the second highest dose (3×10^{13} vg) stayed in a well-controlled therapeutic range (<6 mg/dl). Mice receiving lower vector doses (3×10^{12} and 1×10^{13} vg) showed moderate correction of hyperphenylalaninemia, with significant long-term efficacy of the single AAV infusion.

Figure 2b shows the kinetics of serum Phe in female PKU mice after receiving 1×10^{13}, 3×10^{13} or 1×10^{14} vg of AAV5/CAG-mPAH. The vector administration was effective in the female PKU mice, too, but the dose–response and duration were different from the male mice; that is, about three times more vector was required for the female mice to exhibit an equivalent reduction in serum Phe (Figure 3). At 4 weeks postinfusion when the reduction was at its maximum, 1×10^{13} vg of AAV5/CAG-mPAH lowered serum Phe by 50% in the female mice, while the same level of reduction was achieved by 3×10^{12} vg in the males. Similarly, an 80% reduction was achieved by 3×10^{13} vg in the females, whereas only 1×10^{13} vg were required in the males. Complete correction of hyperphenylalaninemia was achieved by 1×10^{14} vg in the females, while it was achieved by 3×10^{13} vg as well as 1×10^{14} vg in the males. As for duration, the therapeutic effect did not persist in the female PKU mice as seen in the males. Serum Phe levels in each female cohort remained low until 8 weeks post-gene transfer, but gradually rose thereafter. With vector doses of 1×10^{13} and 3×10^{13} vg, serum Phe was greater than 20 mg/dl at 20 weeks, and returned to the pretreatment level at 40 weeks. With the highest dose (1×10^{14} vg), serum Phe was kept below 10 mg/dl until 16 weeks, then gradually increased and returned to the pretreatment level at 40 weeks.

Although we did not kill the animals for enzyme assay, previous studies on adenoviral-mediated gene transfer to PAH^{enu2} mice allowed us to estimate the PAH activity accomplished by our vector. These studies showed, in good agreement, that the threshold PAH activity to correct hyperphenylalaninemia was about 10% of normal mice.[4,5] As shown in Figure 3, male PKU mice given 3×10^{12} vg and females given 1×10^{13} vg of the vector showed 50–60% reduction in serum Phe; we speculate that these mice would express about 5% of normal PAH activity. On the other hand, male PKU mice given 3×10^{13} or 1×10^{14} vg and females given 1×10^{14} vg completely recovered from hyperphenylalaninemia, hence their liver PAH activities would be 10% of normal or greater. Male PKU mice given 1×10^{13} vg (ca. 90% reduction in serum Phe) and females given 3×10^{13} vg of AAV (ca. 80% reduction) would have 5–10% of normal PAH activity.

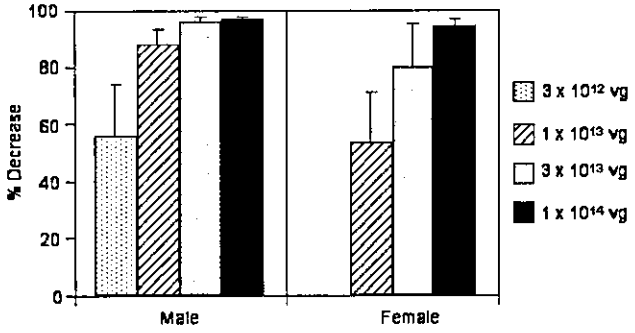

Figure 3 Vector dose-dependent reduction of serum Phe in PKU mice. Percent reduction of serum Phe was calculated by the following formula: {(serum Phe at week 0)−(serum Phe at 4 weeks)} × 100/(serum Phe at week 0). Bars represent the mean±s.d. of % reduction of serum Phe in PKU mice treated with 1×10^{12} vg (dotted bar), 1×10^{13} vg (hatched bar), 3×10^{13} vg (gray bar), or 1×10^{14} vg of AAV5/CAG-mPAH (black bar).

Correction of hypopigmentation

Associated with extended reductions in serum Phe, hypopigmentation in the AAV-treated PKU mice was ameliorated. While the coat color of untreated mice remained grayish brown, hair darkening in the mice receiving higher vector doses was observed 2 weeks post-transduction, and the mice grew black hair in 4 weeks which was indistinguishable from that of wild-type (WT) BTBR mice (Figure 4). Male PKU mice with reduced serum Phe retained black hair throughout the observation period, while female PKU mice lost pigmentation as the therapeutic effect diminished.

Recovery from hypoactivity following PAH gene transfer

Along with persistent correction of hyperphenylalaninemia and hypopigmentation, we observed behavioral

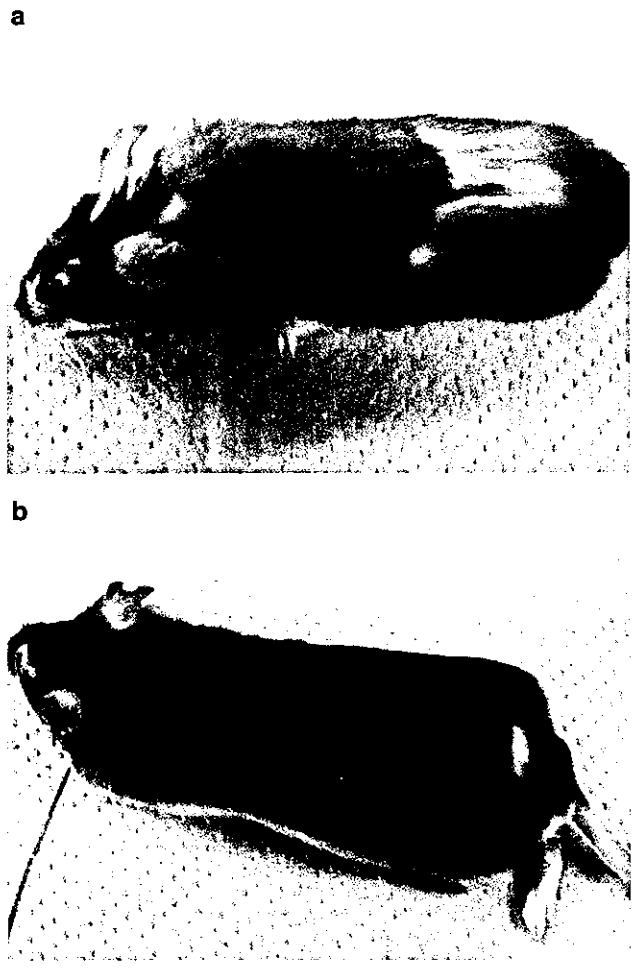

Figure 4 Correction of hypopigmentation in PKU mouse following PAH gene transfer. (a) Untreated PKU mouse showing grayish brown hair, easily distinguished from wild-type and PAH$^{+/-}$ heterozygous BTBR mice. (b) By 8 weeks after PAH gene transfer, the PKU mouse with complete correction of hyperphenylalaninemia recovered black coat color and was indistinguishable from normal BTBR mice.

