Significant Inhibition of Corneal Scarring In Vivo with Tissue-Selective, Targeted AAV5 Decorin Gene Therapy

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PURPOSE. This study tested a hypothesis that tissue-selective targeted decorin gene therapy delivered to the stroma with adeno-associated virus serotype 5 (AAV5) inhibits corneal fibrosis in vivo without significant side effects.

METHODS. An in vivo rabbit model of corneal fibrosis was used. Targeted decorin gene therapy was delivered to the rabbit cornea by a single topical application of AAV5 (100 μ L; 6.5×10^{12} μ g/mL) onto the bare stroma for 2 minutes. The levels of corneal fibrosis were determined with stereomicroscopy, slit lamp biomicroscopy, α -smooth muscle actin (α SMA), fibronectin, and F-actin immunocytochemistry, and/or immunoblotting. CD11b, F4/80 immunocytochemistry, and TUNEL assay were used to examine immunogenicity and cytotoxicity of AAV5 to the cornea. Transmission electron microscopy (TEM) was used to investigate ultrastructural features. Slot-blot-quantified the copy number of AAV5-delivered decorin genes.

RESULTS. Selective decorin delivery into the stroma showed a significant ($P < 0.01$) decrease in corneal haze (1.3 ± 0.3) compared with the no-decorin-delivered control rabbit corneas (3 ± 0.4) quantified using slit lamp biomicroscopy. Immunostaining and immunoblot analyses detected significantly reduced levels of α SMA, F-actin, and fibronectin proteins (59%–73%; $P < 0.001$ or < 0.01) in decorin-delivered rabbit corneas compared with the no-decorin-delivered controls. The visual clinical eye examination, slit lamp clinical studies, TUNEL, CD11b, and F4/80 assays revealed that AAV5-mediated decorin gene therapy is nonimmunogenic and nontoxic for the cornea. TEM studies suggested that decorin gene delivery does not jeopardize collagen fibrillogenesis as no significant differences in collagen fibril diameter and arrangement were observed in decorin-delivered and no-decorin-delivered control corneas.

CONCLUSIONS. Tissue-targeted AAV5-mediated decorin gene therapy is effective and safe for treating corneal fibrosis in vivo. (*Invest Ophthalmol Vis Sci.* 2011;52:4833–4841) DOI: 10.1167/jovs.11-7357

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Corneal scarring is the third leading cause of global blindness and affects 1.3 million Americans every year.^{1,2} The corneal blinding disorders have a great cumulative socioeconomic impact on society. A variety of factors, such as infection, trauma, and chemical and mechanical injury to the eye have been shown to cause fibrosis or scarring in the cornea.^{3–5} Among the most common eye surgeries, photorefractive keratectomy (PRK) has been implicated in the corneal haze that occurs in a significant portion of patients with high myopia due to epithelial injury and abnormal wound healing in the cornea after excimer laser utilization.^{6,7} Nevertheless, PRK is a very safe and precise laser eye treatment among the laser eye surgeries currently available for vision correction.⁷ The current conventional drug therapies for treating corneal scarring require repeated applications, provide short-term benefit, cause many side effects, and are often ineffective in eliminating corneal scarring. At present, no efficacious, long-term treatments for curing corneal scarring without causing side effects are available. Corneal transplantation is a viable treatment for the restoration of vision in patients with severe corneal scarring.

Gene therapy is an attractive approach for the treatment of ocular surface disorders such as corneal scarring.^{8–13} The cornea is an ideal organ for gene therapy because of its immune-privileged status and accessibility for treatment with gene therapy reagents and visual monitoring. Various viral and non-viral vectors have been tested for delivering genes to the cornea.^{8–13} The proof of concept for treating corneal scarring with gene therapy has been demonstrated by delivering the herpes simplex virus thymidine kinase gene with retroviral vector after keratectomy, followed by topical application of ganciclovir in a PRK-induced corneal fibrosis rabbit model.^{14,15} Despite the gene therapy promise shown by this study for treating corneal disorders, not much progress has been made. Among viral gene therapy vectors, retrovirus and adenovirus have been shown to cause multiple side effects that raise safety concerns and sharply limit their clinical application. The adeno-associated virus (AAV) vectors are highly efficient in transducing corneal cells that are nonpathogenic to patients and safe for the environment. Recently, we found the AAV serotypes 5, 8, and 9, among many tested AAV serotypes, to be highly efficient and safe for delivering foreign genes into rodent, rabbit, equine, and human cornea in vitro and in vivo.^{11,12} Other investigators have found AAV vectors effective for delivering genes to the cornea.^{16,17} Using a green fluorescent protein marker gene, we optimized site-selective, tissue-targeted controlled gene transfer approaches for delivering therapeutic genes into the stroma of the normal and damaged (hazy and neovascularized) rabbit cornea in vivo, employing a combination of AAV5 and custom vector-delivery techniques.^{18,19} No apparent side effects were observed in the rabbit eye. Our recent findings and the available literature prompted to hypothesize that AAV-mediated targeted delivery

of antifibrotic genes in the stroma can effectively treat corneal fibrosis without causing significant side effects.

