Phenotype correction in retinal pigment epithelium in murine mucopolysaccharidosis VII by adenovirus-mediated gene transfer

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ABSTRACT We have studied the use of adenovirus-mediated gene transfer to reverse the pathologic changes of lysosomal storage disease caused by β-glucuronidase deficiency in the eyes of mice with mucopolysaccharidosis VII. A recombinant adenovirus carrying the human β-glucuronidase cDNA coding region under the control of a non-tissue-specific promoter was injected intravitreally or subretinally into the eyes of mice with mucopolysaccharidosis VII. At 1–3 weeks after injection, the treated and control eyes were examined histochemically for β-glucuronidase expression and histologically for phenotypic correction of the lysosomal storage defect. Enzymatic expression was detected 1–3 weeks after injection. Storage vacuoles in the retinal pigment epithelium (RPE) were still present 1 week after gene transfer but were reduced to undetectable levels by 3 weeks in both intravitreally and subretinally injected eyes. There was minimal evidence of ocular pathology associated with the viral injection. These data indicate that adenovirus-mediated gene transfer to the eye may provide for adjunctive therapy for lysosomal storage diseases affecting the RPE in conjunction with enzyme replacement and/or gene therapies for correction of systemic disease manifestations. The data also support the view that recombinant adenovirus may be useful as a gene therapy vector for retinal degenerations that result from a primary genetic defect in the RPE cells.

The mucopolysaccharidoses (MPSs) represent a broad spectrum of inherited metabolic diseases caused by deficiencies in one of several enzymes responsible for the degradation of mucopolysaccharides. The MPS syndromes show progressive clinical involvement and are characterized by coarse faces, dysostosis multiplex, joint abnormalities, and various degrees of central nervous system (CNS) abnormalities. Depending on the particular types of MPS, ocular features may include corneal clouding, retinal degeneration, optic atrophy, and glaucoma. These phenotypic abnormalities are a result of lysosomal accumulation of glycosaminoglycans that eventually leads to cell and organ dysfunction.

Genetic mutations that cause a severe reduction in the level of β-glucuronidase (β-D-glucuronoside glucuronosohydrolase, EC 3.2.1.31) produce MPS VII or Sly syndrome (1, 2). Canine and murine models of this disease have been described (3, 4). The MPS VII dog and mouse share many common features with human patients suffering from MPS VII including dwarfism, skeletal deformities, and premature death. The mouse model for MPS VII results from a recessive allele of the structural gene for β-glucuronidase, gus^{mps}, that abolishes production of the enzyme (5). In addition to systemic manifestations, homozygous gus^{mps} mice develop late-onset photoreceptor cell degeneration that becomes evident by 4 months of age (6, 7). Cone photoreceptor loss appears to precede rod photoreceptor cell loss (7). β-Glucuronidase has been shown

to be essential for retinal pigment epithelium (RPE) to degrade glycosaminoglycans of the interphotoreceptor matrix and its deficiency results in lysosomal storage in RPE, but not in photoreceptor cells (8). Failure of RPE to catabolize glycosaminoglycans also leads to altered distribution of chondroitin sulfate proteoglycans in the interphotoreceptor matrix, which may be detrimental to photoreceptor cells (7). Thus it appears that the primary biochemical defect in the retina resides in the RPE, and photoreceptor degeneration results from failure of the photoreceptor-supportive functions of the RPE.

Like most lysosomal hydrolases, β-glucuronidase can either be synthesized endogenously or can be acquired through a secretion/recapture mechanism using the mannose-6-phosphate receptor (9). Cells that are deficient in β-glucuronidase because of gene mutations are still able to endocytose the normal enzyme from external medium when available and correct their disease phenotype (10). Thus bone marrow transplantation or tissue grafting with normal cells or genetically modified cells in adult animals corrects the lysosomal storage in most organs (6, 11–15). However, signs of disease remain in the CNS and in the retina. This suggests that systemically administered therapies may not be sufficient to correct the disease phenotype in these tissues.

We (16) and others (17) have found that recombinant adenovirus can be an effective vehicle for delivering a foreign gene to ocular tissues, including the corneal endothelium, iris pigment epithelium, RPE, and photoreceptor cells. To investigate the therapeutic potential of adenovirus-mediated gene transfer for treatment of retinal degeneration with a presumed RPE origin, we delivered a recombinant adenovirus carrying the gene for human β-glucuronidase to the eyes of MPS VII mice. This report describes the results of these experiments.

