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Inhibition of Choroidal Neovascularization by Intravenous Injection of Adenoviral Vectors Expressing Secretable Endostatin

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Endostatin is a cleavage product of collagen XVIII that inhibits tumor angiogenesis and growth. Interferon α2a blocks tumor angiogenesis and causes regression of hemangiomas, but has no effect on choroidal neovascularization (CNV). Therefore, inhibitors of tumor angiogenesis do not necessarily inhibit ocular neovascularization. In this study, we used an intravenous injection of adenoviral vectors containing a *sig-mEndo* transgene consisting of murine immunoglobulin κ-chain leader sequence coupled to sequence coding for murine endostatin to investigate the effect of high serum levels of endostatin on CNV in mice. Mice injected with a construct in which *sig-mEndo* expression was driven by the Rous sarcoma virus promoter had moderately high serum levels of endostatin and significantly smaller CNV lesions at sites of laser-induced rupture of Bruch's membrane than mice injected with null vector. Mice injected with a construct in which *sig-mEndo* was driven by the simian cytomegalovirus promoter had ~10-fold higher endostatin serum levels and had nearly complete prevention of CNV. There was a strong inverse correlation between endostatin serum level and area of CNV. This study provides proof of principle that gene therapy to increase levels of endostatin can prevent the development of CNV and may provide a new treatment for the leading cause of severe loss of vision in patients with age-related macular degeneration. *(Am J Pathol 2001, 159:313–320)*

Neovascularization plays a role in several disease processes including tumor growth, arthritis, diabetic retinopathy, and neovascular age-related macular degeneration. However, it is not clear if the molecular signals that mediate the neovascular process in these different settings are similar or different. Some differences are likely, because endothelial cells in different parts of the body have different characteristics.[1] Also, surrounding cells and extracellular matrix, which vary among different tissues, participate in the pathogenesis of neovascularization resulting in tissue-specific aspects. Thus, although it is widely accepted that neovascularization occurs because of altered balance between stimulators and inhibitors, the specific stimulators and inhibitors may vary depending on the setting.

The potential for tissue-specific features makes it hazardous to predict how a protein or drug will affect neovascularization in one disease process based on its effects in another. Transforming growth factor-β and thrombospondin inhibit neovascularization in some settings and stimulate it in others.[2–5] The retina and choroid are highly specialized tissues with vasculatures that have unique features making it particularly difficult to predict their response to purported stimulators and inhibitors. Tissue inhibitor of metalloproteinase-1 has been touted as an inhibitor of neovascularization,[6] but it stimulates vascular endothelial growth factor-induced neovascularization in the retina.[7] Interferon α2a causes dramatic involution of hemangiomas[8] and inhibits iris neovascularization in a model of ischemic retinopathy,[9] which led to the prediction that it would inhibit choroidal neovascularization (CNV). However, a multicenter, randomized, placebo-controlled trial demonstrated that patients with CNV who received interferon α2a do not have any involution of CNV and end up with worse vision than those treated with

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placebo.[10] Therefore, testing in relevant animal models is necessary to predict the effect of proteins or drugs on ocular neovascularization.

Endostatin is a cleavage product of collagen XVIII that inhibits tumor angiogenesis resulting in inhibition of tumor growth.[11] Gene transfer provides a strategy to achieve sustained release of endostatin and can circumvent difficulties arising from handling the protein. Adenoviral vectors transduce the liver efficiently after systemic administration, and consequently they can produce high serum levels of encoded secreted proteins. In this study we performed intravascular injections of adenoviral vectors containing a transgene consisting of murine immunoglobulin (Ig) κ chain signal sequence coupled to sequence coding for murine endostatin. This allowed us to determine the effect of high serum levels of endostatin in a model of CNV.

Materials and Methods

Generation of Adenoviral Vectors

The adenoviral vectors used in the experiments described here have the following genes deleted: E1A, E1B, E2, and E3. The expression cassettes have been placed in the position of the E1A gene. Construction of Av3mEndo, in which the Rous sarcoma virus promoter drives expression of murine endostatin fused to the IgGκ signal peptide, has been described.[12] Av3CsmEndo is identical to Av3mEndo except that the simian cytomegalovirus promoter was substituted for the Rous sarcoma virus promoter. Av3Null and Av3CsNull are identical to Av3mEndo and Av3CsmEndo, respectively, but lack the cDNA inserts.

