Ocular Adenovirus Gene Transfer Varies in Efficiency and Inflammatory Response

Teresa Borrás,* Ernst R. Tamm,† and J. Samuel Zigler, Jr.*

**Purpose.** To study the effects of adenoviral gene transfer to the tissues of the anterior segment in vitro by rat and monkey lens organ cultures and in vivo by single injection into the anterior chamber of rabbits.

**Methods.** In vitro, intact lens cultures were exposed to 1 to 4 × 10^8 pfu AvLacZ4 and AvLuc1 in TC199 medium containing no serum or growth factors. AvLacZ4 and AvLuc1 are replication-deficient adenovirus vectors, carrying the reporter genes *Escherichia coli* LacZ and firefly luciferase, respectively. In vivo, the anterior chambers of eight rabbits were injected once with 20 μl AvLacZ4 (8 × 10^8 pfu) and evaluated 48 hours after injection. Enzyme activity of the reporter genes was measured biochemically and histochemically.

**Results.** In organ cultures, adenovirus delivers reporter genes efficiently to the ciliary processes but penetrates poorly into the capsulated lenses. Viral receptors, however, are present in rat lens epithelium, as in primary trabecular meshwork and other lens cell lines. In vivo, gene transfer was evident in corneal endothelium, iris anterior surface, and trabecular meshwork. Presence of the virus did not affect lens transparency or provoke external discomfort signs. Infected corneal endothelial cells were swollen and partly detached; 3 of 8 infected eyes showed a severe inflammatory response in chamber angle, anterior uvea, and limbal conjunctiva.

**Conclusions.** These findings reveal the distinct gene transfer potential of each of the tissues of the anterior segment and emphasize the need to address the inflammatory response to these first-generation adenoviral vectors. Invest Ophthalmol Vis Sci. 1996;37:1282–1293.

Gene transfer techniques offer the possibility of delivering a recombinant protein to cells to replace a defective gene product.¹⁴ No less important, gene transfer techniques offer the possibility of temporarily providing a given tissue with beneficial molecules by supplementing an enzyme concentration or manipulating a metabolic pathway.⁴⁵ In animal models, gene targeting of a protein to a particular organ also may contribute to elucidating its putative involvement in the mechanisms associated with the organ’s proper or altered function.

A substantial proportion of the diseases leading to blindness in the adult population are the result of malfunction of tissues of the anterior segment. Thus, cataract alone is the most common cause of blindness worldwide,⁶ whereas glaucoma is the second most important cause of nonremedial blindness in the United States.⁷ Although numerous studies are in progress to identify and map defective genes linked to both diseases in humans, little is known about what proportion of cases would include a genetic component. To date, no gene has been identified definitely as responsible for a human anterior segment disease. For juvenile glaucoma, a link has been established with chromosome 1q.⁸⁹ For cataracts, assignments to chromosomes 1p and 17q have been described recently,¹⁰¹¹ and mutations in crystallin genes have been associated with hereditary lens opacities in animal models.¹²¹⁴ While the search for genetic identification continues, other molecular mechanisms responsible for the development of these diseases are being unraveled. To ex-

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From the *Laboratory of Mechanisms of Ocular Diseases and the †Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland.


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Reprint requests: Teresa Borrás, National Institutes of Health, National Eye Institute, Building 6, Room 237, 9000 Rockville Pike, Bethesda, MD 20892.

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plore alternative treatments for ocular diseases and to anticipate the delivery of therapeutic proteins to the eye, we have investigated the feasibility of targeting cells of the anterior segment with recombinant genes.

Of the putative vectors to deliver exogenous genes, only viruses have evolved to carry their genomic coded proteins naturally to host cells. Among the recombinant viral systems available, replication-deficient adenovirus has the ability to express its genes in differentiated, nonreplicating cells.\textsuperscript{15} The virion enters the mammalian cell by binding to a host receptor which, in HeLa cells, exists in approximately \(10^5\) copies per cell.\textsuperscript{16} Adenoviral vectors can be produced at high titers and do not have significant potential for integration or insertional mutagenesis.\textsuperscript{17} Because of the low proliferative activity of most ocular cells, the adenovirus-mediated gene therapy approach may become an effective in vivo treatment for various diseases of the anterior segment.

