In Vivo Transfer of a Reporter Gene to the Retina Mediated by an Adenoviral Vector

Tiansen Li,* Michael Adamian,* Dorothy J. Roof,* Eliot L. Berson,* Thaddeus P. Dryja,* Blake J. Roessler,† and Beverly L. Davidson†

Purpose. The ability of replication-deficient adenovirus to mediate gene transfer to retinal cells was evaluated.

Methods. A replication-deficient adenoviral vector, AdCMV βA.ntlacZ, which contains the bacterial β-galactosidase (lacZ) reporter gene, was injected into the subretinal space of normal, rd, and rds strains of mice at various ages. The efficiency and duration of transgene expression were assessed by histochemical examination and transmission electron microscopy.

Results. AdCMV βA.ntlacZ was effective in mediating gene transfer to the retinal pigment epithelial cells, rod and cone photoreceptor cells, and cells in the inner nuclear layer of the retina for periods of up to 1 month. Gene transfer to retinal pigment epithelial cells occurred at much lower viral titers than was required for gene transfer to photoreceptor cells. The extent to which photoreceptor cells could be transduced varied with the age of the animals and the conditions of the photoreceptor cells: greater numbers of photoreceptor cells were transduced in 5- to 7-day-old pups and in mice at the initial stages of photoreceptor degeneration than in normal adult mice. No evidence of gross pathogenic effects or viremia in recipient mice was observed.


Retinitis pigmentosa (RP) is the name given to a group of inherited retinal degenerative diseases. Mutations in three genes—opsin, the β subunit of rod cGMP phosphodiesterase, and peripherin/rds—have thus far been identified to cause RP.1–5 Each of these genes is expressed primarily or specifically in the photoreceptor cells. In the rd and rds mouse models for RP, which carry null alleles of the genes for the β subunit of rod cGMP phosphodiesterase and peripherin, respectively, germline introduction of functional gene constructs rescued the photoreceptor cells from genetically programmed degeneration.6,7 This raises the possibility that specific and local in vivo introduction of genes into the photoreceptor cells as a replacement for certain mutant alleles could potentially be a viable approach to treatment. Such gene replacement therapy for recessive RP, as well as potential therapies aimed at alleviating the effects of dominant RP alleles, such as antisense, antigen, or ribozyme strategies,8–11 must have as a prerequisite the ability to deliver genetic elements to retinal cells in vivo.

Physical, chemical, and biologic methods have been evaluated for in vivo transfer of foreign genes into somatic tissues. Among these, the use of gene transfer vectors derived from retroviruses proved to be effective in mediating stable gene transfer to a wide range of tissues, but only if the target cells were undergoing cell division. However, cells in the adult neural retina are postmitotic and are therefore not receptive to retrovirus-mediated gene transfer. Replication-deficient herpes simplex virus 1 (HSV-1) may hold promise for gene transfer to postmitotic neurons,12 but the current versions of HSV-1–based vectors, most of which have a single immediate early gene inactivated, are cytotoxic.13 In comparison, replication-deficient...
adenoviruses are less cytotoxic and can be prepared and delivered to tissues in vivo at high titers resulting in high levels of gene transfer without severe tissue damage. Recent work on adenovirus-mediated gene transfer to the brain encourages the idea that adenoviral vectors may also be useful for gene transfer to the neural retina. In the present study we explored the use of replication-deficient adenovirus as a means to transfer a foreign gene to retinal cells in normal mice as well as in the rd and the rds mice. Our data suggest that adenoviral vectors are useful for somatic gene transfer to the retina.

METHODS

Animals

The C.B-17 mice, which differ from the Balb/c strain only at the Igh-1 locus, were obtained originally from Charles Sidman (Jackson Laboratories, Bar Harbor, ME). The C.B-17 mice suffer no retinal degeneration. The FVB/n mice homozygous for the rd allele were obtained from Jackson Laboratories. The homozygous rd mice in a Balb/c background were provided by Richard Sidman and Macy Tang (New England Primate Center). Animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Preparation of Viral Suspension for Injection

The method for adenovirus preparation was modified from that described by Graham and van der Eb. The construction of AdCMVβA.vlactZ was essentially as previously described except that the nuclear localization sequence from the SV40 large T antigen was fused to the 5′ end of the lactZ gene. AdCMVβA.vlactZ were amplified on permissive 293 cells (obtained from American Type Culture Collection, [ATCC] CRL 1573) in Dulbecco’s modified Eagle medium supplemented with 10% bovine calf serum. Subconfluent cells were infected with AdCMVβA.vlactZ at 10 plaque-forming units (pfu)/cell, harvested 30 hours later and the virus purified by CsCl gradient centrifugation. Desalting was then carried out by column chromatography using a Sephadex G-25 column (NAP-10 column, Pharmacia) equilibrated with minimal essential medium. The effluent containing the virus was centrifuged through a Centrifugal concentrator (Amicon) to reduce the volume to about 100 µl. Viral titers of the concentrated stock were typically between 10^11 to 10^12 pfu/ml. Fresh virus preparations were used immediately after dilution in minimal essential medium for subretinal injections.

