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Inhibition of corneal neovascularization with endostatin delivered by adeno-associated viral (AAV) vector in a mouse corneal injury model

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Abstract

The use of a recombinant adeno-associated viral (rAAV) vector carrying endostatin gene as an anti-angiogenesis strategy to treat corneal neovascularization in a mouse model was evaluated. Subconjunctival injection of recombinant endostatin-AAV was used to examine the inhibition of corneal neovascularization induced by silver nitrate cauterization in mice. The results showed that gene expression in corneal tissue was observed as early as 4 days after gene transfer and stably lasted for over 8 months with minimal immune reaction. Subconjunctival injection of a high-titer rAAV-endostatin successfully inhibited neovascularization. Immunohistchemistry staining of CD 31 and endostatin showed that the treatment significantly inhibits angiogenesis in cornea. We concluded that the rAAV was capable of directly delivering genes to the ocular surface epithelium by way of subconjunctival injection and was able to deliver sustained, high levels of gene expression *in vivo* to inhibit angiogenesis.

Introduction

Corneal neovascularization (NV) is usually associated with inflammatory or infectious disorders of the ocular surface. It is often accompanied by stromal edema, lipid deposits, keratitis, or scarring, all of which frequently lead to vision impairment or blindness [1]. Pathological angiogenesis is also thought to play an important role in corneal allografts by facilitating the exposure of antigens in the donor cornea to the immune system [2]. Many medical and surgical treatments, including use of steroids [3–5], use of non-steroidal anti-inflammatory agents [6–8], argon laser photocoagulation [9], photodynamic therapy [1, 10], and limbal stem cell transplantation [11, 12], have been effective in inhibiting corneal neovascularization in both animal and human models. However, further investigation is needed to determine whether anti-angiogenic therapy is effective for treatment against actively growing and established corneal neovascularization.

Promising anti-angiogenic agents include: (a) naturally occurring inhibitors of angiogenesis, such as angiostatin [13] and endostatin; (b) specific inhibitors of endothelial cell growth, such as TNP-470 [14] and thalidomide [15]; (c) agents that

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neutralize angiogenic peptides such as antibodies or soluble receptors against FGF [16] and VEGF [17] and (d) anti-adhesion molecules and antibodies [18–20].

Endostatin is a 20 kDa C-terminal fragment of collagen XVIII. The anti-angiogenic activity of endostatin is a complex process that results in the inhibition of endothelial cell adhesion, migration and proliferation, as well as in the induction of apoptosis [21]. Most studies on application of endostatin in disease treatment involve cancer therapy. Recombinant endostatin protein has been administered intravenously [22] or subcutaneously [23]. Different gene constructs have been used in different cancer model by intratumor injection [24], subcutaneous administration [25] or low voltage electroporation [26]. The endostatin gene was even retrovirally transduced to tumor cells and the transduced cells were subsequently xenotransplanted in a murine tumor model [27]. Kurosaka et al. [28] reported that systemic administration of endostatin can inhibit pannus formation and bone destruction in arthritis. Jung et al. [29] showed that recombinant endostatin protein inhibits the proliferation of neovascularization in a human placental vessel vein disk model. Endostatin has been the first endogenous angiogenesis inhibitor to enter a phase II clinical trail for evaluation of biological effectiveness in patients with refractory tumors [30, 31].

Adeno-associated viral (AAV) vectors are derived from the non-pathogenic, replication-defective parvovirus that contains a single-stranded DNA genome [32–35]. The vectors are able to effectively transduce dividing and non-dividing cells both *in vitro* and *in vivo* [36], offering stable gene transfer either by integrating into the host's chromosomes or by remaining as an episome [37, 38]. The lack of cytotoxicity and minimal cellular immune response after AAV-mediated *in vivo* gene transfer also contribute to the success of long-term gene delivery in a variety of tissues [39, 40].