MATERIALS AND METHODS

Preparation of Viral Vectors. Adenovirus carrying the β-glucuronidase expression construct was generated by homologous recombination. First, the cDNA coding for human β-glucuronidase was inserted into the adenoviral shuttle vector pAdRSV4 (18, 19) to generate pAdRSVβ-gluc. The cDNA was inserted between a non-tissue-specific Rous sarcoma virus viral promoter and a simian virus 40 polyadenylylation signal to form an expression cassette. The pAdRSV4 vector contains adenovirus DNA sequences flanking the expression cassette that facilitate homologous recombination. pAdRSVβ-gluc DNA was linearized by restriction enzyme digestion and was cotransfected with adenovirus 5 sub360 (E1 gene deleted) DNA into 293 cells. Virus plaques were screened by restriction map and Southern blot analyses, and the recombinant virus (Ad.RSVβ-gluc) was amplified by routine methods (20). After two rounds of purification by CsCl gradient centrifugation, the

Abbreviations: MPS, mucopolysaccharidosis; RPE, retinal pigment epithelium; CNS, central nervous system; wt, wild type; pfu, plaque-forming unit(s).

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virus preparation was dialyzed overnight against Hepes-buffered saline (HBS; pH 7.5) and concentrated by centrifugation through a Centricon-100 (Amicon) concentrator. The titer of the concentrated viral stock on 293 cells was 1×10^{11} plaque-forming units (pfu)/ml. A recombinant adenovirus carrying the *lacZ* reporter gene with a simian virus 40 nuclear localization sequence, AdRSVnlslacZ (21), was similarly prepared.

Animals. *gus^{mps}/gus^{mps}* mice were provided by Edward Birkenmeier of The Jackson Laboratory. These mice were 12–16 weeks of age at the time of experiments. Albino BALB/c mice and pigmented C57BL/6 wild-type (wt) mice were used in pilot experiments.

Injections. The concentrated AdRSVβ-gluc viral stock was diluted to 1×10^{10} pfu/ml with HBS for ocular injections in *gus^{mps}/gus^{mps}* mice. Mice were anesthetized by i.p. injection of sodium pentobarbital. Ocular injections were performed under an ophthalmic surgical microscope. Pupils were fully dilated and a tiny incision was made in the peripheral cornea with a microscalpel. A Hamilton syringe fitted with a 0.5-inch 33-gauge blunt-ended needle (1 inch = 2.54 cm) was inserted tangentially toward the back of the eye while sliding by the lens. The tip of the needle was clearly in view in the process. Injections were deemed intravitreal ($n = 13$) if the needle did not reach the retina and subretinal ($n = 2$) if the needle was pushed until a resistance was felt. Subretinal injections were confirmed by a partial retinal detachment visible under the microscope. Approximately 0.3–1 μl of viral suspension (0.3–1 $\times 10^7$ pfu) was injected per eye. Control eyes were injected with an equal volume of HBS ($n = 4$) or left uninjected ($n = 2$). The number of eyes in each injection protocol and subsequent processing are summarized in Table 1.

By using the same procedure, a reporter gene virus, AdRSVnlslacZ, was injected in albino BALB/c mouse eyes either intravitreally ($n = 3$) or subretinally ($n = 3$) at 0.5×10^7 pfu per eye; albino mice were chosen in this experiment for ease of visualizing the reporter gene product.

In Situ **Enzyme Detection.** To detect β-glucuronidase expression, mice were sacrificed 1 or 3 weeks after injection. Eyes were enucleated and fixed in 4% (wt/vol) paraformaldehyde for 2 h at 4°C followed by cryoprotection by sequential soaking in 10 and 30% (wt/vol) sucrose. Eyes were frozen in OCT compound (Miles) and 10-μm sections were cut. β-Glucuron-

idase activity in tissue sections was detected by using published histochemical procedures (22, 23) except that incubation with naphthol AS-BI β-D-glucuronide and pararosaniline hydrochloride (Sigma) was at room temperature for 3 h. Under these conditions, eyes injected with Ad.RSVβ-gluc displayed intense red reaction product indicative of β-glucuronidase activity. Uninjected wt eyes did not show convincing positive staining after 3 h of incubation but showed positive staining after an overnight incubation. Uninjected or buffer-injected *gus^{mps}/gus^{mps}* eyes showed no red color, indicating no enzyme activity even after an overnight incubation.