recovery in AAV-treated PKU mice. Consistent with previous studies showing abnormal behavior and cognitive deficits in PAH^{enu2} mice,[9,16,17] we found that untreated PKU (PAH$^{-/-}$) mice were relatively hypoactive compared with WT (PAH$^{+/+}$) and heterozygous carrier (PAH$^{+/-}$) animals. The hypoactivity became apparent with aging, and the difference was significant among animals aged 10 months or older. Figure 5 shows the results of behavior tests on the 12-month-old animals. As for total locomotion over 24 h, the untreated PKU mice displayed about 70% of normal activity (Figure 5a, $P<0.01$ by Student's *t*-test). On the other hand, the AAV-administered male mice without hyperphenylalaninemia exhibited significantly higher 24-h locomotion than the untreated mice (Figure 5a, $P=0.001$ by Student's *t*-test). Indeed, the AAV-treated animals showed a normal activity level in this test.

Similarly, PAH gene transfer improved the PKU animals' exploratory activity in a novel environment.

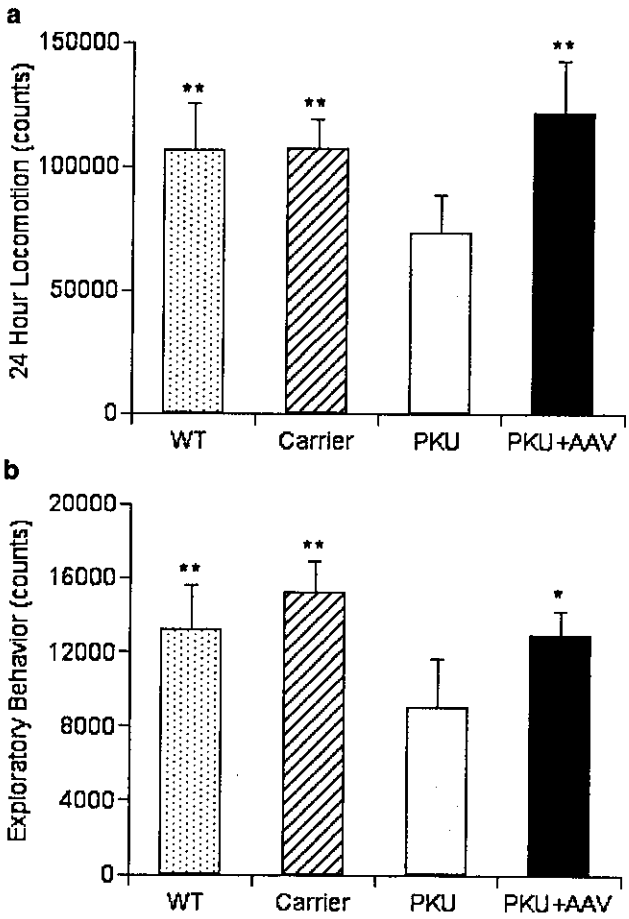

*Figure 5 Recovery from hypoactivity following PAH gene transfer. (a) Total locomotion over 24 h. Mice were placed under an infrared sensor and ambulatory activity was recorded consecutively for 24 h. Wild-type (WT), heterozygous and AAV-treated PKU mice exhibited significantly higher locomotory activity than untreated PKU mice (**$P<0.01$ by Student's t-test). (b) Exploratory behavior. Mice were placed in a novel cage under a sensor and ambulatory activity was quantified during the first 2 h in the chamber. This test showed significantly higher performance by WT, heterozygous and AAV-treated PKU mice than untreated PKU animals (**$P<0.01$ and *$P<0.05$ by Student's t-test). Bars represent the mean±s.d. of WT mice (WT; dotted bar), heterozygous mice (Carrier; hatched bar), untreated PKU mice (PKU; gray bar), and AAV-transduced PKU mice (PKU + AAV; black bar).*

When settled in a novel cage, the untreated PKU males showed 60–70% of normal exploratory activity (Figure 5b, $P<0.01$). On the other hand, PKU mice that had recovered from hyperphenylalaninemia explored as vigorously as WT animals, and their activity level was significantly greater than that of untreated PKU mice ($P=0.015$). These results clearly indicate that the PAH gene transfer improved the CNS function of PKU mice in addition to correction of hyperphenylalaninemia.

Discussion

In this study, we demonstrated that AAV-mediated transduction of the PKU mouse liver brought about a long-term cure of the disease. A single infusion of AAV5/CAG-mPAH completely normalized the hyperphenylalaninemic phenotype in male PKU mice, and the longevity of the therapeutic effect was superior to any other gene delivery vehicle thus far. Although not thoroughly investigated, the result suggests that the transgene was transcriptionally active during observation, and that no significant immune response was elicited against the transduced hepatocytes in the animals. In addition, infusion of very large amounts of AAV did not show any toxicity in the treated mice. The vector safety and viability may be further improved by adopting recently developed purification methods, such as iodixanol gradient, affinity or ion-exchange chromatography.[18–21]

A major problem we encountered in this study was that the same AAV vector was less effective in female PKU mice. About three times more vector was required to achieve an equivalent reduction of serum Phe seen in males, and the therapeutic effect was shorter in duration. The underlying mechanisms for these female-specific phenomena are currently unknown. Davidoff *et al*[22] recently reported similar observations that AAV2- and AAV5-derived vectors less efficiently transduced livers of female mice than males. They suggested that the difference was due to an androgen-dependent pathway for augmenting hepatocyte transduction, but its mode of action is undetermined. Since precise molecular events involved in recombinant AAV-mediated transduction remain obscure, the critical step accounting for the observed sex difference is also a mystery. Androgen may augment the uptake of AAV particles into the cell or traffic them to the nucleus; alternatively, it may stabilize the AAV genome in an episomal state, or enhance vector integration into the host chromosome. Of these possibilities, the last one is less likely, because only a small fraction (<10%) of recombinant AAV genome was reportedly integrated into the mouse hepatocytes.[23] If there is an androgen-dependent mechanism to retain the AAV genome in an episomal state in the liver, lack of such machinery would allow gradual loss of the vector DNA in females, thereby transgene-derived PAH activity would descend over time as we observed. Other possibilities accounting for the lower therapeutic efficacy include transcriptional silencing and an immune response against AAV-transduced hepatocytes, although the latter is unlikely to occur only in female mice.