Several investigators\textsuperscript{18–20} have demonstrated that adenoviral vectors injected into the subretinal space of mice are able to transfer reporter genes into the photoreceptor cell. Recently, they showed that the recombinant enzyme was delivered in active form to the areas of cornea–sclera, cornea endothelium, and lens after intracameral and intravitreal injection.\textsuperscript{21} In rabbits, reverse transcription–polymerase chain reaction analysis demonstrated the presence of the human heme oxygenase mRNA in cornea, iris, ciliary body, and lens after adenovirus intracameral injection.\textsuperscript{22} In this study, we assessed the effect of the uptake of the recombinant adenoviruses Av1LacZ4 and Av1Luc\textsuperscript{25} in vitro and in vivo models. In vitro, we used freshly enucleated rat and monkey lenses in an intact lens organ culture system. We measured the activity of the transferred proteins Escherichia coli LacZ and firefly luciferase in the lens and ciliary processes by biochemical and histologic assays. In vivo, we chose the rabbit model because of eye size and aqueous humor volume. On injection of the adenovirus vectors into the anterior chamber, we performed a detailed morphologic study of the residual cells containing the active delivered enzyme and searched for the appearance of infiltrating leukocytes. We show the importance of using different gene therapy approaches for different tissues of the anterior segment.

**MATERIALS AND METHODS**

All procedures involving animals were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Lens Organ Culture**

Sprague–Dawley rats (each weighing 75 to 100 g and each 3 to 4 weeks old; Taconic Farms, Hamilton, NY) were killed by CO\(_2\) asphyxiation. The eyes were enucleated immediately, and the lenses were dissected carefully and placed in culture as previously described.\textsuperscript{24,25} Lenses from 2-year-old rhesus monkeys (Macaca mutata) were obtained immediately after death from the monkeys used in routine polio vaccine testing at the Food and Drug Administration’s Center for Biologics Evaluation and Research. Each lens was incubated in 2 ml (rats) or 5 ml (monkeys) modified TC-199 medium adjusted to 298 ± 2 mOsm containing no serum or growth factors and maintained at 37°C, 5% CO\(_2\). Lens integrity was evaluated 1 hour after placement in culture by measuring total protein concentration of the media by a modification of the Bradford method.\textsuperscript{26} Lenses whose media measured a concentration higher than 7 μg protein/ml were considered damaged and were discarded.\textsuperscript{27} Lenses with a ring of remaining ciliary tissue attached were chosen for most experiments.

**Cell Cultures**

All cell lines were grown at 37°C, 10% CO\(_2\) in supplemented improved minimal essential media (Biofluids, Rockville, MD) containing 50 μg/ml gentamicin. The rabbit lens epithelial cell line\textsuperscript{28} was supplemented with 10% rabbit serum (Gibco BRL, Gaithersburg, MD). The αTN4 line is a lens epithelial line derived from a transgenic mouse containing the SV40 T-antigen driven by the mouse α-crystallin promoter.\textsuperscript{29} Serum-free medium for culturing αTN4 cells was supplemented with insulin, transferrin, selenious acid, linoleic acid (ITS\textsuperscript{1+}; Collaborative Research, Bedford, MA) to favor the formation of lentoid bodies.\textsuperscript{30} Primary monkey trabecular meshwork cells were sixth-passage cultured from 2-year-old rhesus monkeys (M. mulata). These cells were supplemented with 20% fetal bovine serum (Gibco BRL) and exhibited no replication at this passage level. Rat epithelial explants were dissected from the lenses of 3- to 4-week-old animals and incubated while partially attached in 35-mm dishes (three to four explants per dish) in the serum-free lens organ culture media as described above.

**Preparation of Gene Transfer Vectors**

The adenoviral vectors Av1Luc and Av1LacZ\textsuperscript{46} were obtained under a Material Transfer Agreement between Genetic Therapy (Gaithersburg, MD) and the National Eye Institute and propagated as described on permissive 293 cells (ATCC 1573-CRL, batch F-11973). Both vectors were replication deficient E\textsuperscript{1−}, E\textsuperscript{3−} deletion mutants, expressing the reporter genes firefly luciferase and a nuclear targeted E. coli LacZ under the control of the
rous sarcoma virus promoter. Viruses were purified by
twice banding to equilibrium density in CsCl, 17,31,32 de-
salted by chromatography through Sephadex G-25
(NAP-5; Pharmacia LKB, Uppsala, Sweden) equilibrated
with 0.01 M Tris, pH 8, 0.01 M MgCl₂, 10% glycerol,
and stored in 100 μl aliquots at -70°C until use. Purified
stocks were tiered by plaque assay on 293 cells using 1
hour of serum-free adsorption at day 0, followed by 18
hours adsorption in the presence of 10% serum, removal
of the infective media, and three agar overlays of the
one part 2% sea plaque agarose (FMC, Rockland, ME)
and one part 2× minimum essential medium, 24 mM
MgCl₂, 4% fetal bovine serum, 100 μg/ml gentamicin,
20 μg/ml fungizone (Gibco BRL) at days 1, 5, and 10.
The last agar mixture included 14 mg/ml neutral red
(Sigma Chemical, St. Louis, MO) that facilitated plaque
reading at day 12. Viral titers were typically between 1
to 5 × 10⁶ particle-forming units (pfu)/ml.