The AAV vectors are widely used for treatment of numerous genetic diseases in a wide variety of animal models as well as in human trials. Tsai et al. [41] described delivery of genes into rabbit corneal endothelium through injection of recombinant AAV (rAAV)-Lac Z into the rabbit anterior chamber. Tsubota et al. [42] demonstrated adenovirus gene transfer on the conjunctival epithelium through subconjunctival injection and topical administration of adenovirus-Lac Z. Corneal angiogenesis can be inhibited by intracameral injection of adenovirus-mediated sFlt-1 expression in a rat model [43]. Most gene therapy studies involving endostatin delivered by AAV were on cancer therapy [44]. Auricchio et al. [45] used an AAV vector in the retina to deliver three anti-angiogenic factors including pigment epithelium-derived factor (PEDF), tissue inhibitor of metalloproteinase-3 (TIMP-3), and endostatin to the eyes in a mouse model of retinopathy of prematurity.

In this study, the rapid, efficient recombinant adeno-associated virus-mediated transgene expression in conjunctival cells suggests that AAV may be a useful anti-angiogenic delivery vector. The administration route of subconjunctival injection may help to avoid the endophthalmitits and cataract formation associated with intracameral injection. With only a single injection for gene delivery, endostatin is secreted from the conjunctival tissue around limbal vessels and can successfully suppress corneal neovascularization. This report demonstrated the potential of gene therapy with the AAV-carrying endostatin gene for treatment of corneal neovascularization.

Methods

Construction of AAV vectors carrying the endostatin gene

The conventional plasmid rAAV-CMV-EGFP was used [39, 40]. All rAAV vectors used in this study were from AAV serotype 2. The rAAV-CMV-EGFP (Enhanced GFP) plasmid was constructed by deleting the D-sequence of the 5′ ITR (inverse terminal repeat) with *Msc*I restriction enzyme digestion. The *Msc*I digestion removed the D-sequence and the terminal resolution site (trs) (nucleotides 122–144) of the AAV2 genome. The ITR on the 3′ terminus of the vector remained intact [46]. Plasmid rAAV-CMV-endostatin was constructed by replacing the EGFP gene and the SV40 polyA site of rAAV-CMV-EGFP with the endostatin cDNA coupled with a miniature polyA site (Figure 1a). A standard triple-plasmid cotransfection method was used to produce the AAV vector carrying the endostatin [33]. The

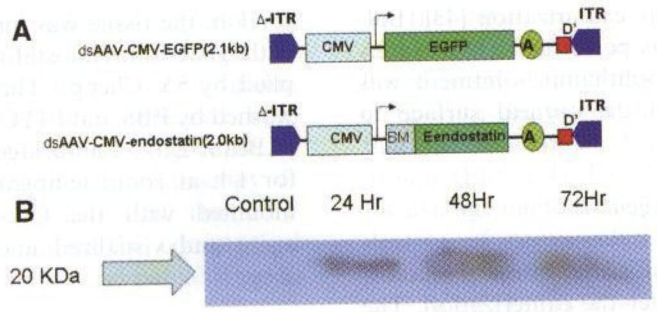

Figure 1. (A) Construct of rAAV-CMV-GFP and rAAV-CMV-endostatin (see text for detail). (B) The 20 kDa endostatin protein expression in 293 cells detected by Western blot analysis.

AAV particles were subsequently purified using the heparin affinity column chromatography (HPLC) technique. The viral titers were in the range of 1 to 3×10^{13} viral particles/ml.

In vitro expression, detection and biological activity of endostatin

For the *in vitro* assays, 2×10^5 293 cells were seeded in 15-cm dishes 1 day before infection. AAV plasmids including rAAV-CMV-EGFP and rAAV-CMV-endostatin diluted in DMEM medium were transfected to the cell culture by $CaCl_2$ method. The GFP expression was examined under fluorescence microscope every other day. The culture media were collected on 1, 2, and 3 days after transfection and western blotting was used to evaluate the secretion of endostatin.

