Delivery of Adeno-Associated Virus Vectors to the Fetal Retina: Impact of Viral Capsid Proteins on Retinal Neuronal Progenitor Transduction

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The development of fetal ocular gene transfer may be useful as a therapeutic tool for the prevention of retinal genetic disorders with congenital or early clinical manifestations. In this study we explored the neural progenitor transduction patterns of adeno-associated virus (AAV) vectors following delivery to the developing retina. Recombinant vectors with the same genome carrying the enhanced green fluorescent protein (EGFP) transgene packaged in capsids of differing serotypes (serotypes 1, 2, and 5, termed AAV2/1, AAV2/2, and AAV2/5, respectively) were created. Delivery of the AAV vectors during early retinal development resulted in efficient and stable transduction of retinal progenitors. Vector surface proteins and the developmental status of the retina profoundly affected viral tropism and transgene distribution. The procedure is not detrimental to retinal development and function and therefore provides a safe delivery vehicle for potential therapeutic applications and a means of assessing the mechanisms of retina development and disease.

A number of retinal diseases such as Leber congenital amaurosis (18), Norrie’s disease (38, 39), microphthalmia (16), and albinism (11, 23) result in blindness or severe impairment of visual function apparent from birth. A number of genes implicated in these diseases have been identified. Their expression profiles, in conjunction with data from human fetal autopsies, suggest that early intervention is critical for preventing the pathological manifestations of these disorders (1, 8, 31, 33). In utero gene therapy is an attractive potential solution for treatment of genetic diseases with fetal onset (44). Proof of principle was demonstrated recently, supporting the effectiveness of in utero gene transfer. Pulmonary epithelium (35), heart tissue (10), fetal peritoneum, and other organ tissues (19, 22, 30) have been successfully transduced with the direct injection of virus and nonvirus vectors. These vectors were used with fetal nonhuman primates to achieve gene transfer in a number of animal models (36, 37).

Gene therapy holds much promise for the treatment of retinal degenerations (6, 13). The retina serves as an ideal target for gene therapy as well as serving as a model system for the assessment of the transduction characteristics of viral vectors (4, 32). Different viral vectors transduce the retina with different cell tropisms and efficiencies (2, 4, 28). Vectors based on adeno-associated virus (AAV) are particularly useful for delivering genes to terminally differentiated neurons of the retina in an efficient nontoxic and stable fashion (7, 17, 42). Furthermore, AAV2-based vectors can transduce a wide variety of dividing and nondividing cells through both random chromosomal integration and episomal transgene expression (15, 27, 40).

The adult retina is a highly structured array of terminally differentiated neurons that differs profoundly from the fetal retina. The developing retina contains a neuroblastic layer, which is characterized by a pseudostratified neuroepithelium of actively mitotic cells (21). Progenitor cells that generate different retinal cell types exit from the cell cycle in an overlapping chronological order (43), allowing for precise analysis. In addition, the retina shares common features with the developing central nervous system (CNS) (14), thus providing an amenable accessible model for the study of virus vector transduction in developing neurons.

Several developmental processes, including mitosis, migration, differentiation, cell death, and synaptic formation, may affect the cellular uptake of a virus vector, the distribution of the particles, and the persistence of transgene expression. In this study, following gene transfer to the developing retina, we compared the neural progenitor transduction patterns and efficiencies of AAV2/1, AAV2/2, and AAV2/5 (AAV2 packaged in capsids of AAV1, AAV2, and AAV5, respectively) carrying a cDNA encoding enhanced green fluorescent protein (EGFP). The delivery of the AAV vectors results in different patterns of onset, cellular specificity, and intensity of transgene expression. The procedure does not interfere with retinal development and function, thus providing delivery vehicles relevant to the treatment of retinal congenital diseases.