The Av3mEndo, Av3Null, Av3CsmEndo, and Av3CsNull vectors were reamplified in S8 cells with 0.3 μmol/L dexamethasone in Richter's conditioned medium containing 5% fetal bovine serum until cytopathic effect was observed. The adenoviral vector titer (particles per ml) and biological titer (plaque-forming units per ml) were determined as previously described.[13] The correct genome structures of each of the purified vectors were confirmed by restriction digests and Southern blot analysis. The Av3mEndo and Av3CsmEndo were confirmed to be negative for replication-competent adenovirus by the Gene Therapy Core Technologies Molecular Core Laboratory at Genetic Therapy, Inc.

Gene Transfer to Mice and Induction of CNV

Viral vectors were injected into the tail vein of adult C57BL/6 mice. Mice were injected with 2×10^{11} particles of either Av3mEndo ($n = 18$) or Av3Null ($n = 17$) or with 6×10^{10} particles of either Av3CsmEndo or Av3CsNull. Four days after viral vector injection, the mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight), pupils were dilated with 1% tropicamide, and krypton laser photocoagulation was used to rupture Bruch's membrane at three locations in each eye of each mouse as previously described.[14] Briefly, krypton laser photocoagulation (100 μm spot size, 0.1 seconds duration, 120 mW) was delivered using the slit-lamp delivery system of a Coherent model 920 photocoagulator and a hand-held cover slide as a contact lens. Burns were performed in the 9, 12, and 3 o'clock positions 2 to 3 disk diameters from the optic nerve. Production of a vaporization bubble at the time of laser, which indicates rupture of Bruch's membrane, is an important factor in obtaining CNV,[14] so only burns in which a bubble was produced were included in the study. A bubble was not produced for one burn in mice injected with Av3mEndo and three burns in mice injected with Av3Null. The cornea of one eye of a mouse that had been injected with Av3mEndo had a corneal scar that prevented visualization for laser and that eye could not be used.

Measurement of the Size of Laser-Induced CNV Lesions

Two weeks after laser treatment, the size of CNV lesions was evaluated by one of two different techniques, measurement of the integrated area of CNV on serial sections as previously reported[15] or measurement of the area of CNV in choroidal flat mounts.[16] For mice injected with Av3mEndo, 10 mice were evaluated by the flat mount technique and 8 by serial sections, and for mice injected with Av3Null, 10 mice were evaluated by the flat mount technique and 7 by serial sections.

Mice used for the flat-mount technique were anesthetized and perfused with 1 ml of phosphate-buffered saline containing 50 mg/ml of fluorescein-labeled dextran (2×10^6 average molecular weight; Sigma, St. Louis, MO) as previously described.[17] 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 (4 to 7, average 5) were made from the edge of the eyecup to the equator and the eyecup was flat-mounted in Aquamount with the sclera facing down and the choroid facing up. Flat mounts were examined by fluorescence microscopy and images were digitized using a 3-charge-coupled device color video camera and a frame grabber. Image-Pro Plus was used to measure the total area of hyperfluorescence associated with each burn, corresponding to the total fibrovascular scar. For mice injected with Av3mEndo, a total of 19 eyes were evaluated (one eye had a pre-existent corneal scar that precluded laser treatment) and there was one burn that had not been associated with a bubble, so that 56 lesions were measured. For mice injected with Av3Null, a total of 20 eyes were evaluated and because there were three burns that had not been associated with a bubble, 57 lesions were measured. The areas within each eye were averaged, and after log transformation, regression analysis with generalized estimating equations was performed using SAS software (SAS Institute Inc., Cary, NC) to compare average areas in eyes of mice receiving Av3mEndo *versus* Av3Null. This analysis adjusts for correlation between right and left eyes of each mouse.

