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# Subconjunctival injection of recombinant AAV-angiostatin ameliorates alkali burn induced corneal angiogenesis

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**Purpose:** To evaluate the effect of subconjunctival injection of recombinant adeno-associated virus (rAAV)-angiostatin in alkali burn-induced corneal angiogenesis.
**Methods:** Adeno-associated viral vector-mediated gene delivery into extraocular tissue was determined by fluorescent microscopy three weeks after subconjunctival injection of viral vector expressing green fluorescent protein (rAAV-GFP). Subconjunctival injection of recombinant adeno-associated viral vector expressing human angiostatin (rAAV-angiostatin) and blank rAAV viral vector (control) was performed in the left eye of male Sprague-Dawley rats (n=6). Alkaline induction of corneal neovascularization (NV) was performed three weeks later. Corneal NV regression was analyzed 7-14 days after alkali burn with slit lamp and digital pictures. Transgenic expression of angiostatin in the cornea, conjunctiva, retina, and muscle insertions was confirmed by reverse-transcriptase polymerase chain reaction (RT-PCR).
**Results:** Transgenic GFP gene expression was detected mainly in the muscle fibers at the extraocular muscle (EOM) insertions after subconjunctival injection. The area of corneal neovascularization was significantly lower in eyes injected with rAAV-angiostatin ($p<0.01$) than in eyes injected with the control virus. RT-PCR demonstrated that the angiostatin gene expression was highly detectable in muscle fibers and not detectable in the conjunctiva, cornea, and retina.
**Conclusions:** Subconjunctival injection of rAAV-angiostatin reduced alkali burn-induced corneal angiogenesis. We proved the concept that ocular gene therapy by subconjunctival injection of adenovirus-associated gene transfer of angiogenesis inhibitors can be a simple and safe treatment modality that can achieve therapeutic levels and long lasting effects in the treatment of corneal NV induced by ocular surface disorders.

Ocular neovascularization (NV) is a sight-threatening condition involved in several pathologic ocular disorders such as corneal neovascularization, retinopathy of prematurity, proliferative diabetic retinopathy, age-related macular degeneration, and neovascular glaucoma. In the cornea, neovascularization involves the formation of new vessels originating from the limbus. A variety of inflammatory, infectious, degenerative, and traumatic disorders of the cornea and the limbal stem cells may induce corneal NV. The major ocular complications include loss of corneal transparency and immune privilege [1].

Several angiogenic factors including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and transforming growth factor (TGF)-$\alpha$ and -$\beta$ have a vital role in corneal neovascularization [1]. Also, several anti-angiogenic factors such as angiostatin [2,3] and pigment epithelium-derived factor (PEDF) [4] may play a role in the control of corneal neovascularization. The regulation of corneal angiogenesis is a complex process which involves the equilibrium between pro- and anti-angiogenetic factors. Loss of equilibrium between these factors will result in abnormal corneal angiogenesis [5]. Unfortunately, at present there is no effective therapy for visually significant corneal NV except more invasive methods such as corneal transplantation. Through the understanding of the physiologic mechanism of corneal transparency and wound healing, modulation of key regulators may be possible and as a result, corneal NV can be reduced.

Several treatment modalities have currently been used for corneal neovascularization including medication (angiostatic steroids, nonsteroidal inflammatory agents, and anti-angiogenesis factors), surgery (limbal transplantation, amniotic membrane transplantation, conjunctival transplantation, and penetrating keratoplasty), electrocoagulation, and laser photocoagulation (argon laser and dynamic phototherapy). Although they have shown to be effective in animal models to inhibit corneal NV, there are still many limitations and complications associated with these treatment modalities [1]. Purified anti-angiogenic peptides have shown potential benefits in corneal NV inhibition in many experimental animal studies. These include prolactin [6], endostatin [7], angiostatin [8], somatostatin [9], TNP-470 [10], cyclosporin A [11], steroids [12], nonsteroidal anti-inflammatory drugs [13], thalidomide [14], methotrexate [15], and culture supernatant of human amniotic cells [16]. However, most in vivo experimental studies

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have tried to suppress corneal NV by exposing the cornea directly to the anti-angiogenic peptide such as in intrastromal injection [17,18] and micropoket assay [19]. These treatment modalities might result in a loss of corneal transparency due to damage of the delicate corneal structure by the procedure itself. Experimental studies with topical eyedrops have shown promising results as an advantageous route for drug delivery to the cornea [20-22]. It is non-invasive, maintains corneal integrity, and results in minimal adverse effects due to systemic administration. At present, the primary limitations for topical application of anti-angiogenetic drugs include difficulty of formulation, low water solubility, low stability in solution, and susceptibility to lose bioactivity during long-term storage [22].

Ocular gene therapy has gained popularity for reasons which include: (1) the eye is an easily accessible target suitable for local administration; (2) local gene delivery has high transduction efficiency; (3) local gene delivery to the eye can be incorporated into routine surgical procedures in humans; and (4) local gene delivery to the eye allows exposure of the target tissue with reduced risk of systemic effects [18]. In this study, we demonstrated that ocular surface gene therapy by subconjunctival injection of adenovirus-associated gene transfer of angiogenetic inhibitors is a simple and safe treatment modality that can achieve therapeutic levels and long lasting effects in the treatment of corneal NV induced by ocular surface disorders.

