Recombinant AAV-Transduced Iris Pigment Epithelial Cell Transplantation May Transfer Vector to Native RPE but Suppress Systemic Dissemination

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Purpose. To determine whether adenoassociated virus (AAV) vectors transduced into iris pigment epithelial (IPE) cells and transplanted into the subretinal space of rats will transfer the AAV genome to the host cells and whether the vectors are disseminated systemically.

Methods. Recombinant (r)AAV was transduced into rat IPE cells and transplanted into the subretinal space of rats. For the control, rAAVs alone were injected subretinally. The transplanted IPE cells were detected by LacZ staining. Immunohistochemistry, electron microscopy, electroretinography, and fluorescein–dextran angiography were performed. DNA was extracted from various organs and blood and examined for the AAV genome by polymerase chain reaction.

Results. No toxicity from rAAV transduction was observed in vitro. LacZ was expressed in the transplanted cells 1 and 2 weeks after transplantation. At 4 and 12 weeks, fewer transplanted cells were detected than at 1 week, and LacZ expression was occasionally detected at the level of host retinal pigment epithelial (RPE) cells. Expression was also detected in ciliary body epithelial cells. The electroretinograms and fluorescein–dextran angiography were only mildly altered. Significantly lower levels of AAV genome were detected in the organs and blood of rats receiving rAAV-IPE cell transplants than with direct intravenous injection of AAV vectors.

Conclusions. AAV-mediated LacZ was expressed in the transplanted cells after subretinal transplantation, and the transplanted IPE cells may transfer the rAAV to host tissues, such as RPE cells, long after the transplantation. This method of gene delivery did not lead to systemic dissemination of the vectors. (*Invest Ophthalmol Vis Sci.* 2006;47:745–752) DOI:10.1167/iovs.05-0398

The adenoassociated virus (AAV) is a single-stranded, nonpathogenic parvovirus,[1] and recent studies have shown that recombinant (r)AAV vectors can deliver target genes efficiently to the brain, retina, and optic nerve for long periods.[2,3] Intravitreal injection of AAV may not deliver the target gene to photoreceptor cells.[4] Conversely, rAAV carrying neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF),[5] basic fibroblast growth factor (bFGF),[6] ciliary neurotrophic factor (CNTF),[7] or X-linked inhibitor of apoptosis[8] have been injected into the subretinal space and shown to rescue photoreceptors from the degeneration induced by different types of toxicity. In our laboratory, we have shown that iris pigment epithelial (IPE) cells transduced with AAV recombined with brain-derived neurotrophic factor (BDNF) can be transplanted into the subretinal space to protect photoreceptors from phototoxicity.[9]

Although the long-term expression of different transgenes has been achieved, cellular and humoral immune responses against the encoded gene products (e.g., RPE65,[10]) have arisen.[11–14] In addition, the AAV genome is disseminated systemically when injected into the subretinal space and can replicate in the presence of wild-type (wt) AAV or helper viruses.[15,16] This fact is important, because wt AAVs have been detected in semen in approximately 18% of the human population,[17] and more than 85% of the population is seropositive for antibodies against AAV.[18] Thus, careful evaluations of the biodistribution of the vector sequences should be performed before transplantation of rAAV.[15]

The purpose of this study was to determine whether AAV vectors transduced into IPE cells and transplanted into the subretinal space of rats disseminate the AAV genome to the host cells, and whether the vectors are disseminated systemically. Because the transgenes may affect the results, we transplanted not only AAV alone but also rAAV-transduced IPE cells. The dissemination of rAAV was determined by PCR and the expression of β-galactosidase. We compared the dissemination of rAAVs that were transduced into IPE cells to those after direct subretinal injections of rAAV alone.

Materials and Methods

All manipulations of the viral vectors were performed in accordance with institutional and national biosafety restrictions. All animals were used after receiving institutional approval and were handled in a humane manner. The procedure complied strictly with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rat strains used were Long-Evans (LE) rats (200–300 g; Imamichi Institute for Animal Reproduction, Ibaraki, Japan) and Sprague-Dawley (SD) rats (Japan SLC, Inc., Shizuoka, Japan).

Virus Preparation

The AAV helper-free system (Stratagene, La Jolla, CA) was used to produce the recombinant AAV vectors.[9] This system produced infectious recombinant rAAV-2 virions without the use of a helper virus.

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Recombinant AAV Preparation, Purification, and ELISA

The genes for BDNF and *Escherichia coli* β-galactosidase (LacZ) were inserted into multiple cloning sites (MCSs) of the AAV to yield AAV-BDNF and AAV-LacZ.[9] The rAAVs were recovered and purified as described.[19] The titer of AAV was determined by enzyme-linked immunosorbent assay (ELISA) for the AAV capsid protein, according to the manufacturer's instruction (Progen Biotechnik, Heidelberg, Germany) and expressed as capsids per milliliter.[20] The vectors were stored at −80°C, and the same stock was used in all experiments.

IPE Cell Preparation

IPE cells from the eyes of LE rats were isolated and cultured as we reported.[21] The medium was changed every 3 days.

AAV Transfection

IPE cells were plated into 24-well plates at a density of 5×10^4 cells/well, and 0.5 mL of AAV-permissive medium (DMEM growth medium supplemented with 240 mM hydroxyurea and 6 mM sodium butyrate) was added. The individual wells were transfected with 1.0×10^9 capsids/mL of AAV-BDNF or AAV-LacZ and incubated for 2 hours at 37°C. After the exposure, H-DMEM (DMEM supplemented with 18% [vol/vol] FBS and 2 mM L-glutamine) was added to each well and incubated at 37°C.