The molecular mechanism of corneal fibrosis has been extensively studied, but is still not fully defined. Scores of studies suggest the role of numerous growth factors and cytokines in the pathophysiology of corneal scarring.^{20,21} Of many cytokines, transforming growth factor (TGF)- β , released from corneal epithelium after eye injury, has been demonstrated to play a central role in the genesis of corneal fibrosis by promoting myofibroblast formation, as well as synthesis of extracellular matrix (ECM) and cytoskeletal proteins.^{19–21} The most convincing support to this notion was provided by the groundbreaking studies performed by Jester et al.²¹ that showed significant inhibition of corneal fibrosis in rabbit eyes by topical application of TGF β neutralizing antibodies. Thus, we hypothesized that targeted delivery of TGF β -antagonizing genes in the cornea is a novel approach for treating corneal fibrosis.

Decorin, a small, leucine-rich proteoglycan, has been shown to inhibit all three isoforms of TGF β —TGF β 1, - β 2, and - β 3—with equal efficiency.^{22–24} Numerous studies have demonstrated the pivotal role decorin plays in suppressing TGF β -regulated fibrosis in the kidneys, lungs, skin, and periodontal ligaments in vivo and in vitro.^{25–32} Recently, we demonstrated that decorin overexpression in human corneal fibroblasts significantly prevents their transformation to myofibroblasts and reduces expression of profibrotic genes when grown in the presence of TGF β 1 in serum-free conditions.³³ This study led us to hypothesize that tissue-selective, targeted decorin gene therapy delivered to the stroma with AAV can retard corneal scarring in vivo without causing side effects. In this study, we tested the efficacy and safety of decorin gene therapy with AAV5 in a laser-induced corneal fibrosis model.

MATERIALS AND METHODS

Animals

Twenty-four female New Zealand White rabbits (Myrtle Laboratories Inc., Thompson's Station, TN) weighing 2.5 to 3.0 kg were used in this study. The Institutional Animal Care and Use Committee of the University of Missouri-Columbia and Harry S. Truman Memorial Veterans' Hospital, approved the study. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbits were anesthetized by intramuscular injection of a mixture of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (10 mg/kg). In addition, topical ophthalmic 0.5% proparacaine hydrochloride (Alcon, Fort Worth, TX) was used for local anesthesia.

Corneal Fibrosis in Rabbit Eyes

A well-established PRK technique was used to produce fibrosis in the rabbit cornea.³⁴ In all 24 rabbits, corneal haze was induced in one eye, and the contralateral eye served as a naïve or PRK-untreated control. In an anesthetized rabbit, 2 to 3 drops of proparacaine hydrochloride solution were instilled on the cornea, and a wire lid speculum was inserted to expose the corneal surface. The corneal epithelium was removed by gentle scraping with a no. 64 Beaver blade (BD Biosciences, Franklin Lakes, NJ), and PRK was performed by creating a 6-mm ablation zone to -9 D with the excimer laser (Summit Apex; Alcon), to produce fibrosis in the cornea, as reported previously.³⁴ This PRK technique has been shown to consistently produce fibrosis and myofibroblasts in the rabbit cornea that peaks at 4 weeks.³⁴

AAV-Decorin Plasmid Generation and Titer Production

The decorin gene was PCR-amplified from rabbit corneal fibroblast cDNA using forward (5'GAT CGC GGC CGC AAT CAT GAC GGC AAC

TCT CAT C3') and reverse (5'GTC AGC GGC CGC GAG TTA CTT GTA GTT TCC GAG C3') primers. The amplified PCR product was cloned into the AAV2 plasmid pTRUF11, containing a hybrid promoter (cytomegalovirus enhancer and chicken β -actin) and the simian virus 40 polyadenylation site, using the *NotI* site. The resultant decorin-cloned plasmid was packaged into AAV5 with a two-plasmid co-transfection method reported previously.³⁵ In brief, approximately 1×10^9 HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (HyClone Laboratories, Inc., Logan, UT), supplemented with 5% fetal bovine serum and antibiotics. A CaPO₄ transfection method was used by mixing an equimolar ratio (1:1) of decorin-cloned AAV2 plasmid and AAV5 rep-cap helper plasmid. This precipitate was applied to the cell monolayer, and the transfection was allowed to incubate at 37°C in 7% CO₂ for 60 hours. The cells were then harvested, lysed by freeze-thaw cycles, and subjected to discontinuous iodixanol gradient centrifugation at 350,000g for 1 hour. This iodixanol fraction was further purified and concentrated by column chromatography on a 5-mL Sepharose column on an FPLC system (HiTrap Q column on a model AKTA system; GE Healthcare, Piscataway, NJ). The vector was eluted from the column using 215 mM NaCl buffer (pH 8.0), and the rAAV peak was collected. The AAV5 decorin (AAV5-dcn) vector-containing fraction was then concentrated and the buffer exchanged in balanced saline (BSS; Alcon) with 0.014% Tween 20, using a concentrator (Biomax 100K; Millipore, Billerica, MA). The vector was titrated for DNase-resistant vector genomes by real-time PCR, relative to a standard.

