Suppression of Choroidal Neovascularization by Adeno-associated Virus Vector Expressing Angiostatin

Chi-Chun Lai, Wei-Chi Wu, Show-Li Chen, Xiao Xiao, Tzung-Chieh Tsai, Shyh-Jer Huan, Tun-Lu Chen, Ray Jut-Fang Tsai, and Yeou-Ping Tsao

PURPOSE. To test the efficacy of a recombinant adeno-associated virus (rAAV) vector that expresses mouse angiostatin in suppressing experimental choroidal neovascularization (CNV) in a rat model.

METHODS. An rAAV vector, rAAV-angiostatin, was constructed to deliver the mouse angiostatin gene. rAAV-angiostatin and a control virus, rAAV-lacZ, were delivered in vivo by subretinal injection in Brown Norway rats, and the delivery was confirmed by reverse-transcriptase polymerase chain reaction (RT-PCR). For a CNV suppression experiment, CNV was generated by focal krypton laser photocoagulation 7 days after the viral vector injection and was evaluated by fluorescein angiography (FA) and histology. Apoptosis in retina was analyzed using the TUNEL assay. Inflammation in the retina was investigated by immunohistochemistry, using antibodies that recognize lymphocytes.

RESULTS. rAAV-angiostatin injection led to sustained expression of the angiostatin gene in chorioretinal tissue for up to 150 days. FA analysis revealed significant reduction of the average sizes of CNV lesions in rAAV-angiostatin–injected eyes when compared with rAAV-lacZ–injected eyes at both 14 (P = 0.019) and 150 (P = 0.010) days after injection. Moreover, histologic analysis of CNV lesions also revealed significantly smaller lesions in rAAV-angiostatin–injected eyes (P = 0.004). As for adverse effects, rAAV-angiostatin injection did not cause inflammation or apoptosis of cells in retina and choroid.

CONCLUSIONS. This is the first report that subretinal injection of rAAV-angiostatin can significantly reduce the sizes of CNV lesions. This and the absence of apoptosis and inflammation in chorioretinal tissue indicate the feasibility of a gene therapy approach for treatment of CNV disease. (Invest Ophthalmol Vis Sci. 2001;42:2401–2407)

A ge-related macular degeneration (ARMD) is a major cause of acquired blindness, which results from development of choroidal neovascularization (CNV) associated with overlying retinal damage.1,2 CNV is also generated in high myopia, angioid streaks, and some inflammatory diseases and after laser photocoagulation.3–5 In the clinical situation, the established treatment for CNV is laser photocoagulation,6,7 and presently only a small percentage of patients are eligible for this treatment. Furthermore, more than 50% of treated CNV has been reported to recur.8,9 The effectiveness of photodynamic therapy on CNV treatment has been demonstrated, yet recurrence of CNV is a major problem in clinical trials.10,11 Improved treatment for CNV is greatly needed.

A recently identified molecule, angiostatin, a 38-kDa internal fragment of plasminogen (Plg; amino acids 98–440) that encompasses the first four kringles of the molecules, was found to have an inhibitory effect on vessel endothelial proliferation in vitro and vessel growth inside tumors.12,13 Subcutaneous bolus injections of purified angiostatin in six different tumor models have been very effective in suppressing primary tumor growth, with no apparent toxicity.14 Until now, observations about angiostatin were centered on its potential as a tumor suppressor. The demonstrated suppression effect on vessel growth suggests that it may also be effective in suppressing CNV, although this potential has not been explored. In practice, long-term maintenance of therapeutic levels of angiostatin in vivo may be critical to arrest disease progression, because ARMD is a progressive disease, and the development of CNV is constantly promoted by angiogenic factors and microenvironmental changes.15–21 However, the delicate tissue and the difficulty in gaining access make repeated subretinal injection of recombinant angiostatin impractical. With the recent advances in gene therapy technique, genes can be delivered locally, and stable gene expression can maintain the level of therapeutic protein in target tissue. Recombinant adeno-associated virus (rAAV) vectors represent a highly efficient gene delivery system that can facilitate long-term transduction and have been used in a wide variety of gene therapy studies.22–25 Recently, we also reported the effective suppression of experimental arthritis and damages induced by cerebral ischemia by rAAV-based gene therapy approaches.26–28 Moreover, the potential of the rAAV vector in gene therapy for ocular diseases has been indicated by the delivery of marker gene by this vector, which achieves long-term and stable gene expression in retinal tissue.29–35