MATERIALS AND METHODS

AAV vectors, plasmid construction, and production and purification of AAV vectors. The AAV2-cytomegalovirus (CMV)-EGFP and the AAV2/1 and AAV2/5 packaging constructs were generated and the vectors were produced and purified as described previously (3, 20, 41). Briefly, AAV vectors were
produced by triple transfection of 293 cells. The first plasmid encoded the EGFP expression cassette packaged between the AAV2 internal terminal repeats. The second plasmid encoded the rep and cap genes (packaging plasmid), and the third, Δψ, encoded the adeno-helper function genes. For production of the AAV2/1 and AAV2/5 vectors, the packaging plasmid contained the cap genes of AAV1 and AAV5, respectively. AAV2/2 was purified by heparin column chromatography. AAV2/1 and AAV2/5 purifications were performed with CsCl gradients. Vector physical titers were assessed by real-time PCR. The infectivity of the preps was assessed by infectious center assay as described before. The copy ratios for infectious versus genomic vectors were 1:34, 1:273, and 1:897 for AAV2/2, AAV2/5, and AAV2/1, respectively. These ratios have been found to be within the range of acceptable preparations for each serotype as assessed by the Vector Core of the University of Pennsylvania. Other quality control studies of these preparations included an endotoxin assay and evaluation of the percentage of empty particles.

AAV vector delivery to mouse retina. Animal studies were performed in accordance with the guidelines for animal research of the University of Pennsylvania. Fetal subretinal injections of AAV2/2, AAV2/5, and AAV2/1 were performed with pregnant C57Bl/6 and CD1 mice on embryonic day 13 (E13) or E14. Mice were mated at night, and evidence of vaginal plugs served to designate E0. Exo utero surgery was performed as described previously (38). Briefly, pregnant mice were anesthetized intraperitoneally with a xylazine-ketamine stock (5 and 20 mg/ml, respectively) at a dose of 0.05 ml/100 g of body weight. A midline laparotomy was performed, and the uterus was exposed. After incision of the uterus wall was performed, the fetuses were released using cotton swabs. These swabs were also used to control bleeding throughout the surgery. One eye of each of the two or three fetuses remaining in the uterine horn was injected. A capillary pipette with a tip diameter of 15 to 20 μm was connected to a pressure microinjection (Harvard Apparatus). Then, 10-micron-thick sections were obtained using a cryostat and EGFP-specific fluorescence was identified by fluorescent microscopy. True EGFP fluorescence was distinguished from background autofluorescence. Cervical dislocation was used to sacrifice the mothers for prenatal and postnatal study. In postnatal studies, the fetuses were delivered by cesarean section on gestational day 19 and fostered to a surrogate mother. Subretinal injections in adults and newborn animals were performed as described previously (5).

Histology. Animals were sacrificed, and the eyes were enucleated. The eyes were fixed overnight in 4% paraformaldehyde in PBS, transferred to 30% sucrose in PBS, and frozen in optimal-cutting-temperature compound (Fisher Scientific, Pittsburgh, Pa.). Then, 10-micron-thick sections were obtained using a cryostat and EGFP-specific fluorescence was identified by fluorescent microscopy. True EGFP fluorescence was distinguished from background autofluorescence by comparing the pattern through a dual filter allowing simultaneous identification of both fluorescein (GFP-specific) and rhodamine (autofluorescence) fluorescent patterns.

Indirect ophthalmoscopy. Mice were anesthetized with avertin, and their pupils were dilated with topically applied 0.5% tropicamide. Indirect ophthalmoscopy was performed using a 90-diopter lens. Photographs were taken with a Kowa Genesis camera (Keeler Instruments, Broomall, Pa.).

ERG. Methods for electroretinogram (ERG) recordings and light stimulus calibration have been described in detail elsewhere (24). ERG analysis was performed and evaluated by an examiner under masked conditions.

Determination of serum-neutralizing antibodies. Following fetal gene transfer, serum samples were collected from 2- to 3-month-old animals to identify serum-neutralizing antibodies to AAV2/1, AAV2/2, and AAV2/5 capsid proteins. The presence of neutralizing antibodies was determined by assessing the ability of serum samples to inhibit transduction of 84-31 cells by reporter viruses. The presence of neutralizing antibodies was determined by assessing the ability of serum samples to inhibit transduction of 84-31 cells by reporter viruses.