Each type of rAAV was added to the medium of cultured IPE cells (AAV-BDNF-IPE or AAV-LacZ-IPE) for 7 days, washed three times with Hanks ' balanced salt solution, (HBSS; TaKaRa, Otsu, Japan) followed by centrifugation at 1000 rpm for 5 minutes at 4°C. When these cells were used for transplantation, they were counted with a cell counter (NucleoCounter; Chemometec, Allerod, Denmark) and prepared so that the final concentration was 2×10^4 cells/μL.

Cell-Viability Test

Cell-viability assays were performed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS; Promega, Madison, WI). Absorbance at 490 nm was measured with a spectrophotometer (Spectra Max Gemini UVmax; Molecular Devices, Sunnyvale, CA).

Quantitative Analysis of Virus Titer in AAV-IPE Cells

The titer of transduced AAV in the AAV-LacZ-IPE cells was estimated by real-time PCR (*Taq*man probe method,[22] using the Smart Cycler System; TaKaRa). Genomic DNA was extracted with a (DNA Extraction Kit; Stratagene) from 5×10^4 AAV-LacZ-IPE cells, which had been infected with 1.0×10^9 capsids/mL of AAV-LacZ.

Real-time PCR was performed according to the manual for the system (Smart Cycler; TaKaRa). PCR amplification was performed with 5′-AGAAGCCTGCGATGTCGGTTT-3′ and 5′-AATGAATCGTCTGACCGA-3′ primers, which were expected to generate a 270-bp LacZ gene DNA fragment. Briefly, 2.0 μL of the 10-fold diluted DNA solution was added to 25 μL of PCR reaction solution, including 2.5 μL of 10× PCR buffer (ExTaq; Takara), 1.5 μL of 2.5 mM $MgCl_2$, 2.0 μL of 2.5 mM dNTP mixture, 2.5 μL of sense and anti-sense LacZ primers (final concentration, 0.3 μM), 0.25 μL polymerase (5 U/μL; ExTaq; TaKaRa), and 10.25 μL of diluted distilled H_2O.

The PCR conditions were 35 cycles for 30 seconds of denaturation at 94°C, 15 seconds of annealing at 60°C, 30 seconds of extension at 72°C, and another 7 minutes of extension at 72°C . After amplification, the PCR products were identified by melting-curve analysis and gel electrophoresis.[23] To test unknown samples, quadruplicates of the standard DNA containing a range from 10 to 10^5 diluted AAV-LacZ solutions were amplified simultaneously, and a standard curve was generated. The titer of the transduced AAV was determined for 5×10^4 AAV-LacZ-IPE cells.

To measure the concentration of rAAV in the supernatant of the cultured cells, the genome of rAAV was amplified by PCR using the supernatants after three washings. Then, the DNA was extracted from 2.0 μL of each wash solution and was amplified by the same method.

Transplantation

For transplantation, 4.0×10^4 rat IPE cells/2 μL of AAV-BDNF-IPE was injected into the subretinal space of 50, AAV-LacZ-IPE into 46, and IPE alone into 17 right eyes of 8-week-old Sprague-Dawley (SD) rats (Japan SLC Inc.). The host rats were anesthetized with pentobarbital sodium (60 mg/kg body weight), the pupils were dilated, and the conjunctiva was cut close to the limbus to expose the sclera. The sclera was punctured with a 30-gauge needle approximately 1 mm from the limbus, and a 32-gauge needle on a syringe (Hamilton, Reno, NV) was passed through this hole and choroid into the subretinal space, to inject the cells or HBSS in a tangential direction while viewing the fundus with an operating microscope. In some of the eyes of the albino SD mice, the injected fluids could be seen in the subretinal region through the translucent sclera and choroid. The area of the transplantation was confirmed by indirect ophthalmoscopy. The site of the AAV-LacZ-IPE transplantation was identified by histochemical evaluation of LacZ expression. The rats with AAV-BDNF-IPE transplantation were used for electroretinography and fluorescein-dextran angiography.

For the control, 2 μL of 1.0×10^6 capsid/mL AAV solution (AAV-BDNF or AAV-LacZ) or HBSS was injected into the subretinal space of the right eye. AAV-BDNF was injected into 18 eyes, AAV-LacZ into 5 eyes, AAV vector alone into 2 eyes, and HBSS into 25 eyes. The successful subretinal injection was confirmed by the presence of a partial retinal detachment by indirect ophthalmoscopy. Six rats with massive subretinal hemorrhage or retinal detachments were discarded.

The rats were kept in a normal light–dark light cycle without immunosuppressants.

DNA Extraction from Organs and Detection of rAAV by PCR

To determine whether rAAV had disseminated, DNA was extracted from the optic nerve, brain, lung, liver, kidney, testis, heart, spleen, skeletal muscle (m. rectus femoris), and blood. The tissues (except blood) were homogenized (Biotron, Göttingen, Germany), and genomic DNA was extracted from each tissue according to the manufacturer's instructions (Stratagene). These organs were obtained from rats that received transplants of AAV-LacZ-IPE cells, AAV-BDNF-IPE cells, or IPE cells alone or age-matched control rats with direct injection of AAV-LacZ, AAV-BDNF, or vehicle. The concentration of DNA was quantified by spectrophotometry (Ultrospec 4000 UV/Visible Spectrophotometer; GE Healthcare, Tokyo, Japan), and the DNA concentration of each organ was equalized to 100 μg/mL.

Blood was drawn from the tail and collected in tubes containing sodium heparin. Genomic DNA was isolated using an automatic DNA purification system (Genextractor TA-100, together with the Genextractin Kit; TaKaRa). PCR was performed as described.[24] Two sets of primers were used; the first set was 5′-TGGAGTTCCGCGTTACATAAC-3′ and 5′-CCGCATCACCATGGTAATAG-3′, which amplified 323 base pairs (bp) of the AAV vector promoter region (CMV); and the second set was 5′-AGAGAGGGAGTGGCCAACTC-3′ and 5′-CATGACCCGTATTACGGTCC-3′, which amplified 359 bp from the AAV inverted terminal repeat (ITR) region.