## METHODS

*Generation of recombinant adeno-associated virus-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'-ACG AAG CTT GGA TCC ATG GAC CAT AAG GAA GTA-3' and 5'-ACG TCT AGA GGA TCC TTA TAT ATT CTA GCG TAA TCC GGA ACA TCG TAG GGT ATG TGG GCA ATT CCC-3', according to a published report [23]. 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 [24], thus placing the angiostatin gene under the transcriptional regulation of the cytomegalovirus immediate early promoter. The recombinant adeno-associated virus (rAAV)-encoding angiostatin was produced by a three-plasmid cotransfection system, and the recombinant AAV was purified by cesium chloride ultracentrifugation twice as previously described [25]. Titers of rAAV-angiostatin were determined by dot blot hybridization using angiostatin cDNA as probes.

*Secretion of angiostatin by rAAV-angiostatin-transduced cells:* Strain 293 human embryonal kidney cells ($1x10^6$) were transduced with $1x10^{10}$ rAAV-angiostatin particles. The conditioned medium was collected 72 h after virus infection and subjected to concentration by lysine-Sepharose as described [23]. 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 [23]

*Animals:* Ten- to twelve-week-old male Sprague-Dawley rats weighing between 250-300 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 using 25 mg/kg intramuscular injection of ketamine (Ketalar; Parke-Davis, Morris Plains, NJ). Topical anesthesia to the ocular surface was performed with proparacaine 0.1% (Allergan Inc., Irvine, CA)

*Subconjunctival injections:* After rats were anesthetized, eyes were gently protruded using a rubber sleeve. Subconjunctival injection was performed in the left eyes by using a Hamilton syringe with 30-gauge tip (Hamilton, Reno, NV) and into the temporal quadrant of the bulbar conjunctiva. Five microliters of viral suspension containing $1x10^{10}$ viral particles of rAAV-angiostatin, which is roughly equal to $1x10^8$ infectious units or $1x10^7$ transduction units, was injected. The same procedure was performed in the left eyes of rats used for localization of transgenic protein expression with rAAV-GFP and the control group eyes that received blank rAAV viral vector. The fellow eyes of all animals were used as the negative control without receiving any procedure. Animals were maintained for three weeks before being used to maximize the rAAV-mediated transgene expression.

*Alkali-induced corneal neovascularization:* After animals were anesthetized, alkali injuries to the control and study eyes were created by 30 s exposure of the central cornea to a 3 mm diameter disk of filter paper soaked in 1 N NaOH followed by rinsing with sterile saline (20 ml). The study eyes (n=6) received a subconjunctival injection of 5 μl of rAAV-angiostatin three weeks before induction of the alkali injury. Similarly, the control eyes (n=6) received a subconjunctival injection of blank rAAV viral vector three weeks before induction of the alkali injury. Injured corneas were then evaluated daily by slit lamp biomicroscopy. Digital photographs of the cornea were stored for masked measurement of the corneal NV area at days 7 and 14 after alkali injury. To take the digital pictures, animals were anesthetized and eyes were gently protruded using a rubber sleeve. Pupils were dilated with 1% tropicamide (1% Mydriacyl; Alcon Laboratories, Hemel Hempstead, UK) to eliminate the iris vessels that might interfere with the corneal NV image.

*Quantification of corneal neovascularization:* All corneas were photographed using a digital camera (Nikon coolpix 995, Tokyo, Japan) connected to a surgical microscope (Carl Zeiss, OPMI-1, Berlin, Germany). Standardized illumination, contrast, and threshold settings are used to capture images, which were stored as files with jpeg extensions on the memory cards of the digital camera and transferred into the computers desktop. Areas containing blood vessels were traced on the computer monitor, calculated with image analysis software, and reported in square millimeters. Briefly, the whole cornea was outlined, and a conversion factor was calculated based on the actual area of the cornea in square millimeters relative to its size on the computer. Each value for the area of

neovascularization was calculated using the conversion factor. The areas of corneal NV were outlined with the computer mouse, and the areas were measured (Image Pro Plus, ver. 3.0 software; Media Cybernetics, Silver Spring, MD). The results expressed in square millimeters.

*Reverse-transcription polymerase chain reaction:* The expression of transgene in the cornea, conjunctiva, retina and extra-ocular muscle (EOM) were confirmed by reverse-transcription polymerase chain reaction (RT-PCR). Three weeks and 16 weeks after subconjunctival injection of rAAV-angiostatin, the animals were killed with an overdose of the anesthetic agent, and the ocular tissue was harvested. The bulbar conjunctiva, cornea, retina, and EOM tissue adjacent to their insertions at the sclera were all carefully dissected. The same procedure was performed for the control group that received rAAV viral vector. EOM bellies were also removed and analyzed with RT-PCR for angiostatin expression. Briefly, the isolated tissues were dissolved in 500 μl TRIzol (Life Technologies, Rockville, MD). Isolated RNA (one μg) was treated with amplification grade DNase I before reverse transcription was performed. cDNA was synthesized using oligo primer [26] and 200 IU transcriptase (SuperScript II; Life Technologies) according to the manufacturer's instruction. PCR amplification was performed with two oligonucleotide primers, 5'-GGT ATG TGG GCA ATT CCC-3' and 5'-CCT GTC AGC GCT GGA GTG-3', which is expected to generate a 500-bp angiostatin DNA fragment. The thermal profile consisted of a 5 min denaturation at 94 °C followed by 35 cycles consisting