In Vitro Adenovirus Gene Delivery

Lenses in organ culture were infected 3 to 4 hours
after enucleation. After confirmation that lenses sus-
tained no damage during dissection, they were trans-
ferred using teflon-covered forceps to fresh 24-well
dishes, 1 ml medium per well containing 1 to 4 × 10⁸
pfu of AvILuc1, AvILacZ4, or viral vehicle. Occasion-
ally, two rat lenses were infected together in the same
well. Viral suspension volumes never exceeded 20 μl
to keep the medium osmolarity between 300 and 310
mOsm. Viral infection was carried out for 24 hours at
37°C, and evaluation of gene transfer was conducted
at the indicated times after infection. For the primary
epithelia explants, a similar protocol was followed us-
ing 35-mm dishes and three to four explants per dish.
Tissue culture cells were grown in the indicated media
and seeded on 6-well dishes to reach 70% to 80%
confluency. Cells were exposed from 1 to 2 × 10⁸
AvILacZ4 pfu at multiplicity of infection 50 to 100 or
to the same volume of viral vehicle under identical
conditions as described for viral propagation. Cultures
were evaluated histochemically 48 hours after infec-
tion.

Luciferase Activity Assay

After viral infection, lenses were washed with phos-
phate-buffered saline (PBS) and separated from the
surrounding ciliary tissue under the dissecting micro-
scope. Both tissues were homogenized separately,
each in 150 μl of lysis buffer (Analytical Luminescence
Laboratory, Ann Arbor, MI) using a plastic disposable
Kontes micropaste in the corresponding Eppendorf
tube. After 10 minutes of incubation on ice, samples
were centrifuged at 14,000g at 4°C for an additional 10
minutes. Supernatants were assayed for activity using a
Luciferase kit (Analytical Luminescence Laboratory)
following the instructions of the manufacturer. Ten to
20 μl of the sample were added to 100 μl of substrate A
and placed within 30 seconds in a Monolight 2010
luminometer (Analytical Luminescence Laboratory)
that automatically injected 100 μl of substrate B. The
emitted flash of light was recorded as light units corre-
sponding to the aliquot assayed.

Enzyme Histochemistry

For whole tissue mounting, intact lens or whole ante-
orier segment were washed briefly with PBS and fixed
for 30 minutes in cold 1% paraformaldehyde contain-
ing 0.2% glutaraldehyde, 0.02% NP40, and 0.01% sodium
deoxycholate. Subsequent β-galactosidase (β-Gal)
activity was assayed by incubating overnight with the
chromogenic X-Gal as described.25 After staining,
tissues were washed again in PBS, postfixed in cold
4% paraformaldehyde for 30 minutes, washed, embed-
ded in paraffin, sectioned at 5 to 10 μm, and counter-
stained with either eosin or eosin–hematoxylin. Fixa-
tion of cell cultures and epithelial explants was per-
formed with 4% paraformaldehyde for either 5 or 20
minutes (cells or explants) and stained with X-Gal as
described. Explants were counterstained further with
hematoxylin and mounted with aqueous Gel/Mount
(Biomed, Foster City, CA) in poly-L-lysine slides
(Sigma Diagnostics, St. Louis, MO). Paraformalde-
hyde solutions were made in PBS, kept at 4°C, and
discarded after 3 weeks.

In Vivo Adenovirus Gene Delivery

New Zealand White rabbits (each weighing 3 kg) were
anesthetized by intramuscular injection containing a
mixture of 35 to 50 mg/kg ketamine and 8 to 12 mg
xylazine. Eyelids were retracted, and 20 μl of AvILacZ4
virus (8 × 10⁸ pfu) were injected into the ante-
orier chamber using a 50 μl Hamilton syringe and a 1
cm beveled 30G 1/2 needle. The injection was per-
formed in a biosafety cabinet (level 2) by inserting the
needle (bevel up) tangentially into the chamber at the
limbus. Eight rabbits received the virus in the right
eye while their contralateral eyes were injected with
20 μl vehicle. Four of the rabbits were treated with
0.25% Fluoromethalone drops (lot no. 5289x; Aller-
gan, Irvine, CA) on both eyes starting immediately
after the injection and continuing three times a day
for the duration of the experiment. Two animals re-
mained uninjected. One animal was left untreated.
Animals were under observation for signs of redness,
swelling, tearing, or excessive blinking. After 48 hours,
all animals were anesthetized and killed by cardiac
puncture with an intravenous overdose of sodium pen-
tobarbital. Aqueous humor was withdrawn and assayed
for viral titer. Eyes were enucleated, and their anterior
TABLE 1. Number of Intact Lens Organ Cultures Showing Reporter Enzyme Activity on Infection With Replication-Deficient Adenovirus