RESULTS

Delivery of AAV vectors to the fetal retina. To transduce the developing fetal mouse retinas, we used exo utero surgical procedures described by Turner et al. (38) (Fig. 1A). A total of 153 fetuses were injected in 35 operations; 85 pups were delivered without complications, and 44 were successfully fostered and survived to adulthood. Following surgery, mouse gross development and growth proceeded normally. Of 44 injected eyes, 39 had no apparent macroscopic or microscopic abnormalities. Finally, the ultimate demonstration of whether the treatment was safe was whether the animals produced after in utero injection were able to reproduce. These animals had normal fertility and produced healthy offspring. No transgene expression was identified in the offspring, indicating that the virus did not target germ line cells.

To evaluate the ability of AAV2/2, AAV2/1, and AAV2/5 vectors to transduce the fetal retina, we performed injections with recombinant viruses carrying an EGFP reporter gene under the control of the CMV promoter (Fig. 1A). Since the neuroblastic layer lies on the outer side of the optic cup, we performed injections in the subretinal space between the retinal pigment epithelium (RPE) and the developing retina. On embryonic days 13 to 14, fetuses received ~0.4 μl of a solution containing genome copies of AAV2/1, AAV2/2, or AAV2/5 (6 × 10⁹, 6 × 10⁶, or 3.4 × 10⁶ copies/μl, respectively) in one eye. Fast-green dye was coadministered with the vectors to verify that the virus was delivered subretinally. In successful injections, dye readily spread behind a wide area of the retina.
We first assessed the onset of EGFP expression by sacrificing fetuses and newborn animals at different time points following gene delivery and analyzing histology under a fluorescent microscope. The onset of EGFP expression was detected in the RPE at 24 h following injection of AAV2/1 (Fig. 1B to D). In AAV2/2- and AAV2/5-transduced retinas, transgene expression was present in the RPE at 8 and 3 days following viral delivery, respectively (Fig. 1E and F). In fetal retinas transduced with AAV2/1, EGFP expression was also present in the neuroblastic layer at 3 days following injection, reaching peak levels of expression in early postnatal life (Fig. 1C). This pattern was not observed with the other vectors.

**Tissue tropism and efficiency of EGFP expression in the adult retina following fetal transgene delivery.** To analyze the transduction profile following fetal gene delivery, we performed histologic and ophthalmoscopic examinations in adult retinas of mice injected in utero after cell division, differentiation, cell death, and synaptogenesis were completed (1 to 3 months postinjection). Following fetal administration of AAV2/1-CMV-EGFP, indirect ophthalmoscopy revealed a strong level of expression in a significant portion of the retina (data not shown). Transduction was observed in RPE cells and in the outer nuclear layer, specifically in cone photoreceptor cells, as judged by morphology (Fig. 2A). In the inner part of the retina, several cells in the outer and inner part of the inner nuclear layer showed positive results. This is consistent with the location of horizontal and amacrine cells, respectively. In addition, ganglion cells showed strongly positive results. Following administration of AAV2/2, the overall transduction efficiency was lower and fluorescence was sometimes difficult to appreciate using ophthalmoscopy (data not shown). Nevertheless, histological analysis revealed EGFP expression in the RPE in a small number of photoreceptor, horizontal, amacrine, Müller, and ganglion cells (Fig. 2B). Transgene expression in ganglion cells resulted in expression in optic nerve tracts in the brain, as has been described previously. This did not cause apparent toxicity for the CNS. Judged on the basis of indirect ophthalmoscopy (Fig. 3A), transduction of the fetal retina with AAV2/5 resulted in widespread EGFP expression throughout the retina. EGFP fluorescence was clearly observed in the RPE, outer plexiform layer, and outer nuclear layer, where strong expression was evident specifically in cones (Fig. 2C).
Neutralizing antibody response and AAV vector readministration. Serum samples from animals injected subretinally with AAV vectors at E13 to E14 were analyzed for anti-AAV1, -AAV2, and -AAV5 antibody at 2 to 3 months after fetal gene transfer. No neutralizing antibodies were detected in any of the treated animals (n = 5 for AAV2/1, n = 5 for AAV2/2, n = 2 for AAV2/5). To determine whether additional transduction events were possible following unilateral fetal subretinal viral administration, 1-month-old animals received a second subretinal injection in the eye contralateral to that injected prenatally. For each AAV vector used, two animals received re-administration. The pattern of transgene expression following readministration was examined histologically 30 days later. Analysis revealed high levels of EGFP expression with all three vectors. Although some photoreceptors were transduced following AAV2/1 readministration, the transgene was localized as previously described (2, 4, 25) (Fig. 2G to I).

