AAV-Mediated Gene Transfer of Pigment Epithelium-Derived Factor Inhibits Choroidal Neovascularization

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Purpose. Adeno-associated viral (AAV) vectors have been used to express several different proteins in the eye. The purpose of this study was to determine whether AAV-mediated intraocular gene transfer of pigment epithelium-derived factor (*PEDF*) inhibits the development of choroidal neovascularization (CNV) in a murine model.

Methods. C57BL/6 mice were given intravitreous or subretinal injections of a PEDF expression construct packaged in an AAV vector (AAV-chicken β-actin promoter-exon 1-intron 1[CBA]-PEDF) or control vector (AAV-CBA-green fluorescent protein[GFP]). After 4 or 6 weeks, the Bruch's membrane was ruptured by laser photocoagulation at three sites in each eye. After 14 days, the area of CNV at each rupture site was measured by image analysis. Intraocular levels of PEDF were measured by enzyme-linked immunosorbent assay.

Results. Four to six weeks after intraocular injection of AAV-CBA-PEDF, levels of PEDF in whole-eye homogenates were 6 to 70 ng. The average area of CNV at sites of the Bruch's membrane rupture showed no significant difference in eyes injected with AAV-CBA-PEDF compared with uninjected eyes. In contrast, 4 to 6 weeks after intraocular injection of 1.5×10^9 or 2.0×10^{10} particles of AAV-CBA-PEDF, the area of CNV at the Bruch's membrane rupture sites had significantly decreased compared with CNV area at rupture sites in eyes injected with AAV-CBA-GFP.

Conclusions. These data suggest that intraocular expression of PEDF or other antiangiogenic proteins with AAV vectors may provide a new treatment approach for ocular neovascularization. (*Invest Ophthalmol Vis Sci.* 2002;43:1994–2000)

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Ocular neovascularization is a major threat to vision and a complicating feature of many eye diseases. In fact, choroidal neovascularization (CNV) complicating age-related macular degeneration (AMD) is the most common cause of severe visual loss in people older than 60 years in developed countries.[1] At best, current treatments merely delay severe vision loss, because they are directed at destroying new vessels and do not address the underlying angiogenic stimuli that frequently cause recurrences.

Currently, there are no antiangiogenic treatments available for patients with ocular neovascularization, but several new approaches hold promise. Orally active drugs that inhibit VEGF receptor kinases cause dramatic inhibition of ocular neovascularization in mice.[2–4] However, before this can be applied in patients, extensive safety data are needed to be certain there are no serious side effects from systemic inhibition of angiogenesis. To avoid these concerns, local delivery of several agents is being investigated. Phase I clinical trials testing the safety and tolerability of intraocular injections of an aptomer that binds VEGF or an anti-VEGF antibody have been completed, and phase II trials are being planned. Preliminary reports suggest that inflammation may occur, particularly after injection of the anti-VEGF antibody, but it is not considered a severe enough problem to discontinue these approaches.[5,6] Endogenous proteins are likely to be better tolerated, and, recently, several proteins with purported antiangiogenic activity have been identified,[7–12] and intraocular injection of each of these alone or in combination could be considered. However, the use of large molecules, such as aptomers or proteins, has a major disadvantage of requiring repeated intraocular injection.

Gene transfer offers an alternative means for local delivery of therapeutic proteins to intraocular tissues. Because the eye is a relatively isolated compartment, intraocular injection of a small fraction of the amount of viral vector used for systemic injections results in transduction of a large number of ocular cells and no transduction of cells outside the eye. Recently, we have demonstrated that intraocular injection of an expression construct for pigment epithelium-derived factor (PEDF) packaged in an adenoviral vector inhibits ocular neovascularization in three different mouse models.[13] This provides proof of concept for the gene transfer approach of treating ocular neovascularization, but adenoviral vectors have features that may limit their use in humans, including some evidence of toxicity and decreased transgene expression to low levels over the course of a few months. It is not yet known whether repeated intraocular injections of adenoviral vectors can be considered. Prolonged transgene expression with no evidence of toxicity has been demonstrated after intraocular injection of expression constructs packaged in adeno-associated viral (AAV) vectors.[14,15] In this study, we tested the effect of intraocular injection of AAV vectors containing expression constructs coding for PEDF.