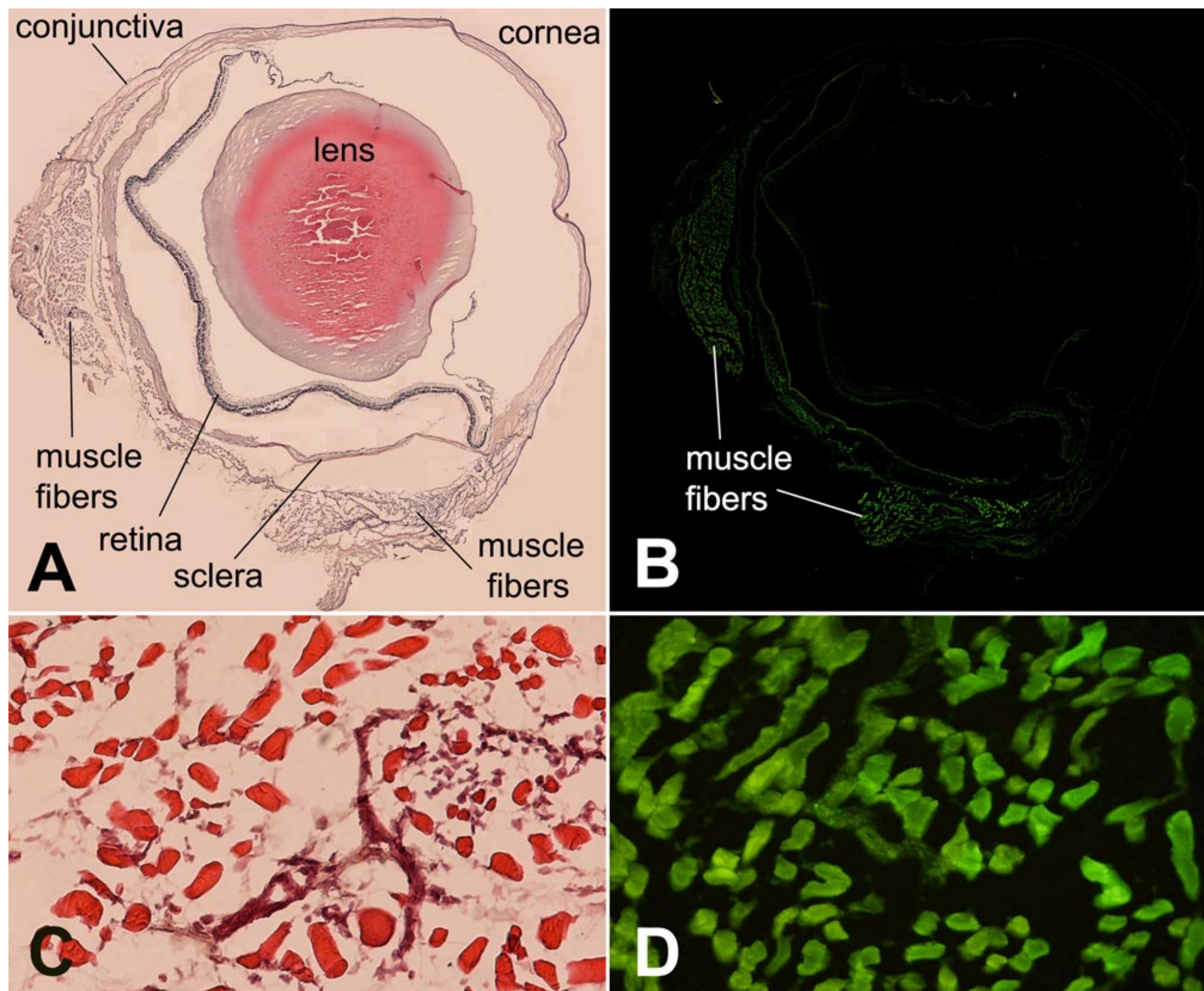


Figure 1. Histopathology and distribution of green fluorescent protein expression after subconjunctival injection of rAAV-GFP. Hematoxylin-eosin staining (**A**) and its corresponding fluorescent microscopy findings (**B**) of GFP overview expression in a rat eye three weeks after subconjunctival injection of rAAV-GFP is shown. GFP expression was distinctly detected in the muscle fibers adjacent to the EOM insertions. No evident green fluorescence was detectable in the conjunctiva, subconjunctival soft tissue, cornea, retina, choroid, or vitreous. Magnification, 40x. **C**: Hematoxylin-eosin staining of muscle fibers adjascent to EOM insertions is shown. Scarce inflammatory cell infiltration can be noted between the muscle fibers. **D**: Corresponding fluorescent microscopy findings of cryo-section in **C**. Note intense expression of GFP by the muscle fibers. Magnification, 400x.

of a 1 min denaturation at 94 °C, a 1 min annealing at 56 °C, and a 1 min 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.

*Statistical Analysis:* Analysis of the significance of differences between two groups was performed by using the paired or unpaired Student's *t*-test (SPSS 10.0 statistical software; SPSS, Chicago, IL). Differences were accepted as significant at $p<0.01$.