Animals

Fifty-two CD-1 mice weighing between 25 and 40 g were used. They included two groups of six mice for GFP experiments, a group of 20 mice divided into three subgroups (n = 7, 7, 6) for the repeated endostatin experiments and a control group with 20 additional mice. The animals were handled in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized by intraperitoneal injection of 300 mg/Kg Tribromoethanol (Avertin). Effectiveness of the anesthesia was monitored by response to tail/footpad pinch/pull. Individual mouse received ear tags for identification purposes. All ophthalmic surgical procedures were performed after 4% Benoxinate hydrochloride (Novesin, CIBA vision Ltd., Hettlingen, Switzerland) had been applied to the eyes for topical anesthesia.

Gene delivery into ocular tissue by way of subconjunctival injection

After systemic and topical anesthesia, AAV-CMV-EGFP, AAV-CMV-endostatin (total volume 5 µl in PBS), or PBS was delivered to the subconjunctival tissue via the indwelling cannula using a Hamilton syringe. Every injection contained 2.5×10^7 viral particles in 5 µl. A cotton strip was used to apply light pressure around the injection site for 2 min to prevent bleeding and backward leaking of the solution. Topical erythromycin ointment was applied to the cornea surface to prevent infection.

The eyes were observed under a surgical microscope every other day after gene delivery. We harvested the ocular tissue after gene delivery at time points of 4 days, 1 and 2 weeks, and 1, 3 and 6 months. The conjunctiva tissue was dissected and wet-mounted on a glass slide for direct evaluation of gene expression of GFP using fluorescent microscopy. The ocular tissue was embedded in OCT compound for frozen section (8 µm thickness) and histological analysis was performed by wet-mount technique and H&E stain.

Induction of corneal neovascularization

The mice were anesthetized with Tribromoethanol 2 weeks after subconjunctival injection of rAAV or vehicle. After topical anesthesia, a 75% silver nitrate/25% potassium nitrate applicator (Graham-Field Surgical, New Hyde park, NY) was applied on the central cornea of the right eye for

2 s to produce a circular cauterization [43]. Bullous saline irrigation was performed after cauterization. Erythromycin ophthalmic ointment was immediately applied on the corneal surface to prevent infection.

Analysis of corneal angiogenesis

The corneas were examined by surgical microscopy every other day after the cauterization. The various stages of neovascularization were then photographed and scored by three independent observers. The corneal angiogenesis was scored using the following grade scale: mild (I), corneas showing 1–6 vertical neovascular vessels on the entire cornea; moderate (II), 7–15 vertical vessels; severe (III), more than 15 vertical vessels; and none (0), no vertical vessels. The grading was performed in a masked manner.

Immunohistochemical analysis of cornea

Immunohistochemical staining of CD31, for vascular endothelial cell proliferation activity was performed on the corneal flat mounts [13]. Briefly, fresh corneas were dissected, rinsed in PBS for 10 min, and fixed in 100% acetone for 30 min. After washing in PBS, non-specific binding was blocked with 0.1 M PBS, 2% Albumin for 1 h at room temperature. Incubation with fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-mouse CD31 antibody (BD Biosciences, Palo Alto, CA) at a concentration of 1:100 in 0.1 M of PBS, 2% Albumin at 4 °C overnight was followed by washes in PBS at room temperature. Corneas were mounted with gelmount (Biomeda, Inc.; Forst city, CA, USA), an antifading agent, and visualized under a fluorescent microscope. The total corneal neovascularization area was outlined using the innermost vessel of the limbal arcade as the border.

The eyes injected with rAAV-CMV-endostatin, rAAV-CMV-EGFP and PBS were enucleated at 7, 14 and 21 days after cauterization. The ocular tissue was immediately frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA, USA) and 8 μm transverse sections were made under cryosection. The sections were examined for the distribution of EGFP and endostatin. After blocking the nonspecific binding with 10% normal mouse serum in 0.1 M PBS at room temperature for 1 h, the tissue was incubated at 37 °C for 2 h with goat anti-endostatin antibody (kindly supplied by SY Cheng). The glass slides were gently washed by PBS and FITC-labeled second antibody (ABcam Ltd., Cambridgeshire, UK) was applied for 1 h at room temperature. The samples were mounted with the Cyc-3 antifading gelmount agent and visualized under a fluorescent microscope.