AAV5 Transduction to Rabbit Cornea

The rabbits were divided into two groups. Group I corneas were exposed topically to 100 μ L decorin gene-expressing AAV5 titer (6.5×10^{12} μ g/mL; $n = 12$) for 2 minutes via a custom cloning cylinder technique, as described recently immediately after PRK surgery.¹⁸ Group II corneas received 100 μ L AAV5 titer (6.5×10^{12} μ g/mL) expressing the green fluorescent protein gene ($n = 12$). The contralateral naïve corneas served as PRK-untreated ($n = 12$) and AAV5-untreated ($n = 12$) negative controls. For each group, slit lamp biomicroscopy was performed in all 12 treated eyes before euthanatization 4 weeks after PRK and vector application. After euthanatization, the 12 corneal tissues were divided for the analyses as follows: 6 for immunocytochemistry and microscopy, 2 for Western-blot, 2 for slot-blot, and 2 for transmission electron microscopy.

Slit Lamp Biomicroscopy in Live Rabbits

The level of corneal haze and health in the eyes of live rabbits was examined by visual clinical and slit lamp microscopic (BX 900 Slit Lamp, Haag-Streit USA, Mason, OH) examinations before PRK and 4 weeks after PRK, as described earlier.³⁴ Grade 0 was a completely clear cornea; grade 0.5 had trace haze seen with careful oblique illumination with the slit lamp biomicroscope; grade 1 was more prominent haze not interfering with the visibility of fine iris details; grade 2 was mild obscuration of iris details; grade 3 was moderate obscuration of the iris and lens; and grade 4 was complete opacification of the stroma in the area of ablation. Haze grading was performed in a masked manner. Optical coherence tomography was performed with a high-definition instrument (Cirrus 3000; Carl Zeiss Meditec, Dublin, CA) in live rabbits under general anesthesia, to analyze corneal thickness. The scans with the best signal strength were selected, and imaging data were analyzed with the optical coherence tomography system software (Cirrus, ver. 3.0; Carl Zeiss Meditec).

Euthanatization and Tissue Collection

Four weeks after PRK and vector application, the rabbits were humanely euthanatized with a pentobarbitone (150 mg/kg) overdose while under general anesthesia. Corneas of six rabbits of each group were removed with forceps and sharp Westcott scissors, embedded in liquid optimal cutting temperature (OCT) compound (Sakura FineTek, Torrance, CA) in a 24 $\times$ 24 $\times$ 5-mm mold (Fisher Scientific, Pittsburgh, PA), and snap frozen, as reported earlier.³⁴ Frozen tissue blocks were

maintained at -80°C for future use. Tissue sections were cut 7 or 20 μm thick with a cryostat (HM 525M; Microm GmbH, Walldorf, Germany), placed on $25 \times 75 \times 1\text{-mm}$ microscope slides (Superfrost Plus; Fisher Scientific), and maintained frozen at -80°C until staining. The remaining six rabbit corneal tissues of each group were immediately either frozen in liquid nitrogen for Western blot ($n = 2$) and slot-blot ($n = 2$) analyses or were fixed in buffer for transmission electron microscopy ($n = 2$).

Immunofluorescence Studies

Immunofluorescence staining for α -smooth muscle actin (αSMA), a marker for myofibroblasts, was performed with mouse monoclonal primary αSMA antibody (1:200 dilution, M0851; Dako, Carpinteria, CA). Tissue sections were incubated with 2% bovine serum albumin for 30 minutes at room temperature and then with αSMA monoclonal antibody for 90 minutes. For detection of the primary antibody, the sections were exposed to Alexa 488 goat anti-mouse IgG secondary antibody (1:1000 dilution, A11001; Invitrogen Inc., Carlsbad, CA) for 1 hour. SMA-positive cells in six randomly selected, nonoverlapping, full-thickness central corneal columns, extending from the anterior stromal surface to the posterior stromal surface, were counted according to a method reported elsewhere.³⁴ The diameter of each column was $400\times$ magnification field.

