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AAV serotype influences gene transfer in corneal stroma *in vivo*

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Abstract

This study evaluated the cellular tropism and relative transduction efficiency of three AAV serotypes, AAV6, AAV8 and AAV9, for corneal gene delivery using mouse cornea *in vivo* and donor human cornea *ex vivo*. The AAV6, AAV8 and AAV9 serotypes having AAV2 plasmid encoding for alkaline phosphatase (AP) gene were generated by transfecting HEK293 cell line with pHelper, pARAP4 and pRep/Cap plasmids. Viral vectors (10⁹ vg/μl) were topically applied onto mouse cornea *in vivo* and human cornea *ex vivo* after removing the epithelium. Human corneas were processed for transgene delivery at day 5 after viral vector application. Mouse corneas were harvested at 4, 14 and 30 days after vector application for AP staining. Transduction efficiency was calculated by quantifying pixels of AP-stained area using Image J software and also confirmed by functional AP enzyme activity in the corneal lysates. Cellular toxicity of the three AAV serotypes was tested with TUNEL assay. Inflammatory response was detected by immunostaining for CD11b and F4/80. All three AAV serotypes successfully transduced mouse and human corneas. The order of transduction efficiency was AAV9>AAV8>AAV6. The transduction efficiency of AAV9 was 1.1–1.4 fold higher (p>0.05) as compared to AAV8 and 3.5–5.5 fold higher (p<0.01) as compared to AAV6. The level of transgene expression for all the three serotypes was greater at 14 days compared to 4 days and this high level of transgene expression was maintained up to the tested time point of 30 days. Corneas exposed to any of the three AAV serotypes did not show significant TUNEL positive cells or any inflammatory response as tested by CD11b or F4/80 staining suggesting that tested AAV serotypes do not induce cell death or inflammation and are safe for corneal gene therapy.

Keywords

AAV6; AAV8; AAV9; Gene transfer; Cornea

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1. Introduction

Gene therapy holds great promise for the treatment of genetic as well as acquired eye diseases. Improved visual function in patients of Leber's congenital amaurosis treated with gene therapy affirms its clinical potential for ocular diseases (Bainbridge et al., 2008; Maguire et al., 2008). For the cornea, gene therapy has been tested in animal models for common clinical disorders such as graft rejection, corneal neovascularization, wound healing and fibrosis (Behrens et al., 2002; Mohan et al., 2005; Cheng et al., 2007; Klausner et al., 2007; Saika et al., 2008; Parker et al., 2009). A variable degree of benefits in treating corneal diseases has been observed in these studies (Behrens et al., 2002; Cheng et al., 2007; Saika et al., 2008; Parker et al., 2009). A variety of factors such as type of vector, degree and duration of therapeutic gene expression and adverse immune response to the gene delivery vector have been proposed to contribute to the success of corneal gene delivery in these studies (Mohan et al., 2005; Klausner et al., 2007).

Vector plays a critical role for the success of gene therapy. Among the various tested viral vectors for ocular gene therapy, adeno-associated virus vector (AAV) is becoming increasingly popular. AAV efficiently transduces mitotic and post-mitotic cells and provides long-term transgene expression with minimal or no inflammatory reaction (Mohan et al., 2005; Verma and Weitzman, 2005). Because of these properties, AAV has been used in many preclinical and clinical studies for ocular diseases (Alexander and Hauswirth, 2008; Surace and Auricchio, 2008). Nonetheless, presence of neutralizing antibodies for AAV2 in humans and low level of transgene expression raise concern about the use of this vector for treating human diseases (Parks et al., 1970; Calcedo et al., 2009). To address the limitations of AAV2, several new AAV serotypes isolated from human and non-human primates have been investigated for their potential as gene delivery vectors (Gao et al., 2002; Gao et al., 2005). Although over 110 AAV serotypes have been isolated, AAV1-9 have been used to generate vectors for gene delivery in ocular and non-ocular tissues. Our previous studies compared the transduction efficiency of AAV5 and AAV2 for delivering genes in the cornea and found enhanced transgene delivery with AAV5 in the rabbit cornea as compared to AAV2 (Mohan et al., 2003; Mohan et al., 2005a). Similar findings have been reported in the retina by Auricchio et al (Surace and Auricchio, 2008). These authors compared the transduction pattern of AAV5 with AAV2 for retinal gene delivery and reported superior efficiency and greater selectivity of AAV5 for photoreceptors (Surace and Auricchio, 2008).