Statistics

Two way analysis of variance was used to calculate the statistical difference in the number of corneal angiogenesis vessels as revealed by microscopy and FITC-CD31. A probability of <0.05 was considered significant.

Results

The recombinant AAV clones expressed efficiently in transfected 293 cells

After *in vitro* transfection of the rAAV-CMV-EGFP or rAAV-CMV-endostatin (Figure 1a) onto 293 cells, the culture medium was collected 1, 2 and 3 days post-transfections. Western blot analysis showed prominent 20 kDa bands of endostatin secreted in the culture media from 293 cells at 24 h, 48 h and 72 h after transfection (Figure 1b). The rAAV-CMV-EGFP transfection examined by microscopy also showed high ($>95\%$) efficiency (data not shown).

The adeno-associated virus (AAV) mediated transgene expressions in the ocular tissue are highly efficient

We used subconjunctival injection as the route of gene transfer of rAAV-CMV-EGFP to examine the expression of the transgene. Gross examination revealed that the conjunctiva and anterior surface of the eyes of the mice were white following injection. Microscopy examination revealed no corneal or conjunctival epithelium inflammation, such as discharge, swollen or fluorescein leakage. The mobility and feeding habits of the mice appeared normal. Dissected and wet-mounted under fluorescent microscopy, the conjunctiva showed green fluorescent protein

(GFP) expression 4 days following injection of rAAV-CMV-EGFP ($n = 8$). We observed no GFP expression in the PBS control group ($n = 6$). GFP gene expression appeared even stronger at 14 days post-injection (Figure 2a, b) and lasted over 6 months. Light microscopy of fresh-frozen sections confirmed that the GFP expression was present within the conjunctival epithelium, on both the bulbar and palpable sides.

Introduction of rAAV-endostatin inhibits neovascularization after silver nitrate cauterization

Two weeks after the gene delivery by subconjunctival injection, silver nitrate cauterization was performed on the central cornea of the mice to induce the wound-healing process. We compared three experimental groups (rAAV-CMV-endostatin, rAAV-CMV-EGFP, and PBS) for gene therapy evaluation. One week after cauterization, we noted prominent neovascularization from the limbal vessels in the rAAV-CMV-EGFP and PBS groups (Figure 3b). Mild neovascularization was observed in the rAAV-endostatin group (Figure 3c). Compared with the normal cornea, without cauterization (Figure 3a), the suppression of corneal neovascularization was due to the subconjunctival injection with rAAV-CMV-endostatin, not of the rAAV-CMV-EGFP or PBS alone. The extent of blood vessel development in cauterized eyes was graded as negative (0) when no new vessels were visible, mild (I) when one to six vertical neovascular vessels appeared on the entire cornea, moderate (II) when 7–15 neovascular vessels grew from the limbus, and severe (III) when more than 15 neovascular vessels were noted on the entire cornea (Table 1). The effect of rAAV-CMV-endostatin on experimentally induced corneal angiogenesis was evaluated by comparing the extent of neovascularization with that in the control AAV-CMV-EGFP or PBS groups (Figure 4). Based on this grading system, the average number of eyes exhibiting the different degrees of neovascularization at different time points (7, 10, 14, and 21 days after cauterization; see Table 1) was evaluated. Here, the rAAV-CMV-EGFP treatment group showed a significantly higher occurrence of severe neovascularization than the rAAV-CMV-endostatin treatment group. Furthermore, in the rAAV-CMV-EGFP treatment group, the number of animals exhibiting severe neovasularization increased over time. In contrast, in the endostatin treatment group, the number of animals exhibiting moderate or severe neovascularization decreased over time while the number of animals showing no neovascularization increased. These opposing trends support the hypothesis that endostatin can inhibit neovascularization induced by silver nitrate cauterization.