Fibronectin immunostaining was performed by incubating the tissue sections in goat polyclonal primary antibody (1:200 dilution, sc6952; Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 90 minutes. For primary antibody detection, the sections were exposed to Alexa 594 donkey anti-goat IgG secondary antibody (1:500 dilution, A11058; Invitrogen) for 1 hour. F-actin staining was performed with Alexa594-conjugated phalloxin (A12381; Invitrogen). The tissues were incubated at 1:100 dilution for 90 minutes followed by subsequent washing with HEPES.

The possibility of immunologic reaction to AAV5-mediated decorin gene therapy was examined by performing CD11b (BDB550282; BD Pharmingen, San Jose, CA) and F4/80 (MCA497; Serotec, Raleigh, NC) immunostaining in rabbit corneal sections using rat anti-mouse antibody. Tissue sections were incubated at room temperature with the CD11b primary antibody at a 1:50 dilution in a $1\times$ HEPES buffer containing 5% BSA for 90 minutes, followed by goat anti-rat IgG secondary antibody (Alexa Fluor 594; Invitrogen) at a 1:500 dilution for 60 minutes. After completion of immunostaining, tissue sections were mounted in medium containing DAPI (Vectashield; Vector Laboratories, Inc. Burlingame, CA), viewed, and photographed under a fluorescence microscope (Leica, Deerfield, IL) equipped with a digital camera system (SpotCam RT KE; Diagnostic Instruments, Sterling, MD).

TUNEL Assay

A TUNEL assay was performed in acetone-fixed rabbit corneal sections using a fluorescent detection assay (ApopTag; Millipore) that detects apoptosis and, to a lesser extent, necrosis according to the manufacturer's instructions. A positive control (4 hours after mechanical corneal scrape) and a negative control (unwounded) were included in each assay.

Immunoblot Analysis

Rabbit corneal tissues were lysed in RIPA protein lysis buffer containing protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN). Protein samples were prepared for electrophoresis by heating at 90°C for 2 minutes followed by centrifugation at 10,000g for 10 minutes. The samples were transferred onto polyvinylidene difluoride (PVDF) membranes (iBlot apparatus; Invitrogen), and the proteins were detected with the following primary antibodies: αSMA (mouse monoclonal, 1:200 dilution; Dako) and β -actin primary antibody (sc-69879; Santa Cruz) followed by alkaline phosphatase-conjugated anti-mouse secondary antibody (Fisher Scientific). After the membrane was washed three times in 0.05% Tween-20 in Tris-buffered saline (pH 8.0)

for 5 minutes each, the blot was developed using the nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) method.

Transmission Electron Microscopy

Rabbit corneas were fixed in a 2% glutaraldehyde and 2% paraformaldehyde solution in 0.1 M Na-cacodylate buffer (pH 7.4), postfixed in 1% osmium tetroxide, sequentially dehydrated in ethanol, and transitionally dehydrated in acetone, followed by infiltration with an Epon and Araldite (Electron Microscopy Sciences, Hatfield, PA) resin mixture. The embedded cornea samples were sectioned at 85-nm on an ultramicrotome (Ultracut UCT; Leica) and transferred onto a 200-mesh copper grid for poststaining with uranyl acetate and Sato's triple lead stain. The samples were then imaged by transmission electron microscopy (1400; JEOL Tokyo, Japan).

Slot-Blot Analysis to Determine Gene Copy Number

The copies of delivered plasmid were determined with slot-blot analysis. The frozen corneal tissues were ground in liquid nitrogen, and DNA was isolated (DNA Easy kit; Qiagen, Valencia, CA). The standards were prepared using 10^4 to 10^{11} copies of decorin gene cloned into the pTRUF11 vector. The DNA probe was prepared by digesting 5 μg of isolated decorin plasmid with the *NotI* restriction enzyme and labeling 1 μg of isolated decorin fragment with digoxigenin (DIG)-labeled UTP (DIG starter kit II; Roche Applied Science). Two microliters of the standard as well as the DNA isolated from corneal tissues were denatured by alkali and heat treatment. Denatured DNA samples were blotted onto nylon membrane with a slot-blot apparatus (Bio-Rad Laboratories, Hercules, CA) and were UV cross-linked. The membrane was hybridized with 300 ng of DIG-labeled probe overnight at 30°C , followed by incubation in 1:5000 anti-DIG-AP antibody. Chemiluminescent detection was used according to the vendor's instructions (Roche Applied Science), and the membranes were exposed to x-ray film. Image J 1.38 X image analysis software was used to determine the number of delivered gene copies in samples by measuring dot intensities in the samples and comparing the data with standards (Image J 1.38X developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>).

Statistical Analyses

The results of corneal haze grading, SMA quantification and collagen fibril diameter were expressed as the mean $\pm$ SEM. Statistical analysis was performed with Student's *t*-test or the Wilcoxon rank sum test. $P < 0.05$ indicated significance.