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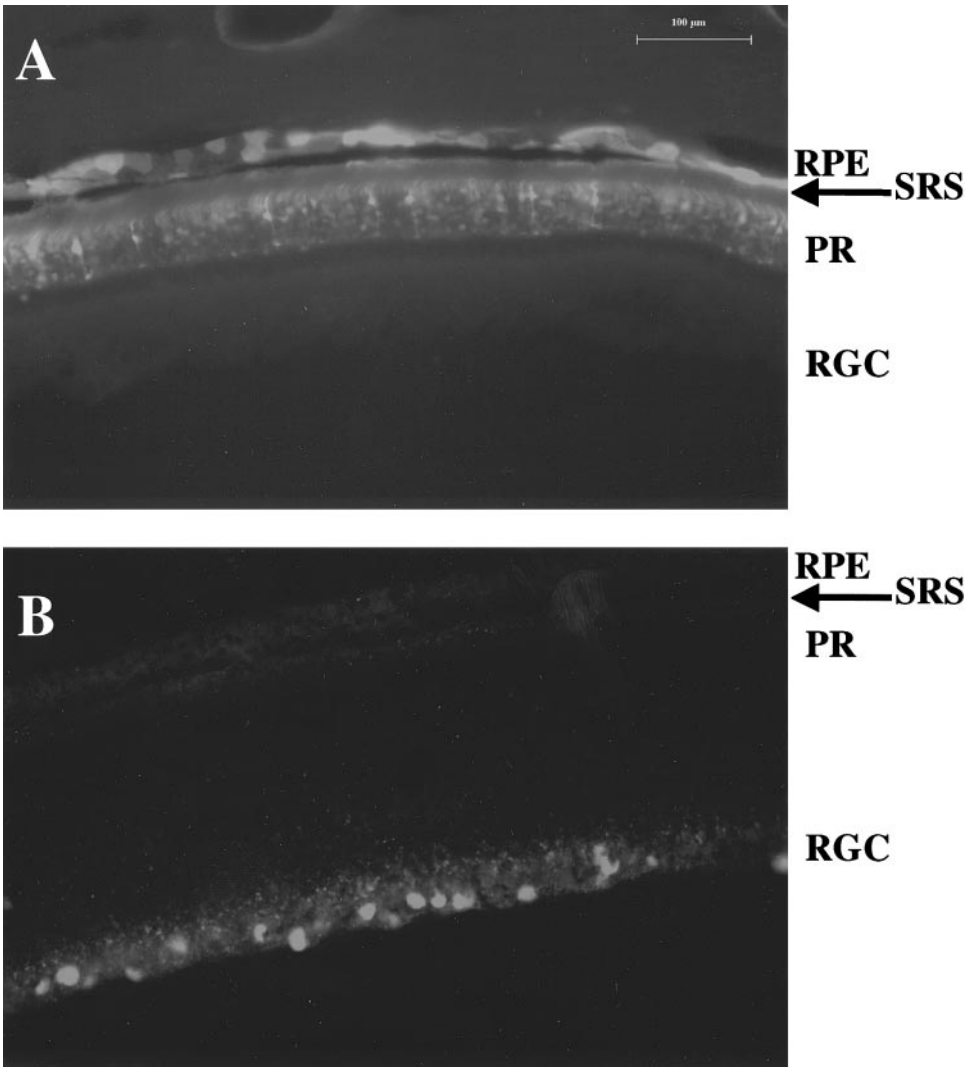

FIGURE 1. Dependence of retinal cell transduction on route of injection of AAV-CBA-GFP vector. Ocular sections were examined by fluorescence microscopy 6 weeks after subretinal (**A**) or intravitreous (**B**) injection of 3×10^9 particles of AAV-CBA-GFP. (**A**) After subretinal injection, prominent transduction occurred in RPE cells and photoreceptors (PR) bordering the subretinal space (SRS), but no detectable GFP appeared in retinal ganglion cells (RGC). (**B**) After intravitreous injection, prominent transduction occurred in retinal ganglion cells (and possibly displaced amacrine cells), but no detectable GFP appeared in photoreceptors or RPE.

MATERIALS AND METHODS

Production of Recombinant AAV Vectors Expressing PEDF

Cloning of human PEDF has been described.[13] Recombinant (r)AAV constructs were based on pTR-UF,[16] a viral vector plasmid in which an expression cassette, consisting of a cytomegalovirus (CMV) enhancer and a truncated chicken β-actin promoter-exon 1-intron 1 (together termed CBA), and a poliovirus internal ribosome entry sequence precede the PEDF cDNA, and a simian virus (SV)40 polyadenylation site follows it. The entire construct is flanked by inverted terminal repeat sequences from AAV-2. AAV-CBA-PEDF vector titers were 1.5×10^{12} or 2.0×10^{13} particles/mL. The control vector (UF12) was constructed identically, except that the coding region for green fluorescent protein (GFP) was substituted for the coding region of PEDF. It was used at 2.4×10^{12} or 4.0×10^{12} particles/mL. Contaminating helper adenovirus and wild-type AAV, assayed by serial dilution cytopathic effects or infectious centers, respectively, were lower than our detection limit of six orders of magnitude below recombinant AAV vector titers.

