In Vivo Gene Transfer Into Murine Corneal Endothelial and Trabecular Meshwork Cells

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Purpose. To determine whether a reporter gene can be introduced into adult mammalian corneal endothelial and trabecular meshwork cells in vivo using a recombinant replication-deficient adenovirus.

Methods. Purified replication-deficient adenovirus containing the cytomegalovirus-promoted Escherichia coli reporter gene, lacZ, was injected into the vitreous cavities or anterior chambers of 30 adult CD-1 mice using the contralateral eyes as controls. LacZ expression was assessed histochemically in enucleated eyes from 2 to 21 days after injection using the β-Galactosidase (β-Gal) assay.

Results. LacZ expression was demonstrated in corneal endothelial and trabecular meshwork cells for as long as 14 days after injection. β-Gal activity was also observed in lens and iris epithelial cells. There was no toxicity of the adenoviral vector demonstrated histologically, and no nonocular tissues expressed lacZ as measured by β-Gal assay.


Recently, successful in vivo gene transfer into adult murine retinal photoreceptors and retinal pigment epithelial cells was reported with the use of an adenoviral vector.1,2 As an incidental finding, gene expression also was observed in anterior segment cells after injection into the posterior segment. The ability to transfer genes to the corneal and trabecular meshwork endothelial cells eventually may have application in the treatment of inherited and acquired anterior segment diseases, such as glaucoma, posterior polymorphous dystrophy, congenital hereditary endothelial dystrophy and Fuchs' endothelial dystrophy. Although specific gene defects for these disorders have not yet been identified, investigators have recently identified a region on the long arm of chromosome 1 that contains a gene sequence responsible for an autosomal dominant form of juvenile open-angle glaucoma4 and a region on the long arm of chromosome 20 that contains a gene responsible for posterior polymorphous dystrophy,5 an autosomal dominant disorder of the corneal endothelium.

The current study was designed to develop a technique for in vivo transfer of a reporter gene (lacZ) into adult murine corneal endothelial and trabecular meshwork cells, to establish the stability of gene transfer by determining the time course of expression of the gene, to evaluate the toxicity of gene transfer in the target tissues, and to determine whether gene expression could be found in other organs after intraocular injection.

METHODS

The recombinant adenovirus, Ad.CMVlacZ, (generously provided by James Wilson, MD, PhD) was pre-
pared, purified, and titered to $10^{11}$ particles/ml using 20% sucrose in phosphate-buffered saline (PBS) as previously described. This adenovirus lacks the viral E1 sequence necessary for replication and, instead, contains the cytomegalovirus (CMV)-promoted Escherichia coli reporter gene lacZ. The same stock of Ad-CMVlacZ was used for all experiments. All manipulations of the adenovirus were made in accordance with institutional and national biosafety guidelines.

All animals were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with federal, state, and local regulations. Thirty adult CD-1 mice ages 4 weeks to 3 months (Charles River Company, Wilmington, MA) were anesthetized with 0.5 to 0.6 g/kg of avertin (Aldrich Chemical, Milwaukee, WI) administered intraperitoneally. In each mouse, one eye was injected with the adenovirus, and the other eye was injected with a control solution consisting of 20% sucrose in PBS. After lateral canthotomy, a 30-gauge needle was inserted into the vitreous cavity by a pars plana approach, and 2 to 3 µl of transfection solution was injected. Anterior chamber injections of 2 to 3 µl of the same transfection solution were made through the peripheral cornea. All injections were monitored by direct visualization through the operating microscope.

Animals were killed from 2 to 21 days after injection, and eyes were enucleated. Eyes were immediately fixed for 1 hour at 4°C in PBS containing 4% paraformaldehyde. They were then washed with PBS and reacted 12 hours at 37°C with 1 mg/ml of X-Gal (5-bromo-4-chloro-3-indolyl galactopyranoside; Sigma Chemical, St. Louis, MO) in a solution containing 10 mM K4Fe(CN)6, 10 mM K3Fe(CN)6·3H2O, 2 mM MgCl2, 0.01% sodium deoxycholate, and 0.02% NP40 in PBS. X-Gal-reacted eyes were examined in whole-mount fashion for β-Gal activity and then postfixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) and embedded in acrylicamide for 24 hours (as described elsewhere). Acrylamide-embedded eyes were immersed in optimum cutting temperature medium (Baxter, McGraw Park, IL) and frozen in liquid N2 for cryosectioning.

X-Gal-reacted eyes were sectioned at 8 to 20 µm at −32°C. Sections were examined both before and after counterstaining with polychrome or hematoxylin and eosin. Specific cell types were identified on the basis of their morphology and location on examination of serial sections. Blue staining after X-Gal reaction was graded as heavy (2+), weak (1+), or absent (—). Hematoxylin and eosin-stained sections were used to examine tissue for an inflammatory cell infiltrate. To screen for potential toxic effects and the transmission of virus to other tissues, the hearts, lungs, livers, and spleens from experimental animals were harvested, and sections were reacted as described above.