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rats</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>40</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>Vehicle</td>
<td>19</td>
<td>0</td>
<td>19</td>
</tr>
<tr>
<td><strong>Infected cultures</strong></td>
<td>Lens</td>
<td>CilPro</td>
<td>Lens</td>
</tr>
<tr>
<td>L/Cp dissected*</td>
<td>25</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Lens, no Cp†</td>
<td>11</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>L/Cp no dissected</td>
<td>4</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td><strong>Monkeys</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>26</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>Vehicle</td>
<td>16</td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>

* Tissue separated into lens (L) and ciliary processes (Cp) before measuring enzyme activity.
† Cultures containing only lens tissue.

segments and lenses were dissected, fixed, and processed as described. For every anterior segment, an average of 10 histologic cuts per each quadrant was analyzed morphologically.

RESULTS

Biochemical Evaluation of β-Gal and Luciferase Gene Transfer to Rat and Monkey Lenses in Organ Culture

Rat lens preparations with and without attached ciliary processes were infected with $2.5 \times 10^8$ pfu of AvlLuc1 for 24 hours and then maintained for as long as 2 weeks with a change of fresh media every 3 days. Enzyme activity, though variable, was detected in lens and ciliary processes homogenates with maximum activity observed 4 days after infection. Results are summarized in Table 1, and the representation of one experiment is shown in Figure 1. Gene transfer into the lens was approximately 1000 times less efficient than gene transfer to the ciliary tissue. Under similar conditions, of a total of 40 lens organ cultures infected with AvlLuc1 or AvlLacZ4, 32 were positive. From the 25 cultures separated into lens and ciliary tissue after organ culture, 12 lenses and 25 ciliary processes were positive. From the 11 cultures containing only lens tissue, 3 were positive and 8 were negative. In no case was activity detected in the 19 mock-infected tissues.

Adenovirus-mediated gene transfer also occurred in 20 of 26 lens organ cultures from 2-year-old rhesus monkeys (Table 1).

To determine whether a shorter exposure to the virus still resulted in detectable gene delivery, the same number of pfu per lens was applied to the organ cultures for only 1 hour instead of the standard 24 hours. On analysis, 48 hours after infection, we found that lenses and ciliary processes infected with the virus for 1 hour contained approximately one tenth the amount of the delivered protein that was obtained when exposing the tissues for 24 hours. Furthermore, the viral titer of the suspension also influences gene transfer efficiency. Rat lens organ cultures 3 to 4 weeks of age infected with $10^7$ pfu showed no luciferase activity, whereas those infected with $\approx 10^8$ were positive.

Gene transfer also occurred in 20 of 26 lens organ cultures from 2-year-old rhesus monkeys (Table 1).

![LENS](image1)

**LENS**

![CILIARY](image2)

**CILIARY**

**FIGURE 1.** Biochemical evaluation of the luciferase activity of lens and ciliary processes from rat organ cultures. Freshly dissected tissue was infected with $2.5 \times 10^8$ pfu of AvlLuc1 (clear bars) or virus vehicle (crossed bars) for 24 hours and harvested at the indicated times. Each bar represents an individual lens or their corresponding ciliary processes (when present). Luciferase light units correspond to total counts per each isolated tissue.
suggesting that a minimal multiplicity of infection is needed to assure gene delivery (results not shown). All lenses remained fully transparent during the duration of the experiment, even at the maximal number of viral particles used ($6 \times 10^8$). Previous control experiments showed that the addition of as much as $15 \mu l$ of viral suspension (containing 10% glycerol) to the 1 ml lens culture medium did not raise the osmolarity above 310 mOsm. Maintaining the osmolarity between 298 and 315 mOsm appears to be critical to the maintenance of lens transparency in organ culture.

Localization of a Nuclear Targeted LacZ Gene Transferred to Rat Intact Lens Organ Cultures

Tissue localization of LacZ expression in organ culture was studied by enzyme histochemistry. Between 48 and 72 hours after infection with AvIacZ4, the enzyme activity of the LacZ gene product was detected after incubation of the intact tissue with the X-Gal substrate. In wholemount preparations, activity was located on the part of the ciliary processes that remained attached to the lens by the zonular fibers. Staining was localized to the tissue around the lens but apparently not inside (Fig. 2a). Disrupted lens exposed to X-Gal substrate gave the same result, indicating that negative staining inside the lens was not caused by poor X-Gal penetration. In mock-infected tissue, no enzyme activity was observed after overnight incubation with the substrate (Fig. 2b). Light microscopy observation of numerous frozen and paraffin-embedded slides confirmed that the concentration of the β-Gal product was localized to the cells of the ciliary epithelia (Figs. 2c, 2d). No cells inside the capsulated lens, either from the central epithelium or from the differentiating fibers at the equator, showed expression of the LacZ gene by this method.