Mouse Model of Laser-Induced CNV

Adult C57BL/6 mice were given either an intravitreous injection of UF12 or AAV-CBA-PEDF by previously published techniques.[13] Intravitreous injections were performed with a Harvard pump microinjection apparatus and pulled glass micropipets. Each micropipet was calibrated to deliver 1 μL of vehicle containing the appropriate number of viral particles, on depression of a foot switch. The mice were anesthetized, pupils were dilated, and under a dissecting microscope, the sharpened tip of the micropipet was passed through the sclera, just behind the limbus into the vitreous cavity, and the foot switch was depressed. Subretinal injections were performed using a condensing lens system on the dissecting microscope, which allowed visualization of the retina during the injection. The pipet's tip was passed through the sclera posterior to the limbus and was positioned just above the

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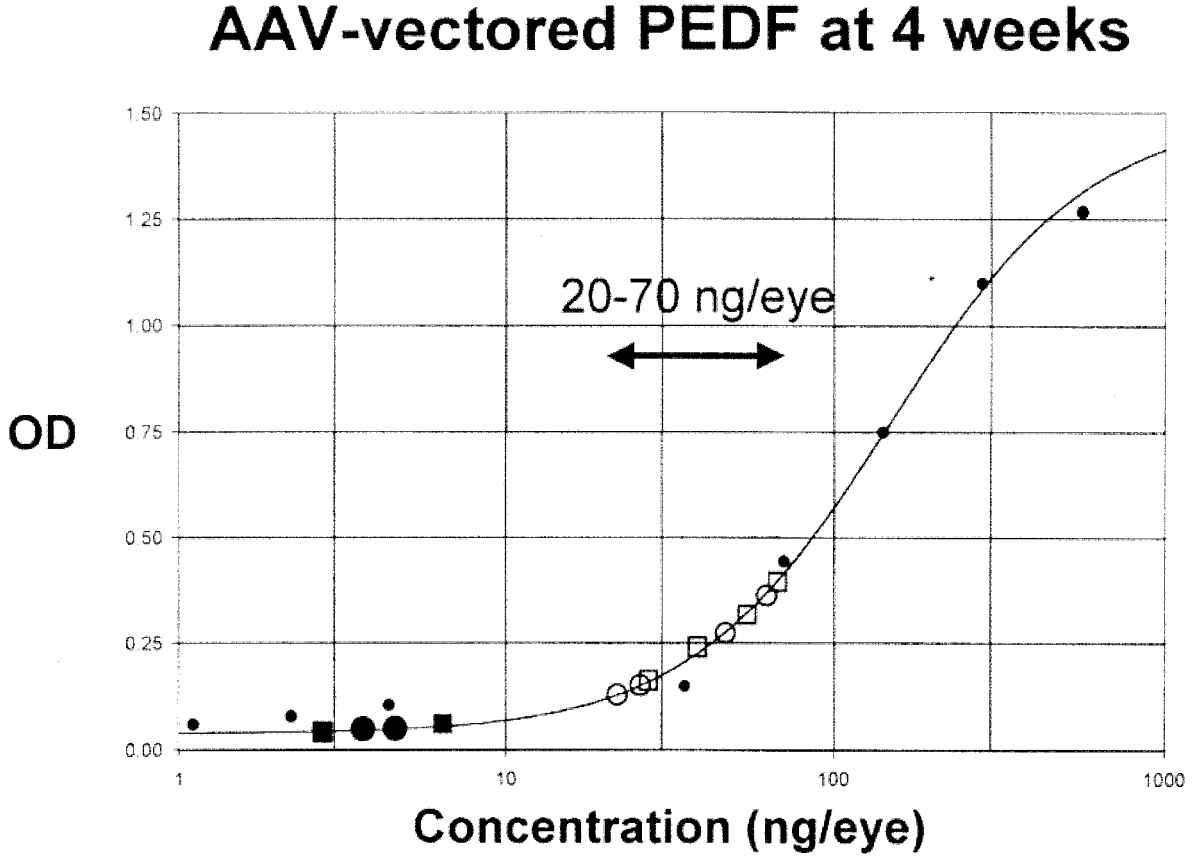