In this study, to establish the potential of combining the capability to arrest vessel growth by angiostatin and stable gene delivery by an rAAV vector in therapy for CNV diseases, an rAAV vector delivered an expression construct of the mouse angiostatin gene into the subretinal space, and CNV formation was induced by laser photocoagulation. The effectiveness of gene therapy on CNV formation was evaluated by fluorescein angiogram (FA) and histology.

MATERIALS AND METHODS

Generation of rAAV-Angiostatin
cDNA coding for mouse angiostatin was amplified by polymerase chain reaction (PCR) using the mouse plasminogen cDNA (American Type Culture Collection [ATTC], Rockville, MD; number 63112) as the template and two oligonucleotide primers 5'-ACGAGCTTGGATCCATGGACCAATAGGAAGTA-3' and 5'-ACGTCTAGAGGATCCTT-
ATATATCTAGGGTAATCCGGAACATCGTAGGTGTTGGGCAATTCCG-3', according to a published report. The PCR product was verified for its DNA sequences and cloned into an AAV vector plasmid pXX-UF1 to replace the green fluorescent protein (GFP) gene, thus placing the angiostatin gene under the transcriptional regulation of the cytomegalovirus immediate early promoter. The rAAV encoding angiostatin and the Escherichia coli lacZ gene were produced by a three-plasmid cotransfection system, as previously described. The recombinant AAV was purified by cesium chloride ultracentrifugation twice, as described. Titer s of rAAV-lacZ and rAAV-angiostatin were determined by dot blot hybridization using lacZ DNA and angiostatin cDNA as probes, respectively.

**Secretion of Angiostatin by rAAV-Angiostatin–Transduced Cells**

Strain 293 human embryonal kidney cells (10^6) were transduced with 10^10 rAAV-angiostatin or rAAV-lacZ particles. Conditioned medium was collected 72 hours after virus infection and subjected to concentration by lysis-Sepharose as described. Proteins were then separated by acrylamide electrophoresis, transferred to nitrocellulose membrane, and identified by immunoblot using monoclonal antibody 12CA5 which detects the hemagglutinin (HA) tag.

**Animals**

Brown Norway pigmented rats weighing between 200 and 250 g were used. The animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rats were anesthetized with intramuscular injection of 0.15 ml/kg of an equal-volume mixture of 2% lidocaine (Xylocaine; Astra, Astra Söderås, Sweden) and 50 mg/ml ketamine (Ketalar; Parke-Davis, Morris Plains, NJ).

**Subretinal Injections**

After rats were anesthetized, pupils were dilated with 1% tropicamide (1% Mydriacyl; Alcon Laboratories), and the eyes were gently protruded using a rubber sleeve. The eyes were then covered with sodium hyaluronidase (Healon; Pharmacia and Upjohn, Uppsala, Sweden) and a transparent disc to serve as a contact lens and allow surgery to be performed under an operating microscope. After a 90° peritomy in the temporal quadrant, a 1.5-cm 33-gauge blunt-tip syringe (Hamilton, Reno, NV) was inserted tangentially to the posterior pole of the eye and 2 µl of viral suspension containing 10^10 viral particles, which is roughly equal to 10^8 infectious units or 10^7 transduction units, was injected. Subretinal injection was confirmed by identifying a retinal detachment roughly the size of a quarter of retina. In a similar fashion, the contralateral eye was injected with rAAV-lacZ.