Mice used to measure the integrated area of CNV on serial sections were sacrificed 2 weeks after laser treatment and eyes were rapidly removed and frozen in opti-

mum-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 *Griffonia simplicifolia* lectin B4 (GSA; Vector Laboratories, Burlingame, CA) that selectively binds to vascular cells. Slides were incubated in methanol/H_2O_2 for 10 minutes at 4°C, washed with 0.05 mol/L 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 mol/L TBS, they were incubated with avidin coupled to alkaline phosphatase (Vector Laboratories) for 45 minutes at room temperature. After being washed for 10 minutes with 0.05 mol/L TBS, slides were incubated with Histomark Red (Kirkegaard and Perry) to give a red reaction product that is distinguishable from melanin. Some slides were counterstained with Contrast Blue (Kirkegaard and Perry).

To perform quantitative assessments, GSA-stained sections were examined with an Axioskop microscope and images were digitized using a 3-charge-coupled device color video camera and a frame grabber. Image-Pro Plus software was used to delineate and measure the area of GSA-stained blood vessels in the subretinal space. For each lesion, area measurements were made for all sections on which some of the lesion appeared and added together to give the integrated area measurement. Measurements were analyzed as described above for area measurements on flat mounts.

Comparison of Mice Injected with Av3mEndo to Those Injected with Av3CsmEndo

Mice were injected in the tail vein with 2×10^{11} particles of Av3mEndo ($n = 10$) or Av3Null ($n = 9$), or they were injected with 6×10^{10} particles of Av3CsmEndo ($n = 11$) or Av3CsNull ($n = 11$). A no injection control group ($n = 11$) was also included. Four days after injection, Bruch's membrane was ruptured with laser in three places in each eye of each mouse as described above. Seven days after injection, blood was drawn from the tail vein of 47 of the 52 mice (despite multiple attempts, serum could not be obtained from five mice) and serum was stored at −80°C for enzyme-linked immunosorbent assays (ELISAs). Eighteen days after injection and 14 days after laser, the area of CNV was assessed on choroidal flat mounts as described above.

ELISAs for Endostatin

Endostatin serum levels were determined with a murine endostatin ELISA kit (Accucyte murine endostatin; CytImmune Sciences, College Park, MD) according to the manufacturer's instructions.

Measurement of β-Galactosidase Activity

Livers and eyes were snap-frozen after removal from mice. On the day of the assay, livers or eyes were homogenized in lysis buffer [40:1 v/v 1× reporter lysis buffer (Promega, Madison, WI) and protease inhibitor cocktail (Sigma, St Louis, MO)]. Protein content was determined by Bradford assay (BioRad, Hercules, CA). β-galactosidase activity was determined using the Galacto-Light system (Tropix, Bedford, MA).

Results

Construction of Recombinant Adenoviral Vectors Expressing Murine Endostatin

The construction and characterization of Av3mEndo has been described.[12] The supernatant from Av3mEndo-transformed S8 cells contains a 20-kd protein, the expected size of endostatin, that potently inhibits vascular endothelial growth factor-165-induced migration of human umbilical vein endothelial cells. An ELISA assay demonstrated that 10^6 Av3mEndo-transduced Hep3B cells secrete 1 to 2 μg of murine endostatin per 24 hours.

Intravascular Injection of Av3mEndo Inhibits Development of CNV

In initial experiments, the amount of CNV at sites of laser-induced rupture of Bruch's membrane was compared in mice injected with Av3mEndo and mice injected with Av3Null. The amount of CNV was assessed by two different techniques; measurement of the area of CNV perfused by fluorescein-labeled dextran on choroidal flat mounts and measurement of the area of CNV on serial sections through the entire lesion, a technique that we have used in previous studies.[15,18] Computerized image analysis performed by investigators masked with respect to treatment group showed that the mean area of perfused CNV lesions on flat mounts was significantly less in mice injected with Av3mEndo compared to Av3Null-injected controls (Table 1). The integrated area of CNV obtained by adding together the area of CNV on each serial section, which assesses size in three dimensions, confirmed that there was significantly less CNV at sites of Bruch's membrane rupture in mice injected with Av3mEndo compared to Av3Null injected-mice (Table 1). Differences are larger between the treated and untreated groups using the first technique, possibly because with the second technique, we measured the entire hyperfluorescent area and in treated eyes, vessels did not always occupy the entire area of hyperfluorescence (Figure 1E). Also, the second technique ignores any difference in the thickness of CNV. Therefore, the second measurement technique underestimates treatment effect, but because both techniques provide similar information, only choroidal flat mounts were used in subsequent experiments. Also, in subsequent experiments, some mice that did not receive any vector injections were treated with laser to evaluate for a possible null vector effect.