## RESULTS

*Localization of transgenic protein expression and histopathology analysis:* Three weeks after subconjunctival injection of rAAV-GFP, fluorescence microscopy of a rat eye cryosection demonstrated that rAAV-GFP effectively infected muscle fibers at the site of the subconjunctival injection. Normal background autofluorescence signal was detected in the sclera and RPE/photoreceptor complex in the normal control eyes and blank rAAV viral vector controls (data not shown). Intensive GFP gene expression can be detected at the EOM insertion fibers of the rAAV-GFP infected eyes (Figure 1B,D). No evident expression of GFP was identified at the conjunctiva, cornea, sclera, choroid, or retina. This demonstrated that subconjunctivally injected rAAV infected structures nearby the location of injection, respecting anatomic and physiologic boundaries (Figure 1B). rAAV-GFP eyes, rAAV viral vector control eyes, and normal control eyes were sectioned, and histopathology analysis with hematoxylin-eosin showed corneas devoid of vessels and eyes with only minor inflammatory cell infiltration in the rAAV-GFP group when compared with both rAAV viral vector eyes and normal control eyes. This demonstrated that the subconjunctival injection of rAAV-mediated gene transfer did not induce any significant changes in the normal physiology of treated eyes (Figure 1).

*Alkali burn-induced corneal neovascularization in rat model:* After the alkali burn, the injured central corneal stroma appeared opaque and edematous. Opacification and edema increased during the post-burn normal saline irrigation. Twenty-