**Reverse Transcription–Polymerase Chain Reaction**

The expression of transgene in chorioretinal tissue was confirmed by reverse transcription–polymerase chain reaction (RT-PCR). The animals were killed with an overdose of anesthetic agent and the eyes enucleated. After the removal of anterior segment and vitreous, each remaining eye cup was dissolved in 500 ml TRIzol (Life Technologies, Rockville, MD). One microgram isolated RNA was treated with amplification grade DNase I before reverse transcription was performed. cDNA was synthesized using oligo(dT) primer and 200 IU transcriptase (SuperScript II; Life Technologies) according to the manufacturer’s instruction. PCR amplification was performed with two oligonucleotide primers, 5'-GGTATGTTGGCAATTCCG-3' and 5'-CTGTAGCC-GCTGGAGGTG-3', which are expected to generate a 500-bp angiostatin DNA fragment. The thermal profile consisted of a 5-minute denaturation at 94°C followed by 35 cycles consisting of a 1-minute denaturation at 94°C, a 1-minute annealing at 56°C, and a 1-minute extension at 72°C. The PCR products were separated by 1% agarose gel electrophoresis. The amplicons were then stained with ethidium bromide and photographed. As a control, the reverse transcriptase was removed from the RT-PCR to rule out the possibility that gene amplification products were derived from amplification of contaminated angiostatin DNA.

**Generation of CNV by Laser Photocoagulation**

Laser photocoagulation was performed 7 days after virus injection according to a published method, with modification. After the rats were anesthetized, pupils were dilated with 1% tropicamide (1% Mydriacyl; Alcon Laboratories). A small piece of transparent sheet (3M, Minneapolis, MN) approximately 3 mm in diameter was attached to the cornea by sodium hyaluronidase (Healon; Pharmacia and Upjohn) to serve as a contact lens. Krypton laser (Novus Omni; Coherent, Palo Alto, CA) irradiation was delivered through a slit lamp (Carl Zeiss, Oberkochen, Germany). Laser parameters used were as follows: spot size of 100 µm, power of 120 to 160 mW, and exposure duration of 0.1 second. An attempt was made to break Bruch’s membrane, as clinically evidenced by central bubble formation, with or without intraretinal or choroidal hemorrhage. Four lesions were created between the major retinal vessels in each fundus.

**Fluorescein Angiography**

The CNV lesions were studied at 14 and 150 days after laser photocoagulation by FA, with a digital fundus camera (Retinal Angiography; Heidelberg Engineering, Heidelberg, Germany). Fluorescein sodium (10%; 0.1 ml/kg; Fluorescein; Alcon, Fort Worth, TX) was injected into the tail vein of the anesthetized rats. Late-phase angiograms were obtained 8 minutes after injection, and digital fundus pictures of bilateral eyes were taken within 1 minute. The mean area of CNV was derived from measurement of all the CNV lesions by an ophthalmologist (W-CW) who was masked to the treatment of the eyes. In each eye the areas of CNV on FA were measured with image analysis software (Retina Angiography Area Measurement program; Heidelberg Engineering). The areas were outlined with the computer mouse, using the option provided by the software, and the results were expressed in square millimeters. The software was developed for the estimation of sizes of lesion in human eyes. The estimated sizes of lesion in the rat eye are not the actual size. Because the estimated sizes should be proportional to the actual sizes, they are used for comparing lesion size differences between different treatment groups.

**Histopathology Analysis**

For histologic analysis, eyeballs were harvested and fixed in 4% paraformaldehyde at 4°C for 24 hours. The fixed tissues were embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin. All the identifiable CNV lesions were measured for their size. When the size measurement was performed for a particular lesion, areas of the lesion on consecutive sections were measured to select the section that contained the largest area of the lesion. This area was used to represent the size of that particular lesion. In each eye analyzed, an effort was made to locate and measure all the CNV. Measurement was performed by a pathology technician who was masked to the treatment of the eyes. To determine the size of the lesion, the microscopic images of retina and a microslide scale were imported into a desktop computer. The areas of CNV were outlined with the computer mouse, and the areas were measured (Image Pro Plus, ver. 3.0 software; Media Cybernetics, Silver Spring, MD) and the results expressed in square millimeters.