In genetic treatment of autosomal and acquired disorders, sex-dependent transduction raises a novel issue. Development of more efficient vectors may over-

come this problem, or other approaches can be considered. In terms of PKU, the disease-associated pathology is caused by accumulated Phe in the body fluids. Thus, it can be prevented by 'heterologous gene therapy', ie targeting tissues other than hepatocytes. Several investigators have exploited this strategy because of difficulties with liver transduction and safety concerns. Christensen *et al*[24] transduced primary keratinocytes with genes for PAH and GTP cyclohydrolase I, which is the rate-limiting enzyme in BH_4 biosynthesis. They showed that the cells cleared excess Phe in the culture medium, and suggested that engraftment of enough of these cells may function as a metabolic sink for detoxification. Harding *et al*[25] investigated the potential of skeletal muscle as a PAH-expressing organ. Using a transgenic technique, they created mice expressing PAH in the skeletal muscle but not in the liver. These mice showed hyperphenylalaninemia at baseline, but serum Phe significantly decreased when the animals were supplemented with BH_4. A similar approach to bone marrow cells was unsuccessful,[26] and careful consideration is required in translating these transgenic studies into human applications.

A novel finding in this study was that AAV infusion lead to behavioral improvement in addition to correction of hyperphenylalaninemia and hypopigmentation. To our knowledge, this is the first demonstration that a gene-based approach to PKU actually benefited CNS function. It has been reported that free amino acid and amine contents are dramatically reduced in the PAH^{enu2} mouse brain, as in untreated human PKU patients.[27,28] Presumably, the observed hypoactivity in older PKU mice was associated with the abnormal synthesis of biogenic amines, whereas the abnormality was reversed in AAV-treated PKU animals with normal serum Phe. We speculate that the behavioral recovery in these mice represents an analogous situation in which dietary restriction of Phe can improve some neuropsychiatric symptoms in untreated PKU patients. It is of particular interest whether an earlier genetic intervention can prevent irreversible neuronal defects in PKU and preserve more sophisticated CNS function such as memory. The AAV vectors and PAH^{enu2} mice will provide an attractive system to address such prompting questions.

Materials and methods

AAV vector construction

To isolate murine PAH cDNA (GenBank Accession # NM008777), liver mRNA was prepared from a C57BL/6J mouse (from Clea Japan, Tokyo, Japan) with Isogen reagent (Nippon Gene, Toyama, Japan) and an mRNA Purification kit (Amersham Pharmacia Biotech, Little Chalfont, UK). The PAH cDNA was cloned by reverse transcriptase-directed polymerase chain reaction using a Superscript II cDNA synthesis kit (Invitrogen, Grand Island, NY, USA). The CAG promoter was derived from pCAGGS (a gift from Dr J Miyazaki, Osaka University, Osaka, Japan).[14] The AAV5 vector plasmid pAAV5LacZ and a helper plasmid 5RepCapA were generous gifts from Dr JA Chiorini (National Institutes of Health, Bethesda, MD, USA).[12] To construct a recombinant AAV5 vector plasmid for PAH expression, the expression cassette of pAAV5LacZ was replaced with the CAG promoter, the murine PAH cDNA and the SV40 late polyadenylation signal, and the plasmid was referred to as pAAV5/CAG-mPAH (Figure 1).

Recombinant AAV stocks were propagated according to an adenovirus-free, three-plasmid transfection protocol described previously.[15] Briefly, subconfluent 293 cells (4×10^8 cells per 10 trays) in Cell Factories 10 (Nunc, Roskilde, Denmark) were cotransfected with 650 μg of the vector plasmid pAAV5/CAG-mPAH, 650 μg of the AAV helper plasmid 5RepCapA and 650 μg of the adenoviral helper plasmid pLadeno1 (identical to pVAE2AE4-2 in Matsushita *et al*;[15] kindly provided by Avigen, Alameda, CA, USA) by using the calcium phosphate precipitation method for a period of 6 h. Cells were harvested 72 h after transfection and lysed by three freeze–thaw cycles. The crude viral lysate was incubated with Benzonase (Merck KGaA, Darmstadt, Germany) and centrifuged. Finally, the clear supernatant was subjected to two rounds of CsCl density-gradient ultracentrifugation for purification. The physical titer of the viral stock was determined by DNA dot blot and hybridization with the murine PAH cDNA probe, along with plasmid standards. Typically, we obtained 5×10^{13} vg of AAV5/CAG-mPAH from a culture container (10 trays).

Transduction of mouse liver

All animal experiments were carried out in accordance with our institutional guidelines. PAH^{enu2} mice were generous gifts from Dr T Shiga (University of Tsukuba, Tsukuba, Japan), and a colony was established at Jichi Medical School (Tochigi, Japan). PKU mice used for *in vivo* gene transfer were 5–7 weeks of age. Mice were anesthetized with isoflurane inhalation followed by laparotomy. A 300 μl of saline suspension containing 3×10^{12}–1×10^{14} vg of AAV5/CAG-mPAH was slowly injected into the portal vein using an insulin syringe with a 29-gauge needle (Terumo, Tokyo, Japan).

Serum Phe assay

Serum Phe was measured by an enzymatic microfluorometric assay using an Enzaplate PKU-R kit (Bayer Medical, Tokyo, Japan). Mice were tail phlebotomized and the blood was spotted onto a mass-screening grade paper filter (#545, provided by Advantec Toyo, Tokyo, Japan). A 3 mm diameter disc was punched out from the dried blood spot and placed in a 96-well plate. Phe was eluted from the disc and incubated with Phe dehydrogenase, an NAD-dependent enzyme, and resazurin. The enzyme reaction produces NADH, which in turn converts resazurin to resorufin with the aid of diaphorase. The resultant resorufin was measured on a Fluoroskan Ascent plate reader (Labsystems, Helsinki, Finland) with a 544/590 nm filter set.

Mouse behavior tests

Mice were tested at 12 months of age. To measure locomotory activity over 24 h, the home cage of the mouse was placed under an infrared sensor that detects thermal radiation from animals (Supermex; Muromachi Kikai, Tokyo, Japan).[29] Ambulation was scored by a personal computer interfaced to the sensor. Alternatively, exploratory behavior was tested by placing the mouse in a novel cage under the infrared sensor.

Ambulatory activity was quantified during the first 2 h in the chamber.