Figure 1A shows the typical appearance of laser-induced CNV in a choroidal flat mount of a no-injection

Table 1. Mice Injected with Av3mEndo Develop Less CNV than Mice Injected with Av3Null after Rupture of Bruch's Membrane

Area of perfused CNV on choroidal flat mounts						Integrated area of CNV on serial sections through entire lesions					
Vector	Mice	Eyes	Lesions	Area (10^{-3} mm^2)	*P*	Vector	Mice	Eyes	Lesions	Integrated area (10^{-2} mm^2)	*P*
Av3mEndo	10	19	56	13.73 ± 1.36	0.0003	Av3mEndo	8	15	44	5.88 ± 0.91	<0.0001
Av3Null	10	20	57	29.41 ± 2.19		Av3Null	7	13	37	12.58 ± 2.21	

Using two different approaches to quantitate the amount of CNV, mice injected intravascularly with Av3mEndo, which expresses endostatin, showed a highly significant decrease in the size of CNV lesions at sites of rupture of Bruch's membrane compared to mice injected with null vector. *P* values are for the difference in CNV size between Av3mEndo-injected and null vector-injected mice and were calculated by regression analysis with generalized estimating equations.

control mouse. The CNV had a similar appearance in mice injected with Av3Null (Figure 1B), but appeared smaller in mice injected with Av3mEndo (Figure 1C). Serial sections stained with GSA show the CNV as red-stained tissue in the subretinal space. The section through the largest part of a typical CNV lesion in a mouse injected with Av3Null is shown in Figures 1F (low power) and 1H (high power). The distance between the arrows represents the approximate diameter of the lesion and the arrowheads along the superior border help to point out the thickness. The section through the largest part of a typical CNV lesion in a mouse injected with Av3mEndo is shown in Figure 1G (low power) and 1I (high power). Note that the approximate diameter and thickness are less than that in the typical lesion of a null vector-injected mouse (1F).

Inverse Correlation between Endostatin Serum Levels and Area of CNV

Characterization of a second vector construct, Av3CsmEndo, demonstrated that intravascular injection of 6×10^{10} particles resulted in ~10-fold higher maximal