**In Situ TUNEL Labeling**

In TUNEL analysis, eyeballs were harvested and marked for orientation and 5-µm sagittal sections were prepared after fixing in 4% paraformaldehyde at 4°C for 24 hours, embedded in paraffin, and sectioned. Sections were dewaxed in xylene and progressively hydrated. The
Primary retinal vessels were harvested 14 days (lane c) and 5 months (lanes d–f) after the infection of rAAV-lacZ (lanes a–d) and rAAV-angiostatin (lanes b, c, e and f), and total RNA was extracted. RT-PCR was then performed. For samples shown in lanes c and f, reverse transcriptase was excluded from the RT-PCR. M, molecular weight maker.

**Immunohistochemistry**

Eyeball sections were prepared as described for the TUNEL assay. Endogenous peroxidase was inactivated by immersing the sections with 3% H2O2 for 5 minutes at room temperature and washed in distilled water. Endogenous peroxidase was inactivated by incubating the sections with 3% H2O2 for 5 minutes at room temperature and washing them in distilled water. The sections were incubated with 5× c cacodylate buffer in a moist chamber for 1 hour at 37°C and washed in PBS. They were treated with peroxidase-conjugated streptavidin for 30 minutes at room temperature and washed with PBS. Diaminobenzidine was used as a chromogen. Counterstaining was performed with methyl green.

**Statistical Analysis**

The Wilcoxon signed-rank nonparametric test was used to test for statistical difference in the number and mean area of CNV lesions as revealed by FA and histology. The results were derived on computer (Prophet, ver. 5.0 software; BBN Systems and Technologies, Cambridge, MA).

**RESULTS**

**Synthesis and Characterization of rAAV-Angiostatin**

An rAAV vector delivering the angiostatin gene was synthesized. rAAV-angiostatin was then used to transduce the 293 cell strain in tissue culture, and the existence of angiostatin in culture supernatant was confirmed by immunoblot after angiostatin was concentrated by lysine-Sepharose, using the method described in a published report.13 As revealed in Figure 1, the 12CA5 antibody recognized angiostatin, which indicates the capability of rAAV-angiostatin to transduce cells into secreting angiostatin.

To confirm the gene delivery by rAAV-angiostatin in vivo, RT-PCR was performed to detect angiostatin mRNA in the chorioretinal tissue of virus-injected animals. Chorioretinal tissue was isolated, and total RNA was extracted from the retina of animals. RT-PCR analysis of angiostatin mRNA in chorioretinal tissue. Eyeballs were harvested 14 days (lanes a–c) and 5 months (lanes d–f) after the injection of rAAV-lacZ (lanes a–d) and rAAV-angiostatin (lanes b, c, e and f), and total RNA was extracted. RT-PCR was then performed. For samples shown in lanes c and f, reverse transcriptase was excluded from the RT-PCR. M, molecular weight maker.

**Prevention of CNV by rAAV-Angiostatin**

The potential suppression effect of rAAV-angiostatin on laser photocoagulation–induced CNV was investigated in Brown Norway rats. Twenty rats were included in the experiment. rAAV-lacZ virus (1011 particles per eye) was injected into the subretinal space of the left eyes of animals, and rAAV-angiostatin virus (1011 particles per eye) was injected in the right eyes. Seven days after rAAV injection, laser photocoagulation was performed in all the eyes (four laser burns per eye). Fourteen days and 150 days after laser photocoagulation, animals were subjected to FA examination.