RESULTS

In Vivo Gene Transfer to Corneal Endothelial and Trabecular Meshwork Cells

Forty-eight hours after injection, whole-mount preparations of mouse eyes injected intravitreally or intracameraly with Ad.CMVlacZ and reacted with the β-Gal substrate, X-Gal, revealed a dark blue ring corresponding to trabecular meshwork and speckled staining within the cornea (Fig. 1A). Light microscopy of frozen sections further localized gene expression to corneal and trabecular meshwork endothelial cells and to cells lining Schlemm's canal (Figs. 1B to 1D). β-Gal activity also was noted in lens epithelial cells after intracameral injections only (Fig. 1E). After intracameral injection, β-Gal activity was observed in the conjunctiva and corneal epithelium, presumably because of leakage of virus at the injection site. No β-Gal activity was detected in cells within the retina or retinal pigment epithelium or in the ocular tissues of control eyes.
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/J-Gal Activity Diminishes Over Time

From 2 to 5 days after injection, /?-Gal activity was observed throughout the anterior segment in ocular whole-mount preparations in all eyes injected intravitreally or intracamerally with Ad.CMVlacZ. Histologic analyses at this point revealed that, after intravitreal injection, trabecular meshwork cells and corneal endothelial cells exhibited strong /?-Gal activity. From day 6 to day 14, transgene expression was found in 6 of 9 eyes injected intravitreally and all eyes injected intracamerally. The intensity of staining (reflecting gene expression) also diminished rapidly during this time. After day 14, no /?-Gal activity was present. Results are contained in Table 1.

Local and Systemic Pathogenicity of Ad.CMVlacZ

Histologically, the anterior chamber appeared normal on polychrome, and hematoxylin and eosin staining of representative frozen sections. LacZ was expressed in low levels in other ocular tissues that came into contact with the transfection solution, such as iris, lens epithelium, sclera, conjunctiva, and extraocular muscles. There was no evidence of any cellular inflammatory reaction as judged by an absence of polymorphonuclear or round cell infiltrate on hematoxylin and eosin staining of the eyes. In addition, for the entire study period, there was no light microscopic evidence of toxic or cytologically disruptive effects caused by the virus in any eyes examined.

Throughout the study period, there was no clinical evidence of toxic effects from the virus. The mice appeared healthy and showed no alterations in food or water consumption, and they bred normally. No histochemical evidence of virally transmitted /?-Gal activity was detected in nonocular tissues, including heart, lung, liver, or spleen.
**TABLE 1. Gene Expression Over Time**

<table>
<thead>
<tr>
<th>Day</th>
<th>Intraocular Injection* (N = 30)</th>
<th>Intensity</th>
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<tbody>
<tr>
<td></td>
<td>Number of Eyes (%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8/8 (100)</td>
<td>2+</td>
</tr>
<tr>
<td>3</td>
<td>2/2 (100)</td>
<td>2+</td>
</tr>
<tr>
<td>4</td>
<td>2/2 (100)</td>
<td>2+</td>
</tr>
<tr>
<td>5</td>
<td>2/2 (100)</td>
<td>2+</td>
</tr>
<tr>
<td>6</td>
<td>2/4 (50)</td>
<td>2+</td>
</tr>
<tr>
<td>7</td>
<td>2/2 (100)</td>
<td>1+</td>
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</tr>
<tr>
<td>21</td>
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* Anterior chamber injections analyzed on day 2 (n = 1), day 7 (n = 1), and day 11 (n = 2); all other data points represent intravitreal injections.

**DISCUSSION**

The current study demonstrates that an adenoviral vector can be used to transfer a functional gene in vivo into adult mammalian corneal endothelial and trabecular meshwork cells by an intravitreal or intracameral approach. Thompson et al reported in vivo gene transfer to rabbit corneal epithelium and endothelium using a retrovirus vector and plasmid DNA. However, retroviral integration and gene transfer require mitosis of the target tissue (induced experimentally in this study by mechanical trauma), thus reducing the usefulness of this vector in postmitotic target cells, such as adult corneal endothelial and trabecular meshwork cells. Feldman et al used a herpes virus vector to demonstrate in vivo gene transfer to corneal endothelial cells. As an advantage, herpes virus vectors can integrate exogenous genes into the host genome, thereby providing longer potential expression than adenoviruses, which do not integrate their DNA into the host genome. However, even replication-deficient herpes viruses have been shown to be cytotoxic, raising concerns over the suitability of this viral vector for gene transfer in therapeutic application.