Eighty-six percent of corneas in control group (PBS injection) developed moderate to severe angiogenesis 7 days after cauterization. The neovascularization did not show any regression during the more than 3 weeks of observation. The subconjunctival injection of rAAV-CMV-EGFP did not affect the development of cauterization-induced angiogenesis, as moderate to severe angiogenesis was present after cauterization. There was no statistically significant difference between the rAAV-EGFP and PBS ($p = 0.914$) groups. Fourteen days after the silver nitrate cauterization, only mild angiogenesis was observed in the

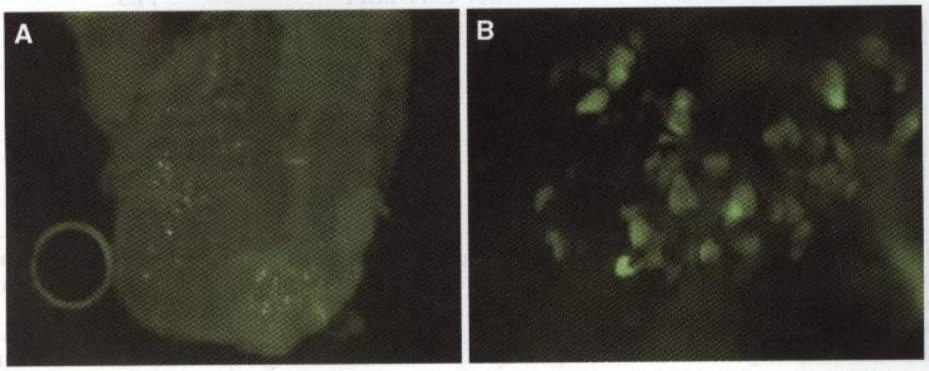

Figure 2. Expression of GFP 2 weeks after delivery to subconjunctival tissue. (A) Original image (10×) and (B) enlargement (400×).

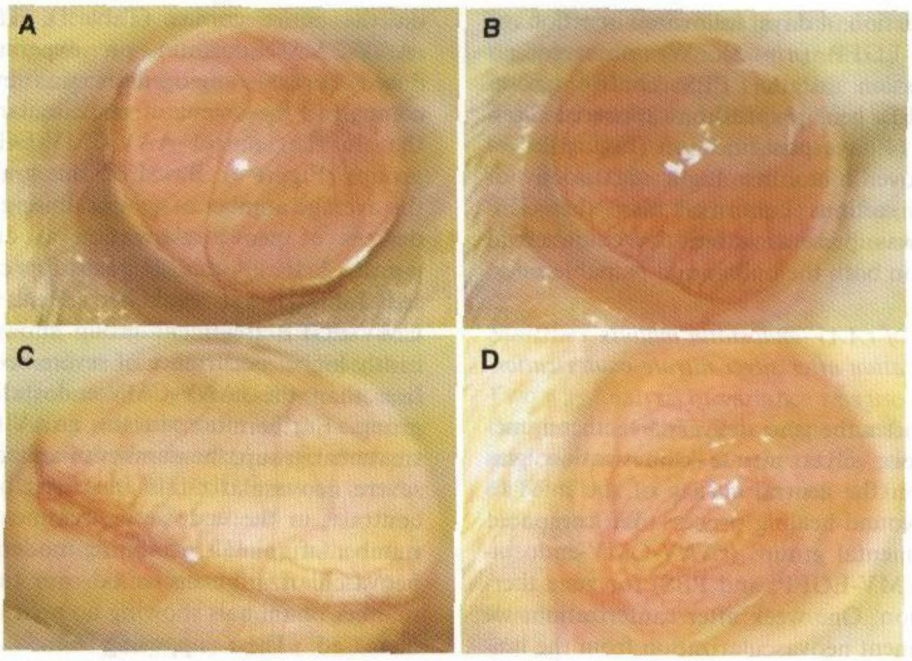

Figure 3. Results of gene therapy with rAAV-CMV-endostatin 1 week after silver nitrate cauterization. (A) Normal eye without cauterization. (B). Prominent corneal neovascularization in the PBS treatment group. (Same results obtained from the rAAV-CMV-EGFP group). (C) Significantly less severe neovascularization in the rAAV-endostatin treated group. (D) Control group without treatment after cauterization.