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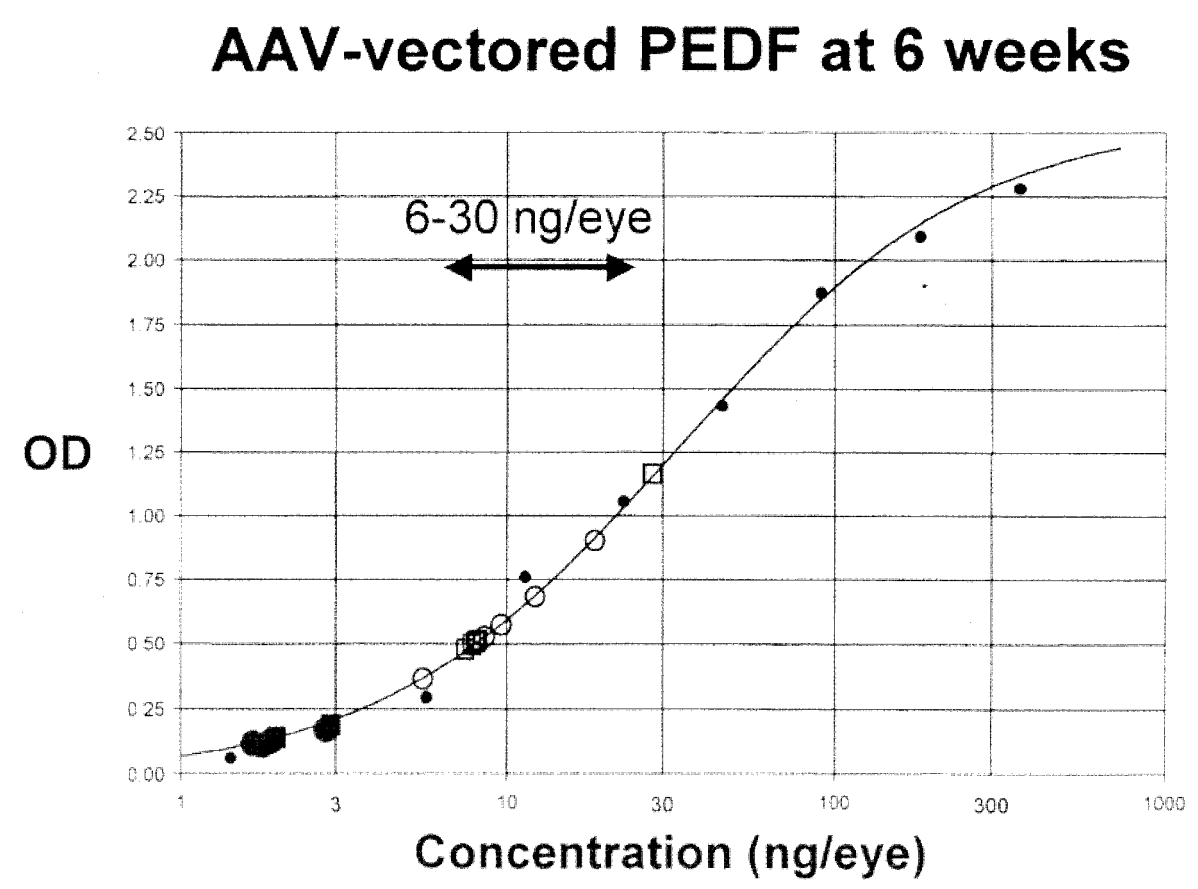

retina. Depression of the foot switch caused the jet of injection fluid to penetrate the retina. The blebs were quite uniform in size, and in each case, two of the laser burns were encompassed by the bleb, and one was outside the region of the bleb.

Two independent experiments were performed. In the first, mice were given intravitreous or subretinal injection of 1 μL containing 1.5×10^9 particles of AAV-CBA-PEDF or 4.0×10^9 particles of control vector, and 4 weeks after injection, the Bruch's membrane was ruptured with laser photocoagulation at three locations in each eye. Some mice were killed without treatment with laser photocoagulation, to measure ocular PEDF levels by ELISA. In the second experiment, mice were given intravitreous or subretinal injection of 1 μL containing 2.4×10^9 particles of control vector or 2.0×10^{10} particles of AAV-CBA-PEDF, and then 6 weeks after injection, the Bruch's membrane was ruptured by laser photocoagulation at three sites in each eye, as previously described.[17] Briefly, laser photocoagulation (532-nm wavelength, 100-μm spot size, 0.1-second duration, and 120-mW intensity) was delivered using the slit lamp delivery system and a handheld cover slide as a contact lens. Burns were performed in the 9, 12, and 3 o'clock positions two to three disc diameters from the optic nerve. Production of a vaporization bubble at the time of laser, which indicates rupture of the Bruch's membrane, is an important factor in obtaining CNV,[17] and therefore only burns in which a bubble was produced were included in the study.