Acknowledgements

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Successful Gene Transfer Using Adeno-Associated Virus Vectors into the Kidney: Comparison among Adeno-Associated Virus Serotype 1–5 Vectors in vitro and in vivo

Shin-ichi Takeda[a,c] Masafumi Takahashi[a,e] Hiroaki Mizukami[b] Eiji Kobayashi[a] Koichi Takeuchi[d] Yoji Hakamata[a] Takashi Kaneko[a] Hisashi Yamamoto[c] Chiharu Ito[c] Keiya Ozawa[b] Kenichi Ishibashi[c] Toshiyuki Matsuzaki[f] Kuniaki Takata[f] Yasushi Asano[c] Eiji Kusano[c]

Divisions of [a]Organ Replacement Research and [b]Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, [c]Division of Nephrology, Department of Medicine, and [d]Department of Anatomy, Jichi Medical School, Tochigi, [e]Department of Organ Regeneration, Shinshu University Graduate School of Medicine, Matsumoto, and [f]Department of Anatomy and Cell Biology, Gunma University School of Medicine, Maebashi, Japan

Key Words

β-Galactosidase · Dependovirus · Epithelial cell · Gene therapy, adeno-associated virus serotype 1–5 vectors · Viral vectors, gene therapy

Abstract

Background/Aim: Gene transfer into the kidney has great potential as a novel therapeutic approach. However, an efficient method of gene transfer into the kidney has not been established. We explored the transduction efficiency of renal cells in vitro and in vivo using adeno-associated virus (AAV) serotype 1–5 vectors encoding the β-galactosidase gene. ***Methods:*** In the in vitro study, rat kidney epithelial cell line NRK52E cells were transfected with AAV serotype derived vectors. In the in vivo study, AAV serotype derived vectors were selectively injected into the kidney using a catheter-based gene delivery system in rats and mice mimicking the clinical procedure. The efficiency of gene expression was histologically evaluated on the basis of the β-galactosidase expression. ***Results:*** AAV serotype 1, 2, and 5 vectors transduced in rat kidney epithelial cell line NRK52E cells in vitro, whereas AAV serotype 3 or 4 vectors showed no transduction. In addition, the kidney-specific injection of AAV serotype 2 vectors successfully transduced in tubular epithelial cells, but not in glomerular, blood vessel, or interstitial cells in vivo, whereas the rest of the serotypes showed no transduction. ***Conclusion:*** Since kidney-specific gene delivery via the renal artery by catheterization is highly feasible in humans, these findings provide useful information for promising strategies in renal gene therapy.

Introduction

Gene transfer into the kidney has a great potential as a novel therapeutic approach for renal diseases. However, the ability to pursue gene therapy for renal diseases is substantially limited, because there is no efficient and adequate local gene delivery system into the kidney and because of the potential adverse effects on renal functions

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Masafumi Takahashi, MD, PhD
Division of Organ Replacement Research, Center for Molecular Medicine
Jichi Medical School, Minamikawachi-machi
Tochigi 329-0498 (Japan)
Tel. +81 285 58 7446, Fax +81 285 44 5365, E-Mail masafumi@jichi.ac.jp

associated with systemic gene therapy. A few reports have been published on gene delivery into the kidney using recombinant virus vectors in experimental models [1, 2]. The direct injection of retrovirus vectors into the kidney has been reported to transduce in tubular epithelial cells, but not in glomerular or vascular cells [3]. Because retrovirus vector mediated gene transduction requires target cell replication, the use of retrovirus vectors in renal gene therapy is still restricted. The adenovirus vector is an alternative vector system that results in high gene transduction in both dividing and nondividing cells. Several investigations demonstrated that adenovirus vectors were successfully transduced in renal cells [4–6]. However, the clinical application of adenovirus vectors also has been limited by cytotoxicity, immune responses, and short-term transgene expression.

Adeno-associated virus (AAV) vectors have a number of attractive features for the clinical application of renal gene therapy: no cytotoxicity, the ability to transduce in both dividing and nondividing cells, low immune responses, and long-term transgene expression [7–9]. When the experiment was started, there were six primary isolates of AAV (AAV serotypes 1–6) [8, 10–14], although the cap sequence of serotype 6 is strongly related to that of serotype 1 [8]. Two novel serotypes were recently isolated from monkeys [15]. The distinct AAV serotypes were shown to have different tropisms for the cells and tissue. For instance, the AAV serotype 1 vectors could efficiently transduce the skeletal muscles [16], whereas AAV serotype 5 vectors could efficiently transduce liver [17] and central nervous system [18]. Although these differences in the tropism might be due to the receptors and processes during AAV transduction, the precise mechanisms are unknown. In terms of AAV vector mediated gene transfer into the kidney, it is not known whether any of the serotypes of AAV vectors can achieve transduction. In the present study, we explored the transduction efficiency of renal cells in vitro using AAV serotype 1–5 vectors. To achieve efficient gene transduction in vivo, we developed a catheter-based injection system that can be inserted into the kidney of rats and mice and tested these distinct AAV serotype vectors.

Materials and Methods

Cell Culture and Reagents

Normal rat kidney cell lines (NRK52E and ACTT-CRL1571) displaying a tubuloepithelial phenotype [19] were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. The cells were plated onto 60-mm dishes (Falcon, Franklin Lakes, N.J., USA) at a density of 3×10^6 and cultured at 37°C in 5% carbon dioxide and 95% air. The primary antibody against aquaporin-1 (AQP-1) was prepared with rabbit immunization, targeting for rat AQP-1 (234–247; Arg-Ser-Ser-Asp-Phe-Thr-Asp-Arg-Met-Lys-Val-Trp-Thr-Ser). The reagents were obtained from Sigma (St. Louis, Mo., USA) unless otherwise indicated.

Experimental Animals

Twelve male Lewis rats (initial body weight 110–150 g), originally purchased from Japan SLC (Shizuoka, Japan) and maintained at our animal center, and 10 male BALB/c mice (initial body weight 27–33 g), purchased from CLER Japan (Tokyo, Japan), were used in this study. The animals had free access to standard chow and drinking. All experiments in this study were performed in accordance with the Jichi Medical School Guide for Laboratory Animals.

Preparation for AAV Vectors

AAV vectors encoding the β-galactosidase (β-gal) gene were produced based on plasmid transfection [10]. Briefly, subconfluent 293 cells were cotransfected with AAV vector plasmid, AAV helper plasmid, and adenovirus helper plasmid by a calcium phosphate precipitation method. Recombinant AAV was harvested by three cycles of freezing/thawing. The vector solution was then purified twice on a gradient. The vector titer was determined by quantitative dot-blot hybridization of DNase-treated stocks.