Gene Transfer to Ocular Primary Cell Cultures and Cell Lines

To determine the reason for the poor penetrance of the virus into the intact lens, we examined the capacity for viral uptake (presence of viral receptors) of isolated ocular cells. The N10003 untransformed rabbit lens epithelial cell line and the αTN4 mouse lens epithelial cell line, infected at a multiplicity of 100, yielded detectable expression of the reporter gene in approximately 80% of the cells 48 hours after infection (Figs. 3a, 3c). Staining was concentrated in the cell nuclei, indicating that the enzyme activity corresponded to the nuclear targeted transferred gene and not to the presence of a putative endogenous enzyme. Lentoid bodies, present in the serum-free αTN4 line, were particularly active. Based on a comparative evaluation of the total cell number between infected and control cultures, there were no evident cytotoxic effects caused by virus infection or the expression of the reporter gene in the rabbit cell line. However, αTN4-infected cells exhibited some reduction in number, possibly because of the presence of the SV40 T antigen in the genome of this cell line. The SV40 T antigen has been shown to share some of the adenovirus E1A functions, including transactivation of the adenovirus E2 promoter. This function could promote some
Ocular Gene Transfer in Organ Culture and In Vivo

FIGURE 3. Histochemical evaluation of β-Gal activity in ocular cells. Bright-field micrographs (b, phase-contrast) of ocular cells infected with to 1 × 10⁷ pfu of AV1LacZ4 (left column) or virus vehicle (right column) and assayed 48 hours after infection. (a,b) Rabbit lens epithelial cell line N10005. (c,d) Transgenic mouse lens epithelial cell line αTN4 showing lentoid body formation. (e,f) Rat primary lens epithelial explant counterstained with hematoxylin after the enzyme assay. (g,h) Monkey primary trabecular meshwork cells at sixth passage.

lytic activity of the E1A-deleted, replication-deficient adenoviral vectors.

Infectivity of primary lens cells in the absence of serum or growth factors was tested by exposing the freshly dissected rat lens capsule–epithelium layer to virus in the lens culture media. In these close-to-natural conditions, the nonreplicating lens cells in the explant were able to internalize the virus and express its reporter gene (Fig. 3e).

In addition, sixth-passage primary monkey trabecular meshwork cells were able to express efficiently the reporter gene at stages at which replication no longer occurs (Fig. 3g). These results suggest that, in culture, most cell types from the anterior segment of the eye are capable of adenoviral uptake. Expression of viral genes occurs in cells in nonproliferative conditions similar to those found in vivo. No detectable activity of the reporter gene was present in any of the mock-infected cells (Figs. 3b, 3d, 3f, 3h).

Gene Transfer to Rabbit Anterior Segment In Vivo

Infection of rabbits with the adenovirus vector containing the E. coli LacZ gene through the aqueous
humor rendered the following results. After overnight X-Gal treatment, wholemounts of the anterior segment revealed intense staining in the cornea, iris, and ciliary tissue in 7 of 8 infected eyes (Fig. 4a). Control wholemounts from uninfected and vehicle-injected animals showed staining of the ciliary and iris epithelium (Fig. 4b). Presence of the virus in the aqueous humor did not have an effect on lens transparency, nor did it cause abnormal external signs. Neither group of animals showed signs of eye irritation such as redness, swelling, abnormal tearing, or blinking as a consequence of the viral or vehicle injection. Histologic examination, though, reveals a severe inflammatory reaction in 3 of 8 infected animals. Of the three, two belonged to the group treated with the antinflammatory drug. Histology of infected eyes confirmed expression of the transferred gene in anterior segment cells of the seven positive animals (Figs. 4c, 4e, 4f, 4g, 4i, 4j, 4l, 4m, 4n). In these, β-Gal could be visualized in cells of the cornea endothelium, but never in cells of the corneal stroma or corneal epithelium (Fig. 4c). In two animals, only a few corneal endothelial cells (2%) stained, whereas in the five other animals, most of the cells were positively labeled. These corneal endothelial cells were swollen markedly and were detached partially from the Descemet’s membrane (Figs. 4e, 4f). In three animals, such a detachment occurred throughout the entire endothelial layer of the cornea endothelium (Fig. 4c). Staining of iris and ciliary body epithelial layers also was observed in all infected eyes. In addition, the cells covering the anterior stroma were stained, as were several macrophages in the stroma. No staining was observed in the sphincter muscle, in resident iris stromal cells, or in vascular endothelial cells (Fig. 4g). In the chamber angle, pronounced β-Gal activity was seen in most of the trabecular meshwork cells of all positive animals (Figs. 4i, 4j, 4l, 4m). This staining was confined to the inner parts of the meshwork, whereas staining in cells next to the aqueous plexus or in endothelial cells of the plexus was observed in only half the infected eyes (Figs. 4j, 4m). In five animals, most of the resident cells of the anterior choroid were intensely labeled. In the two most intensely labeled eyes, no staining was observed inside the lens.