The cellular entry of AAV depends on host-cell receptor and viral capsid interaction (vanVliet et al., 2008). The capsid region of different AAV serotypes shows structural variations that enable each serotype to bind to different cell surface receptors. For example, AAV serotypes 1, 2 and 3 use heparin sulfate proteoglycan (Summerford and Samulski, 1998; Negishi et al., 2004), serotypes 4, 5 and 6 use sialic acid (Wu et al., 2006) whereas serotype 8 or 9 use laminin receptors for gaining cellular entry (Akache et al., 2006). Thus, pseudopackaged AAV vectors have been generated employing transencapsidation of AAV2 genome into the capsid of AAV1-9. Initial studies testing these next generation vectors for ocular gene delivery demonstrated far superior transduction efficiency for retina with AAV4 and AAV5 in comparison to AAV2 (Surace and Auricchio, 2008). Subsequent studies with AAV6, AAV7, AAV8 and AAV9 further revalidated the above finding for retinal tissue (Surace and Auricchio, 2008). Similarly, AAV8 and 9 vectors showed 5–100 fold higher transduction efficiencies for cardiac muscle (Wang et al., 2005), brain (Broekman et al., 2006), skeletal muscle (Wang et al., 2005), lung (Limberis et al., 2006) and liver (Gao et al., 2006). These studies prompted us to hypothesize that the relative efficiency of AAV-mediated gene transfer in the cornea depends on serotype. Our *in vitro* study supported this hypothesis as different levels of gene transduction were observed with three tested AAV serotypes in human corneal fibroblasts (Sharma et al., 2010). However, literature survey

shows that AAV transduction efficiency noted *in vitro* may not follow similar pattern *in vivo*. Therefore, this study tested the transduction efficiency of AAV6, AAV8 and AAV9 for corneal gene delivery using *in vivo* mouse model and *ex vivo* human cornea.

2. Materials and methods

2.1. AAV vector production

Plasmids pCMV-Cap6, pMT-Rep2 and AAV genomic plasmid, pARAP4 were obtained from Dr Dusty Miller, Fred Hutchison Cancer Research Center, Seattle WA. Plasmids pAAV2/8, pAAV2/9 were provided by Dr James M. Wilson, Gene Therapy Program, Division of Medical Genetics, Department of Medicine, University of Pennsylvania, Philadelphia PA. AAV vectors were generated using adenovirus free system following previously published protocol (Ghosh et al., 2006). Briefly, human embryonic kidney (HEK) 293 cells were co-transfected with AAV2-based genomic vector pARAP4, AAV Rep/Cap plasmids and adenovirus helper plasmid (pHelper; Stratagene La Jolla, CA, cat.# 240071). The pARAP4 expresses heat stable placental alkaline phosphatase (AP) under the regulation of Rous sarcoma virus (RSV) promoter/enhancer and simian virus 40 (SV40) polyadenylation sequence. AAV6 was generated using four plasmids viz pHelper, pCMV-Cap6, pMT-Rep2 and pARAP4. These plasmids were used at a ratio of 3:3:1:1 respectively. For AAV 8 and AAV9 production, pAAV2/8 and pAAV2/9 encoding for AAV2rep and AAV8 or AAV9cap were used along with pHelper and pARAP4 at a ratio of 3:3:1 respectively. The virus containing cell lysate was harvested at 62 h post-transfection. Recombinant viral stocks were purified by two sequential rounds of CsCl gradient ultracentrifugation. Collected viral fractions were pooled and dialyzed through two rounds of HEPES-buffered saline. Viral titer was determined by dot blot analysis using DIG labeled probes (Roche Applied Science, Indianapolis, IN).