The PCR amplification conditions for the first primer set were: 95°C for 10 minutes; 35 cycles at 95°C for 1 minute, 57°C for 30 seconds, and 72°C for 2 minutes and an extension at 72°C for 7 minutes (GeneAmp PCR System 9600; Applied Biosystems, Kanagawa, Japan). The amplification conditions for the second set of primers were: 95°C for 10 minutes; 35 cycles at 95°C for 1 minute, 56°C for 30 seconds, and 72°C for 2 minutes; and an extension at 72°C for 7 minutes.

Then, 0.5 μL of genomic DNA was added to 10 μL of PCR reaction solution including 1.0 μL of 10× buffer (ExTaq; TaKaRa), 0.80 μL of dNTP mixture (2.5 mM each), 0.5 μL of each primer, and 0.05 μL of polymerase (5 U/μL; ExTaq; TaKaRa), and 6.7 μL of distilled water. The PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and viewed with a UV transilluminator.

For the positive control, we extracted DNA from the retina after injection of AAV-LacZ into the subretinal space on the indicated days. For the negative control, we examined the DNA from untreated retinas. The methods were same as described earlier.

Light and Electron Microscopic Examinations

The eyes were enucleated, immersion-fixed in 4% paraformaldehyde, and embedded in paraffin. Tissue blocks were sectioned at 3 μm along the vertical plane through the optic nerve head and the transplant and nontransplant sites. The sections were stained with hematoxylin-eosin (H-E).

Some of the tissues were removed from the paraffin blocks and refixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The tissues were dissected, postfixed in phosphate-buffered 1% osmium tetroxide (pH 7.4), and dehydrated in an ascending series of ethanol. The specimens were embedded in Epon 812, and ultrathin sections were stained with a uranyl and lead salt solution and observed by microscope (TEM-100CX; JEOL, Tokyo, Japan).

Other eyes were initially fixed as described, cut into smaller pieces, and postfixed in phosphate-buffered 1% osmium tetroxide (pH 7.4). The tissues were dehydrated in an ascending ethanol series and embedded in Epon 812. Ultrathin sections were cut and stained with a uranyl and lead salt solution. These tissues were obtained 2 weeks after transplantation.

Histochemical Evaluation for LacZ Expression

Animals were killed at 1, 2, 4, and 12 weeks after AAV-LacZ-IPE transplantation, and the eyes were enucleated and fixed immediately in 4% paraformaldehyde in PBS (pH 7.5) for 16 hours at 4°C. The eyes were cryoprotected by successive incubations in 10%, 20%, and 30% sucrose dissolved in saline for 16 hours at 4°C. The tissues were immersed at the optimal temperature in 5% CMC compound (Finetec Co., Tokyo, Japan) and frozen in acetone in a dry-ice bath. The frozen eyes were sectioned at 10 μm with a film transfer kit on a cryostat (Cryofilm Transfer Kit; Finetec Co. on a cryostat, Leica CM3050; Finetec, Co.) at −16°C. The frozen sections were stained for 2.0 hours at 37°C in a solution containing 1.0 mg/mL β-gal (β-gal Staining Kit; Invitrogen, Carlsbad, CA) and counter stained with H-E. The sections were examined and photographed (model BX50 microscope; Olympus, Tokyo, Japan).

Electroretinography

Electroretinography (ERG) and FITC-dextran fluorescein angiography were performed on rats that received transplants of AAV-BDNF-IPE cells or with IPE cells only. ERGs were recorded simultaneously from both eyes at 6 months after transplantation. The number of SD rats examined was four for AAV-BDNF-IPE and five for IPE only.

A Ganzfeld stimulator was used to stimulate the eyes, and a data-acquisition system (Universal Testing and Analysis System-Electrophysiology 3000, UTAS-E 3000; LKC Technologies, Inc., Gaithersburg, MD) was used to record the ERGs. The rats were anesthetized with pentobarbital sodium (60 mg/kg body weight), and the pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine. The rats were dark-adapted for 40 minutes at 37°C,[25] and contact lenses carrying the electrodes were placed on the corneas. The reference electrodes were inserted subcutaneously on the nose. Stimuli were presented at intensities of 0.01, 2.4, and 44.0 cd-s/cm^2, and the ERGs recorded with 44.0 cd-s/m^2 were used for comparisons.

The a-wave amplitude was measured from the baseline to the trough of the corneal negative wave, and b-wave amplitudes from the corneal negative trough to the major corneal positive peak.

Fluorescein-Dextran Labeling of Retinal Vessels

After the ERGs, rats were perfused with 2×10^4 molecular weight FITC-dextran (Sigma-Aldrich, St. Louis, MO) through the heart to fill the retinal and choroidal vessels. The eyes were enucleated and fixed in 2% paraformaldehyde for 24 hours at 4°C.[26] The cornea and iris were removed and flat wholemounted sections of retina-choroid-sclera were made and examined under a fluorescence microscope (Leica stereomicroscope, MZ APO; Leica Microsystems AG, Heidelberg, Germany) with ultraviolet illumination (360/40 nm, 420 nm). The images were captured with a black-and-white charge-coupled device [CCD] camera (Hamamatsu Photonics, Bridgewater, NJ) coupled to a computer (Macintosh G3; Apple Computer, Cupertino, CA) equipped with software (IPLab 3.5/J software; Scanalytics, Inc., Fairfax, VA).[27]

Statistical Analyses

The Fisher protected least significant difference (PLSD) or χ^2 tests were used to determine the significance of any differences. $P < 0.05$ was considered to be statistically significant.