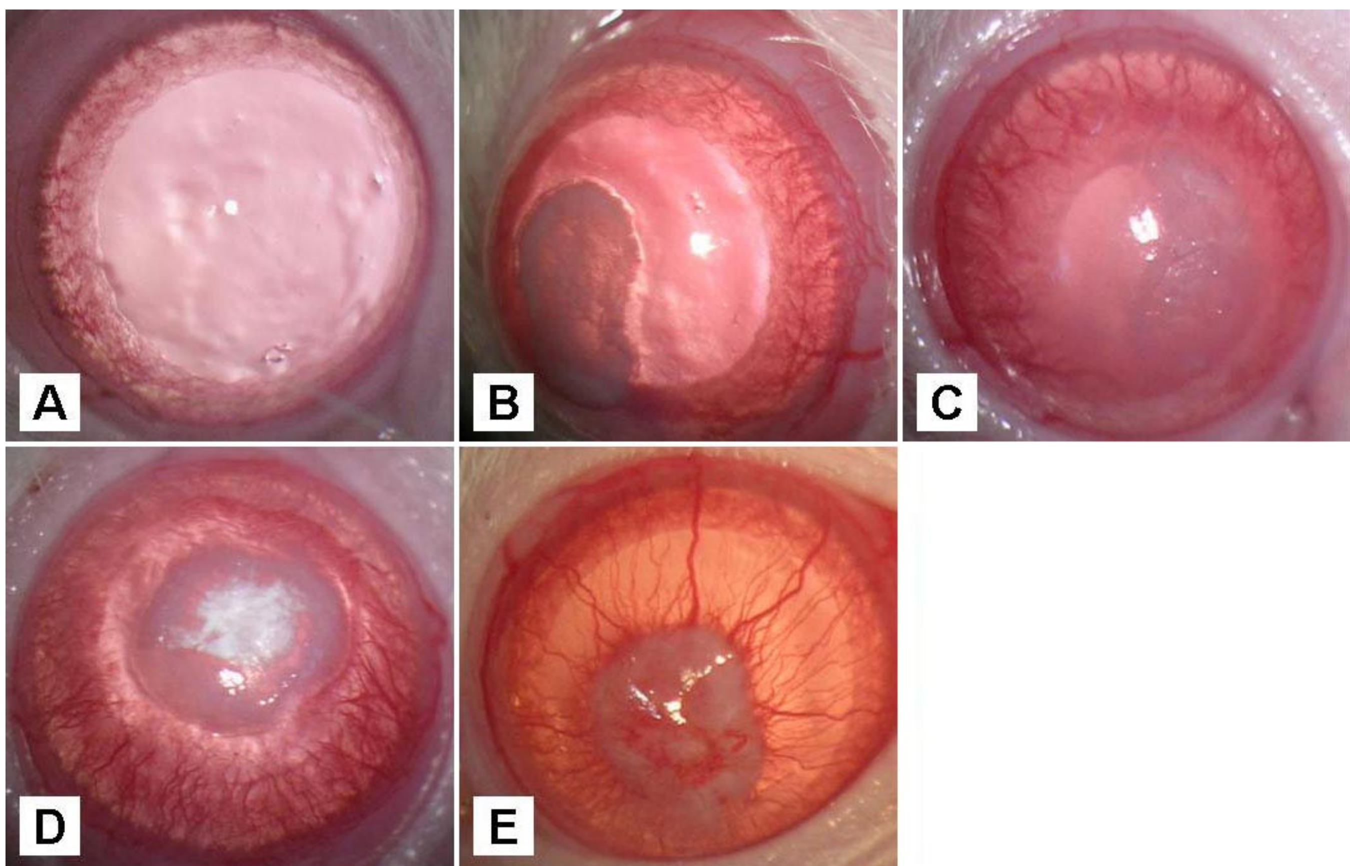


Figure 2. Alkali burn-induced corneal neovascularization in the rat animal model. Rats received alkali burns on the central cornea of their left eyes. Corneal NV was monitored by slit lamp examinations. **A**: In the Control treatment a normal and clear cornea was shown before induction of alkali burn. **B**:An opaque central cornea was observed after exposure to 1 N NaOH at PD0. **C**: At PD3, an initial peripheral NV ingrowth toward the central cornea with increased central corneal opacification was observed. **D**: At PD7, dense NV ingrowth, reaching the central cornea was observed. Note an elevated pannus surrounding the area of central epithelial defect. **E**: At PD14, cornea NV maturation with decreased vessels caliber was observed. Note that the central corneal scarring and pannus formation were stationary.

four h after wounding, an acute inflammatory response was noted. Neovascularization, originating from the limbal vessels and extending toward the central corneal burn, was clearly visible by 72 h. The onset of peripheral NV occurred clinically on postoperative day 2 (POD2), reaching maximum proliferation across the entire cornea from the limbus by POD7. The following days showed that the progressive decrease of the inflammatory activity could be characterized by a decrease in the central corneal epithelial defect, decreased haze, and corneal edema. Neo-vessel maturation and corneal scarring were evident by POD14. Under light microscopy, a large number of vessels and inflammatory cells were observed in the cornea after the alkali burn. Although there were variations between individual animals in the progression of NV, the NV growth from limbal vessels toward the central cornea was relatively uniform around the circular corneal injury in all ani-