2.2. AAV transduction of mouse and human corneas

All animals and human corneas were treated in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the declaration of Helsinki. Six to eight weeks old female C57 mice were used for the study. Mice were anaesthetized with intramuscular injection of ketamine (130 mg/kg) and xylazine (8.8 mg/kg). Topical solution of 1% proparacaine hydrochloride (Alcon, Ft. Worth, TX) was instilled to each eye for local anesthesia. Alcohol soaked gauze was applied on each cornea for 20 seconds and epithelium was removed by gentle scraping with a #64 Beaver blade (Becton–Dickinson, Franklin Lakes, NJ) under an operating microscope. Two microliters of viral vector (viral titer 10^9 genomic copies/ μ l) was directly applied to the corneal stroma for 2 minutes after drying the cornea with merocel sponge. Animals were divided into 3 groups. The eyes of Group 1 received AAV6 vector, Group 2 received AAV8 vector and Group 3 received AAV9 vector. Animals of each group were sacrificed at 4, 14 or 30 days after AAV application.

For *ex vivo* human cornea application, the stroma of donor human corneas were incubated with AAV serotypes (viral titer 10^9 vg/ μ l) in a humidified CO₂ incubator at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum for 12 hours. Thereafter, the tissues were washed with HBSS twice and were incubated for another 5 days in growth medium.

2.3. Tissue Embedding

Mouse and human corneal tissues were embedded in liquid OCT compound (Sakura FineTek, Torrance, CA) within a 15 mm $\times$ 15 mm $\times$ 5 mm mold (Fisher, Pittsburgh, PA) and snap frozen as reported previously (Mohan et al., 2003). The frozen tissue blocks were

maintained at -80°C . Seven micron thick tissue sections were cut with a cryostat (HM 525M, Microm GmbH, Walldorf, Germany) and maintained frozen at -80°C until staining.

2.4. Alkaline phosphatase detection, localization and quantification in corneal tissues

Corneal sections were washed with HEPES buffer. Cytochemical staining was performed by incubating the corneal sections with a mixture of BCIP (5-Bromo-4-Chloro-3'-Indolylphosphate p-toluidine) and NBT (Nitro-Blue Tetrazolium) at 37°C for 10 minutes. The AP-stained corneal stroma appeared as dark blue. The nuclei were stained using nuclear red fast solution (Sigma Aldrich Inc., St. Louis, MO). To determine the levels of transgene delivery in the cornea mean pixel area of AP staining in six randomly selected, non-overlapping, full-thickness corneal sections ($4 \times 10^4 \mu\text{m}^2$) was quantified.

2.5. Alkaline phosphatase enzyme activity in corneal lysates

The corneal lysates were prepared using RIPA buffer (Tris 50mM, NaCl 150mM, NP40 1%, Na-deoxycholate 0.5% containing 1X protease inhibitor). The protein content for each sample was determined using the Bradford assay. AP activity in corneal lysates was determined by a spectrophotometric assay using StemTAG alkaline phosphatase activity assay kit (Cell Biolabs, Inc., San Diego, CA) following the manufacturer's protocol. Controls were a blank sample (no corneal lysate) and corneal lysate from control eyes. The optical density for AP activity was read at 405 nm. The p-nitrophenol was used for plotting the standard curve. The AP activity was expressed as μM of p-nitrophenol generated/ μg protein. AP activity assay was performed on three corneas for each time point and each sample was analyzed in duplicate.

2.6. TUNEL assay

For TUNEL assay, tissue sections were fixed in acetone at -20°C for 10 min, dried at room temperature for 5 min, and then placed in PBS balanced salt solution. Fluorescent ApopTag apoptosis detection assay (Chemicon international, Temecula CA) that predominantly detects apoptosis and to a lesser extent necrosis, was performed following the manufacturer's instructions. Appropriate positive (corneal scrape) and negative (unwounded) controls were included in each assay.