Results

Virus Titer and Cell-Viability Assay

The titers, determined by ELISA, were 2.2×10^{12} capsids/mL for the pAAV vector only, 1.3×10^{11} capsids/mL for AAV-BDNF, and 3.9×10^{10} capsids/mL for AAV-lacZ. The infectious center assay[28] was performed on HT1080 cells, a highly permissive cell line.[9] Representative data of transduced AAV-LacZ are shown Figure 1A. The results showed that the 1.0×10^9 capsids/mL of AAV-LacZ solution transduced 5.8×10^6 capsids into 5×10^4 rat IPE cells. The titer of the AAV transduced cells was calculated to be 1.2×10^2 capsids/cell, so that approximately 4.8×10^6 capsids of each recombinant AAV were injected into the subretinal region in 2 μL of the rAAV-IPE solution. AAV-BDNF and pAAV were also transduced at approximately the same concentration. For control, we also injected approximately 2.0×10^6 capsids/eye of rAAV directly into the subretinal space.

Approximately 0.6×10^6 capsids/mL were found in the supernatant of the culture medium. There were 0.09×10^6 capsids in the first HBSS wash and 0.06×10^6 capsids in the second wash. AAV was not detected in the third wash solution. PCR on the supernatant with both primer sets did not detect the rAAV or AAV vector after two washes (Fig. 1B).

The MTS assay demonstrated that the rAAV transduction was not toxic to the transduced cells in our experimental conditions (Fig. 1C).

Histology of Eye after AAV-IPE Transplantation

Transplanted IPE cells, identified by the characteristic blue-green expression of LacZ, were observed in the subretinal space around the transplant area 1 week after transplantation. Some of the cells at the level of photoreceptor were also positive for LacZ (Fig. 2A, arrow). LacZ positivity was also observed 2 and 4 weeks after AAV-LacZ-IPE transplantation (Figs. 2B, 2C, respectively), but the blue color appeared to be present at the level of host RPE cells. The transplanted cells were sometimes very difficult to find, probably because this was an allotransplantation with a its slow rejection.[29] These findings were clearer at 12 weeks after transplantation, and LacZ staining was occasionally observed at the level of host RPE cells (Fig. 2D). The number of transplanted cells at the transplant site was fewer at 4 and 12 weeks after transplantation than at 1 week. After 12 weeks, it was difficult to find transplanted cells.

Some of the cells in the ciliary body close to the transplant site were also positive for LacZ 4 weeks after transplantation

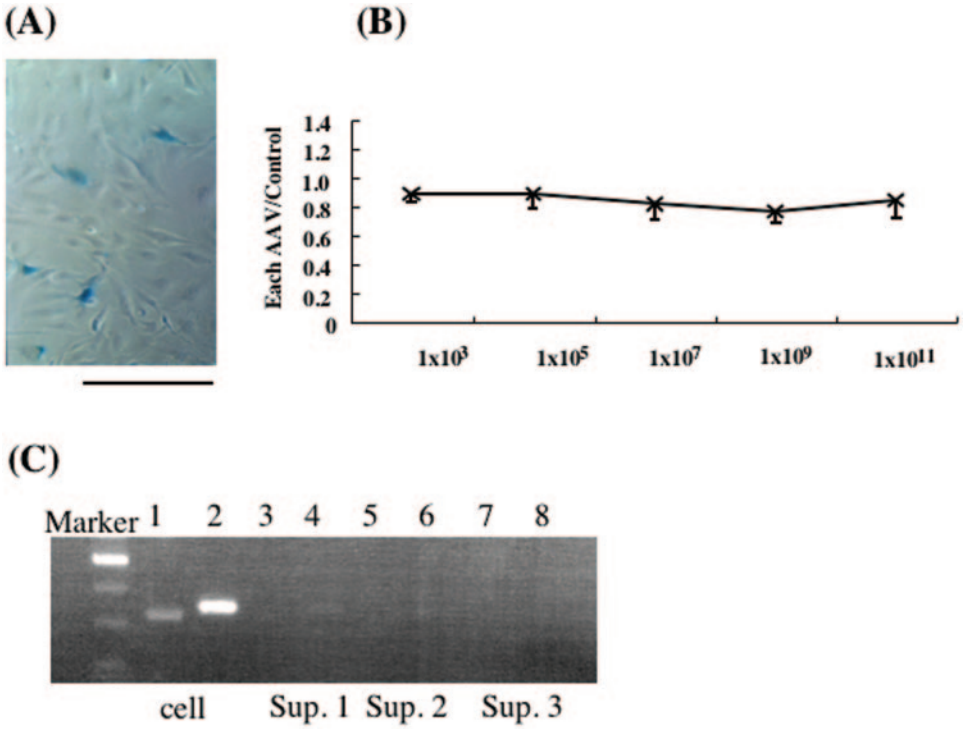

FIGURE 1. (**A**) Rat IPE cells 7 days after transduction by 1×10^{10} capsids/mL of AAV-LacZ. Bar, 50 mm. (**B**) Representative results (AAV-BDNF-IPE) of cell viability assays. The viability was assayed by MTS 7 days after transduction. When AAV-BDNF was transduced into rat IPE cells at the indicated concentrations, no significant difference in viability was found at each concentration, relative to that of non-transduced IPE cells. Similar results were obtained with AAV-LacZ. (**C**) rAAV was not detected in the supernatant of the transplanted cells. *Lanes 1* and *2*: results of PCR using two sets of primers of AAV serotype 2; *lanes 3* and *4*: result of PCR using the supernatant of the first wash; and *lanes 5–8*: results of the second and third washes.

(Fig. 2E). There were no β-gal-positive cells in the anterior segments and in control eyes.