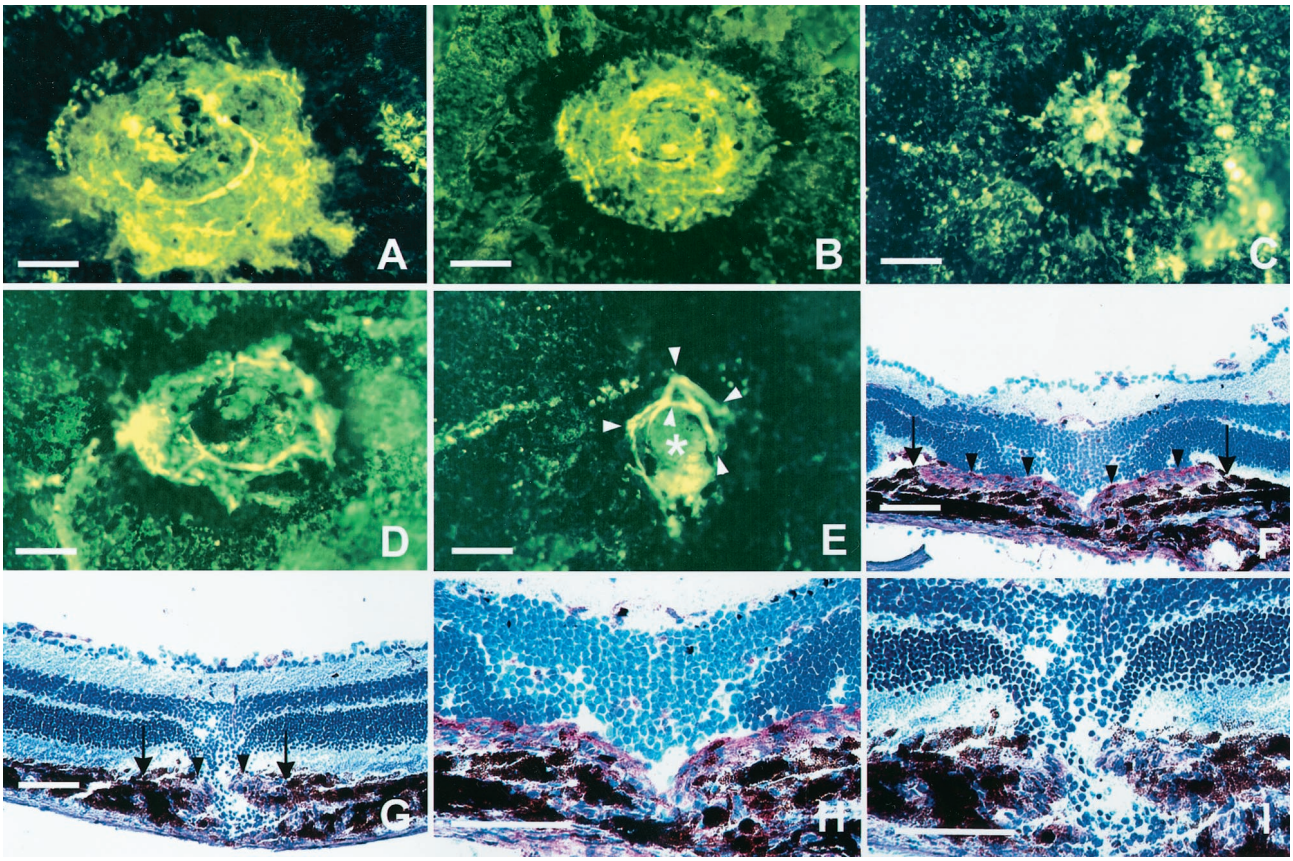

Figure 1. Intravascular injection of adenoviral vectors containing the *endostatin* gene inhibits CNV. Four days before laser-induced rupture of Bruch's membrane, mice were given no injection (**A**) or were injected into the tail vein with one of the following vector constructs: Av3Null (**B**, **F**, and **H**), Av3mEndo (**C**, **G**, and **I**), Av3CsNull (**D**), or Av3CsmEndo (**E**). Fourteen days after laser, some mice were perfused with fluorescein-labeled dextran and choroidal flat mounts were evaluated by fluorescence microscopy (**A–E**) and the eyes of other mice were frozen and serial sections through entire CNV lesions were stained with a vascular cell-selective lectin to give a red reaction product and counterstained with Contrast Blue (**F–I**). Injection of vectors containing *endostatin* resulted in smaller CNV lesions (**C**, **E**, **G**, and **I**) compared to no injection (**A**) or injection of null vectors (**B** and **D**), and in many cases there was diffuse hyperfluorescence at the site of rupture of Bruch's membrane (**E**, **asterisk**) with few vascular structures (white **arrowheads**). In **F** and **G**, the **arrows** show the horizontal extent of the CNV and the **arrowheads** the vertical extent. Scale bar, 100 μm.