To detect β-galactosidase (the *lacZ* gene product) activity, mice were sacrificed 1 week after injection and eyes were fixed in 4% paraformaldehyde for 2 h at 4°C. The cornea of each eye was cut open and the whole eye was stained for 2 h at room temperature in 5 mM $K_3Fe(CN)_6$/5 mM $K_4Fe(CN)_6$/2 mM $MgCl_2$/5-bromo-4-chloro-3-indolyl β-D-galactoside (1 mg/ml) in PBS buffer (pH 7.8). After histochemical staining, eyes were cryoprotected, frozen, and cut. Sections were counter-stained lightly with neutral red.

Histology. For light and electron microscopy, eyes were fixed in 2.5% (vol/vol) glutaraldehyde/1% paraformaldehyde in 0.1 M sodium cacodylate buffer for 4 h. After fixation, the cornea was dissected and the lens was removed. The corneas and eye cups were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. The tissues were then washed in cacodylate buffer, dehydrated through a graded series of ethanol solutions, and embedded in Epon resin. Sections were cut, stained with methylene blue, and examined under a light microscope. Ultra-thin sections (60–80 nm) were cut and stained with uranyl acetate and lead citrate. Sections were viewed under a JEOL 100C electron microscope.

RESULTS

Expression of the Transferred Genes and Localization of the Gene Products. Treated *gus^{mps}/gus^{mps}* eyes were examined for β-glucuronidase activity *in situ* by histochemical staining at 1 and 3 weeks after intravitreal injection of Ad.RSVβ-gluc. At 1 week after injection, enzyme activity was readily detected in corneal endothelial cells (Fig. 1*A*). Although heavy pigmentation somewhat obscured the red color reaction product in pigmented tissues, enzyme activity was also identifiable in
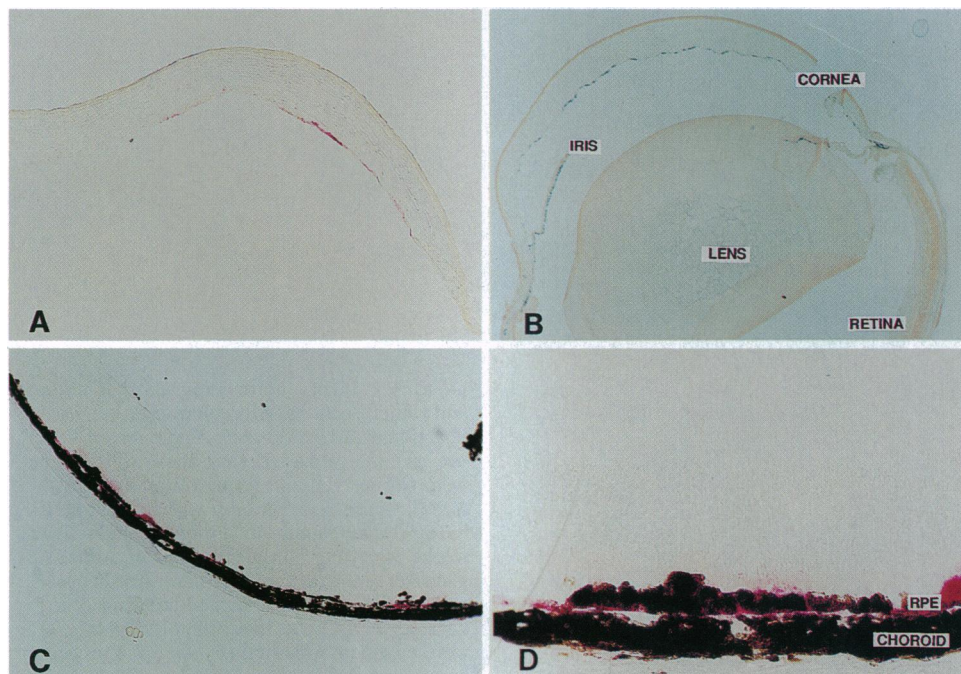