In the three animals affected with inflammation, chamber angle and ciliary body stroma were filled with numerous polymorphonuclear leukocytes and mononuclear cells (Figs. 4j, 4m). Some of these cells expressed β-Gal activity. In some parts of the circumference, the chamber angle contained fibrin with enmeshed leukocytes. Inflammatory cells similarly were found in the adjacent scleral and conjunctival stroma. In these animals, resident scleral fibroblasts were stained in the limbus area as well as endothelial cells, perivascular macrophages of collector channels, and episcleral vessels (Figs. 4j, 4m, 4n). In one animal, cells of the limbal conjunctiva epithelium stained positively for β-Gal. In all these eyes, the iridial processes showed a marked edema with extravasation of blood and occasional formation of Greff’s vesicles. Such edema also was observed in one control eye.

Histology of the control eyes showed activity of β-Gal in the pigmented ciliary epithelium (Fig. 4k) and FIGURE 4. Histochemical evaluation of β-Gal activity in the anterior segments of albino rabbit eyes. Animals were injected into the anterior chamber with 8 × 10⁷ pfu of AvlLacZ4 (a,c,e,f,g,i,j,l,m,n) or virus vehicle (b,d,h,k). (a,b) Frontal photographs of the anterior segment upon removal of the lens. (c to n) Light micrographs of 10 μm meridional paraffin sections through different tissues of the anterior eye segment counterstained with eosin or eosin plus hematoxylin (d). (c to f) Cornea. In the cornea of virus-treated animals, activity for reporter gene is only present in cornea endothelial cells (c,e,f). Infected cells that stain blue for β-Gal (c,e,f) are marked swollen when compared with endothelial cells of the contralateral control eye (d) and partially detached (e). In some of the virus-treated eyes, almost the entire endothelium is detached from Descemet’s membrane (c, arrow). (g,h) In the iris, positive expression of the reporter gene in virus-treated eyes is seen in the cell layer covering the anterior stroma (g, arrow). In contrast, virus-treated (g) and vehicle-treated (h) contralateral eyes show positive staining for endogenous β-Gal in the iris epithelium facing the posterior chamber. (i to m) In the chamber angle of virus-treated eye (i,j,l,m), numerous cells express β-Gal activity, whereas no such activity is seen in control eyes (k). In most of the eyes, almost all trabecular meshwork cells express the reporter gene (i,j,arrow). Some of the eyes (j,m) show, in addition to staining of the resident trabecular meshwork cells and aqueous plexus endothelium (j, arrow), a severe inflammation of the chamber angle, with infiltration of numerous polymorphonuclear granulocytes, plasma cells, and mononuclear cells. (n) Episcleral vessels. In the eyes with inflammation, staining is seen in endothelial cells of episcleral vessels (arrow). In addition, numerous leukocytes are seen in limbal sclera and conjunctiva. Magnifications: (c, g to k), ×48; (d to f, l to n) ×100.
pigmented epithelium of the iris (Fig. 4h). When the X-Gal treatment was reduced to 5.25 hours, staining from control tissues was reduced to a weak signal on the iris epithelium whereas the staining pattern of the infected tissues did not change (not shown).

Analysis of the aqueous humor for the presence of viral particles was performed at the conclusion of the experiment by serial dilution and plaque assay on 293 cells. Infected eyes contained an average of $4 \times 10^5$ pfu/ml or a total of approximately 800 of the $8 \times 10^8$ particles injected per anterior chamber. No virus was detected on the aqueous humor from contralateral eyes.