Detection of β-Gal Expression

Detection of the β-gal expression was described previously [20, 21]. Briefly, samples of the kidney from rats and mice were embedded in OCT compound (Miles Laboratories, Elkhart, Ind., USA), frozen in liquid nitrogen, and cut into thin (10–20 μm) sections. The sections were fixed with 0.2% glutaraldehyde for 5 min at room temperature, washed three times in 0.1 *M* of phosphate-buffered saline (PBS; pH 7.4) for 5 min, transferred to X-gal staining solution [1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-*D*-galactopyranoside, 2 m*M* $MgCl_2$, 5 m*M* potassium hexacyanoferrate [III], 5 m*M* potassium hexacyanoferrate [II] trihydrate] at 37°C for 2 h, and then counterstained with kernechtrot solution. To detect β-gal expression in cultured cells, the cells were washed with PBS three times, fixed with 0.2% glutaraldehyde for 5 min at room temperature, washed with PBS three times, and reacted with β-gal staining solution.

Immunohistochemistry

Fresh-frozen kidney sections were fixed with 100% acetone for 20 min at room temperature, washed, and transferred to X-gal staining solution as described above. Using a standard avidin-biotin complex technique, the sections stained with X-gal were blocked with 1.5% H_2O_2 for 10 min, 5% bovine serum albumin/5% horse serum for 20 min, and avidin/biotin (Vector Laboratories, Burlingame, Calif., USA) and incubated for 2 h with rabbit anti-rat AQP-1 antibody (dilution 1:1,000), followed by biotin-conjugated antirabbit IgG (Rockland Immunochemicals, Gilbertsville, Pa., USA, dilution 1:250) for 1 h and horseradish peroxidase-labeled streptavidin (Vector Laboratories; dilution 1:200) for 30 min. Immunoreactive cells were detected with 3,3′-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan). To estimate the transduction efficiency, X-gal-positive cells of AQP-1-positive cells were quantified using a grid point counting method. The percentages of cells double positive for both X-gal and AQP-1 were calculated.

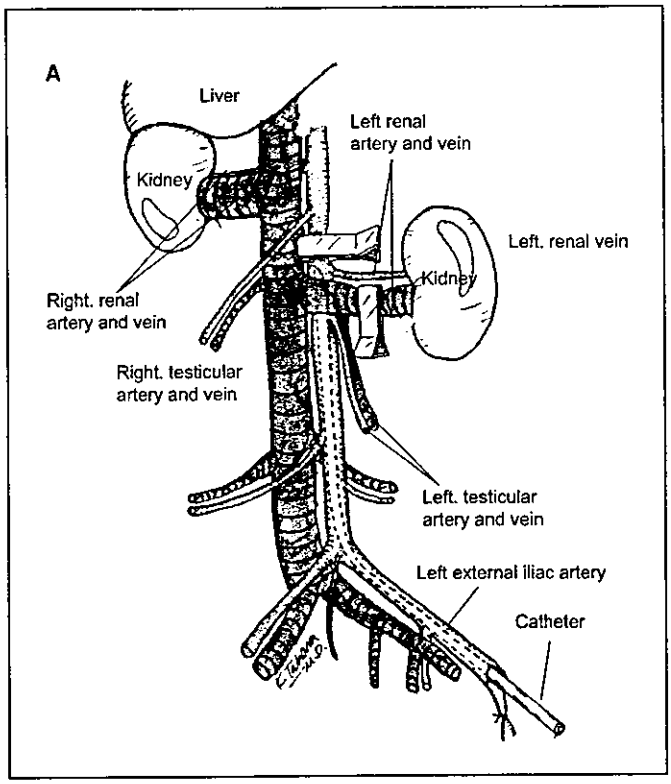

Fig. 1. Procedure for catheter-based gene delivery into the kidney. **A** A 2-Fr flexible catheter is inserted via rat left iliac artery and abdominal aorta, and the tip is placed into the left renal artery. After the aorta is clamped just above the left renal artery, AAV solution is injected after saline to wash out blood through the catheter. The left renal vein then is clamped for 10 min. **B**, **C** The color of the kidney was dark-red before (**B**; arrows) and pale after (**C**; arrows) injection of AAV solution into mice.

In vitro Gene Transduction in NRK52E Cells

The cells were plated on 24-well dishes (Falcon) at 5×10^4/well. After incubation for 16 h, 1.5×10^{10} vector genomes of each of the AAV serotype 1–5 vectors were added to each well (3×10^5 particles/cell). The cells were cultured for 48 h, and the β-gal gene expression was evaluated histologically.

In vivo Gene Transduction into the Kidney

Gene delivery was performed with each of the AAV serotype 1–5 vectors (5.0×10^{11} and 1.0×10^{11} vector genomes in rats and mice, respectively). To achieve efficient gene delivery into the kidney in vivo, we developed a catheter-based gene delivery system for use in rats and mice that mimicked the clinical procedure. The flexible Solo-Cath catheter (2 and 1 Fr for rats and mice, respectively; Solomon Scientific, Plymouth Meeting, Pa., USA) was inserted via the left iliac artery and the abdominal aorta, and the tip was placed beneath the left artery (fig. 1A). After the aorta was clamped just above the left renal artery, 1 ml (rats) or 0.5 ml (mice) of AAV vector solution was injected after 1–2 ml of saline to wash out blood via the catheter. The left renal vein then was clamped for 10 min. Catheter and clip were removed. Figures 1B and C show the color of the kidney before and after the injection of AAV vector solution into the left kidney of mice. The color of the injected kidney changed from dark-red to pale. Histological analysis confirmed that no injury occurred