2.7. Cd11b and F4/80 Immunostaining

Immunofluorescent staining for CD11b (BD Pharmingen, San Jose, CA) and F4/80 (Serotec, Raleigh, NC) was performed using rat anti-mouse antibodies. Tissue sections ($7 \mu\text{m}$) were incubated at room temperature with the primary antibody at 1:50 dilution in $1 \times$ HEPES containing 5% BSA for 90 minutes and with secondary antibody goat anti-rat IgG (AlexaFluor 594, Molecular Probes, Eugene, OR) at a dilution of 1:500 for 60 minutes. Vectashield mounting medium containing DAPI (Vector Laboratories, Inc. Burlingame, CA) was used to visualize nuclei in the tissue sections. The sections were viewed and photographed under a Leica fluorescent microscope (Leica, Wetzlar, Germany) equipped with a digital camera (SpotCam RT KE, Diagnostic Instruments Inc., Sterling Heights, MI, USA).

2.8. Statistical analysis

The results were expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis between various AAV serotypes was performed using two-way analysis of variance (ANOVA) followed by Bonferroni test. For comparing different time points within a serotype, one way ANOVA followed by Tukey's multiple comparison test was used. A p value <0.05 was considered as statistically significant.

3. Results

3.1. Level, localization and duration of AAV-mediated gene transfer in mouse cornea

Alkaline phosphatase (AP) staining representing level and localization of gene transfer in corneal sections of mouse eyes treated with AAV9, AAV8 and AAV6 is shown in Figures 1–3. As evident from these figures a statistically significant transgene delivery was noted in the anterior stroma below the epithelium of mouse corneas treated with AAV than the control corneas ($p < 0.01$ or $p < 0.05$). No AP staining was detected either in the epithelium or endothelium suggesting that vector-delivery technique plays a critical role in tissue-selective gene delivery as reported earlier (Mohan et al 2005a).

Figure 1 shows transgene delivery in the mouse cornea with AAV9 vector examined on 3 selected time points (4, 14 and 30 days). The testing of AAV9-mediated gene transfer earlier than day 4 time point was not done because our earlier experiments showed that the onset of gene expression with AAV2 and AAV5 starts from day 3 (Mohan et al 2005 and 2005a). The AAV9 showed several fold higher transgene expression in mouse stroma on day 14 and 30 compared to day 4. The amount of transgene delivery was significantly higher at day 14 and 30 compared to day 4 ($p < 0.01$; shown by * in Figure 4). This suggests that AAV9 mediated transgene expression reached its peak on or before day 14 and continued to express up to the longest tested time point of 30 days.

Figure 2 demonstrates the level and localization of AAV8 mediated transgene expression in mouse corneal sections collected on day 4, 14 and 30. AAV8 serotype also showed significantly high amount of transgene delivery into keratocytes of the mouse cornea on day 14 and 30 compared to day 4 ($p < 0.05$; shown by Ψ in Figure 4). The transduction characteristics exhibited by the serotype 8 was similar to the serotype 9 showing peak transgene expression on tested day 14 that continued up to day 30.

Figure 3 depicts the gene transfer data collected with AAV6 from corneal sections of mouse eyes treated with AAV6 at day 4, 14 and 30. Contrary to AAV serotypes 8 and 9, serotype 6 showed low to moderate level of transgene delivery in the mouse stroma and the transgene expression was localized more towards the posterior stroma. The transgene expression on day 14 and 30 was more than the day 4, however, it was statistically insignificant ($p > 0.05$).

3.2. Cytochemical quantification of AAV transduction efficiency in mouse cornea

The amount of transgene delivery quantified using Image J program showed relative transduction efficiency of the tested AAV serotypes and has been shown in Figure 4. The three AAV serotypes showed significant transgene delivery as compared to control corneas. The highest transgene delivery was noted with AAV9 and the lowest transduction efficiency was noted with AAV6. The relative order of transduction efficiency was AAV9 > AAV8 > AAV6. The AAV9-treated corneas showed significantly higher transgene expression compared to AAV6 (3.5–5.5 fold, $p < 0.01$; shown by Ω in Figure 4) and AAV8 (1.1–1.4 fold, $p > 0.05$ not significant) at day 14 and 30. Relative comparison between AAV serotype 8 and 6 was also significantly greater (3.1–4 fold, $p < 0.05$; shown by ϕ in Figure 4) at day 14 and 30.