AAV-CMV-endostatin group. The AAV-CMV-endostatin group showed significant regression of angiogenesis ($p < 0.05$), demonstrating the efficacy of gene therapy with AAV carrying the endostatin gene. The protein of endostatin in the conjunctiva and the limbus tissues in the rAAV-CMV-endostatin group was localized by immunohistochemistry study (Figure 5).

To examine the molecular marker for angiogenesis, we performed immunohistochemistry stain with CD31, a marker for endothelial proliferation. The results showed that there was less CD31 protein in eyes of the rAAV-endostatin group (Figure 6a), whereas eyes with no rAAV-endostatin exhibited significant CD31 protein expression (Figure 6b).

Table 1. Number of corneas showed grade of neovascularization at different time points after silver nitrate ($AgNO_3$) cauterization

	rAAV-CMV-endostatin[b]				rAAV-CMV-EGFP [c]				PBS			
Grading[a]	0	I	II	III	0	I	II	III	0	I	II	III
Days												
7	1	9	8	2	0	1	5	14	0	0	5	15
10	*1*	*8*	*6*	*2*	*0*	*1*	*1*	*15*	*0*	*0*	*2*	*15*
14	2	11	1	1	0	0	1	14	0	0	1	14
21	5	7	0	1	0	0	0	13	0	0	0	13

[a] According to the grading system: 0, none vertical vessel; I, comprised corneas showing 1–6 vertical neovascular vessels on the entire cornea (Mild); II, 7–15 vertical vessels (Moderate); III, more than 16 vertical vessels (Severe). The ratios were given as the average from three independent observers in a masked manner.

[b] $p < 0.001$, when comparing neovascularization grading between the treatment group of AAV-CMV-endostatin and AAV-CMV-EGFP. $p < 0.001$, when comparing neovascularization grading between the treatment group of AAV-CMV-endostatin and PBS.

[c] $p = 0.914$, when comparing neovascularization grading between the treatment group of AAV-CMV-EGFP and the controlled group of PBS.

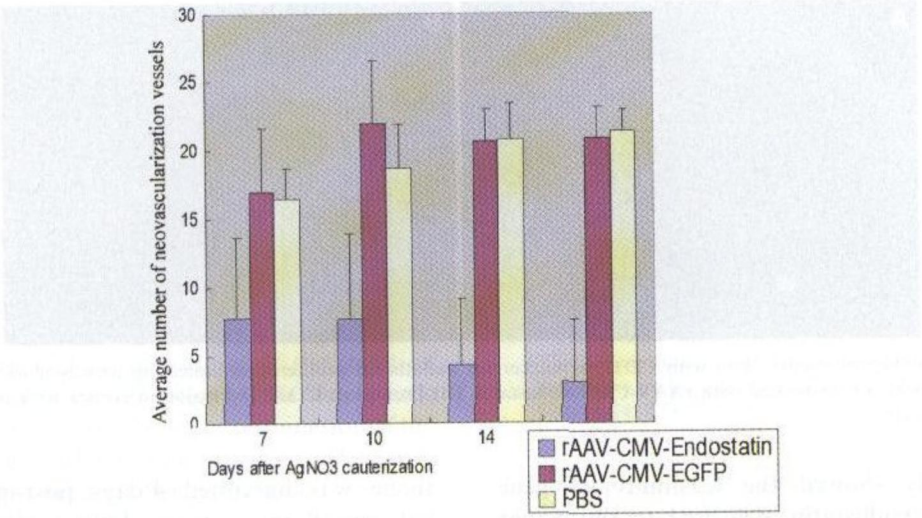

Figure 4. Average number of neovasculation vessels in corneas treated with PBS, rAAV-CMV-GFP and rAAV-CMV-endostatin. Vertical bars denote standard deviation of the mean.