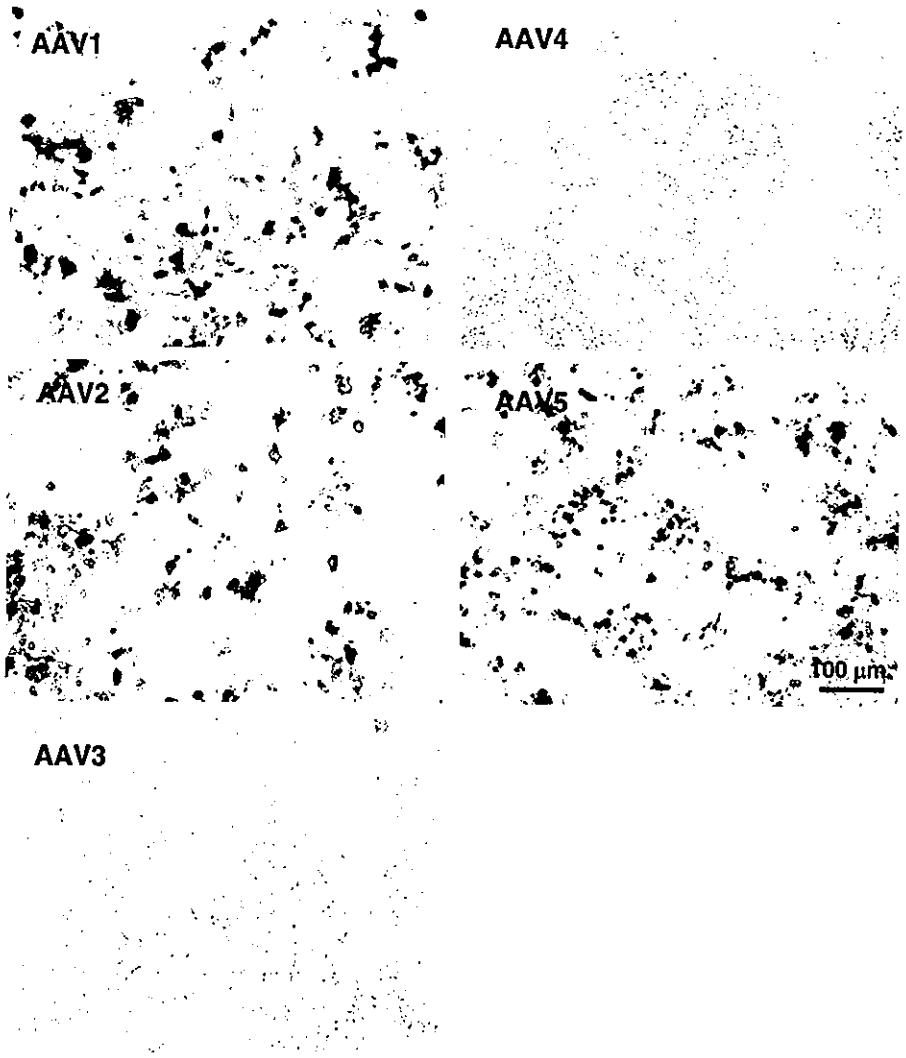

Fig. 2. In vitro transduction of NRK52E cells by AAV serotype derived vectors. NRK52E cells were transduced with AAV serotype 1–5 vectors for 48 h, and the β-gal expression was evaluated. Efficient gene transduction is observed in the cells transduced with AAV serotype 1, 2, and 5 vectors. The results are representative of two independent experiments.

in associationwith this procedure in the injected kidney. Each serotype of AAV vectors was delivered into 2 rats and mice each which were sacrificed on day 14 to evaluate which serotype of AAV vectors had the ability for gene transduction. To investigate long-term gene transduction, additional 2 rats were transduced by AAV2 vectors and sacrificed on day 28.

Results

In vitro Transduction by AAV Serotype Derived Vectors in NRK52E Cells

To evaluate the efficiency of AAV serotype derived vectors for gene transfer into renal cells, we used rat epithelial cell line NRK52E cells as an in vitro model. The cells were exposed to AAV serotype derived vectors encoding the β-gal gene for 48 h, and the β-gal expression was evaluated. The AAV serotype 1, 2, and 5 vectors efficiently transduced these cells (fig. 2). These observations suggest that AAV vectors are useful for gene delivery in renal cells in vitro and that the transduction efficiency might differ among AAV serotypes.

In vivo Gene Transduction by Distinct AAV Serotype Derived Vectors into the Kidney

The vector solutions were selectively injected into rat kidneys using the procedure as described in Materials and Methods, and the animals were sacrificed and the β-gal expression evaluated 14 days after gene delivery. The injection of AAV serotype 2 vectors showed β-gal expression, whereas the rest of the serotypes showed no expres-

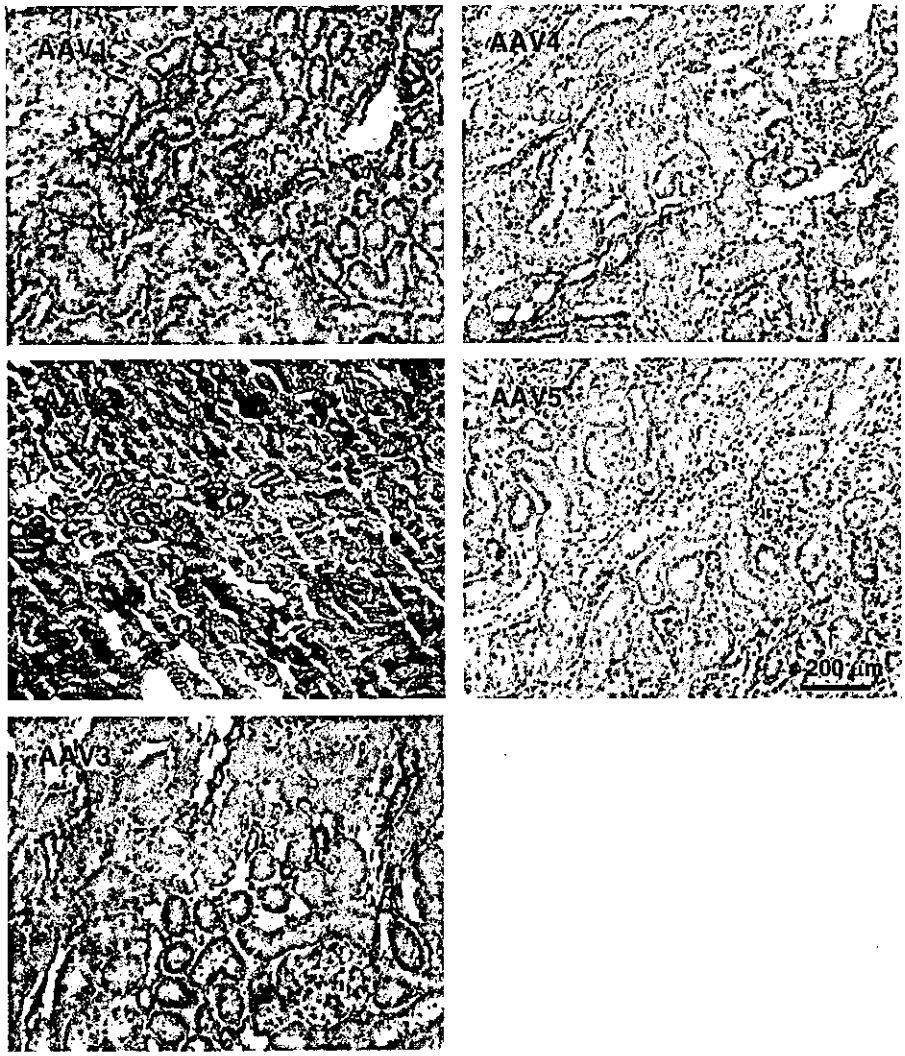

Fig. 3. In vivo transduction by AAV serotype derived vectors into the rat kidney. Solutions with AAV serotype 1–5 vectors encoding the β-gal gene were selectively injected into rat kidney. The animals were sacrificed on day 14, and the kidney sections were evaluated for β-gal expression. Injection of AAV serotype 2 vectors resulted in gene transduction, whereas AAV serotype 1, 3, 4, and 5 vectors showed no transduction. The results represent two independent experiments.