3.2. Functional assay quantifying AAV transduction efficiency in mouse cornea

The biological function of delivered transgene was determined using AP enzyme functional assay. The transduction efficiencies of the tested serotypes were also compared by quantifying the AP enzyme activity in the corneal homogenates. Figure 5 demonstrates the AP enzyme activity in the corneal homogenates prepared from mouse eyes treated with selected AAV serotypes at 3 tested time points. All tested serotypes demonstrated markedly

higher AP enzyme activity at the 3 selected time points (4, 14 and 30 days) compared to controls. The level of enzyme activity at day 14 and day 30 was significantly higher as compared to day 4 in AAV8 and AAV9 treated corneal homogenates ($p < 0.05$; shown by Ψ in Figure 5).

Amongst the three serotypes, AAV9 treated corneal homogenates showed highest levels of AP enzyme activity and it was 2.5 fold ($p < 0.05$; shown by * in Figure 5) higher as compared to AAV6 and 1.5 fold ($p > 0.05$; not significant) higher than AAV8. Comparative analysis between AAV8 and AAV6 revealed that AP enzyme activity was 1.7 fold higher ($p < 0.05$; shown by * in Figure 5) for AAV8 treated corneas than AAV6.

3.4 Gene transfer in human cornea with AAV serotypes

Figure 6 demonstrates gene transfer efficiency of the three tested AAV serotypes in human cornea *ex vivo*. Serotype 9 showed the highest and serotype 6 showed the lowest amount of gene transfer in the human cornea. AAV 8 serotype demonstrated significantly higher gene delivery compared to AAV 6 but moderately less than AAV9. This experiment was performed because tested AAV serotypes showed different transduction efficiency for mouse cornea *in vivo* and human cornea *in vitro* (Sharma et al., 2010). Thus, we wanted to know whether selected AAV serotypes transduce donor human cornea according to transduction pattern that was observed *in vitro* or *in vivo*. This data suggests that AAV serotypes follow transduction pattern noted for the mouse cornea *in vivo* suggesting that *in vivo* testing is critical for establishing vector efficacy for gene therapy. Limited availability of donor human corneas restricted us from performing statistical analysis.

3.5. Effect of titer on AAV- mediated gene transfer in mouse cornea

To test the effect of viral titer on the level of transgene expression, we applied 1,000 times diluted AAV9 to the mouse cornea. As shown in Figure 7, mouse cornea treated with the low titer AAV9 serotype (10^6 genomic copies/ μ l) resulted in a notable decrease in the level of transgene expression.

3.6. Effect of AAV on cell death and immune reaction

Figure 8 shows results of TUNEL staining in control and AAV treated mouse corneas 4 days after epithelial scraping and viral vector application. In control corneas, TUNEL positive cells were mostly noted in corneal epithelium with no or very few TUNEL positive cells in stroma. None of the three AAV serotypes caused any significant change in TUNEL positive cells in mouse corneal sections. To further rule out the possibility of late onset cellular toxicity, we also performed the TUNEL staining at 14 days after AAV applications. No TUNEL positive cells were detected in corneal stroma at 14 days (data not shown) suggesting that tested AAV serotypes do not induce cell death, and are safe for corneal gene therapy.

To test the possibility of inflammatory response due to AAV application, we stained the mouse corneal sections for CD11b, a granulocyte marker, and F4/80, a macrophage marker. As evident from Figure 9, few CD11b or F4/80 positive cells were detected in control corneas at 4 days after epithelial scraping suggesting a normal corneal response to tissue injury. Topical application of AAV6, AAV8 or AAV9 did not cause any significant change in the number of CD11b or F4/80 stained cells. Figure 8 shows a representative CD11b and F4/80 staining in mouse corneas treated with AAV9 at day 4 after vector application. Similar level of positive staining was noted with AAV6 or AAV8 (data not shown).