FIG. 1. Histochemical detection of β-glucuronidase expression in *gus^{mps}/gus^{mps}* eyes injected intravitreally with Ad.RSVβ-gluc (*A*, *C*, and *D*) or β-galactosidase in a BALB/c mouse eye injected intravitreally with AdRSVnlslacZ (*B*). All were processed 1 week after injection. Enzyme activity is visualized by red (β-glucuronidase) (*A*, *C*, and *D*) or blue (β-galactosidase) (*B*) staining. (*A*) Corneal endothelial cells show positive staining for β-glucuronidase in a cross section of cornea. (*B*) Corneal endothelial cells, iris pigment epithelial cells, and cells in the ciliary body are positive for β-galactosidase in a whole eye section. The section was lightly counterstained with neutral red. (*C*) Positive staining for β-glucuronidase in RPE cell layer is uniform across the retina. (*D*) Localization of β-glucuronidase in RPE is more definitive under higher magnification. (*A* and *C*, ×170; *B*, ×70; *D*, ×700.)

Table 1. Summary of injection protocols and histochemical/histological analyses of gus^{mps}/gus^{mps} mice

Injection protocol	Total no. of eyes injected	No. of eyes				
		Histochemistry		Histology		
		1 wk	3 wk	1 wk	2 wk	3 wk
Ad.RSVβ-gluc subretinal	2	—	—	—	—	2
Ad.RSVβ-gluc intravitreal	13	2	2	2	2	5
HBS buffer intravitreal	4	2	—	—	—	2

After injection examinations were done by histochemistry or histology at the week(s) (wk) after injection indicated and involved the number of eyes indicated.

RPE cells (Fig. 1 *C* and *D*) and cells at the posterior surface of the iris (data not shown). Uninjected and HBS-injected gus^{mps}/gus^{mps} eyes were negative for the histochemical staining. The level of enzyme expression as a result of gene transfer appeared to be higher than the endogenous enzyme activity in wt eyes (C57BL/6 and BALB/c) as judged by the time needed for the appearance of positive staining (3 h vs. overnight) and the intensity of color. Enzyme activity was still detectable in injected gus^{mps}/gus^{mps} eyes at 3 weeks after injection (data not shown).

Because the *lacZ* gene product of AdRSVnls*lacZ* is concentrated in the nucleus of the cell in which it is synthesized,

positive staining for β-galactosidase in a cell corresponds to the presence of the transferred gene in the cell, making it an unambiguous marker for direct viral transduction. One week after injection of AdRSVnlslacZ, β-galactosidase activity was detected in the following cell types of intravitreally injected eyes: corneal endothelium, iris pigment epithelium, and the ciliary body. Lens epithelium was free of positive staining cells in most areas, except occasionally small foci of positive cells were seen associated with frayed lens capsules, apparently due to abrasion by the injection needle. Thus an intact lens capsule appeared to prevent virus entry into lens epithelium. Ganglion cells in the neural retina were rarely positive. No positive staining RPE cell was observed (Fig. 1*B*). In contrast, in subretinally injected eyes, the majority of β-galactosidase-positive cells were RPE cells (data not shown). These data are in complete agreement with our previous study (16) on adenovirus-mediated gene transfer using a similar recombinant virus (AdCMVβA.nls*lacZ*) carrying the *lacZ* reporter gene, in which we found that gene transfer by adenovirus required direct exposure of target cells to the virus.

