 injected mice. (K, Q) Higher magnification of boxed areas (H) and (N), respectively. Arrowheads: choroidal blood vessels emanating from tufts. Abbreviations are as in Figure 4. Original magnification: (A–D) ×40; (F–H, L–N) ×10; (I–K, O–Q) ×20.}
\end{figure}
some important problems that still must be resolved. Unlike the human studies referenced herein, high-titer adenovirus vectors have been shown to be immunogenic in animal studies, despite the subretinal space’s being a site of immune privilege.  However, this obstacle has been solved in part by the use of helper-dependent adenovirus vectors that persist in the RPE for more than 6 months.  We did not perform a genome-wide scan to determine whether there is nonspecific down-regulation of mRNA in the RPE in addition to specific silencing of VEGF, although all our experimental data are relative to a nonspecific shRNA, and we did not observe any gross morphologic changes in the RPE in our injected control subjects. Nonetheless, we have begun addressing the possibility that some mRNAs in addition to VEGF may also be targeted by VEGF shRNA. Immunohistochemical responses to long-term expression of shRNA are also possible and need investigation. Although the impetus for the present study was to develop a long-term therapy for AMD, during the course of the study, it was determined that CNV could be stimulated within 5 days in this VEGF165-induced CNV animal model. Hence, whereas this study does not specifically show that VEGF can be silenced over years, the large number of previous studies showing transgene expression from adenovirus extending for years provides plausible support for the hypothesis that the experimental system described herein has significant promise as a mode of treatment for AMD.

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References