In Vivo Delivery

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital. Subretinal injections were performed under an ophthalmic surgical microscope. After making a incision slightly behind the ora serrata with a microscalpel, a Hamilton syringe with a 1.3-cm/33-gauge blunt-ended needle was inserted tangentially toward the back of the eye. Approximately 0.3 to 0.5 µl of viral suspension was injected per eye. Control eyes were injected with an equal volume of minimal essential medium. Proper delivery into the subretinal space was confirmed by the appearance of a partial retinal detachment seen by indirect funduscopy. Intravitreal injection was performed similarly except that the injection needle was inserted vertically through the neural retina.

Eight adult C.B-17 (normal) mice, 30 C.B-17 pups (age 5 to 7 days), 10 rd pups (age 7 days), and 3 adult rds mice (age 2.5 months) were injected subretinally with purified AdCMVβA.vlactZ at high titer (10^11 pfu/ml). This is the highest titer we could routinely obtain in viral preparations. Two 7-day-old C.B-17 pups were injected subretinally with the virus at lower titer (10^8 pfu/ml), and 2 C.B-17 adult mice were injected intravitreally with the low titer virus (10^7 pfu/ml). Control mice were injected with viral suspension medium. Indirect funduscopy after subretinal injection of 0.5 µl showed a partial retinal detachment over approximately one quarter of the retinal area in adult mice (Fig. 1), allowing exposure of retinal pigment epithelium (RPE) and the neural retina to the injected material. This partial retinal detachment usually disap-

**FIGURE 2.** Histochemical examination of retinas injected with AdCMVβA.vlactZ. Transduced cells expressing the lacZ reporter gene can be readily identified by their blue appearance. Representative sections from mice sacrificed 1 to 6 weeks postinjection, counterstained with neutral red to show layers of retinal structure. RPE, retinal pigment epithelium; ONL, outer nuclear layer; INL, inner nuclear layer. (a) Near-uniform labeling of RPE but no photoreceptor cells in an adult C.B-17 mouse retina 1 week after injection. ×1600. (b, c) Gradual reduction in the number of lacZ-positive cells in C.B-17 mouse retinas injected at 5 to 7 days of age and examined at 4 (b) and 6 (c) weeks. ×1600. (d) Abundant labeling of both RPE and photoreceptor cells in an rd/rd mouse retina injected at 7 days of age and examined at 1 week postinjection. ×400. (e) Labeling of photoreceptor cells in an rd/rd mouse retina injected at 2.5 months of age and examined 1 week later. Cells in the inner nuclear layer are also labeled. ×1600. (f) Labeling of the corneal endothelium and the iris pigment epithelium in an adult C.B-17 mouse retina injected intravitreally and examined 1 week later. ×400.
Gene Transfer to the Retina Mediated by Adenovirus

FIGURE 1. A fundus photograph showing partial retinal detachment after subretinal injection of 0.5 μl. Arrowheads delineate the area of detachment. Shown here is a normal adult pigmented mouse retina.

peared within 3 days. In all cases one eye of each mouse was left uninjected to serve as an internal control.

Histochemical Examination

Normal pups were euthanized and eyes were enucleated and processed for histochemical examination at 1, 4, 6, or 8 weeks postinjection. The rd, rds, and normal adult mice were analyzed at 1 week postinjection. Eyes were fixed in 0.5% glutaraldehyde in phosphate-buffered saline for 1 hour at room temperature. The anterior segments and the lens were removed, and the eye cups were incubated at room temperature for 2 hours in a solution of 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, 1 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal) in phosphate-buffered saline at pH 7.8. After staining for lacZ activity, the eye cups were postfixed in 2.5% glutaraldehyde and 1% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) for 1 hour or longer, embedded in acrylamide, frozen in OCT compound, and cryosectioned at 12- to 18-μm thickness. Sections were examined either unstained or counterstained with neutral red.
Electron Microscopy

Histochemically stained rd mouse eye cups were post-fixed in 2.5% glutaraldehyde and 1% formaldehyde in 0.1 M sodium cacodylate buffer for 1 hour, followed by 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. The eye cups were then washed and dehydrated in a graded series of ethanol solutions and embedded in Spurr’s medium. Ultrathin sections (0.1 µm) were cut and stained with uranyl acetate and lead citrate. The sections were then viewed under a JEOL 100C electron microscope.