Mononuclear cells were not detected at the light microscope level in the eyes at 4 and 12 weeks ($n = 4$ at each examination) after transplantation in H-E-stained sections. Electron microscopic examination of tissue obtained from the paraffin blocks showed the apoptotic nuclei of host RPE cells at 1 week after transplantation (Fig. 3A). Electron microscopic examination also showed occasional macrophage-like cells in the area of transplantation 2 weeks after transplantation. These cells contained many cytoplasmic organelles in the cells (Fig. 3B). Of interest, the host RPE cells under these cells closely resembled cytoplasmic organelles. Some of the transplanted cells on the host RPE cells contained many lysosome-like materials, and the cells also showed vacuoles in the cells (Fig. 3C). The host RPE under these cells also contained lysosomal-like materials.

Electroretinography

The amplitudes of the scotopic a- and b-waves elicited by a stimulus of 44.0 cd-s/m^2 from the transplanted right eye were compared to the comparable waves from the nontransplant-recipient control eyes. The mean amplitudes of both waves in

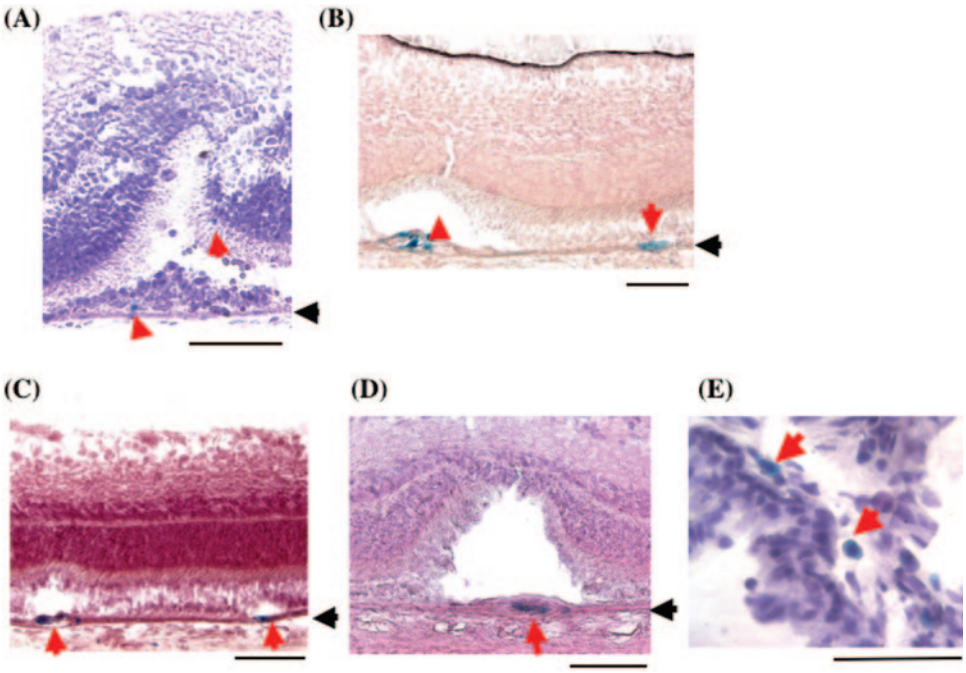

FIGURE 2. (**A**) LacZ staining of transplanted IPE cells 1 week after transplantation. A few *blue-green*-positive cells were present among the subretinally transplanted cells (*red arrowhead*). A *blue-green*-staining cell was also observed in the cells in the outer nuclear layer (*red arrow*). (**B**) Two weeks after transplantation, *blue-green* staining was observed not only in transplanted cells (*red arrowhead*), but also at the level of the host RPE (*red arrow*). (**C**) Four weeks after transplantation, *blue-green* staining was also observed at the level of host RPE (*red arrows*). (**D**) Three months after transplantation, the *blue-green*-positive cells (*red arrow*) were also present at the level of the host RPE. (**A–D**, *black arrow*) RPE. (**E**) LacZ staining in the cells (*red arrows*) of the ciliary body close to the transplant site. Bars, 50 μm.