sion (fig. 3). Transduction was observed in cortical tubular epithelial cells, but not in glomeruli, endothelial cells, smooth muscle cells, or interstitial cells. We further confirmed the transduction efficiency with AAV serotype derived vectors into the murine kidney. Consistent with the experimental findings in rats, only AAV serotype 2 vectors showed β-gal expression in the tubular epithelial cells in the kidneys of mice, whereas the other serotypes showed no expression (fig. 4).

We next evaluated the long-term gene transduction by AAV serotype 2 vectors and demonstrated β-gal expression in the rat renal cortex 28 days after gene delivery (fig. 5A). No β-gal expression was observed in extrarenal organs, including liver or skeletal muscle. Immunohistochemical analysis revealed coexpression of β-gal with a proximal tubule marker, AQP-1 [22], suggesting AAV serotype 2 vector mediated gene transduction into the proximal tubular cells. The proportions of gene transduction were 3.2 and 4.0% of the proximal tubular cells.

Discussion

The two major findings of this study are that, in vitro, the AAV serotype 1, 2, and 5 vectors transduced gene expression in kidney epithelial cells, whereas AAV serotype 3 and 4 vectors showed no transgene expression; in vivo, only the AAV serotype 2 vectors transduced gene expression in the proximal tubule of kidney when the vectors were selectively injected into the kidney of rats and

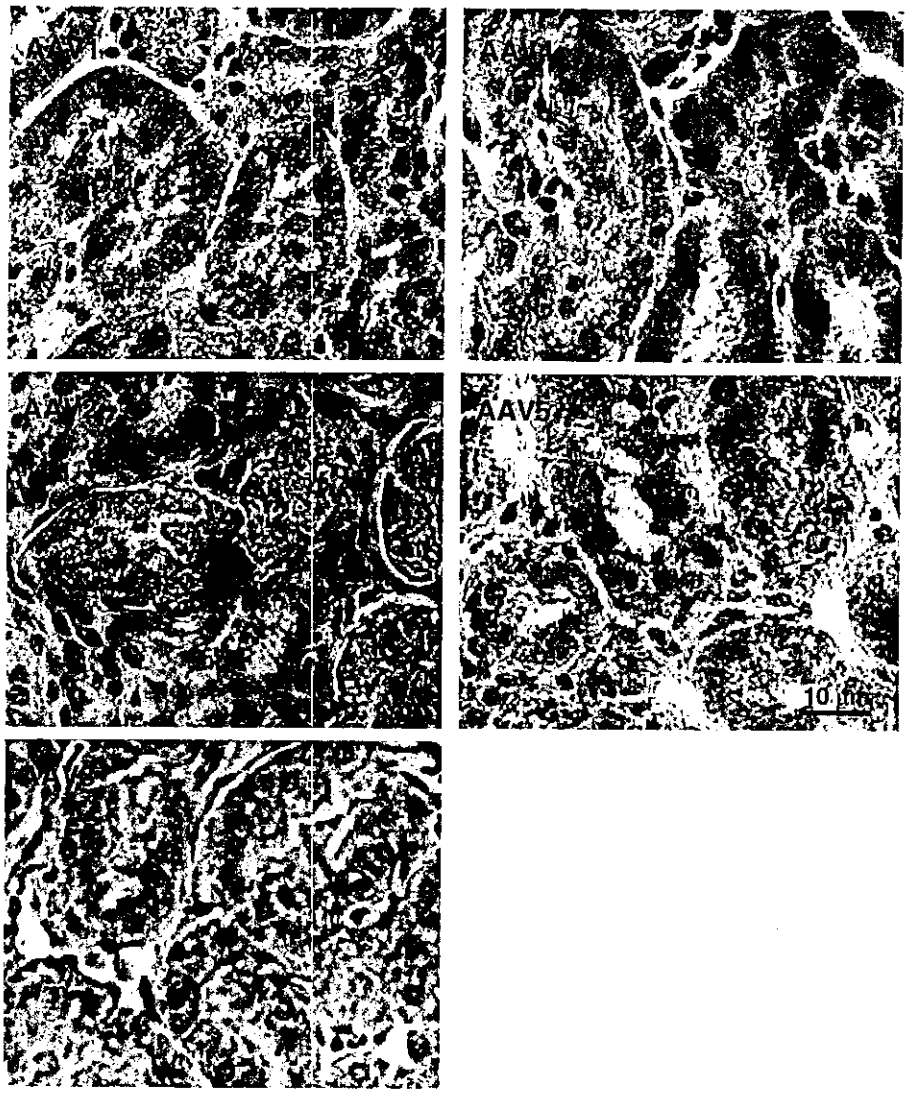

Fig. 4. In vivo gene transduction by distinct AAV serotype vectors into the mouse kidney. Transduction of AAV vectors and evaluation of β-gal expression in mice are essentially described in the figure 1 legend. Consistent with the experiments in rats, the injection of AAV serotype 2 vectors showed gene transduction in the kidney of mice, whereas AAV serotype 1, 3, 4, and 5 vectors showed no transduction. The results are representative of two independent experiments.

mice using a catheter-based procedure. These findings suggest that the transduction efficiency might differ among AAV serotype vectors and that kidney-specific gene delivery using the AAV serotype 2 vectors has the potential for renal gene therapy. The difference in the relative transduction efficiency was not due to a difference in the potency of the serotype-derived vectors, because the capacity of each of the vector stocks was tested and confirmed in vitro and in vivo (data not shown).

Gene transfer into the kidney has a great potential as a novel therapeutic approach. However, efficient gene transduction to the kidney has not been established. Adenovirus-mediated gene delivery is a feasible strategy for gene transduction in the kidney, because it transduces efficient gene expression in the kidney in several experimental models [4–6]. However, there are substantial limitations associated with the clinical application of adenovirus vectors. First, adenovirus vectors show short-term expression (weeks to months) of the transduced gene, because adenovirus does not integrate into the host genome. Second, adenovirus vector mediated gene transduction may cause adverse inflammatory and immunologic responses. Moreover, the risk of recombination with wild-type and generation of replicative mutant virus has not been eliminated.