RESULTS

In Vivo Gene Transfer to Retinal Cells

At 1 week postinjection of AdCMVβA.ntlacZ, gross inspection of the eye cups after histochemical staining with X-gal showed lacZ activity in an area encompassing 25 to 50% of the retina. Light microscopic examination of multiple serial sections from this area demonstrated lacZ gene expression (blue-staining nuclei) in several retinal cell types, including RPE cells, photoreceptor cells, and to a lesser extent, cells in the inner nuclear layer (Figs. 2a to 2e). Control eyes injected with suspension medium or eyes left uninjected were all negative for lacZ activity. Throughout the blue-staining areas in viral injected eyes, lacZ-positive RPE cells were close to one another, indicating nearly 100% staining areas in viral injected eyes, lacZ-positive photoreceptor cells and lacZ-positive RPE cells were close to one another, indicating nearly 100% of the RPE cells were transduced (Figs. 2a, 2d). Adenovirus-mediated gene transfer to RPE was reproducible in mice of different strains and ages. Gene transfer to photoreceptor cells, however, was most efficient in 5- to 7-day-old pups and in mice whose photoreceptor cells were in the early stage of degeneration (Figs. 2d, 2e). Comparatively, lacZ staining of photoreceptor cells in normal adult retina was either absent or limited to very small areas where the retinal structures were apparently injured by the injection needle (Fig. 2a; additional data not shown). The regions occupied by lacZ-positive photoreceptor cells and lacZ-positive RPE cells essentially overlapped, with the latter consistently occupying a wider area. However, in the center of most abundant photoreceptor cell labeling, there were fewer and sometimes no labeled RPE cells, with positive RPE cells increasing in abundance away from this region.

To determine whether RPE cells and photoreceptor cells could be transduced at lower multiplicity of infection, a low titer viral suspension (10⁷ pfu/ml) was injected subretinally in 7-day-old normal pups. Upon examination 1 week postinjection, we found near uniform labeling of the RPE cells but no labeling of photoreceptor cells. As a comparison, injection of the virus at 10⁸ pfu/ml intravitreally resulted in efficient gene transfer to the corneal endothelium and the iris pigment epithelium (Fig. 2f) and retinal ganglion cells, but not to cells in the inner and outer nuclear layers of the retina (data not shown).

The percentage of lacZ-positive cells of all types decreased over time. In normal mice injected at 5 to 7 days of age, the abundance of labeled photoreceptor cells at 1 week postinjection was similar to injected rd retina but decreased on average to less than half of that at 4 weeks postinjection (Fig. 2b). At 6 weeks postinjection, only few and scattered labeled cells were seen (Fig. 2c).

Electron Microscopy Analysis

Precipitates of X-gal reaction product are electron dense and can be visualized using transmission electron microscopy. To determine whether both rod and cone photoreceptor cells could be transduced by AdCMVβA.ntlacZ, EM analysis was done on histochemically stained rd mouse retinas injected with AdCMVβA.ntlacZ 7 days earlier. X-gal precipitates were evident in both rod and cone photoreceptor cells (Figs. 3a, 3b) and in RPE cells (Fig. 3b).

Pathogenicity of Subretinally Administered AdCMVβA.ntlacZ

Photoreceptor outer segments were shorter or absent in the areas exposed to the virus when examined at 1 week postinjection. There was no apparent infiltration by inflammatory cells. In adjacent areas not exposed to the virus, the retinal structures appeared indistinguishable from uninjected control eyes. Some of these local pathologic changes could be attributed to the effect of retinal detachment, as control eyes injected with suspension medium also displayed some shortening of outer segments, although to a lesser extent. The absence of labeled RPE cells in some regions with abundant labeled photoreceptor cells might reflect acute cell death due to high multiplicity of infection and subsequent replacement with replicating adjacent RPE cells. Retinas examined at 8 weeks postinjection showed a full complement of retinal cell layers and inner and outer segments. In some sections, however, there were areas with reduced thickness of the outer nuclear layer and shortened inner and outer segments compared to adjacent areas.