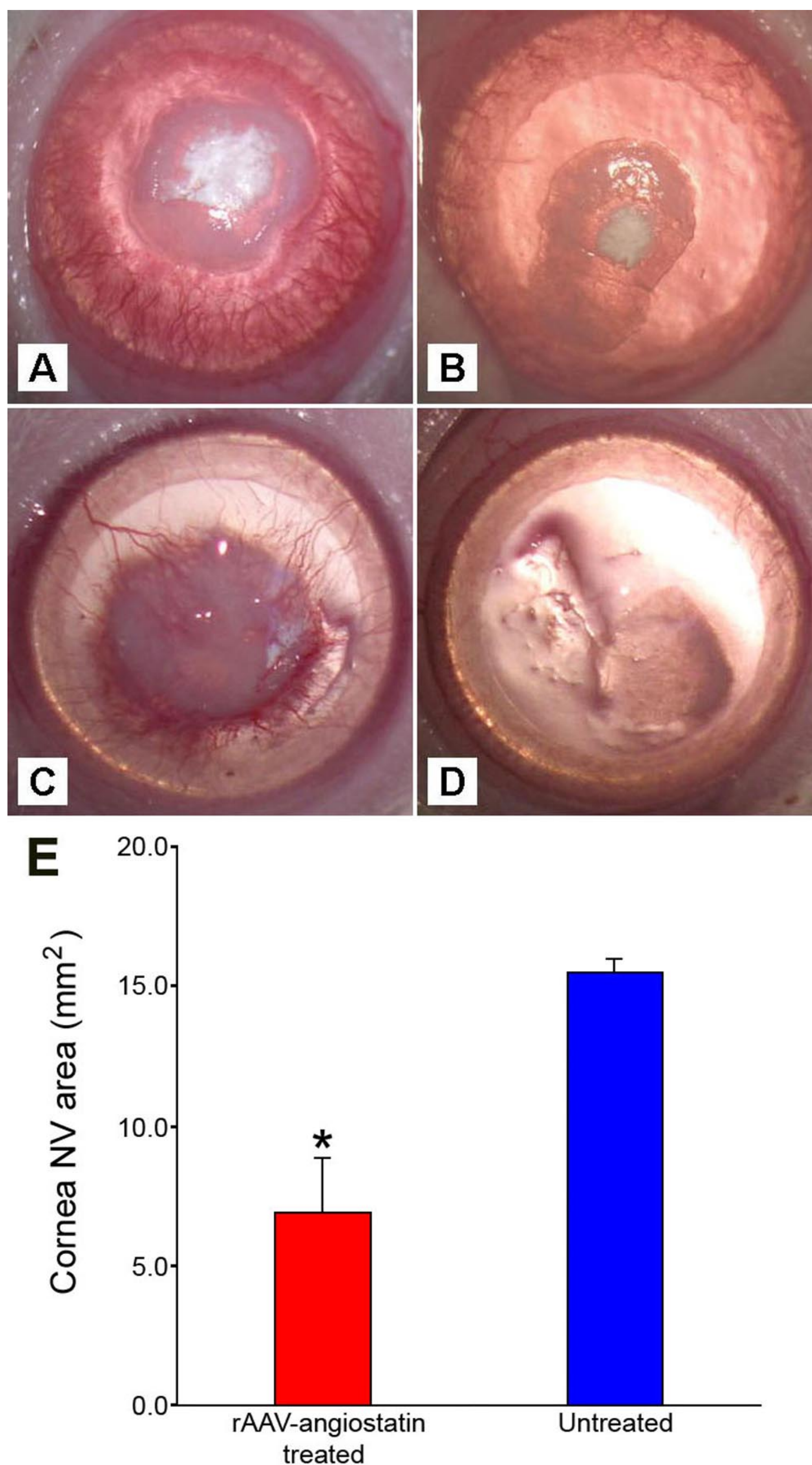


Figure 3. Effect of subconjunctival injection of rAAV-angiostatin on alkali burn-induced cornea neovascularization regression. One week before infliction of the alkali burn with 1 N NaOH in rats, the rats were assigned randomly either to a treatment group or control group. The treatment group received subconjunctival injection of rAAV-angiostatin three weeks before the alkali burn, and the control group received blank rAAV viral vector treatment. Corneal NV was examined daily by slit lamp for two weeks. Representative photographs of alkali burn-induced corneal NV at days 7 and 14 are shown. **A**: Dense NV is growing toward the central cornea. Seven days after the alkali burn, there was severe corneal opacity and epithelial defects in the rAAV viral vector control eye. **B**: The rAAV-angiostatin injected eye seven days after the alkali burn is shown. Note the scarcity of corneal NV and the mild to moderate corneal opacity. **C**: The rAAV viral vector control eye at day 14 follow-up is shown. Corneal NV and pannus maturation were evident. **D**: The rAAV-angiostatin injected eye 14 days after the alkali burn is shown. Not only the corneal NV was absent, but also the central corneal opacity was mild. **E**: The corneal NV area (mean±SD, n=6) measured at seven days after exposure to the alkali burn is shown. The rAAV-angiostatin treated group demonstrated significantly smaller NV area compared with the rAAV viral vector control group (the asterisk means that $p<0.05$).

mals. These results show that the alkali burn-induced corneal NV is a simple and useful animal model for corneal NV (Figure 2).

*Effect of subconjunctival injection of rAAV-angiostatin on corneal neovascularization regression:* In our model, alkali burn-induced corneal NV was induced three weeks after subconjunctival injection of rAAV-angiostatin. Corneal NV formed one week after the injury. At this point, rAAV-angiostatin injection resulted in an obvious regression of corneal NV in the treated group (Figure 3). The mean corneal NV area at POD7 with standard deviation (±SD) was 15.83±0.55 $mm^2$ and 6.87±2.23 $mm^2$ for rAAV viral vector control group and rAAV-angiostatin treated group, respectively. Quantification of the NV area at POD7 confirmed a significantly smaller NV area in the rAAV-angiostatin treated eye than in the controls ($p<0.01$; Figure 3E). The quantification of corneal vascularization were focused on day 7 post-treatment as the vessels were markedly engorged, and the NV area could be easily outlined for area calculation. This data suggests that rAAV-angiostatin effectively induced regression of newly formed vessels in the cornea.

*Angiostatin gene expression in ocular tissue:* RT-PCR was performed on mRNA extracted from harvested ocular tissues three weeks and 16 weeks after the subconjunctival injection of rAAV-angiostatin for angiostatin expression. The examined tissues included pooled corneas, bulbar conjunctiva, EOM insertions, and retina. Angiostatin gene expression was only detected in the EOM insertions. RT-PCR demonstrated that angiostatin gene expression was highly detectable at EOM insertions and not detectable in the cornea, conjunctiva, and retina. EOM bellies were also tested for angiostatin mRNA expression and were not detectable (data not shown). RT-PCR data were consistent with rAAV-GFP expression. These data demonstrated that subconjunctival injection of rAAV-angiostatin effectively induced local production of angiostin, which was mainly produced by infected muscle fibers (Figure 4).

## DISCUSSION

Corneal transparency and avascularity are essential for good vision. A delicate balance between angiogenic stimulators and inhibitors plays a key role in maintaining the homeostasis of angiogenesis. Angiostatin is a 38 kDa internal fragment of plasminogen that was found to have an inhibitory effect on vessel endothelial proliferation in vitro and vessel growth inside tumors [23,27]. Recently, in vivo studies have demonstrated the role of angiostatin in controlling and modulating corneal neovascularization and wound healing [5,28-30]. In this study, we demonstrated that a subconjunctival injection of rAAV-angiostatin effectively induced local production of recombinant angiostatin at therapeutic levels sufficient to reduce alkali burn-induced corneal angiogenesis.