Measurement of the Sizes of Laser-Induced CNV Lesions

Two weeks after laser treatment, the sizes of CNV lesions were measured in choroidal flatmounts.[18] Mice used for the flatmount technique were anesthetized and perfused with 1 mL phosphate-buffered saline (PBS) containing 50 mg/mL fluorescein-labeled dextran (2×10^6 average molecular weight; Sigma, St. Louis, MO), as previously described.[19] The eyes were removed and fixed for 1 hour in 10% phosphate-buffered formalin. The cornea and lens were removed, and the entire retina was carefully dissected from the eyecup. Radial cuts (four to seven; average, five) were made from the edge to the equator, and the eyecup was flatmounted in aqueous medium (Aquamount; BDH, Poole, UK) with the sclera facing down. Flatmounts were examined by fluorescence microscopy (Axioskop; Zeiss, Thornwood, NY), and images were digitized using a three-color charge-coupled (CCD) video camera (IK-TU40A; Toshiba, Tokyo, Japan) and a frame grabber. Image analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was used to measure the total area of hyperfluorescence associated with each burn, corresponding to the total fibrovascular scar. The areas within each eye were averaged to obtain one experimental value, and mean values were calculated for each treatment group and compared by Student's unpaired *t*-test.

Some mice were killed 2 weeks after laser treatment, and eyes were rapidly removed and frozen in optimum cutting temperature embedding compound (OCT; Miles Diagnostics, Elkhart, IN). Frozen serial sections (10 μm) were cut through the entire extent of each burn and histochemically stained with biotinylated *G. simplicifolia* lectin B4 (GSA; Vector Laboratories, Burlingame, CA), which selectively binds vascular cells. Slides were incubated in methanol-H_2O_2 for 10 minutes at 4°C, washed with 0.05 M Tris-buffered saline, pH 7.6 (TBS), and incubated for 30 minutes in 10% normal porcine serum. Slides were incubated 2 hours at room temperature with biotinylated GSA, and after rinsing with 0.05 M TBS, they were incubated with avidin coupled to peroxidase (Vector Laboratories) for 45 minutes at room temperature. After a 10-minute wash in 0.05 M TBS, slides were incubated with HistoMark Red (Kirkegaard & Perry, Cabin John, MD), to give a red reaction product that is distinguishable from melanin, and counterstained with Contrast Blue (Kirkegaard & Perry).

Fluorescence Microscopy after Intravitreous or Subretinal Injection of AAV-CBA-GFP

Adult male C57BL/6 mice were given either a subretinal or intravitreous injection of 3×10^9 particles of AAV-CBA-GFP. Six weeks after injection, mice were killed and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The eyes were removed, the cornea was punctured, and the eyes were immersed in the same fixative for 2 hours at 4°C. After removal of the cornea and lens, eyecups were cryoprotected in 30% sucrose in PBS for 6 to 12 hours and then frozen in OCT. Ten-micrometer frozen sections were mounted on gelatin-coated slides and used immediately or stored at −20°C. Slides were examined by fluorescence microscopy (Axioplan 2; Zeiss).

ELISA for PEDF

Mice were killed, and eyes were removed, quick frozen in 100 μL PBS (pH 7.4) with 0.05% phenylmethylsulfonyl fluoride, and homogenized manually on ice using a ground glass tissue homogenizer followed by three freeze–thaw cycles on liquid nitrogen and wet ice. The homogenate was centrifuged in a refrigerated desktop centrifuge to pellet the insoluble material, and the supernatant was loaded in sample wells for detection by ELISA. PEDF was detected by a sandwich ELISA procedure using a biotin-conjugated antibody and HRP-conjugated avidin for detection. Rabbit anti-PEDF was coated on 96-well, flat-bottomed microtiter plates (Immulon; Thermo Labsystems Oy, Helsinki, Finland) in 0.1 M $NaHCO_3$ overnight at 4°C. The wells were blocked with 10% fetal bovine serum in PBS (pH 7.4) for 2 hours at 37°C. PEDF protein standards and eye extract samples were loaded as 100-μL aliquots into wells, and the plate was kept overnight at 4°C. Detection consisted of a secondary mouse polyclonal anti-PEDF followed by a biotin-conjugated rat anti-mouse IgG (ICN Biomedicals, Costa Mesa, CA) and HRP-conjugated avidin (PharMingen, San Diego, CA). Each step of detection was conducted with plate agitation at room temperature for 1 to 2 hours, and the plate was washed five times between steps. A TMB peroxidase substrate system (Kirkegaard & Perry) was allowed to reach fully developed color, usually after 30 minutes, before the reaction was stopped with 1 M H_3PO_4. The plates were read in an automated microplate reader at 450 nm.