The two recombinant viruses used in this study share the same backbone structure including the promoter for the expression cassette and differ only in the inserted foreign genes. As such, comparison of the labeling patterns of the two viruses after intravitreal injection suggests that the β-glucuronidase activity detected in the RPE cell layer originated from other ocular tissues (i.e., the corneal endothelium, iris pigment epithelium, and cells in the ciliary body) and accu-
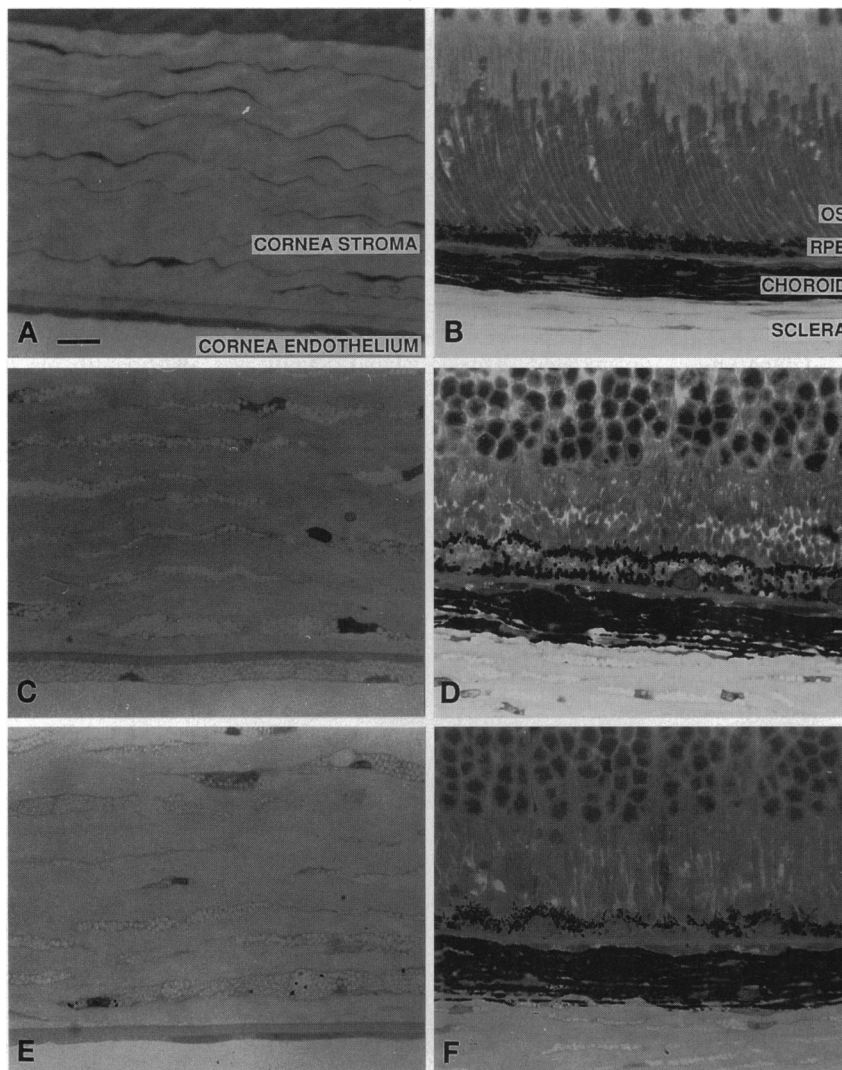

FIG. 2. Light photomicrographs of corneal and retinal sections before and after intravitreal injection of Ad.RSVβ-gluc. Age-matched wt cornea (*A*) and retina (*B*) were shown for comparison. Cornea (*C*) and retina (*D*) from a gus^{mps}/gus^{mps} mouse 3 weeks after injection with HBS buffer display numerous storage vacuoles. Three weeks after intravitreal injection of Ad.RSVβ-gluc, storage vacuoles were diminished in corneal endothelial cells but remained unchanged in the keratocytes (*E*). Storage vacuoles in the RPE were completely cleared (*F*). OS, photoreceptor outer segments. (Bar = 10 μm.)