In the current study, *lacZ* expression was evident in endothelial and epithelial cells directly exposed to the recombinant virus. Both intravitreal and intracameral injections were successful in achieving gene transfer into the corneal endothelium and the endothelial cells of the aqueous outflow tract, including the trabecular meshwork and Schlemm’s canal. *LacZ* expression was seen in lens epithelium, however, only after intracameral injection of recombinant adenovirus. This may relate both to the cellular anatomy of the crystalline lens and to mechanical properties of the injection procedure. Because of the small volume of the anterior chamber, the cells lining this space are exposed to a high concentration of the recombinant adenovirus after intracameral injection. This may be required to penetrate the lens capsule and to achieve transduction of the lens epithelial cells. A similar phenomenon would not be seen after intravitreal injection because there are no epithelial cells adjacent to the posterior capsule. In addition, because of the greater volume of the vitreous space, the injected recombinant adenovirus is diluted, and, therefore, the lens is exposed to a lower concentration relative to intracameral injection.

Differences are also evident between gene transfer efficiencies of the anterior chamber endothelial cells and cells of the neural retina. The neural retina cells have a low efficiency of transduction and do not manifest significant expression levels until 2 weeks after subretinal injection. The difference in transduction in the various cell populations may result from a variety of factors, including target cell susceptibility to adenovirus-mediated transduction, anatomic differences that might alter the diffusion and thus local concentration of virus particles, and differences in the local microenvironment, such as immune surveillance of the injected area. Both the anterior chamber endothelial cells and the posterior segment retinal pigment epithelial (RPE) cells have a similar high surface-to-volume ratio that may account for the high efficiency of gene transfer. Cells of the neural retina and lens epithelium have less surface membrane exposure and are bordered by extracellular matrix structures that may provide diffusion barriers to gene transfer.

Adenovirus-mediated transduction of anterior chamber endothelial and epithelial cells also differs qualitatively from that seen in the neural retina and RPE. Although anterior segment endothelial cells and RPE cells show high efficiency of transduction, there is less stability of the transduced genes in the anterior chamber regardless of the mode of administration. Although both cell types display intense β-Gal activity 48 hours after injection, anterior segment endothelial cells show rapid decline of both intensity of the histochemically detectable reaction product and the number of reacting cells. β-Gal activity is essentially absent after 2 weeks in anterior segment cells. In contrast, RPE cells have been shown to maintain high levels of expression for more than 100 days after injection despite the episomal nature of adenovirus-mediated transduction, β-Gal activity could be lost rapidly in the anterior chamber secondary to immune-mediated clearance. However, this seems unlikely given the lack of an immune cell infiltrate and the knowledge that the anterior chamber, like the subretinal and vitreous spaces, possesses enhanced immune privilege properties because of immune deviation and immunomodulating factors. However, further studies should test the possibility, as others have suggested, that conventional immunity can emerge and
overcome the deviant form of antigen-specific systemic immunity of the anterior chamber.

Once the technology has developed to allow efficient and stable gene transfer to cells of the anterior segment, it will be possible to investigate the potential of gene therapy for inherited and acquired diseases involving either the corneal endothelial or trabecular meshwork cells. Inherited diseases of the corneal endothelium, such as congenital hereditary endothelial dystrophy, posterior polymorphous dystrophy, and Fuchs’ corneal dystrophy, may be caused by a missing gene or a nonfunctional gene product that could be replaced with gene therapy. Noninherited corneal endothelial diseases, such as the iridocorneal–endothelialization syndromes, might theoretically be treated by introducing a gene that acts to stop progression through the cell cycle. Although not fully elucidated, the pathogenesis of glaucoma may be related to trabecular meshwork cell population and function. It is known that trabecular meshwork cell numbers are lower in patients with glaucoma\(^1\) and that they increase after argon laser trabeculoplasty.\(^{15,16}\) If a specific genetic defect is identified, potential exists for replacement gene therapy using techniques described in the current study. The fact that functional genes can be transferred to the lens epithelial cells suggests that inherited forms of cataracts also might be potential targets through the delivery of genes to the anterior chamber.

Direct application of these techniques in gene therapy of the above disorders is limited at the current time by the lack of identification of the specific gene defect involved, methods for specific targeting of a cell type (such as corneal endothelial cells or trabecular meshwork cells), the lack of suitable animal models for disorders of the anterior segment, and the inefficiency, cytopotoxicity, or both, of the vectors already tested.\(^8\)\(^-{10}\) Based on the transient expression noted with the E1 deleted adenovirus used in these experiments, we recommend evaluation of other vectors that may result in more stable gene transfer to the anterior segment.

**Key Words**
adenovirus, corneal endothelial cells, gene transfer, glaucoma, trabecular meshwork

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**References**