Discussion

Ocular neovascularization diseases are major contributors to blindness around the world. Angiogenesis in the eye may be the result of an imbalance between stimulatory and inhibitory factors that presumably results from the elevated expression of local angiogenic factors induced by ischemia. Various angiogenic factors have been proposed to mediate vasoproliferative eye diseases, including bFGF [16], insulin-like growth factor-1, and vascular endothelial growth factor (VEGF) [17]. However, the imbalance responsible for pathologic angiogenesis may also result from downregulation of inhibitors of blood vessel growth. Previous studies have shown that thalidomide [15], curcumin, somatostatin, integrin antagonists, cyclooxygenase inhibitors, prolasctin, octreotide, herbal extracts, matrix metalloproteinase inhibitors, angiostatic steroids [3–5], thrombospondin-2, kringle 1–3, and β-cyclodextrin tetradecasulfate all exert inhibitory effects on corneal neovascularization induced by various methods. When the therapeutic peptides are formulated into eye drops, the stability of the therapeutic peptide and patient's adherence to drug application may be potential problems since long-term treatment may be needed for these chronic diseases. Gene therapy offers solution as genes of therapeutic proteins can be delivered locally and stably expressed so that the level of the proteins can be maintained in target tissues.

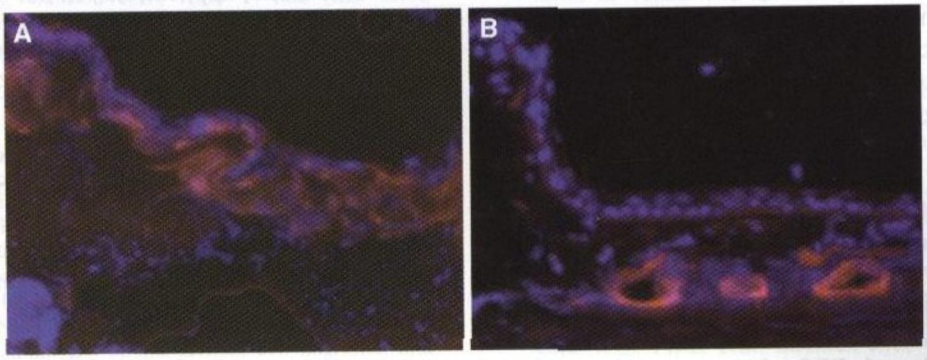

Figure 5. Localization of endostatin expression. (A) In the conjunctiva, endostatin was expressed in the lower fornix. (B) In the cornea, endostatin was expressed around the limbus.

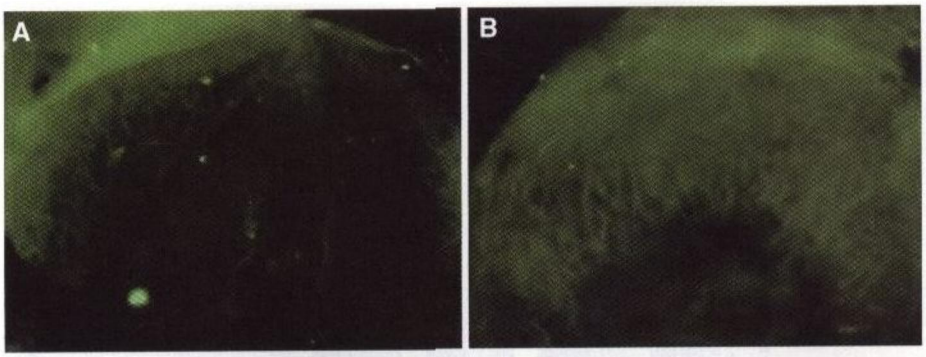

Figure 6. Immunohistochemistry stain with CD31, a marker for endothelial proliferation indicating neovascularization. (A) Less CD31 expression in cornea treated with rAAV-CMV-endostatin. (B) Prominent CD31 expression in cornea with no rAAV-CMV-endostatin treatment.