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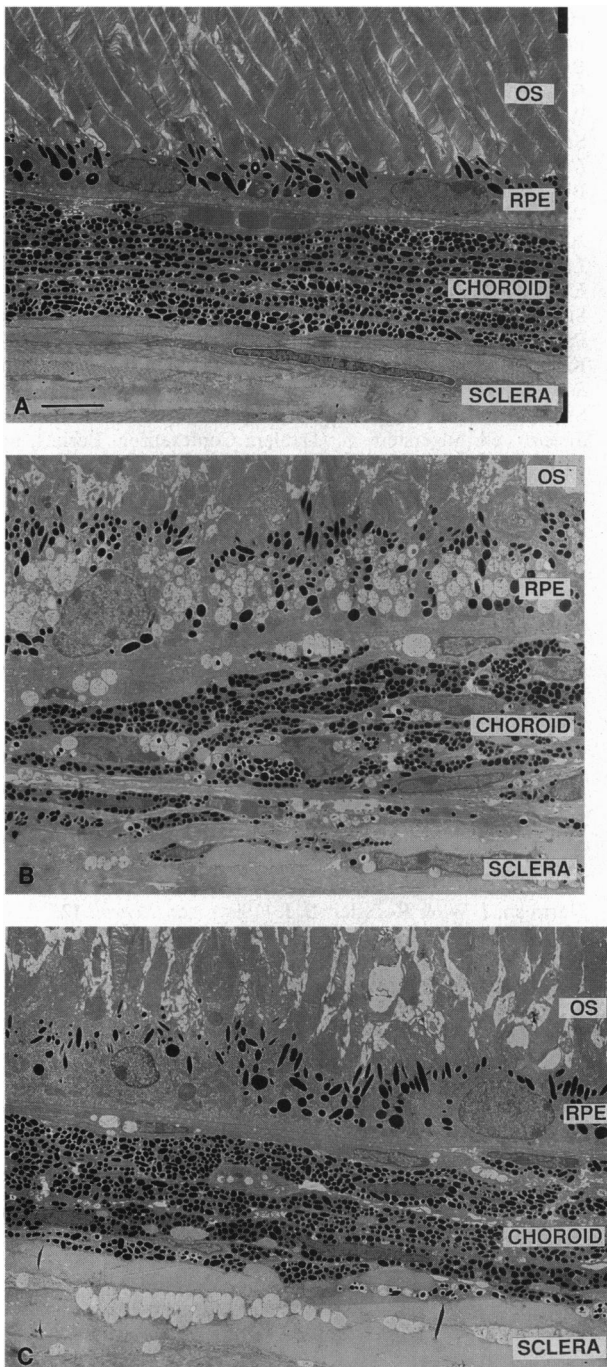*Proc. Natl. Acad. Sci. USA* 92 (1995)   7703

FIG. 3.   Electron micrographs of retinal sections surrounding the RPE cell layer from wt mice and treated and untreated *gus^{mps}/gus^{mps}* mice. (*A*) A wt (C57BL/6) retinal section showing normal morphology of RPE cells and adjacent structures. (*B*) Retinal section from a *gus^{mps}/gus^{mps}* eye 3 weeks after intravitreal injection of HBS buffer showing numerous large storage vacuoles in the RPE cells and cells in the choroid and sclera. (*C*) Retinal section taken 3 weeks after intravitreal injection of Ad.RSVβ-gluc in a *gus^{mps}/gus^{mps}* eye. The RPE cells are devoid of any storage vacuoles and are of normal size and appearance. This is in sharp contrast to cells outside of the RPE cell layer that retain their diseased appearance. The mice in *B* and *C* were littermates. OS, photoreceptor outer segments. (Bar = 5 μm.)

mulated in the RPE cells by the secretion/recapture mechanism.

Correction of the Storage Defect in Treated *gus^{mps}/gus^{mps}* Eyes. Histologic examinations were carried out 1, 2, and 3 weeks after injection of Ad.RSVβ-gluc by light and electron microscopy (see Table 1 and Figs. 2 and 3). Buffer-injected and

uninjected *gus^{mps}/gus^{mps}* eyes showed numerous large lysosomal storage vacuoles within the cytoplasmic space in many cell types, including keratocytes in the corneal stroma, corneal endothelial cells, RPE cells, and cells in the choroid and the sclera. These cells were highly distended in comparison to those in normal eyes. Photoreceptor outer segments were shortened. The RPE cell layer appeared much taller than normal and the pigment granules were displaced to the apical surface of the cells. No vacuolated inclusions were apparent in the photoreceptor layer or the inner retina.

At 1 week after injection of Ad.RSVβ-gluc, pathological findings in treated *gus^{mps}/gus^{mps}* eyes were not appreciably different from untreated *gus^{mps}/gus^{mps}* eyes (data not shown). At 3 weeks, however, the differences between treated and untreated eyes were striking under the light microscope: vacuolated inclusions were cleared from RPE cells across the retina (Fig. 2*F*) in the treated eyes. Under higher magnification, treated *gus^{mps}/gus^{mps}* RPE cells were morphologically indistinguishable from normal RPE cells (Fig. 3). The appearance of *gus^{mps}/gus^{mps}* RPE cells at 2 weeks after intravitreal injection of Ad.RSVβ-gluc was similar to those at 3 weeks (data not shown). The clearance of storage defect in RPE cells also occurred in subretinally injected eyes. However, after subretinal injection, an area of RPE cells could be found that were thin and hypopigmented. Such signs were always observed after subretinal injection of adenovirus, the severity of which correlated with the amount of virus injected (unpublished data and ref. 16). In contrast, the morphology of RPE cells after intravitreal injection of Ad.RSVβ-gluc in *gus^{mps}/gus^{mps}* eyes appeared normal.