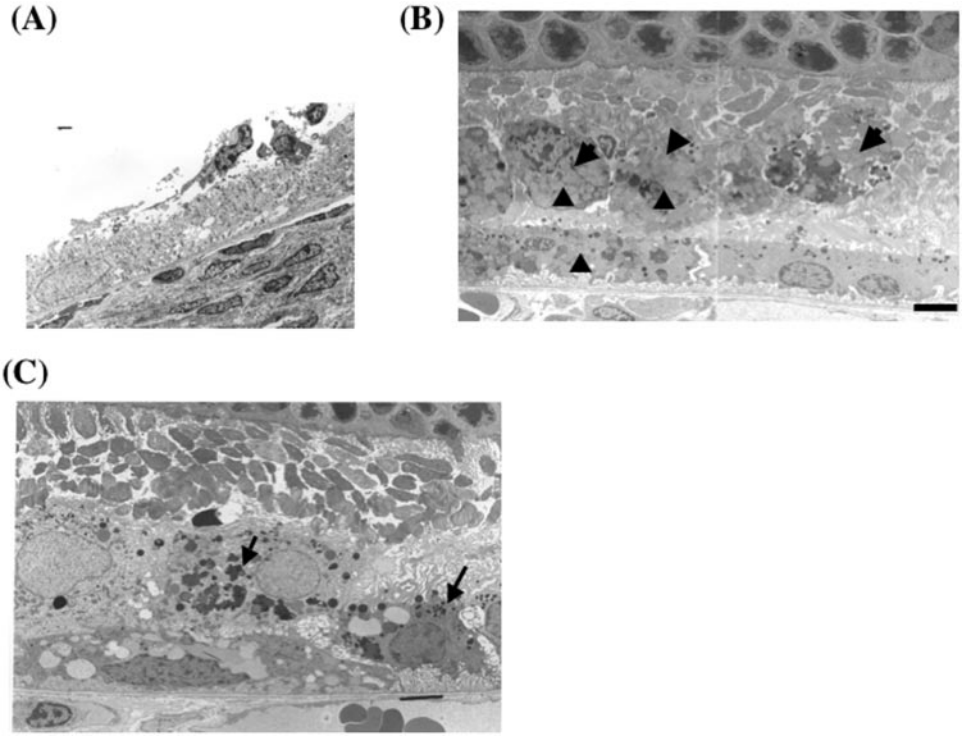

FIGURE 3. Electron microscopic examination demonstrates that cells showing apoptotic nuclei were on the host RPE 1 week after transplantation (**A**). Vacuoles are artifacts due to the refixation of the paraffin blocks with 2.5% glutaraldehyde and 2% paraformaldehyde. Occasional macrophage-like cells are observed in the area of transplantation 2 weeks after transplantation (**B**, *arrow*s). These cells and underlying host RPE contain many cytoplasmic organelles (*arrowhead*s) in the cell body. Some of the possible transplanted cells and host RPE included many lysosome-like materials (**C**, *arrow*s). The host RPE under these cells also included lysosomal-like materials. Bar: (**A**) 2 μm; (**B**) 5 μm; (**C**) 5 μm.

the rats with AAV-BDNF-IPE or IPE transplant-recipient eyes were reduced by approximately 10% to that of the nontransplant-recipient left eye. These results indicated that the differences were not significant between AAV-BDNF-IPE and IPE transplantation (a-wave, $P = 0.559$; b-wave; $P = 0.661$).

Fluorescein–Dextran Angiography

Two of the four rats that had AAV-BDNF-IPE transplantation and three of five rats with only IPE transplantation had almost normal retinal vascular structure (Fig. 4A). Two rats from each type of transplantation had mild disorganization of the retinal vessels at the transplant area (Fig. 4B).

AAV DNA in Other Organs and Blood

AAV DNA was always amplified by the two sets of primers from the DNA extracted from the retina transplanted with rAAV-IPE or rAAV direct injection (Fig. 5). These were used for positive controls of the examination of the frequency of detecting rAAVs. The frequency of detecting rAAV DNA in rats with rAAV-IPE transplantation ($n = 22$) and rats with direct rAAV injection ($n = 20$) at 1 week are shown in Tables 1 (blood) and 2 (optic nerve, whole brain, lung, liver, kidney, testis, heart, spleen, and muscle). AAV DNA was detected in only 1 (4.5%) of 22 rats treated with rAAV-IPE transplantation, and in 14 (70%) of 20 rats treated by direct rAAV injection 1 week after treatment (Table 1; number in parentheses represents the result using ITR primers). The incidence of AAV DNA after rAAV-IPE transplantation was significantly lower than that after direct rAAV injection ($P < 0.0001$).

AAV DNA was not detected in the blood at 4 and 12 weeks after rAAV-IPE cell transplantation. AAV DNA was also not detected in the blood of untreated or vehicle injected rats.

AAV vector DNA was detected in the two organs of two rats in the group receiving rAAV-IPE transplantation 1 week after

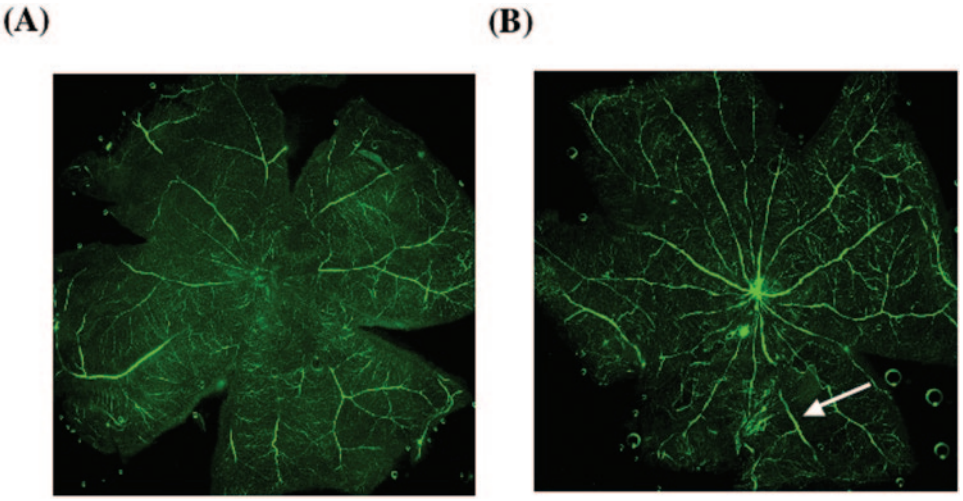

FIGURE 4. (**A**) The results of FITC–dextran angiography. Abnormal new vessels, such as choroidal neovascularization, were not present. (**B**) Mild retina vessel tortuosity (*arrow*) was observed. The changes were observed in some of rats with either AAV-BDNF-IPE or only IPE cell transplants.