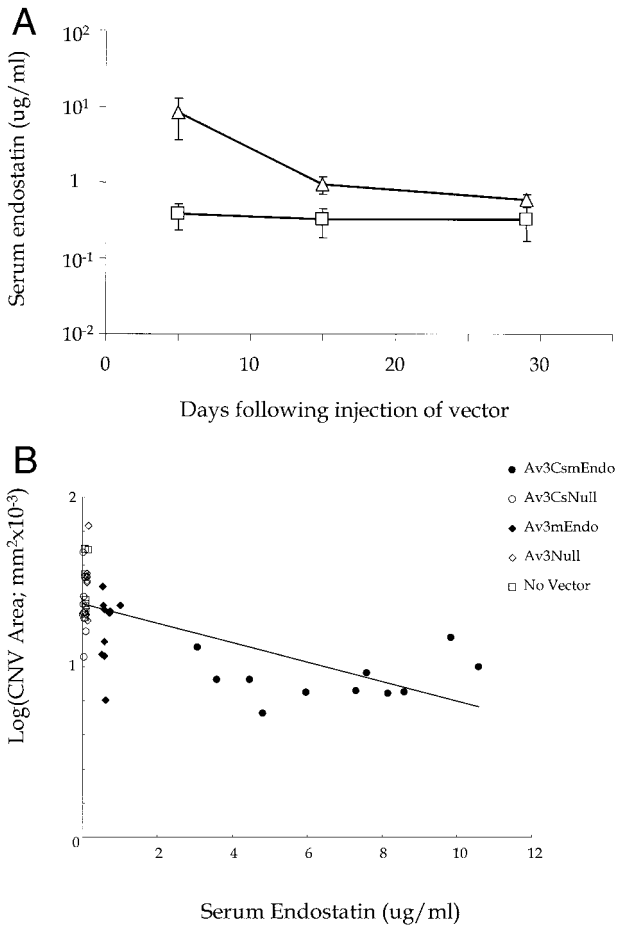

Figure 2. Inverse correlation of size of CNV lesions with endostatin serum level in mice with laser-induced rupture of Bruch's membrane. **A:** Serum levels of endostatin were measured at the time points indicated in nude mice after the injection of 2×10^{11} particles of Av3mEndo, **squares**, or 6×10^{10} particles of Av3CsmEndo. Error bars show standard deviations, $n = 5$. **B:** Four days after intravascular injection of null vectors, Av3mEndo (2×10^{11} particles), or Av3mCsmEndo (6×10^{10} particles), Bruch's membrane was ruptured in three locations in each eye. At 7 days after vector injection, blood was drawn from tail veins and endostatin serum levels were measured by ELISA. Two weeks after laser, mice were perfused with fluorescein-labeled dextran and the area of CNV was measured by image analysis of choroidal flat mounts. The mean area of CNV in each mouse (it was not possible to obtain serum from 5 of 52 mice, and therefore 47 mice were included in the analysis) is plotted against the endostatin serum level. There is a strong negative correlation with $r = -0.66$.

endostatin levels compared to levels in mice injected with the maximum tolerated dose (2×10^{11} particles) of Av3mEndo (Figure 2A). The palindromic series I repeats in the simian cytomegalovirus promoter behave as strong basal enhancers and cyclic AMP response elements boosting expression.[19] The kinetics of expression differ for the two vector constructs, but serum endostatin attains maximal levels 4 to 7 days after intravenous injection and remains greater than background (30 to 150 ng/ml, results not shown) for at least 1 month.

Mice were injected with Av3mEndo or Av3CsmEndo and control groups were injected with matched doses of Av3Null or Av3CsNull. Laser treatment was done on day 4 and serum was obtained 7 days after injection. With investigators masked with respect to vector group and endostatin serum level, the area of CNV was measured on choroidal flat mounts 14 days after laser photocoagulation. The typical CNV lesion in mice injected with Av3CsmEndo (Figure 1E) appeared smaller than those in mice injected with Av3CsNull (1D). Image analysis confirmed that the area of CNV lesions was significantly less in mice injected with either Av3CsmEndo or Av3mEndo compared to controls (Table 2). A plot of the mean area of CNV lesions *versus* endostatin serum level in each mouse showed a strong inverse correlation with $r = -0.66$ (Figure 2B).

Intravascular Injection of Reporter Gene Vector Constructs Results in High Expression in Liver and No Detectable Expression in the Eye

To determine whether systemic administration of adenoviral vectors results in significant transduction of the eye, a group of mice was injected with Av3nBg. This vector expresses β-galactosidase from an Rous sarcoma virus promoter. After 5 days, the mice were sacrificed and β-galactosidase activity was measured in homogenates of the eye and liver using a chemiluminescence assay. In the livers of mice that received vector, levels of β-galactosidase activity were ~1000-fold higher than uninjected controls, whereas in the eye, the levels of this enzyme activity were similar between vector-injected and control animals (Figure 3). The absence of detectable β-galactosidase activity in the eye after administration of an adenovirus expressing this enzyme suggests that the anti-angiogenic effect after intravascular injection of endostatin vectors is because of systemically produced rather than locally produced endostatin.

Table 2. Both Av3mEndo and Av3CsmEndo Inhibit the Development of CNV after Rupture of Bruch's Membrane

Vector	Mice	Eyes	Lesions	Area (10^{-3} mm^2)	*P*
Area of perfused CNV on choroidal flat mounts					
Av3CsmEndo	11	22	66	8.87 ± 0.85	*<0.0001, **<0.0001
Av3mEndo	10	19	55	18.36 ± 2.24	*0.0013, **0.0004
Av3CsNull	11	21	62	24.41 ± 2.92	*0.22
Av3Null	9	17	48	32.91 ± 4.87	*0.89
No vector	11	21	59	31.71 ± 3.98	

*, For difference from no vector controls; **, for difference from corresponding null vector control by regression analysis with generalized estimating equations.