In FA examination 14 days after viral vector injection, the average number of CNV lesions was 2.50 ± 0.70 per eye in eyes injected with rAAV-angiostatin and 3.50 ± 0.85 per eye in eyes injected with rAAV-lacZ (P = 0.059). The average number of CNV lesions was 3.20 ± 0.65 per eye in eyes injected with rAAV-angiostatin and 3.50 ± 0.53 per eye in eyes injected with rAAV-lacZ at 150 days after viral vector injection (P = 0.18). The suppression of the number of CNVs by angiostatin gene transfer was not significant. However, angiostatin gene transfer seemed to reduce the size of the CNV lesions. As shown in Table 1, the average size of CNV lesions in rAAV-angiostatin–injected eyes was significantly less than in rAAV-lacZ–injected eyes at both 14 (P = 0.019) and 150 (P = 0.010) days. Moreover, in many instances, the leakage spots in rAAV-angiostatin–injected eyes had less fluorescein intensity in the center of each CNV lesion and thus seemed to have formed an
The suppressive effect of angiostatin gene transfer on CNV formation did not lead to detectable apoptosis of chorioretinal cells. In the TUNEL assay and the already-suppressed lesions remained stable after a lengthy period.

To further establish the potential of angiostatin gene therapy as a remedy for CNV diseases, we also evaluated the potential side effects of angiostatin. TUNEL analysis was used to investigate whether apoptosis was induced by angiostatin. For TUNEL assay and immunohistochemical analysis, a separate group of five animals were injected in the subretinal space with rAAV-angiostatin in the right eye and with rAAV-lacZ in the left eye. TUNEL analysis was performed 10 days after rAAV injection. No apoptosis was identified in any area of the retinal and choroidal tissues in either rAAV-lacZ–injected eyes (Fig. 4a) or rAAV-angiostatin–injected eyes (Fig. 4b). Figure 4c shows a DNase-treated sample from an rAAV-angiostatin injected eye that stained positive in the TUNEL assay and served as a positive control. These indicate that angiostatin transgene expression did not lead to detectable apoptosis of chorioretinal cells.

Immunohistochemical analysis with antibodies that recognize lymphocytes was used to evaluate inflammation. The angiostatin injection was also found not to induce apoptosis or inflammation in chorioretinal tissue. Whether rAAV-angiostatin–based gene therapy is the solution for CNV diseases requires further investigation. Nevertheless, our results provide important evidence indicating the potential of this approach.

Under the same experimental conditions, we injected the eyes of experimental group had significantly smaller average sizes of CNV lesions on FA, when compared with the eyes in the control group. Histologic analysis also revealed significantly smaller lesions in rAAV-angiostatin–injected eyes. rAAV-angiostatin injection was also found not to induce apoptosis or inflammation in chorioretinal tissue. Whether rAAV-angiostatin–based gene therapy is the solution for CNV diseases requires further investigation. Nevertheless, our results provide important evidence indicating the potential of this approach.

To further confirm the effect of gene therapy on formation of CNV lesions, all the eyes were harvested and fixed immediately after the second FA analysis performed at 150 days after laser photocoagulation. Histologic analysis of CNV lesions was then performed. Altogether, there were 20 eyes used in both the angiostatin group and the lacZ control group. Fifty-nine and 67 CNV lesions were identified and measured in the angiostatin and the lacZ control groups, respectively. In animals injected with rAAV-lacZ, FA revealed prominent CNV formation (Fig. 3a). Histology of the same lesion showed damage to Bruch’s membrane and prominent CNV formation at and around the membrane (Fig. 3c). In contrast, in eyeballs injected with rAAV-angiostatin, FA revealed less prominent CNV formation (Fig. 3b) and histology of the same lesions showed smaller ones (Fig. 3d). The individual sizes of the lesions were then determined, and results revealed significant reduction of the size of lesions in rAAV-angiostatin–injected eyes. The average size of lesions in rAAV-angiostatin–injected eyes was 0.0155 ± 0.0056 mm² and that of lesions in rAAV-lacZ–injected eyes was 0.0253 ± 0.0092 mm² (P = 0.004). This further confirmed the suppressive effect of angiostatin gene transfer on CNV formation.