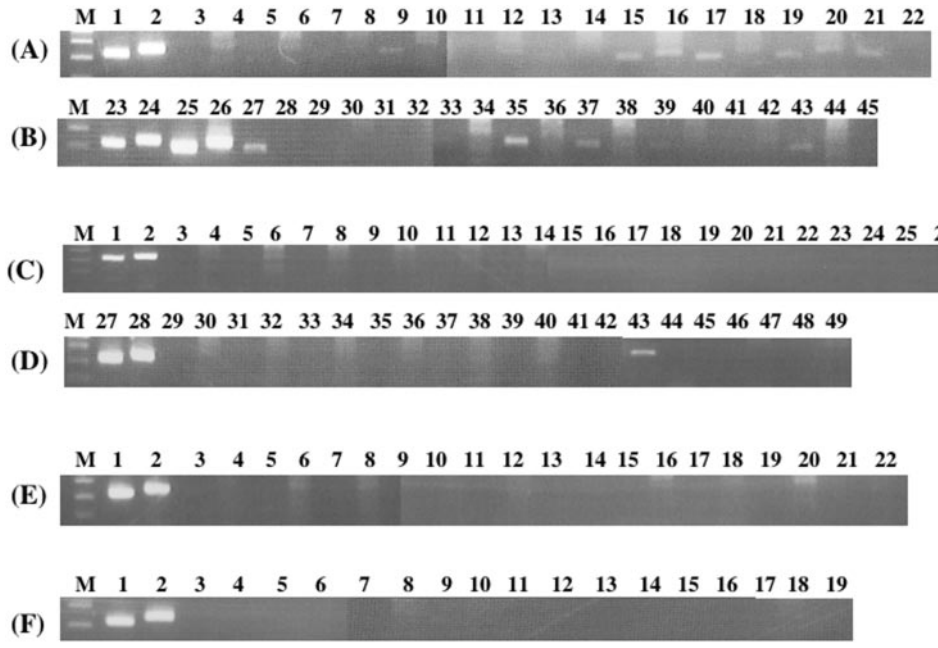

FIGURE 5. Results of PCR using DNA extracted from blood are shown. Two sets of primer, CMV and ITR, which amplified 323 and 359 bp, respectively, were used. Positive control using DNA extracted from retina after direct injection of AAV-LacZ or rAAV-IPE cell transplants for each primer is shown in *lanes 1* and *2* in (**A**), (**C**), (**E**), and (**F**), *lanes 23* and *24* in (**B**), and *lanes 27* and *28* in (**D**). *Odd numbered lanes*: results of CMV primer; *even numbered lanes*: results of ITR primer. The results of (**A**) and (**B**) are from the samples of rats with rAAV injected directly into the subretinal space; (**C**, **D**) transplants of rAAV-IPE cells; (**E**) vehicle injection; and (**F**) transplant-recipient control. Positive amplifications were determined in *lanes 4*, *6*, *9*, *10*, *15*, *16*, *17*, *18*, *19*, *20*, *21*, *25*, *26*, *27*, *34*, *35*, *37*, *39*, *43* in (**A**) and *43* in (**D**).

transplantation (Table 2, $n = 8$). Conversely, AAV DNA was detected from every organ examined in 10 of 12 rats that had direct rAAV injection (Table 2; number in the parentheses represents the results with ITR primers).

AAV DNA was not detected in the organs of rats treated with rAAV-IPE transplantation 3 months after transplantation (Table 2).

There were no differences in the detection of AAV genomes between AAV-LacZ and AAV-BDNF transplanted eyes. The AAV genome was not detected in the blood of four randomly selected rats by PCR before we performed this experiment. Vector DNA was also not detected in the three rats treated with HBSS injection or with only IPE ($n = 3$) transplantation.

DISCUSSION

Although accumulating evidence suggests that most of the transduced AAV DNA is not integrated into the host genome, some is integrated and induces deletions in the chromosome.[30,31] We did not find any changes in the cell morphology and cell viability of the rat IPE cells at successive passages after transduction. These findings were also made when we used human IPE cells (data not shown). In contrast, when rAAV was transduced into a human ARPE-19 cell line at a density of less than 70%, the viability was significantly lower than at higher densities (data not shown). The possibility of cell damage as well as phototoxicity using AAV-green fluorescein protein (GFP) has been reported.[32] Thus, the conditions used to transduce and maintain the rAAVs are important.

TABLE 1. The Number of Samples with AAV Genome Detected in Blood by PCR

Time after Transplantation	rAAV or rAAV-IPE	AAV Genome Detected in Blood	Total Sample (*n*)
1 w	rAAV	14 (14)	20
	rAAV-IPE	1 (1)*	22
	IPE	0	8
	HBSS	0	9
1 mo	rAAV-IPE	0	53
	HBSS	0	16
3 mo	rAAV-IPE	0	17

The AAV genome was detected either by CMV primer or ITR primer (in parenthesis) from the AAV vector in blood samples rAAV indicates recombinant AAV constructed by the *LacZ* or *BDNF* gene. rAAV-IPE indicates rAAV transduced into rat IPE.

* Significantly less AAV genome detected in rAAV-IPE at 1 week after transplantation, compared with that of direct injection of rAAV.

Although it has been reported that the use of three immunosuppressive agents increased the survival time of transplanted cells, the increase was for only 4 weeks.[33] On the other hand, cyclosporine did not prevent the disruption of RPE allografts in the subretinal space.[34] Thus, to avoid inserting another variable into this study, we elected not to use any immunosuppressants.

After the transplantation of AAV-LacZ-IPE into the subretinal region, LacZ expression was observed in the transplanted cells 1 and 2 weeks after transplantation. However, at 4 and 12 weeks, fewer transplanted cells were observed at the transplant site, and LacZ staining was observed occasionally at the level of the host RPE cells. The expression of LacZ in both transplanted cells and the RPE was not extensive. As we reported in an earlier study,[9] less than 15% of rat IPE cells appeared to be transduced by AAV-LacZ in in vitro experiments after transduction, and the same findings may be observed in these in vivo experiments.

Host RPE cells have been reported to phagocytose not only the subretinally injected pigment of RPE cells[35] but also transplanted dead allogenic cells.[36] Even with autologous cells, the transplanted cells will undergo apoptosis unless they attach to the basement membrane.[37] We found apoptotic host RPE cells that appeared as if they were being engulfed 1 week after transplantation, and we found macrophage-like cells in the subretinal transplant area, and these cells, and also underlying host RPE cells, contained many cytosomal inclusions. Some of the cells contained lysosome-like material. These observations suggest that the host RPE may be activated by the transplanted cells or the transplantation procedure.