Results

Localization of Expression of the AAV-Vectored Transgene

Six weeks after subretinal injection of AAV-CBA-GFP, fluorescence microscopy showed prominent fluorescence from GFP in photoreceptors and RPE cells (Fig. 1A). In contrast, 6 weeks after intravitreous injection of AAV-CBA-GFP, there was prominent expression of GFP in ganglion cells (and possibly displaced amacrine cells), but no detectable expression in RPE cells or photoreceptors (Fig. 1B).

Expression of PEDF in Mice after Intravitreous or Subretinal Injection of AAV-CBA-PEDF

Mice given an intravitreous or subretinal injection of AAV-CBA-PEDF showed levels of human PEDF ranging from 20 to 70 ng/eye 4 weeks after the injection (Fig. 2A). In a second series

Figure 2. Intraocular levels of human PEDF 4 and 6 weeks after intraocular injection of control vector or AAV-CBA-PEDF. (**A**) C57BL/6 mice were given a subretinal (*squares*) or intravitreous (*circles*) injection of control vector (*filled*) or AAV-CBA-PEDF (*open*). Four (**A**) or 6 (**B**) weeks after injection, the mice were killed, and PEDF levels were measured in whole-eye homogenates by ELISA. The optical density (OD) of the standard concentrations (*small filled circles*) were plotted to generate the standard curve. The PEDF levels in eyes injected with control vector were below the limit of detection, and the levels in eyes injected with AAV-CBA-PEDF ranged from (**A**) 20 to 70 ng at 4 weeks (**B**) and from 6 to 30 ng at 6 weeks.

of mice, the range of PEDF was 6 to 30 ng/eye 6 weeks after intravitreous or subretinal injection of vector (Fig. 2B). All mice given intravitreous or subretinal injections of control vector had undetectable levels of PEDF. Given the variability from injection to injection, these ranges of PEDF are likely to be the same at 4 and 6 weeks after injection, and they are well above the background levels observed in control eyes. Subretinal and intravitreous injection of PEDF vector produced similar and overlapping levels of protein expression.

Effect of Intravitreal or Subretinal Injection of AAV-CBA-PEDF on CNV

Figure 3 illustrates representative flatmounts and cross-sections from the group of mice treated with laser 6 weeks after vector injection. Mice that did not receive an intraocular injection showed large areas of CNV at sites of rupture of Bruch's membrane (Figs. 3A, 3B). Control mice that received an intravitreous (Figs. 3C, 3D) or subretinal (Figs. 3E, 3F) injection of 2.4×10^9 particles of UF12 showed areas of CNV that were very similar to those in uninjected mice. Mice that received an intravitreous (Figs. 3G, 3H) or subretinal (Figs. 3I, 3J) injection of 2×10^{10} particles of AAV-CBA-PEDF showed visibly smaller areas of CNV than did uninjected mice or mice injected with UF12.

Measurement of the area of CNV by image analysis in each of the groups showed that there was no significant difference between the mean area in uninjected mice and mice given an intravitreous or subretinal injection of empty virus. Mice treated with laser 4 (Fig. 4A) or 6 weeks (Fig. 4B) after intravitreous or subretinal injection of AAV-CBA-PEDF showed significantly smaller mean areas of CNV than did mice injected with control vector.

Discussion

Current treatments for CNV are ineffective, because they are directed at ablating the new vessels, but do not address the underlying angiogenic stimuli. Using adenoviral vectors, we have recently demonstrated that two proteins that have previously been shown to inhibit tumor angiogenesis, endostatin and PEDF, also inhibit ocular neovascularization.[13,20] These studies also provide proof of concept for use of gene transfer to treat ocular neovascularization. In the present study, PEDF inhibited ocular neovascularization, independent of the vector used to express the PEDF.

Another recent study has demonstrated that systemic administration of recombinant PEDF protein inhibits retinal neovascularization in the murine model of oxygen-induced ischemic retinopathy.[21] In that study, the minimum effective dose of PEDF protein was approximately 5 μg, administered by daily intraperitoneal injections. Assuming that 5 μg was the steady state, whole-animal level and correcting for the fractional volume of the eye relative to the whole body (both conservative assumptions), the threshold level of PEDF necessary to inhibit retinal neovascularization is estimated at approximately 2 ng/eye. All PEDF vector-treated eyes exceeded this level, usually by one order of magnitude or more. Therefore, the ocular levels of PEDF after gene transfer in our study that resulted in inhibition of CNV are likely to be above the therapeutic level for inhibition of retinal neovascularization.