Retina function is normal following fetal gene transfer. ERGs were measured to determine whether the surgical procedure or the vectors were detrimental to retinal development and function (Fig. 4). For 2- to 3-month-old animals, the saturating amplitudes of the rod a-wave, which are proportional to the magnitude of the averaged rod photoreceptor photocurrent, revealed no differences between AAV vector-injected eyes and control noninjected eyes in rod photoreceptor function (Fig. 4). Similarly, scotopic and photopic b-waves, which reflect primarily the currents of the rod-bipolar cells and cone on bipolar cells of the inner retina, did not differ in treated and untreated eyes. These ERG results show that neither the vector nor the surgical technique itself was detrimental to retinal function.

Tissue tropism and efficiency of EGFP expression in the adult retina following gene delivery at birth. Since retinal differentiation is completed postnatally, we wanted to assess virus efficiency and tropism in adult animals (postnatal day 30 [P30]) following subretinal administration at birth (P0). Subretinal delivery of AAV2/1 resulted in highly efficient and robust transduction of RPE and photoreceptor cells and less efficient transduction of the inner nuclear layer (Fig. 2D). In contrast, transgene expression in animals injected with AAV2/2 was significantly lower but detectable in a subset of all retina cell types (Fig. 2E). Delivery of AAV2/5 at birth resulted in specific expression in both RPE and photoreceptors, with the highest level of EGFP expression seen in cones. Expression was also detected in a large subset of Müller cells (Fig. 2F).

The inner retina, expression consistent with the location of horizontal cells was localized to cells found in the outer part of the inner nuclear layer. Additional EGFP expression was also detected in the inner plexiform layer and in cells on the inner margin of the inner layer, which were most likely amacrine cells. Ganglion cells also gave strongly positive results, and fluorescence was also observed in the optic nerve, the optic tract, and the lateral geniculate nucleus (data not shown). By 3 months, there was no difference in the level of EGFP expression between tissues exposed to any of the three vectors, suggesting that transgene expression was stable over time. Similar patterns of retinal transduction were observed using AAV2/1 and AAV2/5 at a dose of 1.5 log units less then what was administrated here (data not shown).

Tissue tropism and efficiency of EGFP expression in the adult retina following gene delivery at birth. Since retinal differentiation is completed postnatally, we wanted to assess virus efficiency and tropism in adult animals (postnatal day 30 [P30]) following subretinal administration at birth (P0). Subretinal delivery of AAV2/1 resulted in highly efficient and robust transduction of RPE and photoreceptor cells and less efficient transduction of the inner nuclear layer (Fig. 2D). In contrast, transgene expression in animals injected with AAV2/2 was significantly lower but detectable in a subset of all retina cell types (Fig. 2E). Delivery of AAV2/5 at birth resulted in specific expression in both RPE and photoreceptors, with the highest level of EGFP expression seen in cones. Expression was also detected in a large subset of Müller cells (Fig. 2F).