**Investigation on the Presence of Inflammation and Apoptosis in rAAV-Angiostatin–Injected Animals**

To further establish the potential of angiostatin gene therapy as a remedy for CNV diseases, we also evaluated the potential side effects, such as apoptosis and inflammation, that may be induced by angiostatin. TUNEL analysis was used to investigate whether apoptosis was induced by angiostatin. For TUNEL assay and immunohistochemical analysis, a separate group of five animals were injected in the subretinal space with rAAV-angiostatin in the right eye and with rAAV-lacZ in the left eye. TUNEL analysis was performed 10 days after rAAV injection. No apoptosis was identified in any area of the retinal and choroidal tissues in either rAAV-lacZ–injected eyes (Fig. 4a) or rAAV-angiostatin–injected eyes (Fig. 4b). Figure 4c shows a DNase-treated sample from an rAAV-angiostatin injected eye that stained positive in the TUNEL assay and served as a positive control. These indicate that angiostatin transgene expression did not lead to detectable apoptosis of chorioretinal cells.

Immunohistochemical analysis with antibodies that recognize lymphocytes was used to evaluate inflammation. The

**TABLE 1.** Mean Area of CNV with or without rAAV-Angiostatin Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>14 Days*</th>
<th>150 Days*</th>
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<tr>
<td></td>
<td>Angiostatin</td>
<td>LacZ</td>
</tr>
<tr>
<td>Mean area of CNV (mm²)†</td>
<td>1.13 ± 0.43</td>
<td>1.56 ± 0.69</td>
</tr>
<tr>
<td>Eyes (n)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>P</td>
<td>0.019</td>
<td>0.010</td>
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* Days after injection of rAAV-angiostatin or rAAV-LacZ.
† Sizes, estimated by computer, do not represent the actual size of the CNV lesion (Wilcoxon signed-ranks nonparametric test).

Data are means ± SD.

**FIGURE 2.** Suppression of laser photocoagulation-induced CNV by rAAV-angiostatin, identified by FA. Rat eyes were injected in the subretinal space with rAAV-angiostatin (a, b) or rAAV-lacZ vectors (c, d). Laser photocoagulation was performed 7 days after rAAV injection. FA examinations were performed 14 (a, c) and 150 (b, d) days after laser photocoagulation.
nasal quadrants and those in temporal quadrants. Our observation indicated that angiogenesis outside the area of angiostatin gene–transduced cells is also suppressed. This is conceptually acceptable, because angiostatin is a secretory factor, as confirmed in Figure 1, and may diffuse within retina throughout the whole retina and suppress angiogenesis everywhere. It could be argued that the secreted angiostatin originating from a restricted area of retina would set up a diffusion gradient of angiostatin in the subretinal space. However, under certain conditions lesions exposed to higher concentrations of angiostatin may be of similar size to lesions exposed to lower concentrations. One possible condition is that the gradient of angiostatin is not steep. If the half-life of angiostatin is much longer than the time required for it to be transported to a remote area, the accumulation of angiostatin will reduce the ratio between the areas proximate and remote to the bleb.

**Figure 3.** Histopathology of rAAV-angiostatin–transduced retina. After the second FA analysis performed at 150 days after laser photocoagulation, animals were killed and the eyes enucleated, and randomly sampled eyeballs were subjected to histology examination. (a) FA analysis of eyes injected with rAAV-lacZ. (b) FA analysis of eyes injected with rAAV-angiostatin. (c) Histology of the CNV lesion marked by the arrow in (a); (d) histology of the CNV lesion marked by arrowhead in (b). Scale bar, 25 μm.

**Figure 4.** Identification of apoptosis and inflammation in choroidoretinal tissue. TUNEL analysis was performed in tissue under the bleb from an eye injected with rAAV-lacZ (a) and an eye injected with rAAV-angiostatin (b). (c) Tissue section from the same source as in (b), except that it was treated with DNase I before labeling. (d, e) Immunohistochemical analysis using antibody recognizing lymphocyte common antigen in a normal naive eye (d), an eye injected with rAAV-lacZ (e), and an eye injected with rAAV-angiostatin (f). Scale bar, 5 μm.
Another possibility is that the size reduction of CNV lesions may not be proportional to the concentration of angiostatin. There may be multiple mechanisms involved in CNV formation. The concentration of angiostatin in remote areas may be high enough to inhibit CNV growth by inhibiting some of these mechanisms. The concentration in areas proximate to the bleb may be higher, but still may not inhibit other mechanisms and further reduce the size of the lesions. We do not have evidence to support these possible explanations. However, the available evidence does not exclude these possibilities.