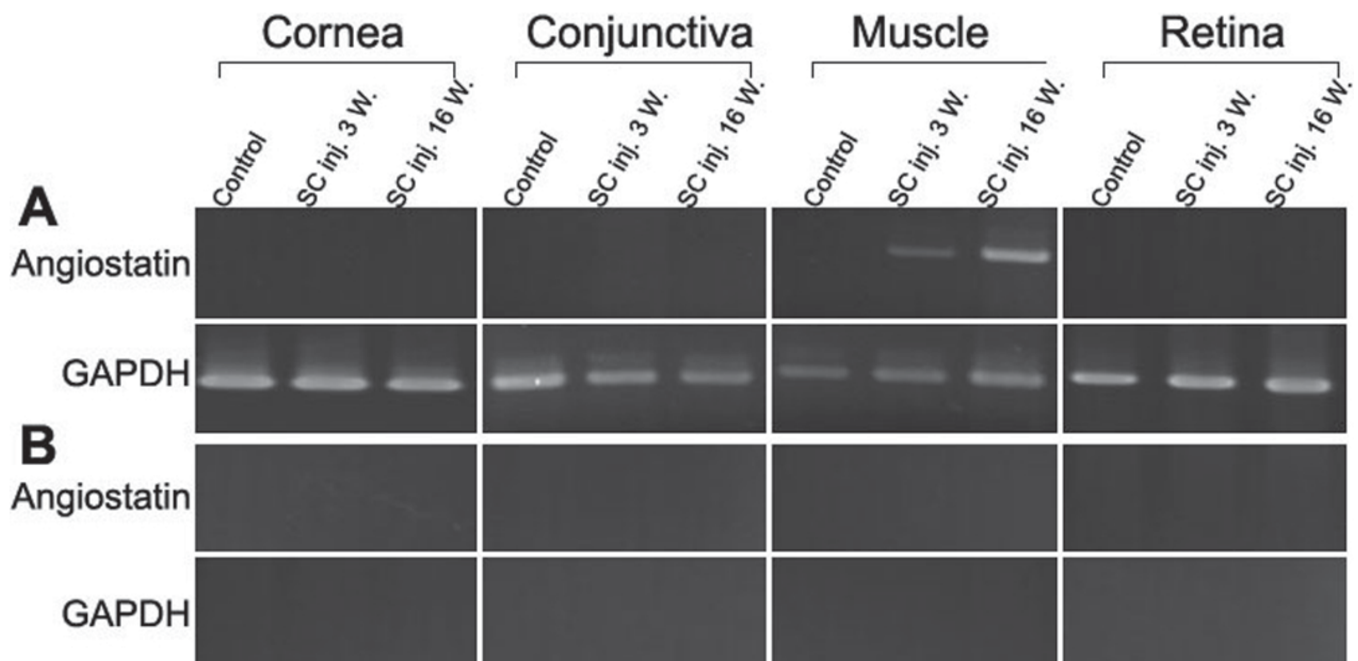


Figure 4. Angiostatin gene expression in ocular tissue by reverse-transcription polymerase chain reaction. RT-PCR analysis was performed on mRNA extracted from harvested ocular tissues three weeks and 16 weeks after the subconjunctival injection of rAAV-angiostatin. **A**: Total RNA extracted from pooled corneas, bulbar conjunctiva, EOM insertions, and the retina. PCR results for angiostatin with reverse transcriptase were shown. Expression of angiostatin mRNA was only detectable at EOM insertions. Mild expression was detected at the three-week follow up and highly detectable at the 16 week follow up. Angiostatin mRNA was not detected in the conjunctiva, cornea, and retina. The result of the rat GAPDH (465 bp) confirmed the relative amounts and fidelity of the total RNA samples. These data demonstrated that a subconjunctival injection of rAAV-angiostatin effectively induced local production of angiostatin, which was mainly produced by rAAV-infected muscle fibers. **B**: PCR without reverse transcriptase of extracted RNA for angiostatin and rat GAPDH (465 bp) was performed as control.

Purified anti-angiogenic peptides have shown potential benefits in corneal NV inhibition in experimental animal studies. However, there are several limitations to clinical use of the recombinant protein treatment. First, the requirement of prolonged administration increases the treatment cost for patients. Also, it is difficult to produce the recombinant protein due to physical properties and variations in the purification procedure. Moreover, relatively large quantities present difficult manufacturing and economic considerations. Lastly, there exists the challenge of the long-term storage of bioactive protein and the cumbersome daily administration. Thus, an alternative strategy to obviate these problems is gene therapy, where protein is generated in vivo rather than delivered through exogenous recombinant protein.

Major determinants for the success of gene therapy are the use of a novel vector and a gene delivery technique. Several non-viral and viral vectors have been tested in pre-clinical models for ocular gene delivery. Non-viral methods have obtained popularity because of its relative safety, capacity to transfer large genes, site-specificity, and its non-inflammatory, non-toxic, and non-infectious properties. However, low transfection efficiency and relatively poor transgene expression tend to limit the utility of the non-viral methods [18]. In our animal model, we used viral vectors as they typically offer higher transduction efficiency and long-term gene expression. However, they may be associated with some limitations including toxicity, immunogenicity, restricted target cell specificity, inability to transfer large genes, and high cost [18].

In our study, recombinant adeno-associated virus (rAAV) vectors were used since they typically provide a highly efficient gene delivery system that can facilitate long-term expression of foreign genes. The rAAV is also an ideal vector for human gene therapy because of its non-pathogenic nature, ability to infect wide variety of cells including non-dividing cells and hematopoietic stem cells, and its tendency to not induce inflammation or cytotoxicity [18,25,31]. Recently, we reported the effective suppression of experimental arthritis and the reduction of infarction size induced by cerebral ischemia by rAAV-based gene approaches [24,32,33]. In ocular gene therapy, we have successfully suppressed choroidal neovascularization by rAAV-expressing angiostatin [34]. The potential of rAAV vectors in the gene therapy of ocular tissues has been implicated by the delivery of marker genes by this vector, which achieved long-term and stable gene expression in retinal tissues [26,35,36]. Our present study demonstrated the potential of rAAV vectors in ocular surface gene therapy. We successfully reduced alkali burn-induced corneal angiogenesis by subconjunctival injection of rAAV-angiostatin.