FIG. 3. EGFP expression following fetal delivery of AAV2/5-CMV-EGFP. Injections were performed on E13. (A) Indirect fluorescent ophthalmoscopy. EGFP expression was evident throughout the retina on P90. Arrowhead, optic disk. Small arrows indicate borders of the EGFP-expressing region. (B) Histological appearance of the retina shown in panel A.
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months from experiments with a-titudes (mean ± standard deviations) of ERG components at 2 to 3

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Traces at the top row of the graphs represent scotopic b-waves elicited

dash estimated to isomerize 9 rhodopsin molecules per rod; the arrow at the left indicates the amplitude (rod b_{max}). The middle row shows ERGs evoked by a flash estimated to isomerize 100,000 rhodopsin molecules per rod. The initial, cornea-negative de

300

100 µV

200 ms

rod b_{max} untreated

rod a_{max} untreated

cone b_{max} untreated

rod b_{max} treated

rod a_{max} treated

cone b_{max} treated

ERG component

FIG. 4. No adverse effect on visual function after fetal subretinal injection of AAV vectors. (A) ERG results for a 3-month-old mouse following fetal delivery on E13 of AAV2/5-CMV-EGFP to the treated eye. Histological analysis results for this animal are shown in Fig. 3. Traces at the top row of the graphs represent scotopic b-waves elicited by a flash estimated to isomerize ~9 rhodopsin molecules per rod; the arrow at the left indicates the amplitude (rod b_{max}). The middle row shows ERGs evoked by a flash estimated to isomerize ~100,000 rhodopsin molecules per rod. The initial, cornea-negative de

response magnitude (µV)

rod b_{max}

rod a_{max}

cone b_{max}

ERG component

rod b_{max}

rod a_{max}

cone b_{max}

respond eye

untreated eye

Treated eye

untreated eye

Response magnitude (µV)

rod b_{max}

rod a_{max}

cone b_{max}

A

B

contrast, AAV2/1 administration at P0 resulted in strong and specific expression of EGFP in rod photoreceptors in adulthood (Fig. 5B). Administration of AAV2/2 into the fetal retina resulted in delayed transgene expression and lower transduction efficiency in comparison with results seen with the other two serotypes, although gene delivery of both fetuses and newborn animals resulted in transduction of several retinal cell types. Fetal gene transfer of AAV2/5 resulted in early onset of transgene expression in the RPE (Fig. 5A); however, onset began 2 days later than that seen with AAV2/1. The expression pattern of AAV2/5 after fetal gene transfer resulted in transduction of ganglion cells, amacrine cells, and cone photoreceptors (Fig. 5A), while delivery of AAV2/5 at birth resulted in transduction of photoreceptors and Müller cells (Fig. 5B).

Interestingly, the patterns observed following fetal gene transfer with AAV2/1 and AAV2/5 are consistent with retinal cell genesis. The cells that showed robust EGFP expression were those that were exiting the cell cycle at the stage of injection, as deduced from cell cycle studies performed by other investigators (43). This implies that the targeted cells were pluripotent progenitors, which were able to generate different retinal cell types. To explain this, one could hypothesize that in postmitotic cells the number of viral genomes (expressing EGFP) is stable over time. In contrast, cells which are still actively mitotic show decreasing transgene expression due to loss of the viral genomes or to a different pattern of gene silencing. Alternatively, the receptor responsible for viral binding and entry may not be present on an early progenitor but may be expressed on a progenitor committed to the generation of a specific retinal cell type. This might explain the transgene expression pattern observed in Müller cells following fetal gene delivery of AAV2/2. Müller cell progenitors were actively dividing at the time of fetal gene delivery. In addition, the susceptibility to viral transduction changes with time, and at a certain point, some cells are no longer transduced. Indeed, the inner retina and the ganglion cell layer are not transduced when AAV2/1 and AAV2/5 are administered in adult retina, even when virus is administered intravitreally (4). To summarize these findings, we have identified different transgene expression patterns dependent on both the type of virus administered and the stage of retinal differentiation at the time of virus delivery. This suggests that the combination of the presence of virus receptors and the stage of differentiation controls the final outcome. As described above, a unique aspect of the developing retina is that there are waves of differentiation of particular cell types occurring over a short period of time. Future studies could deliver viruses at defined fetal timepoints to decipher the specific roles of cellular receptors and the stage of differentiation in the final transgene expression patterns. Results of such a study would be relevant to development of fetal gene delivery techniques for every organ system.