RESULTS

Biomicroscopic Quantification of Corneal Fibrosis

Figure 1 shows slit lamp- and stereo-biomicroscopy images depicting corneal haze in no-decorin-delivered (control) and decorin-delivered rabbit corneas. The PRK-treated rabbit eyes that received AAV5-gfp vector showed a strong fibrotic response in the cornea as evident from the cloudiness (Figs. 1A, 1B). The PRK-treated rabbit eyes that received AAV5-dcn vector showed a substantial decrease in corneal cloudiness (Figs. 1C, 1D). The PRK- or AAV5-untreated negative control rabbit corneas showed no corneal haze (Fig. 1E). The quantification of haze inhibition after decorin gene therapy was performed in a blinded fashion by three researchers. Figure 1F shows the mean haze score in decorin-delivered and no-decorin-delivered rabbit corneas observed 4 weeks after vector application. The no-decorin-delivered rabbit corneas showed an average haze score of 3 ± 0.4 , whereas decorin-delivered rabbit corneas showed a significantly ($P < 0.01$) lower corneal haze score of 1.3 ± 0.3 (Fig. 1F). Optical coherence tomography did not

FIGURE 1. Representative stereomicroscopy (A, C, E) and slit lamp (B, D) biomicroscopy images showing haze levels in no-decorin-delivered control (A, B) and decorin-delivered (C, D) rabbit corneas. Haze was produced with PRK surgery, AAV5 viral titer ($6.5 \times 10^{12} \mu\text{g/mL}$) was applied immediately to the cornea for 2 minutes after PRK, and corneal tissues were imaged 4 weeks after PRK (the time at which peak in haze is reported). (F) Quantification of corneal haze. Significantly less haze ($P < 0.01$) was observed in decorin-delivered corneas than in no-decorin-delivered corneas. No haze was observed in the contralateral naïve rabbit corneas (E). dcn, decorin.

detect any significant difference in corneal thickness among the naïve, no-decorin-delivered, and decorin-delivered rabbit corneas (data not shown). These findings suggest that AAV5-mediated decorin gene therapy is highly efficient in preventing corneal fibrosis and appears safe for the rabbit cornea.

Immunohistochemical Determination of Corneal Fibrosis

Myofibroblast formation is a hallmark of corneal fibrosis and is characterized by the expression of cytoskeletal proteins, such as α SMA, F-actin, and fibronectin. We evaluated the inhibitory effects of decorin gene delivery on corneal fibrosis by immunostaining rabbit corneal tissues for those three proteins. Figure 2 shows the immunostaining for α SMA (Figs. 2A, 2B), F-actin (Figs. 2C, 2D), and fibronectin (Figs. 2E, 2F) in decorin-delivered and no-decorin-delivered rabbit corneas. The rabbit corneas subjected to PRK without AAV5-dcn vector treatment showed intense α SMA immunostaining in the anterior stroma confirming the presence of myofibroblasts and development of haze (Fig. 2A). Conversely, decorin-delivered rabbit corneas showed a significant decrease in α SMA expression (Fig. 2B), suggesting that AAV5-mediated decorin gene therapy effectively attenuates fibrosis in the rabbit cornea in vivo. The antifibrotic effects of decorin gene therapy were revalidated by F-actin (Figs. 2C, 2D) and fibronectin (Figs. 2E, 2F) immunocytochemistry. Both, additional tested fibrotic parameters showed similar levels of decrease as noted for SMA, providing further support that AAV5-delivered decorin in the stroma modulates ECM proteins and wound healing and inhibits scar formation in the rabbit cornea. Phalloidin staining also de-

tected the filamentous actin of the epithelial borders (Figs. 2C, 2D) that used anti-mouse monoclonal SMA antibody was able to recognize feebly (Figs. 2A, 2B). The naïve rabbit corneas did not show any positive staining of tested antigens in the stroma (data not shown).

The quantification of α SMA immunostaining in the rabbit corneas subjected to PRK with AAV-dcn or AAV-gfp treatment is shown in Figure 3. As evident from the data shown in Figure 3, AAV5-mediated decorin gene therapy given by a single 2-minute topical application caused a statistically significant decrease in α SMA (59%; $P < 0.001$). The digital quantification of F-actin and fibronectin immunocytochemistry data showed a significant decrease in F-actin (72%; $P < 0.001$) and fibronectin (64%; $P < 0.01$) expression in the rabbit cornea in vivo.

Immunoblot Quantification of Corneal Fibrosis

The efficacy of AAV5-mediated decorin gene therapy on corneal fibrosis was also examined by α SMA immunoblot analysis. Figure 4 shows results of α SMA Western blot performed with protein lysates of the decorin-delivered and undelivered rabbit corneas collected 4 weeks after PRK (haze is at its peak at this time point). The quantification of immunoblot data with Image-J demonstrated a statistically significant reduction in the expression of α SMA in decorin-delivered corneal samples (67%; $P < 0.01$) compared to no-decorin-delivered control corneal samples (Fig. 4). The β -actin of the same intensity band in each lane confirmed equal loading of protein samples. These data further indicate that AAV5-mediated decorin gene therapy is significantly effective in reducing corneal fibrosis in a rabbit model.