4. Discussion

Efficient and targeted gene delivery is critical for the success of gene therapy. Pseudopackaged AAV vectors have been developed with the aim of achieving higher gene delivery efficiency and broader tissue tropism while still retaining the safety profile of AAV2. Multiple studies have demonstrated superior transduction efficiency of these next generation vectors for a wide variety of tissues including ocular tissue (Alexander and Hauswirth, 2008; Surace and Auricchio, 2008). In the present study, we demonstrate the ability of AAV6, AAV8 and AAV9 to deliver transgene in mouse cornea *in vivo*. Our technique of topical gene delivery in combination with epithelial scraping successfully achieved selective gene delivery in the anterior portion of corneal stroma suggesting its possible clinical application in corneal disorders associated with anterior stroma abnormalities. Other techniques of gene delivery such as subconjunctival injection have also been tested for AAV application to rodent cornea (Lebherz et al., 2008). However, the high and more cornea selective transgene expression could be achieved with topical gene delivery technique used in this study.

Pseudopackaged AAV vectors display unique tropism and transduction efficiency for specific tissues. Among the 7 tested serotypes, Bish et al showed AAV9 as the most efficient for gene delivery in rodent heart whereas it was the least effective serotype for transducing neurons (Howard et al., 2008). Similarly, AAV8 demonstrated remarkable efficiency for gene delivery in liver (Gao et al., 2006) whereas it transduced vascular endothelial cells with very low efficiency (Denby et al., 2005). Furthermore, different cell types within the same tissue also show variable response to AAV serotype-mediated gene expression. For example, AAV1, AAV4 and AAV6 have shown higher transduction efficiency for retinal pigmented epithelium than AAV5. However, AAV5 had more robust expression in the photoreceptors. On the other hand, AAV7, AAV8 and AAV9 showed equal preference for retinal pigmented epithelium and photoreceptors (Surace and Auricchio, 2008). These studies lead us to propose different AAV serotypes may have unique transduction efficiencies for the cornea. We tested this hypothesis by quantifying transduction efficiency of the three serotypes for corneal gene delivery. Our results demonstrate that amongst the three tested serotypes, AAV9 is most efficient for corneal gene delivery whereas AAV6 shows the lowest transgene delivery. AAV mediated gene transfer involve multiple steps beginning with binding to cell surface receptors, endocytosis, trafficking to the nucleus, viral uncoating and synthesis of double-stranded DNA. Any of these processes may contribute to the higher gene transduction efficiency noted for AAV9 in the present study. Further, multiple studies have reported that peak of AAV-mediated gene expressions may take a few days to weeks depending on tissue-type (Bennet et al., 1997; Gao et al., 2006; Lebherz et al., 2008;). Nonetheless, our data suggests that AAV-mediated gene expression in the eye may follow a similar pattern for various ocular tissue as transgene expression detected in the cornea with selected AAV vectors was found very similar to the retina (Bennet et al., 1997).

Although serotype AAV6, AAV8 and AAV9 share 85% homology in the capsid region (Akache et al., 2006; Pacak et al., 2006), they bind to different cell surface receptors. The AAV6 uses $\alpha 2,3$ - or $\alpha 2,6$ -linked sialic acid receptor (Seiler et al., 2006) whereas AAV8 and AAV9 use laminin receptors for cellular entry (Akache et al., 2006). It is likely that receptor(s) for AAV6 may not be present in the anterior stroma and this may explain the noted low level of transgene expression with AAV6. On the other hand, higher level of corneal gene delivery noted in the present study with AAV9 and AAV8 as compared to AAV6 may be due to higher laminin receptor expression in cornea. However, other unique properties of AAV8 and AAV9 such as rapid uncoating and higher stability of double stranded DNA may also contribute to the higher level of transgene delivery noted with

AAV9 and AAV8. Future studies will be needed to fully understand infection kinetics of these AAV serotypes in corneal tissues.