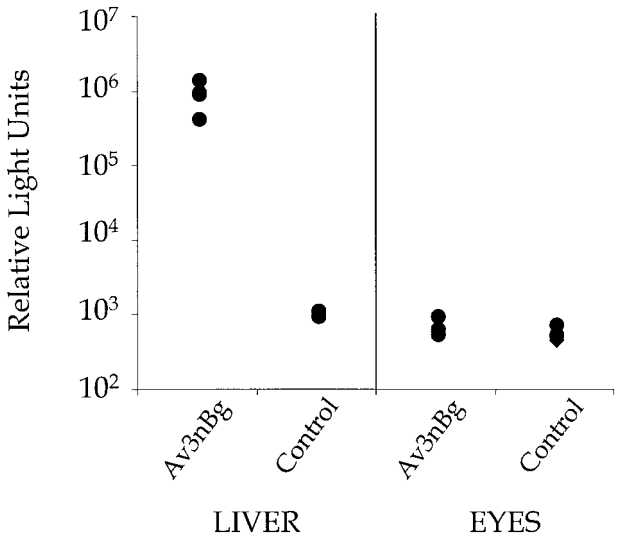

Figure 3. Intravascular injection of Av3nBg results in high levels of β-galactosidase activity in the liver and levels in the eye that are not greater than background. Five days after intravascular injection of 2×10^{11} particles, mice were sacrificed and the liver and eyes were rapidly removed and β-galactosidase activity was measured in liver and eye homogenates. β-galactosidase activity was three orders of magnitude higher in the liver than in the eye where it was the same as that in eyes not injected with Av3nBg.

Discussion

In this study, we have demonstrated that intravenous injection of adenoviral vectors containing an expression construct for endostatin results in significant prevention of laser-induced CNV in mice. There is a strong negative correlation between the area of CNV and the serum level of endostatin. Previous studies have suggested that liver cells are preferentially transduced by adenoviral vectors injected into the venous circulation.[20,21] In the current study, we injected β-galactosidase reporter gene packaged in our vectors and showed high expression in the liver with no detectable expression in the eye. Secretion of endostatin into the circulation by transduced liver cells provides a useful experimental approach to investigate the effect of endostatin on neovascularization in remote vascular beds, because it eliminates any possible confounding effect from local injection and minimizes the possibility of any effect of the viral vector. A remote possibility is that the inhibition of CNV is because of a nonspecific effect of increased protein in the circulation. However, this is very unlikely; in fact, in other experiments we injected adenoviral vector containing an expression construct for another protein and this did not result in decreased CNV (data not shown). Therefore, our data provide strong evidence that endostatin is a good inhibitor of CNV and that its inhibitory activity is dependent on its serum concentration.

Intravascular injections of large doses of recombinant endostatin inhibit tumor growth in mice and even cause tumor regression.[11] Endostatin-treated tumors are much less vascular, suggesting that inhibition of tumor angiogenesis is the basis for the tumor regression. Gene transfer provides a means to achieve sustained release of endostatin and avoid storage, potential loss of activity, and multiple injections of recombinant protein. Two studies have explored nonviral approaches for *endostatin* gene transfer in tumor models.[22,23] Intramuscular injection of a synthetic polymer containing an expression plasmid encoding a secretable form of mouse endostatin resulted in peak serum levels reported as 8 ng/ml, which is less than the range of endogenous endostatin levels in our experiments. This suggests that the increase in endostatin levels were quite modest, but they resulted in decreased growth of subcutaneous renal cell carcinoma by 40%, and a sixfold decrease in lung metastases.[22] Similar results were obtained in nude mice implanted with MDA-MB-435 tumors in which liposomes complexed to plasmids encoding angiostatin or endostatin were injected.[23]