There were no fatalities due to subretinal administration of AdCMVβA.ntlacZ. The development of mouse pups injected with AdCMVβA.ntlacZ appeared similar to that of uninjected littermates. At the time of sacrifice, the injected eyes were of comparable size to the contralateral control eyes. To determine if the adenovirus could spread outside of the eyes, serum samples were taken at 1 and at 4 weeks after injection and inoculated onto cultures of 293 cells. The cultures were serially passaged 5 times and did not show any
Gene Transfer to the Retina Mediated by Adenovirus

FIGURE 3. Transmission electron microscopy analysis of an X-gal-stained retina. Shown is a section of FVB/n rd/rd mouse retina injected with AdCMVβA:nlacZ at 7 days of age, sacrificed and processed for lacZ activity 7 days later. LacZ-expressing cells are identified by electron-dense particles concentrated around their nuclear membranes. (a) Rod and cone photoreceptor cells are distinguishable by their heterochromatin patterns. Representative cone (C) and rod (R) photoreceptor cells containing X-gal precipitate. (b) A RPE cell intensely labeled with lacZ reaction product. A pyknotic cell is seen (arrow). Scale bar, 10 μm.

evidence of viral infection. Mouse brain, lungs, and liver tissues taken at the time of sacrifice and histochemically stained for lacZ activity were negative for X-gal-stained cells. These data suggest that adenovirus injected into the subretinal space does not readily spread through systemic circulation.

DISCUSSION

In the current study, we have demonstrated that a recombinant human adenovirus can mediate the transfer and expression of a foreign gene to retinal cells under conditions that cause neither a severe disruption of retinal structure nor any apparent systemic toxicity. Importantly, both rod and cone photoreceptor cells are receptive to gene transfer, as are RPE cells. Photoreceptor cells are currently the primary target of gene transfer, because the mutant genes causing RP identified so far are all expressed primarily in the photoreceptor cells. However, given the close dependence of photoreceptor structure and function on RPE cells, future research may reveal a role for RPE cells in some forms of RP, thus making them a target for gene transfer as well. Targeting RPE cells with recombinant adenovirus has the advantage that a lower viral titer can be used, resulting in even lower cytotoxicity and higher efficiency of gene transfer.

In these experiments adenovirus-mediated gene transfer compares favorably with HSV-1-derived vectors with respect to efficiency of photoreceptor cell transduction and pathogenicity. In an earlier set of experiments, the efficiency of HSV-1 gene transfer to photoreceptor cells was consistently low, possibly because of the inability to purify HSV-1 to very high titers. Furthermore, even at the relatively low titer of $10^7$ to $10^8$ pfu/ml, there were gross pathogenic effects as evidenced by failure of eye development in some cases and neurologic symptoms after subretinal injection in young pups (ref 23 and unpublished data). Thus, currently, adenovirus-mediated gene transfer appears to be technically superior to HSV-based vectors for the introduction of foreign genes into the retina.

In vivo gene delivery to photoreceptor cells presents a significant challenge relative to other cell types in the eye. In normal adult mice, subretinal injection of AdCMVβA:nlacZ at $10^{11}$ pfu/ml results in essentially 100% transduction of RPE cells, with no gene
transfer to photoreceptor cells. In 5- to 7-day-old pups and in mice carrying mutations that cause retinal degeneration, however, AdCMVβA.nlacZ readily transduced photoreceptor cells. In contrast, RPE cells, the corneal endothelium, and the iris pigment epithelium were efficiently transduced at a titer of 10^8 pfu/ml, and the transfer efficiency was not related to age or mutations affecting the retina. One common feature shared by the developing retina and pre-degenerate mutant mouse retina is that the photoreceptor outer segments are shorter or absent. This suggests that the long, densely packed outer segments and/or the healthy interphotoreceptor matrix in normal adult mouse retina may act as a physical barrier to virus-mediated gene transfer.

The number of cells in the retina expressing the reporter gene decreased over time, as has been reported for similar adenovirus-mediated gene transfer experiments in other tissues. Adenovirus DNA reportedly remains episomal in infected cells, with the loss of transgene expression due to loss of transferred DNA or its inactivation without DNA loss. Alternatively, the possibility that some transduced cells were lost because of immune reactions against transduced cells could not be excluded. Thus, for practical gene therapy applications, vectors need to be generated that will increase the stability of transgene expression.

In summary, our data demonstrate a novel method for gene transfer to the photoreceptor cells in developing mice and in mouse models of RP. Thus, recombinant adenovirus containing the complementary DNAs for the β subunit of rod cGMP phosphodiesterase or peripherin may be effective in rescuing photoreceptor degeneration after transduction into the rd and rds mice, respectively. Such investigations should provide direct information on the feasibility of this approach to treating inherited retinal diseases in humans. Additionally, these techniques should be generally applicable to basic research into retinal cell physiology and the regulation of photoreceptor cell-specific gene expressions.

Key Words
adenoviral vector, viral transduction, gene transfer, gene therapy, retinitis pigmentosa

Acknowledgments
The authors thank Xandra Breakefield for helpful discussions and Shizuo Mukai for photographing the fundus. They also thank Yuko Soneoka and Mike Imperiale for assistance with recombinant adenovirus production.

References
Gene Transfer to the Retina Mediated by Adenovirus