Although we could not clearly distinguish the LacZ-expressing cells from the transplanted IPE cells, the LacZ-expressing cells at the level of RPE may be transplanted AAV-LacZ-IPE

TABLE 2. The Number of Samples with AAV genome Detected in Each Organ by PCR

Time after Transplantation	rAAV or rAAV-IPE	Optic Nerve	Brain	Lung	Liver	Kidney	Testis	Heart	Spleen	Muscle	Total (*n*)
1 w	rAAV	10 (4)	10 (3)	10 (3)	10 (3)	10 (3)	10 (4)	10 (3)	10 (3)	10 (3)	12
	rAAV-IPE	0	0	1 (0)*	0*	0*	0*	0*	0*	1 (1)*	8
	IPE		0	0	0	0	0	0	0	0	3
	HBSS	0	0	0	0	0	0	0	0	0	3
3 mo	rAAV-IPE	0	0	0	0	0	0	0	0	0	6

The AAV genome was detected either by CMV primer or ITR primer (in parenthesis) from each organ. Abbreviations are as described in Table 1.

* Significantly less AAV genome was detected in rAAV-IPE at 1 week after transplantation when compared with that detected after direct injection of rAAV.

cells, probably ingested by host RPE and detected by electron microscopic or histochemical examination. Alternatively, the genome of AAV-LacZ may be transferred to host RPE cells. As reported by Braun et al.,[36] replacement of the transplanted cell with host RPE is unlikely to occur. Further examination will clear up these alternative explanations.

AAV-GFP can also be transferred from transduced cells to nontransduced cells.[32] A few cells in the photoreceptor layer that showed LacZ staining may have had a transfer of AAV-LacZ from the transplanted cells. However, there is a possibility that the LacZ-expressing cells were transplanted rAAV-LacZ-IPE cells that migrated. Earlier, we transduced a BDNF-expressing plasmid vector into RPE or IPE cells and transplanted them into the subretinal region of rats. These transplanted cells were able to rescue photoreceptors from phototoxicity during the early days after transplantation, but the rescue was significantly reduced 3 months after transplantation.[38] One of the reasons for the rescue effect of AAV-BDNF-IPE cells from phototoxicity 3 months after allotransplantation[9] may be the transfer of AAV-BDNF from transplanted cells to the host RPE or surrounding cells.

Although we showed that the subretinally transplanted cells may remain in the transplant area,[39] some cells in the ciliary body showed LacZ staining. This indicates that transplanted cells can migrate to other regions, as reported.[40] Alternatively, these results may be due to the dissemination of AAV-LacZ either during the transplantation procedure or after transplantation despite washing the cells three times, and rAAV was not detected in the wash solution by PCR.

Although prolonged transgene expression has been demonstrated with no evidence of toxicity after intraocular injection of expression constructs packaged in AAV vectors,[41] AAV-mediated RPE65 gene delivery induced inflammation in the eyes of cats.[10] Accumulating evidence also indicates that administration of rAAV may initiate detectable levels of cellular and humoral immune responses to its transduced neoantigen in vivo, probably by activation of CD40L-dependent T cells.[13,14] We found occasional macrophage-like cells in the transplant area soon after transplantation, and this suggests that the subclinical inflammation is mainly due to the surgical procedure, as we reported.[42] Otherwise, our AAV-BDNF-IPE transplantation did not induce inflammation during the 6-month follow-up as shown by ERGs, FITC–dextran angiography, and histologic examinations. We suggest that the mild retinal vessels tortuosity is due to the transscleral transplantation procedure and not to BDNF expression from the transplanted cells, because similar findings were also observed in the rats with IPE-only transplantation (data not shown). These findings may also account for the slight depression in the ERGs. Alternatively, the retina in the transplant area was sometimes thinner than that of the nontransplant area (data not shown) and the results may affect the amplitude of ERG.

Although exposure of mature sperm to rAAV is unlikely to lead to germline transmission in mice,[43] as mentioned in the introduction, AAV has been detected in the semen in approximately 18% of the human population.[17] rAAV is also reported to replicate in the presence of wild-type AAV and of a helper virus.[15] Systemic AAV dissemination after rAAV subretinal injection has also been reported.[16] These results show that direct rAAV subretinal injection and systemic AAV dissemination may not always be safe. Our results clearly demonstrated that the systemic dissemination of rAAV was far less with the transplantation of rAAV-IPE than after direct rAAV injection. Serum testing also showed normal findings relative to those of normal rat serum (data not shown). Our results showed that rAAV-IPE transplantation may not only deliver target genes to the regions but could also be performed safely. However, AAV genome was detected in approximately 80% of the blood and organ samples of rats treated with direct AAV subretinal injection (data not shown). The results of AAV dissemination were similar in organs and blood. These findings indicate that AAV dissemination may be mediated by the blood.[11]

We transplanted the cells transsclerally because of the size of the rat's eye. This method affect the distribution of the transplanted cells, with leakage into the choroid and systemic dissemination. Other methods of delivery, such as a transvitreal approach, may have dispersed cells into the vitreous or ciliary body. Our experiments are now being extended to larger animals (monkeys) with transvitreal injections into the subretinal region.

We and several other authors have reported that BDNF-expressing cells may rescue photoreceptors from various toxicities.[9,44,45] One of the important factors for clinical application is to generate BDNF-expressing cells that are as safe as possible. Our results showed that subretinal transplantation of rAAV-IPE will lead to the expression of transgene in the subretinal space but may also result in the transfer of rAAV to host RPE or ciliary body cells around the transplant site for long periods after transplantation. This method may be a safer way to deliver target genes to treat the retina than is direct injection from the viewpoint of systemic dissemination.

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