FIGURE 2. Effect of AAV5-mediated decorin gene delivery on markers of corneal fibrosis, as detected by immunostaining in rabbit corneal tissue. Immunohistochemistry showing levels of α SMA (A, B), F-actin (C, D), and fibronectin (E, F) in no-decorin-delivered control and decorin-delivered rabbit corneal tissue, collected at 4 weeks after PRK-induced corneal fibrosis. Blue: DAPI-stained nuclei; green: α SMA staining; red: F-actin and fibronectin staining. Decorin-treated corneas showed significant decreases in α SMA ($P < 0.001$) and notable decreases in F-actin and fibronectin levels in the stroma compared with that in control corneas. Scale bar, 100 μ m.

Effects of AAV5-Mediated Decorin Gene Transfer on In Vivo Cytotoxicity and Immunity

The CD11b (activated granulocytes marker) and F4/80 (macrophage marker) immunostaining and TUNEL assay were used to analyze the immunogenicity and cytotoxicity of the used AAV5 vector for corneal decorin gene therapy. The anti-mouse

CD11b and F4/80 antibodies known to cross-react with rabbit antigen were used for the study.^{36,37} Furthermore, to ensure their reactivity to the rabbit corneal tissues, alkali burn rabbit corneas collected 4 hours after 30 seconds of 1 M sodium hydroxide application on the cornea were used as a positive control. Figure 5 shows results of CD11b and F4/80 immunostaining detected in the alkali-burn (Fig. 5, left), no-decorin-delivered (Fig. 5, middle), and decorin-delivered (Fig. 5, right) rabbit corneas. The positive control alkali-burn corneal sections showed numerous CD11b⁺ and F4/80⁺ cells, confirming that used antibodies recognize rabbit antigens. The naïve (data not shown), no-decorin-delivered (Fig. 5, middle), and decorin-delivered (Fig. 5, right) rabbit corneal sections showed two to six CD11b⁺ or F4/80⁺ cells. The detection of occasional and statistically insignificant CD11b⁺ or F4/80⁺ cells in the naïve, no-decorin-delivered, and decorin-delivered rabbit corneas suggests that AAV5-delivered decorin gene therapy does not induce significant immune response in the cornea.

The in vivo cytotoxicity of AAV5-mediated decorin gene therapy for the cornea was analyzed by TUNEL assay. Figure 6 shows the results of TUNEL staining in rabbit corneas. Detection of one to four TUNEL⁺ cells (shown in red) in the stroma

FIGURE 3. The effect of AAV5-mediated decorin gene therapy on fibrosis in rabbit corneal tissues quantified by counting α SMA-positive cells at 400 $\times$ magnification. Decorin treatment significantly ($P < 0.001$) decreased α SMA-positive cells.

FIGURE 4. Western blot analysis for α SMA, to detect the effect of AAV5-mediated decorin gene therapy on fibrosis in rabbit corneas. A significant (75%–86%) decrease in the expression of α SMA was detected in decorin-delivered corneas compared with control corneas, suggesting that AAV5-mediated decorin gene therapy is highly efficient in treating corneal fibrosis. β -Actin was used to confirm equal loading of protein in each well and to normalize the data.

FIGURE 5. Effect of AAV5-decorin gene therapy on immune response in rabbit corneas using CD11b and F4/80 immunocytochemistry. The lack of a significant difference in the presence of CD11b- and F4/80-stained cells in the stroma of no-decorin-delivered and decorin-delivered rabbit corneas suggests that AAV5-mediated decorin gene therapy does not induce an immune response in the cornea. *Blue*: DAPI-stained nuclei; *red*: CD11b or F4/80 stained cells. Alkali-burned rabbit corneal tissue sections at 4 hours were used as the positive control. Scale bar, 100 μ m.

of the naïve (data not shown), no-decorin-delivered (Fig. 6A), and decorin-delivered (Fig. 6B) rabbit corneas suggests that the AAV5 vector is noncytotoxic to keratocytes. The 6 to 10 TUNEL⁺ cells observed in the corneal epithelium were due to its replenishment. The presence of similar number of DAPI-stained nuclei in no-decorin-delivered (Fig. 6, left) and decorin-delivered (Fig. 6, right) rabbit corneas also allows us to infer that optimized AAV-based decorin delivery into stroma does not alter keratocyte population. These results suggest that the defined gene transfer modality is safe for in vivo corneal gene therapy.