The demonstration that AAV-mediated intraocular expression of PEDF reduces CNV at sites of rupture of the Bruch's membrane is important with regard to practical aspects of treatment development. Patients with age-related macular degeneration (AMD) are at risk for the development of CNV for many years, and long-term treatment is needed. Prolonged intraocular transgene expression has been achieved with AAV

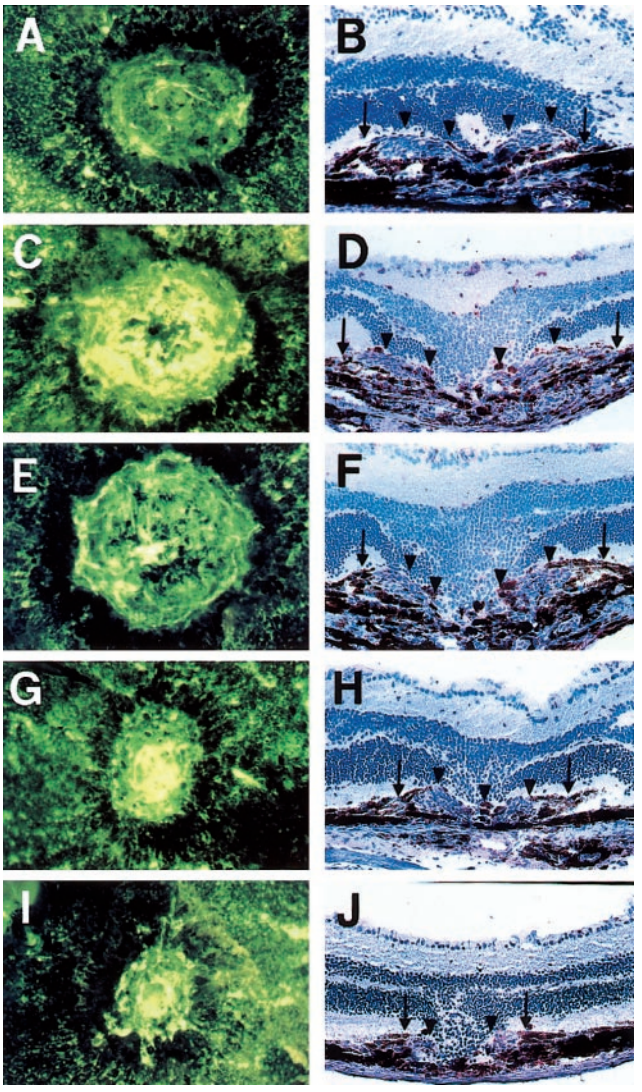

Figure 3. Smaller CNV lesions in eyes injected with AAV-CBA-PEDF compared with eyes injected with control vector. C57BL/6 mice were given an intravitreous or subretinal injection of control vector or AAV-CBA-PEDF. Six weeks after injection, the Bruch's membrane was ruptured with laser photocoagulation at three sites in each eye. Two weeks after rupture of the Bruch's membrane, the mice were perfused with fluorescein-labeled dextran, and choroidal flatmounts were prepared (**A**, **C**, **E**, **G**, **I**), or eyes were frozen and serial sections were stained with GSA lectin B4, which stains vascular cells, and counterstained with hematoxylin and eosin (**B**, **D**, **F**, **H**, **J**). The section showing the maximum diameter (*arrows*) for each CNV lesion is shown, and the thickness is indicated by the *arrowheads* along the surface. (**A**) Fluorescence microscopy shows a large CNV lesion at the rupture site of the Bruch's membrane in an eye that did not receive any injections. (**B**) A frozen section through the center of a CNV lesion in another uninjected eye shows a large maximum diameter (*arrows*). The lesion was thick, as shown by the *arrowheads* along its surface. Large CNV lesions were observed in eyes that received intravitreous (**C**) or subretinal (**E**) injection of control vector. A frozen section through the center of a CNV lesion in different eyes that received intravitreous (**D**) or subretinal (**F**) injection of control vector shows that the lesions had large maximum diameters (*arrows*). Small areas of CNV were observed in eyes that received intravitreous (**G**) or subretinal (**I**) injection of AAV-CBA-PEDF. A frozen section through the center of CNV lesions in different eyes that received intravitreous (**H**) or subretinal (**J**) injection of AAV-CBA-PEDF shows that the lesions had small maximum diameters (*arrows*).