Notably, the overall distribution of EGFP-expressing cells following fetal and newborn gene transfer was significantly larger than that observed after delivery to the adult retina. This effect may be due to the fact that the RPE and the developing neural retina are physiologically detached or less tightly attached in the fetus, thus allowing for a better distribution of the vectors following injection. The diffuse and widespread pattern of gene expression following fetal delivery may also be due to a higher ratio of viral particles to tissue as well as to the particular cellular arrangement of the retina during retinogenesis. In the mammalian CNS, neurogenic mitosis occurs on the luminal surface of the neural tube (ventricular surface) and the daughter cells migrate inward to a more superficial layer at the end of the cell cycle. This movement of dividing neuron progenitors (called interkinetics migration [21]) occurs in the outer retina where the injections were performed. Therefore, a transduced cell in the outer retina may differentiate and migrate to the opposite vitreal side before completion of retina lamination and this would increase the distribution of trans-
gene across the retina. Of all of the vectors, AAV2/5 had a particularly wide expression after fetal subretinal injection (Fig. 3). This may be due to the ability of AAV2/5 to diffuse through the extracellular matrix as has been observed after administration in adult retina and CNS (12, 26, 42).

In this report, we have described the effect of AAV capsid on targeting different retinal cell types during retinal development. We have also demonstrated that the AAV tropism is affected by the developmental stage of gene delivery. This is crucial to the design of therapeutic strategies, for which the expression of therapeutic genes requires efficient and primary transduction of the affected cells. Obviously, it will be important in future studies to incorporate a variety of safety features to assure that only the targeted cells are transduced and that transgene expression in these cells can be maintained at appropriate levels. For such purposes, it will be important to include cell-specific and/or -inducible promoters. Such mechanisms could, for example, prevent expression of the transgene by ganglion cells. This would prevent inadvertent delivery of transgene product to the brain (through ganglion cell axons which target the brain through the optic nerve).

While further studies must be undertaken to fully evaluate the risks and benefits of fetal gene transfer, there were no obvious toxic effects of the treatment on development of the eye or of the entire fetus in mice. These animals grew up without negative consequences for their ability to reproduce. In addition, the treated eyes retained normal retinal function through adulthood, as shown by the ERG data. Further, there were no indications of humoral immune response to the virus vectors when injections were administered subretinally. This may reflect the “immune privilege” of the fetus and specifically of the retina.

The ability to deliver transgenes somatically to the retina of the fetus promises to be useful not only for gene therapy applications but especially for treatment of diseases resulting from developmental defects. Although the technique should be applicable to treatment of humans, clearly there are abundant ethical and safety issues that would have to be addressed before such an approach was considered. This technology would also be useful for studies of the mechanisms of retinal development and disease. For example, the method may serve as an alternative (and faster) approach than conventional transgenic techniques and may provide a means of assessing gene function selectively in a subset of retinal cells. Somatic gene delivery has an additional benefit: the contralateral eye can be used as an internal control.

In conclusion, we demonstrate that AAV-based recombinant vectors can efficiently transduce retinal neuroprogenitors in a stable and nontoxic fashion. Different AAV vectors can be used to transduce particular progenitor cells destined to become specific retinal cell types. Delivery of the virus in utero is compatible with readministration of virus later, in adulthood. In addition to the therapeutic implications for the retina, the results also suggest that AAV-based vectors can be successfully used for fetal gene transfer to other portions of the CNS and, potentially, other organ systems.

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REFERENCES


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