One of the most interesting findings of the present study is that it highlights the importance of *in vivo* testing for evaluating transduction efficiency of AAV vectors. *In vivo* topical application of AAV9 and AAV8 has shown high level of transgene expression in mouse cornea as well as in human cornea *ex vivo* whereas in a recently published study from our lab low level of transgene expression was noted *in vitro* in cultured human corneal fibroblasts treated with AAV9 and AAV8 (Sharma et al., 2010). Corneal fibroblast culture conditions are reported to modulate laminin (integrin $\alpha 3 \beta 1$ and $\alpha 3 \beta 1$) expression (Masur et al., 1993; Stepp, 2006). Since AAV9 and AAV8 use laminin as receptors for cellular entry, difference in laminin expression may explain the noted higher *in vivo* transduction efficiency of AAV9 and AAV8. Another important finding of this study is that the amount of transgene delivery in the cornea also depends on the concentration of virus titer. As evident from Figure 7 low AAV9 viral titer (10^6 genomic copies/ μ l) showed significantly less transgene delivery in the mouse stroma compared to the high AAV9 viral titer (10^9 genomic copies/ μ l). This study also reveals that duration of AAV application impacts level of transgene expression in the cornea. The donor human corneas incubated for longer duration (12 hours) showed higher level of transgene expression in the stroma of the human cornea compared to the shorter exposure of AAV titer (2 minutes) on the mouse cornea at early time points (day 4). In spite of the enhanced transgene expression noted in human corneas with longer application time, the order of transduction efficiency of the three tested AAV serotypes (AAV9>AAV8>AAV6) remained unaltered (Figure 6) suggesting that transduction characteristics of the tested AAV serotypes is not affected by the duration of AAV exposure.

Safety and toxicity of vectors are important factors for determining the clinical usefulness of gene therapy. Although AAV is characterized by low immune response and low level of toxicity, some tissues such as neurons and glia have shown a decrease in cellular viability when exposed to high doses of AAV suggesting that AAV-mediated toxicity may vary amongst the different tissue types (Howard et al., 2008). This study tested the toxicity of the three selected AAV vectors for the cornea. Eyes exposed to any of these AAV serotypes did not show significant TUNEL positive cells or any inflammatory response (Figures 8 and 9). None of the tested serotype showed large numbers of CD11b or F4/80 stained cells suggesting that tested AAV serotypes are safe for corneal gene therapy. Nonetheless, further studies will be needed to establish the safety of AAV for corneal gene therapy in clinical settings.

In conclusion, tested AAV6, AAV8 and AAV9 vectors efficiently delivered foreign genes in the cornea *in vivo*. The AAV9 and AAV8 showed the high transduction efficiency followed by AAV6. This study highlights the importance of *in vivo* testing of AAV vectors for their future application for corneal gene therapy.

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Fig. 1.

Representative images showing gene transfer into keratocytes of the mouse cornea *in vivo* with AAV9. The purple alkaline phosphatase cytochemical staining shows gene transfer in the stroma of mouse cornea collected 4, 14 and 30 days (d) after AAV9 administration. Control (con) represents corneal sections treated with naked viral vector. Nuclei are stained red with nuclear red fast solution. Scale bar denotes 50 μ m.

Fig. 2.

Representative images showing gene transfer into keratocytes of the mouse cornea *in vivo* with AAV8. The purple alkaline phosphatase cytochemical staining shows gene transfer in the stroma of mouse cornea collected 4, 14 and 30 days (d) after AAV8 administration. Control (con) represents corneal sections treated with naked viral vector. Nuclei are stained red with nuclear red fast solution. Scale bar denotes 50 μ m.

Fig. 3.