**DISCUSSION**

The eye is one of the organs most accessible to gene therapy. Although a gene defect has not been characterized fully for diseases of the anterior segment, the development of a system to target the delivery of beneficial proteins to these tissues could provide an alternative approach to ocular drug therapy. Assessing delivery of recombinant proteins through different routes of entry is of utmost importance. Assessing their expression in cells of the anterior segment would further contribute to determining their usefulness in other applications. For instance, cells of the anterior segment are easily accessible to surgeons during procedures such as corneal transplants, cataract extractions, and trabeculotomies. Transfer and expression of genes (coding for antioxidants, antiproliferative products, cytokines, transport, or extracellular matrix proteins) to those cells could contribute to a successful recovery. We studied the feasibility and effects of delivering reporter genes carried by these viral vectors to the anterior segment of rat, monkey, and rabbit eyes.

Our first set of experiments in rat lens organ cultures used a very sensitive enzyme assay, the expression of the firefly luciferase gene. By this method, we detected viral expression in the lens to values 10 to 15 times over the control, whereas enzyme activity values of the surrounding ciliary tissue were approximately $10^4$ times higher. Total activity peaked at approximately 4 days after infection. Because protein synthesis remains constant for the duration of the experiment (T. Kamiya, personal communication, 1995), the lower activity observed with time may have been caused by a decay in the foreign gene transcription, a common occurrence seen for gene transfection in culture.

The activity of the ciliary processes was approximately 1000-fold the activity of the lens. When the lower sensitivity enzyme histochemistry method was used, activity inside the lens was not observed. Nevertheless, histologic staining corresponding to values 1000 times lower than the ones observed in the ciliary processes would not have been detected by this method. Although we cannot rule out the possibility of ciliary tissue contamination of the lens during the biochemical assay, we think viral infection of the intact lens is indeed occurring, though at a much lower efficiency. Once the capsule of the rat lens was disrupted, cells of the epithelial layer showed an efficient uptake of the vector, indicating the presence of natural, not serum-induced, adenoviral receptors in their membranes. In addition, lens cell lines from other species (rabbit and mouse) also showed high expression of the foreign gene on viral infection. Altogether, these results clearly indicate that the lens capsule is the barrier responsible for the low gene transduction observed in the intact lens. This finding limits the usefulness of gene delivery to the lens using these vectors to those proteins needed in very small concentrations.

On the other hand, the high activity found in the ciliary processes, when maintained in an aqueous humor-like medium, makes this tissue an attractive target for gene therapy. Furthermore, the ciliary processes attached to the lens by the zonula appear ideal for gene therapy of the lens by the neighboring approach. Delivering genes to this tissue—for example, those coding for antioxidant enzymes—could help to provide a protective environment for the lens by lowering the level of $\text{H}_2\text{O}_2$ in the aqueous humor. In addition, our tissue culture results with different ocular cell types, grown under different conditions, show that these viruses have a good spectrum of infectivity with respect to the cells of the anterior segment.

Our in vivo experiments demonstrate the feasibility of gene transfer through injection into the aqueous humor. Rabbits were chosen as the animal model because their aqueous humor volume is similar to that of humans. Because of the lens capsule leakiness to proteins during inflammation, and with the idea of favoring viral penetrance into the lens, a group of rabbits purposely was not treated with the anti-inflammatory drug. Our results demonstrate that in a volume approximately one tenth that of the aqueous humor, a suspension containing 10% glycerol can be taken readily by the animal. After recovering from anesthesia, rabbits had no external signs of discomfort such as abnormal tearing or blinking.

Histology indicated that the majority of the infected animals (7 of 8) showed pronounced specific staining for $\beta$-Gal in the trabecular meshwork, the corneal endothelium, the anterior surface of the iris, and the anterior choroid. In our opinion, the activity in all these tissues was caused by virus uptake and reporter gene expression. The high uptake of the virus in the resident trabecular meshwork cells indicates the presence of numerous adenovirus receptors and offers this tissue as one of the easiest targets for gene deliv-
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ery. The same seems to be true for the corneal endothelium; however, the marked cellular changes, including detachment of infected cells, denote an adverse effect that must be overcome before adenovirus transfer can be used routinely for this tissue. Detachment and subsequent loss of cells is a characteristic sign of endothelial damage that can occur after various forms of insults to the corneal epithelium, including infection with replication-competent viruses. In addition, three of the infected eyes had severe inflammation of the chamber angle and the anterior ciliary body. No signs of such toxicity were observed in a recent study, when adenovirus was injected into the anterior chamber of mice. In this study, however, histology was only performed in frozen sections; hence, cytologic details easily might have been missed. At present, we do not know the reason for the variability in inflammatory response. A likely possibility could be the high number of first-generation adenovirus (8 × 10⁹ pfu) injected per eye. First-generation adenovirus lacks the viral E3 region whose gene product has been associated with a host-defense mechanism to the adenoviral infection. Other authors have shown the same type of vectors to the lung of primates have shown the presence of infiltrates at the highest dose of the virus in animals that otherwise appear clinically well. In a study of humans, a pulmonary infiltrate developed in the patient who received the highest intrapulmonary dose (2 × 10⁹ pfu) in the area of virus administration. If the concentration used in these experiments (8 × 10⁹ pfu) were at or near the limit for provoking an inflammatory response, the variability observed would be expected. Another possible reason for the variable inflammatory response could be the presence of wild-type adenovirus in our replication-deficient viral stocks. Experiments are in progress to investigate these possibilities.