Partial phenotypic correction was also seen in corneal endothelial cells (Fig. 2 *A*, *C*, and *E*). The clearance of storage vacuoles was not as complete as noted in the RPE, with some cells retaining small amounts of storage vacuoles. The keratocytes in the corneal stroma did not appear to respond to the treatment (Fig. 2 *C* and *E*).

The area corrected as a result of gene transfer was limited to the region delineated by the blood–retinal barrier, as the storage defect remained unchanged in tissues outside of the RPE cell layer (choroid and sclera). These cells are in sharp contrast to the RPE cells that have been cleared of vacuolar inclusions (Fig. 3).

DISCUSSION

In this investigation we examined direct intraocular delivery of an adenoviral vector designed to provide for replacement gene function in the MPS VII mice. These mice develop retinal degeneration resulting from a primary defect in the RPE cells. The study was carried out based on the observation that replication-defective recombinant adenovirus could efficiently transduce foreign genes into several ocular tissues, including the RPE cells (16). Our results show that intraocular delivery of the recombinant adenovirus directing the expression of β-glucuronidase (Ad.RSVβ-gluc) could result in rapid and significant (Figs. 2 and 3) correction of the lysosomal storage phenotype in RPE cells.

Interestingly, both intravitreal and subretinal injections corrected the RPE disease phenotype, yet the results of gene transfer by AdRSVnls*lacZ* presented here and in a previous study (16) showed that RPE could be transduced only when directly exposed to the virus, i.e., by subretinal injection of the virus. Thus, our data suggest that the accumulation of β-glucuronidase activity in the RPE after intravitreal injection (Fig. 1 *C* and *D*) and correction of the disease phenotype in RPE cells (Figs. 2 and 3) were probably due to uptake of β-glucuronidase synthesized from transduced cells located elsewhere in the eye. As seen in Fig. 3, this occurred to an extent that was sufficient to reverse the disease phenotype in the RPE. Given the fact that β-glucuronidase can be secreted and recaptured

through receptor-mediated endocytosis, such findings were not unexpected. Since subretinal injection causes a much greater disturbance in the retinal structure than intravitreal injection, the latter provides a preferred route of delivery. It is not clear, however, why corneal endothelium and keratocytes responded less well to the treatment.

Because the short life span of gus^{mps}/gus^{mps} mice was a limiting factor, we did not extend the after-injection treatment period beyond 3 weeks. Therefore, the duration of β-glucuronidase gene expression after a single injection is not known. However, β-galactosidase expression has been noted to persist for up to several months (16, 17) after adenovirus-mediated gene transfer. The duration of expression in the eye is longer than that noted in liver or lung (24) but is comparable to that noted in the CNS (25, 26). This may be due to the level of immune surveillance; both the eye and the CNS are "immune privileged" sites. The extended level of transgene expression after adenovirus gene transfer to the eye, coupled to the time required for reaccumulation of storage product after extinction of transgene expression (if it occurs completely), may impart a level of biological efficacy not noted in other systems by using gene transfer with first-generation adenovirus vectors. Adoption of newer versions of adenovirus vectors that have the potential to be less immunogenic (27–29) could allow readministration of virus or significantly prolong expression, thereby enhancing the benefit of this approach. Although not investigated in this work, we speculate that photoreceptor structure and function may improve if followed for longer periods after correction of the RPE phenotype.

The ocular involvement in MPS VII is not responsive to systemic treatment by enzyme therapy, presumably due to the blood–retina barrier (13). Our data show that adenovirus-directed gene transfer to ocular tissues may be useful in this condition, and possibly in other lysosomal storage diseases with ocular phenotypes in conjunction with systemic enzyme or gene therapies. These data may also have broader implications in the treatment of retinopathies with an RPE origin. For example, age-related macular degeneration is thought to result from life-long accumulation of lipofuscin in secondary lysosomes in the RPE cells (30) that ultimately involves photoreceptor cells. If enzymes can be identified that aid in the clearance of lipofuscin in the RPE cells, then the ability of adenovirus to express high levels of lysosomal enzymes, even if transiently, may allow "rejuvenation" of RPE cells and thus provide a potential therapy for this clinically important condition.

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