The pathophysiology of CNV disease is a combination of ischemia and a persistent Bruch’s membrane defect that may stimulate constant expression of angiogenic factors.2 Long-term presence of therapeutics seems to be necessary to overcome the progressive nature of CNV. Previously, genes delivered by rAAV into the retinas of experimental animals have been shown to be very stable.26 In this study, angiostatin gene expression remained detectable up to 150 days (Fig. 1b). To further confirm the stable expression of the angiostatin gene, we injected recurrent CNV by repeated laser photocoagulation in rAAV-angiostatin–injected animals at 150 days after the injection of rAAV-angiostatin. Our preliminary data indicate that rAAV-angiostatin also protected animals from recurrent CNV induced by repeated laser photocoagulation (Tsao et al., unpublished results, 2000). Such stable gene expression should be able to satisfy the requirement for persistent levels of therapeutic proteins in therapy for CNV diseases.

Although rAAV-angiostatin injection can reduce the size of CNV lesions, the reduction of the number of CNV lesions by gene therapy was not significant in our study. In this study, a high-energy laser beam was used to generate Bruch’s membrane defects, and the effect was evident from the higher induction efficiency of the CNV lesion than has been reported previously.35–37 CNV lesions from such damage may be difficult to prevent completely by gene therapy. However, most CNV diseases involve subtle membrane damage, and gene therapy by angiostatin may still be effective. Moreover, there may be room for improvement in the virus titer and injection technique in the future.

In this study, we observed very little spontaneous regression of CNV. This may be because of the high-energy laser beam used to generate Bruch’s membrane damage. Our results were statistically significant. Whether the sample size of 20 animals is large enough is debatable. However, since this report was first submitted, more than 60 animals have been subjected to the same experimental protocol, and we have determined that the findings described in this article are reproducible.

The potential of angiostatin in tumor suppression has been fully explored, and the capability of suppressing vascular endothelial cell growth has been proposed as the mechanism.3–14 Unlike tumor suppression, the therapeutic goal of CNV diseases is not to starve proliferating tumor cells but to prevent the synthesis of leak-prone neoangiogenesis. In our observation, although angiostatin gene therapy only partially prevented the formation of CNV, the remaining CNV lesions became smaller and had less fluorescein leakage, indicating a milder vascular defect. From this standpoint, although it did not completely abolish the formation of CNV as revealed by histopathology and FA, rAAV-based gene therapy described herein may still be a potential instrument for the prevention of CNV formation.

The successful gene delivery that suppressed the formation of CNV also provided a powerful instrument for exploring pathogenesis of CNV and developing rational design of therapy in the future. Recently, several potential mechanisms by which angiostatin inhibits endothelial cell migration and/or proliferation were proposed. Binding to ATP synthase,38 upregulating selectin,39 preventing the downregulation of caveolin-1,40 and blocking matrix-enhanced plasminogen activation41 are a few examples. Whether these mechanisms are also involved in the suppression of CNV by angiostatin remains to be determined. Moreover, the effects of angiostatin on the expression and/or function of factors involved in angiogenesis, such as basic fibroblast growth factor, vascular endothelial cell growth factor, and matrix metalloproteinases have not been studied.37,42,43 The rAAV-mediated angiostatin gene delivery shown in this study can be a valuable tool to help address these important issues.

The mechanism through which angiostatin gene therapy reduces fluorescein leakage in FA remains unclear. Retinal endothelial cell tight junctions and adhesion junction complexes are important for the permeability of the retinal capillary vessel wall.44 Whether pathologic changes of endothelial cell tight junctions and adhesion junction complexes can be prevented by angiostatin gene therapy is currently under investigation.

References