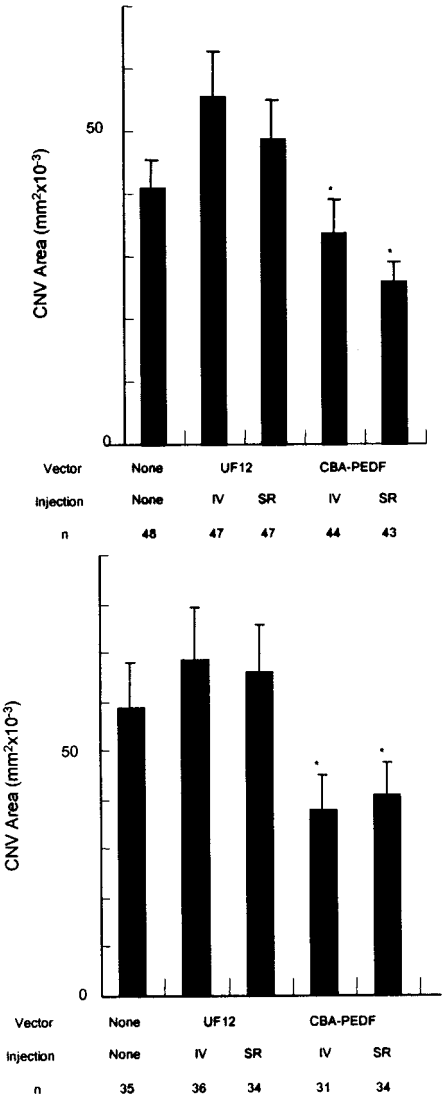

FIGURE 4. AAV-vectored PEDF inhibited CNV. Four weeks after intravitreous (IV) or subretinal (SR) injection of 4.0×10^9 particles of control vector (UF12) or 1.5×10^9 particles of AAV-CBA-PEDF (**A**) or six weeks after IV or SR injection of 2.4×10^9 particles of UF12 or 2.0×10^{10} particles of AAV-CBA-PEDF (**B**), C57BL/6 mice had laser-induced rupture of Bruch's membrane at three sites in each eye. Two weeks later, the mice were perfused with fluorescein-labeled dextran, choroidal flatmounts were prepared, and the area of CNV at each rupture site was measured by image analysis. $^*P < 0.05$ for difference from results of control vector administered by the same route, determined by unpaired *t*-test for samples with unequal variances.

vectors, and therefore they may provide the sustained intraocular production of antiangiogenic proteins that is likely to be needed to counter chronic production of angiogenic stimuli.

PEDF is a particularly appealing therapeutic candidate for patients with AMD. Although CNV is the major cause of severe visual loss in patients with AMD, most moderate loss of vision is due to death of photoreceptors and retinal pigmented epithelial (RPE) cells. PEDF was first identified as a component of conditioned medium of cultured fetal RPE cells that causes neurite outgrowth of Y79 retinoblastoma cells.[22,23] Several studies have suggested that PEDF has neuroprotective activity,[24–29] including protection of photoreceptors separated from the RPE from degeneration and loss of opsin immunoreactivity.[30] Therefore, long-term AAV-mediated expression of PEDF in the eyes of patients with early AMD may slow progression of the degeneration as well as reduce the likelihood of CNV.

Long duration of expression is not the only advantage of AAV vectors. Although use of adenoviral vectors is complicated by significant inflammation, there is no recognized AAV-mediated toxicity. Also, although AV vectors mediate higher levels of transgene expression after subretinal injections than after intravitreous injections,[13,31] there is comparable expression of PEDF after either intravitreous or subretinal injection of AAV-CBA-PEDF. This is probably because AAV-CBA vectors efficiently transduce ganglion cells, whereas adenoviral vectors do not. The attainment of comparable PEDF levels and efficacy after either intravitreous or subretinal injection of AAV-CBA-PEDF is important. From a clinical standpoint, intravitreous injections are easier and less invasive than subretinal injections, and whereas the former can be done in the clinic, the latter necessitates a procedure in the operating room.

The duration of AAV-mediated expression of proteins in the eye is not yet known. In rodents, expression appears to occur for the entire life of the animal (Flannery JG, Hauswirth WW, unpublished data). Although such long-term expression is an advantage from one viewpoint, it also raises potential concerns. If chronic expression of an antiangiogenic agent in the eye has some unsuspected deleterious effect, it may not be possible to halt the expression. Use of promoter systems that allow inducible expression could provide a safety net until the effects of long-term expression of PEDF in the eye are better understood. In any case, although further refinement of the system and more study are needed, the demonstration that AAV-mediated expression of PEDF in the eye inhibits CNV is an important step in the development of antiangiogenic gene therapy for patients with AMD.

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