Gene transfer has great potential in treating ocular surface diseases particularly related to cornea because of its accessibility and immune privilege. Various routes of administration of the reporter gene have been used in experimental studies to test the feasibility of gene transfer to the ocular surface tissues. Intracameral delivery [37], intrastromal application [17,18], topical eyedrops [20,38], and subconjunctival injection [38-41] have demonstrated encouraging results. However, except for topical and subconjunctival administration, all other gene delivery strategies to the cornea are invasive and result in the damage of corneal integrity.

Topical administration is an advantageous route for gene delivery to the ocular surface because it is non-invasive and results in minimal adverse effects due to systemic administration. However, there are many limitations for topical gene transfer. First, optimal concentration for gene transfer may differ for the corneal and conjunctiva epithelium since there are differences in tissue resistance for gene transfer. The corneal epithelium is more resistant and needs a higher concentration for gene transfer whereas the conjunctival epithelium needs a lower concentration and easily develops cellular toxicity [38,42]. The presence of natural barriers such as tight hemidesmosomes and the mucin layer may also limit the feasibility of gene transfer. The mucin layer protects the ocular surface from infection by preventing pathogens from attaching directly to the epithelium. It is probably necessary to rub the ocular surface with a cotton swab to remove the mucin layer so gene transfer to the ocular surface can be enhanced [38]. Lastly, corneal and conjunctival epithelia have a fast cell cycle turnover. Topical treatment may only be effective for superficial cells, and as a result, the cumbersome frequent application would be necessary for prolonged expression of the target gene. Although there is the advantage of avoiding possible toxicity due to prolonged expression by simply stopping the topical eyedrops, if chronic expression is needed, gene transfer to corneal and conjunctival stem cells will be necessary [38,43]. Unfortunately, at present there is no efficient and reliable method for restricted gene transfer to corneal and conjunctival stem cells, and more studies in the future are needed.

Tsubota et al. [38] studied the feasibility of gene transfer to the ocular surface epithelium using replication deficient recombinant adenovirus vector to deliver an exogenous gene to the human corneal and conjunctival cell lines in animal ocular surface epithelium in vivo. They demonstrated that both subconjunctival injection and topical use of high concentration drops resulted in gene transfer to the ocular surface. For topical gene transfer, the turnover of the corneal and conjunctival epithelia was too fast thus frequent applications were necessary for prolonged expression of the target gene. In contrast, the subconjunctival injection resulted in the expression of the transgene in all layers of the conjunctival epithelium, which may extend the duration of expression. However, they observed that if indefinite expression is needed, a gene transfer to corneal and conjunctival stem cells is necessary. Our study demonstrated that gene transfer by subconjunctival injection could achieve therapeutic levels and long lasting effects in the treatment of corneal NV. It has the advantage of preserving corneal integrity and overcome the limitations of topical treatment. Subconjunctival injection of rAAV-angiostatin resulted in diffusion of viral particles into the subconjunctival tissue. All tissues embedded by the viral particles were exposed to viral infection. Similarly to Tsubota and co-workers [38], our data demonstrated differences in ocular tissue resistance to rAAV infection. The corneal and conjunctival epithelia were more resistant to rAAV gene transfer than the EOM fibers. Three weeks after a single dose application of subconjuncti-

val rAAV, GFP expression was not seen by fluorescent microscopy nor was the angiostatin mRNA detectable by RT-PCR in the cornea and conjunctiva (Figure 1 and Figure 4). This was probably due to a high turnover of the corneal and conjunctival epithelia. Our experiment showed that significant rAAV-mediated infection was locally achieved at the insertions of EOM. This was demonstrated by strong GFP expression (Figure 1) and highly detectable amounts of transgenic mRNA (Figure 4), which might be the main source of generating angiostatin protein in vivo.

Saishin et al. [44] have also demonstrated that a periocular injection may provide a potential route to achieve sustained delivery of therapeutic proteins to the eye. Their studies in rodents have suggested that periocular injection of adenoviral vectors containing expression cassettes for anti-angiogenic proteins resulted in high intraocular levels of the proteins and suppression of choroidal NV. However, differences in size and scleral thickness between mouse and human eyes made it difficult to ascertain if a periocular gene transfer was a feasible approach for treating human ocular neovascular diseases. Also, high intraocular levels of the transgenic proteins were an undesirable result since the goal was to achieve a non-invasive and selective gene therapy of ocular surface disorders [44]. In our study, the rAAV-mediated gene transfer succeeded in achieving long lasting infection of EOM fibers with a single subconjunctival injection. Also, it was demonstrated that this procedure spared intraocular structures. A normal background autofluorescence signal was expected in the sclera and RPE/photoreceptor complex as observed in the control eyes [45]. Therapeutic levels of angiostatin were achieved probably due to the expression of the transgene at local muscle fibers of EOM insertions. Since corneal neovessels have their origin at the limbal and conjunctival vessels, continuous exposure of these structures to the secreted transgenic angiostatin may have contributed with the regression of alkali burn-induced corneal NV. In conclusion, we demonstrated that subconjunctival injection of rAAV-mediated gene transfer is a simple, economical, and reproducible method of gene delivery that can successfully achieve therapeutic levels of the transgenic protein for the treatment of ocular surface diseases.

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