Compared to nonviral approaches, *endostatin* gene transfer with recombinant adenoviral vectors results in higher serum levels and more dramatic effects. In one study,[24] peak endostatin serum levels were 1740 ng/ml and growth of subcutaneous breast cancer was slowed resulting in a 60% reduction in tumor size. The effect on pre-established Lewis lung carcinoma tumors was investigated by injecting vector containing the murine *endostatin* gene or control vector 7 days after subcutaneous tumor implantation. There was a 78% reduction in tumor size and complete absence of metastases in the treated group compared to controls, but no tumor regression. In another study,[25] peak serum levels of endostatin were 1770 ng/ml resulting in a 40% inhibition of MC38 adenocarcinoma growth. Using the same Av3mEndo vector reported on in the present study, Chen and colleagues[12] found that compared to Av3Null-injected mice, which all died from liver metastasis 2 months after implantation of poorly differentiated human stage IV colon carcinoma, Av3mEndo-injected mice survived significantly longer, with 25% still alive at 4 months. Although these results are good, they are not comparable to the dramatic tumor regression achieved with injection of 10 to 20 mg/kg/day of recombinant endostatin.[11]

Our data show a strong correlation between endostatin serum levels and prevention of CNV after rupture of Bruch's membrane. To our knowledge, this is the first demonstration of endostatin-induced inhibition of intraocular neovascularization. This is an important finding, because as noted above, there are agents that inhibit tumor angiogenesis and have no effect on CNV. Furthermore, CNV is a major public health problem; it is responsible for the vast majority of severe visual loss in patients with age-related macular degeneration, the most common cause of visual morbidity in patients older than the age of 60 in developed countries.[26] The magnitude of this already huge problem is continuing to increase as our population ages. Our current treatments for CNV are inadequate, because they are all directed at ablation of the abnormal vessels and do nothing to address the stimuli for blood vessel growth. As a result, they are all plagued by recurrences. Treatments that antagonize or neutralize the stimuli for blood vessel growth are urgently needed.

Our data suggest that endostatin could potentially provide a means to counter the strong stimuli for neovascularization that are present in the eyes of patients with

age-related macular degeneration and related diseases. Because sustained high levels of endostatin are needed for the effect, and efficacy increases with increasing concentration, gene transfer offers advantages as a possible therapeutic approach. In this study, we used systemic gene therapy to provide proof of concept. Because endostatin is an endogenous protein and is not known to have toxic effects, it is conceivable that systemic gene therapy could be used in nonmalignant neovascular diseases such as CNV. However, it is also conceivable that high serum concentrations of endostatin could have deleterious effects, and recently patients with systemic sclerosis have been noted to have elevated serum levels of endostatin.[27] Highest levels of endostatin were found in patients with cutaneous scars or ulcers and it was postulated that endostatin could contribute to ischemic complications. Local delivery of vectors to the eye provides an appealing alternative to systemic delivery that deserves investigation.

Adenoviral vectors provide rapid onset of fairly high level expression and therefore are ideal for proof of concept, but do not provide long-term expression, which is disadvantageous for treatment of chronic diseases such as CNV in age-related macular degeneration. Helper-dependent adenoviral vectors provide long-term expression in baboons while maintaining high serum levels of secreted protein,[28]or alternatively adeno-associated virus or lentivirus vectors could be considered. Although many details must be worked out, our data suggest that *endostatin* gene therapy may provide a new approach in patients with age-related macular degeneration who are at high risk for development of CNV.

The implications of our study go beyond using endostatin for prevention of CNV. Experiments are underway to determine whether increasing levels of endostatin after development of CNV results in regression. We have previously demonstrated that vascular endothelial growth factor kinase inhibitors prevent the development of CNV;[15,18] it will be important to determine whether combining endostatin gene therapy with vascular endothelial growth factor kinase inhibitors provides synergy with regard to prevention and/or regression of CNV. Also, there are several other proteins and/or protein cleavage products, in addition to endostatin, that are purported endogenous inhibitors of angiogenesis including angiostatin,[29] antithrombin III,[30] platelet factor-4,[31] and pigment epithelium-derived factor[32] just to name a few. Additional studies are needed to assess the relative efficacy and safety of these and other proteins alone and in combination, because the present study demonstrates that the general approach of using purported protein inhibitors may have merit for treatment of ocular neovascularization and justifies the enormous amount of work and resources that such studies will require.

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