Histology showed no abnormalities in anterior chamber morphology in most of the control eyes. The edema of the iridal processes in one of the animals probably resulted from a paracentesis effect during injection of the vehicle. It is generally agreed that this is the typical response of the rabbit eye to a sudden lowering in intraocular pressure. Ciliary epithelium of control eyes was thought to be caused by the presence of endogenous enzyme activity. This endogenous β-galactosidase activity in the rabbit was confined to the pigmented layer, in contrast to the most internal nonpigmented layer previously reported.

Despite the presence of inflammation in the anterior segment, we were unable to detect by histologic methods the viral-delivered, recombinant protein inside the lens, in contrast with recently published results in mice. Again, as was observed in vitro, the lens capsule appears to be the main barrier. Furthermore, the extent of the barrier may vary with the species. In a new report in rabbits, after intracameral injection, the presence of the delivered gene mRNA is detected in the lens by reverse transcription-polymerase chain reaction. In agreement with our organ culture results, their positive signal from the ciliary body is much higher than that from the lens, again raising the issue of possible ciliary tissue being carried over with the lens. Nevertheless, both studies indicate that only very sensitive biochemical or RNA amplification techniques are able to detect the delivered product in the lens.

For the ciliary body, the in vivo results are not yet definite. Because of the endogenous background of the rabbit pigmented layer, the question remains whether this tissue is able to take up the virus delivered to the anterior chamber. The high activity observed in the ciliary processes of our organ culture experiments suggests that the lack of activity observed in vivo in the nonpigmented layer is most likely caused by low efficiency of the virus reaching the posterior chamber. Earlier studies had shown that a radioactive metabolite perfused into the anterior chamber of rabbits, with and without pupil dilation, was taken up by the lens in direct proportion to the pupillary area, suggesting limited diffusion from the anterior to the posterior chamber. An unexpected finding was the expression of the reporter gene in cells of the anterior choroid. It seems reasonable to assume that the virus was transported to the choroid by uveoscleral flow. In contrast to primates, in which uveoscleral flow contributes significantly to total aqueous outflow, uveoscleral outflow in rabbits plays only a minor role under physiological conditions. An inflammatory response, however, might have increased uveoscleral flow in our animals. Such an increase has been reported in cynomolgus monkeys after experimental iridocyclitis. β-Gal activity in endothelial cells of episcleral vessels indicates that virus injected into the anterior chamber reaches the vascular system by means of conventional outflow. By 48 hours, the presence of adenovirus in the aqueous humor was reduced dramatically, with approximately 800 pfu of the 800 million injected remaining. At an aqueous humor flow of 2.48 µl/minute for the rabbit, the virus should have disappeared in less than 2 hours.

Further studies are needed before the usefulness of these vectors for gene therapy of the anterior segment can be determined. Our findings show that it is crucial to evaluate the effect of viral uptake on normal cell physiology. For instance, it has been reported that, once inside the host cell, adenovirus transport to the nucleus appears to be mediated by the microtubule-associated proteins. Considering the possible rele-
vance of the trabecular meshwork cytoskeleton in the outflow facility, the general adenovirus effect in trabecular meshwork cells must be studied.

In summary, our results show that the first-generation adenoviruses deliver reporter genes efficiently to cells of the rat ciliary processes, rabbit corneal endothelium, iris anterior surface, and angular plexus meshwork but that they penetrate poorly, if at all, into the capsulated lens. Because of the different efficiency of delivery to the tissues and of the inflammatory response obtained, these results stress the importance of designing customized vectors and delivery conditions for each tissue to be targeted. Thus, approaches for gene therapy of the lens will have to be different from those to target the trabecular meshwork. Control of inflammation by manipulating viral concentrations and modifying existing vectors will require further study. Once such control is accomplished, adenoviral gene transfer in the eye might be a straightforward approach not only to correct a genetic defect but also to deliver putative therapeutic proteins to the anterior segment and to study their involvement in the physiology and gene regulation of eye tissues in vivo.

**Key words**

adenovirus, anterior segment cells, gene transfer, inflammation, lens organ cultures

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