This study showed the feasibility of gene therapy with endostatin, delivered to the ocular surface at conjunctiva using the recombinant adeno-associated viral (rAAV) vector. In 1997, O'Reilly et al. reported that endostatin inhibits cell proliferation, migration and angiogenesis in culture endothelial cells [21]. More recently, Jung et al. showed that endostatin effectively blocks initiation and significantly inhibits subsequent development of angiogenic vessels in human placental endothelial cells by inducing apoptosis [29]. Our study produced similar findings, showing that the gene expression of endostatin inhibits corneal neovascularization following cauterization. Here, we observed less neovascularization in the rAAV-CMV-endostatin treatment group than that in the rAAV-CMV-EGFP treatment group and the control group (PBS). Immunohistochemistry stain with CD 31 supported these results. The endostatin was reported to have no interference on wound healing process in murine cutaneous injury [47]. Our gross examination in our mouse corneal injury model seemed to agree with their observation albeit in different tissue.

The recombinant adeno-associated viral (rAAV) vector proved to be an efficacious means of delivering the endostatin gene. AAV is a non-pathogenic, replication-defective single-strained DNA virus, capable of persisting by integrating into the host genome [37]. The rAAV vector can transduce both dividing and non-dividing cells [36]. To evaluate the immune reaction from the subconjunctival injection of recombinant adeno-associated virus, the combined rAAV-CMV-EGFP and PBS alone was injected into subconjunctival tissue separately. The eyeball tissue was harvested 4 days post-injection and cut into 8 μm sections. Immuno-histochemistry (IHC) stain of CD4 and CD8 lymphocytes indicated that there was no difference in the immune reactions of the groups with rAAV-CMV-EGFP and PBS injection or with no injection (data not shown). Thus efficient and long-term *in vivo* transduction and the lack of both cytotoxicity and cellular immune responses are the hallmark features of AAV-mediated gene transfer [48].

Compared with subconjunctival injection, other traditional methods, such as subretinal, intracameral or intravitreous injections are more invasive to the eye ball and higher technique is needed. They may cause penetrating wound into the anterior chamber of the eye ball, the risk of ocular infection – endothalmitis – is much higher. Cataract formation is also another severe complication from intraocular procedure. The large volume of viral particles may cause aqueous obstruction within the trabacular meshwork. Based on the reasons above, subconjunctival injection is considered better than other traditional applications. In our study, expression with rAAV could be detected 4 days after subconjunctival injection. Gene delivery was performed 2 weeks prior to cauterization, primarily because it takes 10 days for the genes to express at their maximum level. Mice repair neovascularization completely within 2 or 3 months. Such repair in humans, on the other hand, may take years. Moreover, without anti-angiogenesis treatment, there is no regression of vascular neovascularization in corneal wounds in humans; ghost vessels often remain after repair.

In addition, immunohistochemistry stain of CD4 and CD8 indicated that subconjunctival injection of

AAV clones induced only minimal cellular inflammation. Generally, we found that transfer through subconjunctival injection of the rAAV vector leads to stable gene expression in the conjunctival cell for more than 6 months with no complications. Also, the recombinant AAV vector consistently demonstrated superior transduction for faster onset of gene expression both *in vitro* and *in vivo*.

We have observed the transgene, endostatin, can be stably expressed for over 8 months and possibly for longer period of time. The expression of the gene usually is confined to the vicinity of the injection site. The concern of side effect of the therapeutic gene in long-term may rely on the termination of the gene expression after disease recovery. The long-term effect may need further evaluation.

In conclusion, the present study shows the feasibility of gene transfer to ocular surface tissue with rAAV vectors. Subconjunctival injection of rAAV-endostatin may provide an important anti-angiogenesis factor to the ocular surface during the wound-healing process, inhibit neovascularization and reduce corneal damage in injury.

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