Representative images showing gene transfer into keratocytes of the mouse cornea *in vivo* with AAV6. The purple alkaline phosphatase cytochemical staining shows gene transfer in the stroma of mouse cornea collected 4, 14 and 30 days (d) after AAV6 administration. Control (con) represents corneal sections treated with naked viral vector. Nuclei are stained red with nuclear red fast solution. Scale bar denotes 50 μ m.

Fig. 4.

Digital measurement of transgene in the stroma of the mouse cornea delivered with AAV6, AAV8 or AAV9 vector. The AP stained tissue area was quantified digitally by measuring pixels of AP-stained tissue in $4 \times 10^4 \mu\text{m}^2$ tissue area. $\Psi = p < 0.05$ (significance value of AAV8-mediated gene transfer noted at day 14 and 30 compared to day 4), $\ast = p < 0.01$ (significance value of AAV9-mediated gene transfer noted at day 14 and 30 compared to day 4), $\phi = p < 0.05$ (significance value of AAV8-mediated gene transfer compared to AAV6 at day 30), $\Omega = p < 0.01$ (significance value of AAV9-mediated gene transfer compared to AAV6 at day 14 and 30). All AAV treated corneas showed statistically significant transgene delivery in anterior stroma of the mouse cornea compared to control cornea ($p < 0.01$ or $p < 0.05$ not shown in Figure 4).

Fig. 5. Quantification of functional activity of transgene delivered into mouse cornea *in vivo* with AAV6, AAV8 or AAV9 vector. The AP enzyme activity was quantified with spectrophotometer in corneal homogenates prepared 4, 14 and 30 days after vector application. Ψ = $p < 0.05$ (significance value of AAV8 or AAV9-mediated gene transfer noted at day 14 and 30 compared to AAV6, AAV8 or control groups of day 4), * = $p < 0.05$ (significance value of AAV9-mediated gene transfer compared to AAV6 at day 14 and 30 and AAV8 compared to AAV6 at day 30).

Fig. 6. Representative images showing AAV-mediated gene transfer in the human cornea *ex vivo*. The purple alkaline phosphatase cytochemical staining in panels show transgene delivery in the human cornea analyzed 5 days after AAV6, AAV8 or AAV9 treatment. Control (con) represents corneal sections treated with naked viral vector. Nuclei are stained red with nuclear fast dye. Scale bar denotes 100 μ m. The statistical analysis was not performed due to limited availability of donor human corneas.

Fig. 7.

Representative images showing effect of titer on AAV9-mediated gene transfer in the mouse cornea *in vivo*. The purple alkaline phosphatase cytochemical staining shows gene transfer in the stroma of mouse cornea on day 14 after AAV9 application. Upper panel represents corneas treated with the naked AAV9 vector, the middle panel represents the corneas treated with high titer AAV9 vector (10^9 genomic copies/ μ l) and lower panel represents corneas treated with low titer AAV9 vector (10^6 genomic copies/ μ l). Nuclei are stained red with nuclear red fast solution. Scale bar denotes 100 μ m.

Fig. 8.

Representative images of TUNEL assay showing cell death in mouse corneal sections of 4 days that received AAV6, AAV8 or AAV9 vector. Most of the TUNEL-positive cells (Red) were detected in the upper layers of the corneal epithelium that replenishes via apoptosis. Very few TUNEL-positive cells were observed in the mouse stroma where transgene was delivered by the AAV. Nuclei are stained blue with DAPI. Scale bar denotes 100 μ m.

Fig. 9.

Representative images of immunostaining for CD11b and F4/80 of mouse cornea collected 4 days after AAV9 application. The AAV9-treated as well as control corneas showed few (up to 7) CD11b- or F4/80-positive cells in the stroma at 400x magnification. Similar CD11b and F4/80 staining pattern was noted in the cornea treated with AAV6 or AAV8 vector (data not shown). The mouse corneas collected 12 hours after epithelial scrape injury were used as a positive control and showed large number of CD11b+ or F4/80 cells in corneal sections. These sections do not show DAPI-stained epithelium due to scrape. Nuclei are stained blue with DAPI. Scale bar denotes 100 μ m.