Effects of AAV5-Mediated Decorin Gene Transfer on Corneal Collagen Fibrils

Decorin is known to bind to collagen and regulate fibrillogenesis. Collagen fibril diameter and arrangement in the cornea play an important role in corneal transparency. Thus, we used transmission electron microscopy to determine whether decorin gene therapy affects collagen fibril diameter and/or arrangement in the cornea. Figure 7 shows the diameter and arrangement of collagen fibrils in no-decorin-delivered (Fig. 7A) and decorin-delivered (Fig. 7B) rabbit corneas. The lack of a significant difference between the collagen fibril arrangement and diameter in the decorin-delivered and no-decorin-delivered rabbit corneas suggests that AAV5-delivered decorin to the stroma did not jeopardize the geometric arrangement of the collagen fibrils. The quantification of collagen fibril diam-

eter data shown in Figure 7C did not detect any significant differences between the corneas of the two groups.

Determination of Delivered Decorin Gene Copies with AAV5

To confirm the delivery of the decorin gene after a single topical application of AAV5-dcn on the rabbit cornea, we determined the decorin gene copy number by using a slot-blot assay. Figure 8 shows the gene copy number delivered in the rabbit corneas by AAV5 selected for this study. Densitometric quantification of slot blotting data revealed delivery of an average of 10^9 genomic copies of decorin by AAV5 in rabbit cornea.

DISCUSSION

We previously identified the AAV serotypes efficient for delivering genes to the cornea in vivo; optimized minimally invasive delivery techniques for administering vectors preferentially into keratocytes or endothelium in vivo; and using a combination of vector and delivery technique, defined tissue-selective targeted gene therapy approaches for treating corneal disorders and dystrophies.^{8–12,16} In this study, for the first time, we report that tissue-selective targeted decorin gene delivery in the cornea with AAV5 significantly retarded corneal fibrosis in vivo in a well-established laser-based ex-

FIGURE 6. Effect of AAV5-decorin gene delivery on rabbit corneal toxicity tested with TUNEL assay. The low number of TUNEL⁺ cells ($n = 2$ –5) in the stroma of no-decorin-delivered control (A) and decorin-delivered (B) rabbit corneal sections, the comparable number of TUNEL⁺ cells in the epithelium, and no change in overall keratocyte density in the stroma determined with DAPI⁺ nuclei suggests that decorin gene therapy is noncytotoxic and safe for the cornea. *Red*: TUNEL⁺ cells; *blue*: DAPI⁺ nuclei. Scale bar, 100 μ m.

FIGURE 7. Effect of decorin gene therapy on collagen fibril diameter determined by transmission electron microscopy of no-decorin-delivered control (A) and decorin-delivered (B) rabbit corneas. (C) Quantification of collagen fibril diameter in nanometers. No significant difference in the collagen fibril diameter was detected between control and decorin-delivered rabbit corneas. This result suggests that AAV5-delivered decorin did not alter the collagen fibril status in the rabbit cornea. dcn, decorin.

perimental rabbit corneal scarring model. In addition, we report that AAV5-mediated gene therapy was safe for the cornea, as visual and slit lamp clinical eye examinations did not detect inflammation, redness, opacity, mucous, or other discharges in the rabbit eye, and optical coherence tomography imaging did not find any distortion in rabbit corneal thickness or curvature.

Decorin gene transfer has been reported to significantly attenuate fibrosis in many nonocular tissues such as heart, kidneys, and lungs.^{25–32} Recently, we reported significant inhibition of TGF β -induced transformation of human and rabbit corneal fibroblasts to myofibroblasts and profibrogenic genes with decorin gene transfer.³³ Decorin is a small, leucine-rich

proteoglycan that has been shown to play an important role in the regulation of various ocular and nonocular diseases.^{24,38–40} In the cornea, decorin is expressed in the stroma and contributes to the maintenance of clarity and structural integrity of the corneal stroma required for normal vision.^{4,24,41} In addition to decorin, other proteoglycans such as biglycan, fibromodulin, mimecan, keratocan, and lumican are expressed in the cornea and have been shown to play a role in corneal healing and homeostasis.^{42–44} Recently, we investigated the levels and localization of decorin and TGF β in normal and PRK-injured rabbit corneas at various time points, to better understand the function of decorin in the cornea.^{20,24} The results of these studies revealed that decorin plays an important role in preventing scar formation in the cornea by hindering TGF β activity. Decorin, an endogenous inhibitor of TGF β , has been shown to regulate the TGF β signal transduction pathway through its interaction with low-density lipoprotein receptor-related protein-1 and the decorin endocytic receptor.^{24,40} Furthermore, decorin modulates signaling of epidermal growth factor receptors (EGFR) and insulin-like growth factor-I and its receptor.^{24,40} Based on the fact the decorin binds to all three isoforms of TGF β with equal affinity and TGF β is a key modulator of corneal fibrosis, we postulate that observed antifibrotic effects of decorin gene therapy in rabbit cornea in vivo are due to modulation of TGF β signaling by decorin. Future studies will test this hypothesis to gain understanding of the molecular mechanism associated with corneal scar inhibition by decorin.

Numerous viral and nonviral vectors have been tested for delivering therapeutic genes in the cornea. However, AAV vectors have been found most suitable for transducing slow/no dividing corneal endothelium or keratocytes.^{8–12} Our recent studies found AAV serotypes 5, 8, and 9 highly efficient in transducing rodent and rabbit corneas in vivo with no major side effects. In this study, AAV serotype 5 was used for decorin gene delivery because toxicity and safety of AAV5 have been extensively investigated in our laboratory for the cornea during the optimization of targeted gene therapy approaches for the cornea. Another major challenge in the development of tissue-targeted gene therapy for the cornea is the unavailability of a cornea-tissue specific promoter that is not leaky. The studies from several research groups showed promise in the identification of nonleaky cornea cell-specific promoter.^{45–47} To address this limitation, we postulated a few years ago that admin-

FIGURE 8. Slot-blot analysis showing the decorin gene copy number delivered by the AAV5 vector to the rabbit cornea. Densitometric analysis detected 10⁹ copies of the decorin gene delivered by the vector to the rabbit corneas. Left lane: the pTRUF11-dcn plasmid DNA blotted at different concentrations; right lane: corneal DNA samples isolated from two animals that received AAV5-dcn.

istration of vector with minimally invasive surgical techniques can be used for developing targeted gene therapy for corneal diseases because of the physical accessibility of corneal tissue. Thus, several minimally invasive simple surgical techniques were optimized to administer gene transfer vectors into rabbit and rodent stromal keratocytes or corneal endothelial cells.¹⁸ Indeed, introduction of efficient vectors via optimized delivery techniques permitted tissue- and site-selective targeted gene delivery into keratocytes or endothelium.¹⁹ In our optimized corneal gene therapy approaches, targeted gene delivery in the cornea is predominantly dependent on vector delivery techniques.

The safety and toxicity of the vector to the patient and the environment are major concerns with gene-based therapy. Many viral and nonviral gene transfer methods, although efficient for delivering genes to the cornea, cannot be used for patients, because of their toxicity and/or lack of safety. In this study, we used AAV for introducing the decorin gene into the rabbit stroma, because of its nonpathogenicity, low immunogenicity, and safety. Eyes exposed to the AAV5 vector did not show significant keratocyte apoptosis, when examined by TUNEL assay, or infiltration of immune cells in the stroma, when examined with CD11b or F4/80 immunocytochemistry, suggesting that AAV5-mediated decorin gene therapy is noncytotoxic and safe for the cornea. Decorin interacts with collagen to modulate collagen fibril organization, cross-linking, and degradation.^{38–40} In vitro studies have demonstrated that decorin could restrict fibrillar collagens to a thinner and homogeneous diameter whereas in vivo studies of decorin knockout mice detected larger diameter collagen fibrils in skin tissues.^{24,38–40,48} These reports raised a possibility that decorin gene delivery could affect collagen fibril organization in the cornea. We postulated that AAV-mediated decorin gene therapy does not jeopardize corneal collagens, based on the fact that it would require decorin interaction with collagen and de novo collagen synthesis, and collagens in the cornea have an extremely low turnover. No significant differences in collagen fibril diameter and arrangement in decorin-delivered and no-decorin-delivered rabbit corneas observed with transmission electron microscopy supported our hypothesis. Furthermore, the optical coherence tomography biomicroscopy performed in rabbit eyes provided additional support to our hypothesis as no significant change in corneal thickness up to 4 weeks was observed among the naïve, no-decorin-delivered, and decorin-delivered rabbit corneas. Our ongoing corneal gene transfer studies performed in the rodent model show that the AAV-delivered transgene in the stroma continues to be expressed up to 11 months and possibly more (Mohan et al. unpublished data, 2011). This raises the question of whether long-term retention of the AAV-decorin gene in the stroma has any adverse effects on corneal function? Longer duration studies are needed to address such a relevant question. It is worth mentioning that keratocytes in the stroma are slow-dividing cells and tend to stay in the stroma without proliferation under normal or non-wound-healing conditions. Our future studies will examine the effects of long-term expression of AAV-mediated decorin gene transfer in the stroma on corneal homeostasis and wound healing.

In summary, for the first time, this study demonstrates significant inhibition of corneal fibrosis in the rabbit cornea by AAV5-mediated, tissue-targeted gene therapy, with no visual side effects. Furthermore, results of this study support our hypothesis that tissue-selective, targeted delivery of therapeutic genes to the stroma with defined gene therapy approaches can effectively cure ocular surface diseases without causing significant side effects.

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