In contrast to adenovirus vectors, AAV vectors have a number of attractive features for the clinical use of gene therapy. Recombinant AAV vectors are safe, nonpatho-

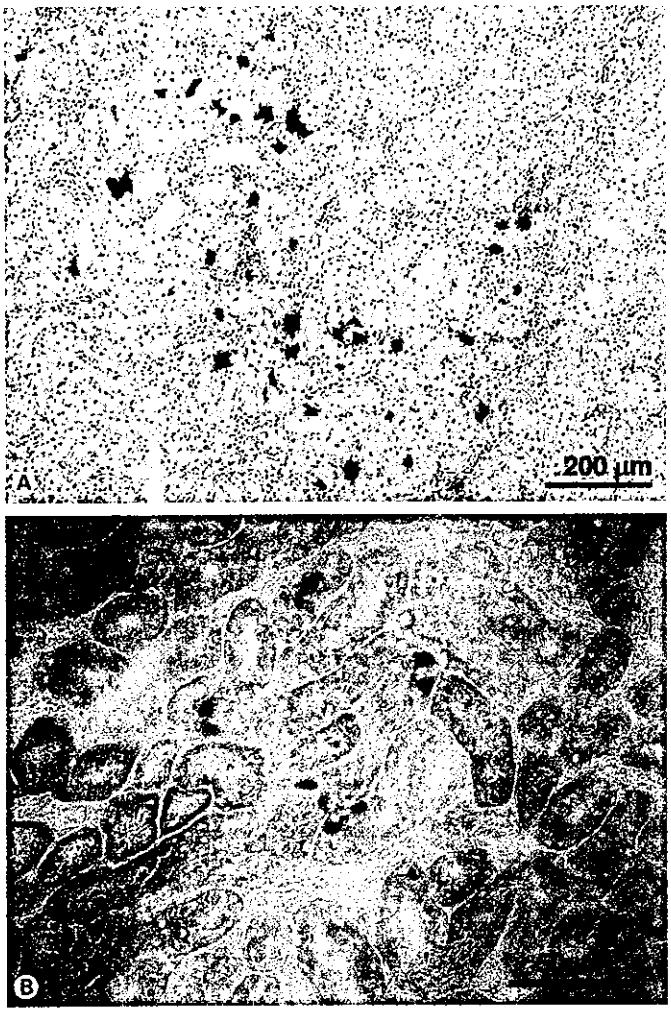

Fig. 5. In vivo long-term transduction by AAV serotype 2 vectors into the rat kidney. Solutions with AAV serotype 2 vectors encoding the β-gal gene were selectively injected into rat kidney, and the rats were sacrificed on day 28. The kidney sections were evaluated for β-gal expression (**A**) and immunohistochemical staining with anti-rat AQP-1 antibody (**B**).

genic, and nonreplicating according to the life cycle of the wild-type virus [23, 24]. Further, unlike the reports using adenovirus vectors, AAV vectors can give rise to long-lasting gene expression without an obvious immune response [9]. An increasing number of reports demonstrated that AAV vectors can transduce into various cell types. Regarding the kidney, the usefulness of the AAV vectors was demonstrated in vitro [25] and in vivo [26]. AAV serotype 2 was used in both studies, because it was representative of AAV and because other serotypes were not focused at that time. However, it is now possible to compare the efficiency of gene transfer into the kidney by using distinct AAV serotype vectors. We, therefore, evaluated the usefulness of AAV serotype 1–5 vectors in renal cells in vitro and in vivo. Interestingly, AAV serotype 1, 2, and 5 vectors transduced renal epithelial cell lines in vitro, whereas only AAV serotype 2 vectors transduced the tubular epithelial cells. Consistent with our findings, AAV serotype 2 vector mediated delivery of reporter genes into tubular epithelial cells was shown in the mouse kidney. Of note, only the tubular epithelial cells, but not the glomerular or vascular cells, were transduced following in vivo AAV serotype 2 vector administration. This means that the tubular epithelial cells are highly susceptible to AAV serotype 2, but the reason for this phenomenon is unclear. Thus far, heparan sulfate as a primary receptor [27] and fibroblast growth factor receptor [28] and αvβ5 integrin [29] as coreceptors have been identified for AAV serotype 2. Therefore, the distribution of these molecules might explain the difference in transduction efficiency. Further investigations are required to determine the precise mechanisms of AAV-mediated gene transfer and to promote gene therapy approaches using AAV vectors in the kidney.

To establish a safe and clinically relevant approach, we developed the catheter-based method of AAV vectors to achieve an organ-specific gene delivery. Alternative approaches for kidney gene delivery have been also demonstrated. Lipkowitz et al. [26] reported that intraparenchymal injection of AAV serotype 2 vectors in the mouse kidney could transduce into tubular epithelial cells. Furthermore, successful gene delivery from renal vein or ureter into the kidney using naked DNA or HVJ liposomes was reported as other approaches for kidney-targeted gene transfer [2, 30, 31]. Since the approach developed in this study mimicked the clinical procedure and might be more physiological than alternative approaches, we believe that this procedure could be useful for future clinical applications.

In summary, we demonstrated that AAV vectors could transduce β-gal gene expression in renal cells in vitro and in vivo. In particular, the catheter-based direct gene delivery of AAV serotype 2 vectors caused successful gene transduction into the kidney tubular epithelial cells of rats and mice. The procedure demonstrated in this study has clinical advantages, because it seems to allow organ-specific gene delivery in the kidney. Since kidney-specific gene delivery via catheterization of the renal artery is highly feasible in humans, our findings provide useful information for promising strategies in renal gene therapy.

Acknowledgments

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RESEARCH ARTICLE

In situ generation of pseudotyped retroviral progeny by adenovirus-mediated transduction of tumor cells enhances the killing effect of HSV-*tk* suicide gene therapy *in vitro* and *in vivo*

Takashi Okada,[1]* Natasha J. Caplen,[2] W. Jay Ramsey,[3] Masafumi Onodera,[4] Kuniko Shimazaki,[5] Tatsuya Nomoto,[1] Rahim Ajalli,[1] Oliver Wildner,[6] John Morris,[7] Akihiro Kume,[1] Hirofumi Hamada,[8] R. Michael Blaese,[9] Keiya Ozawa[1]

[1]*Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan*
[2]*Medical Genetics Branch, National Human Genome Research Institute, National Cancer Institute, NIH, Bethesda, MD, USA*
[3]*Link Pharmaceuticals, Ames, IA, USA*
[4]*Department of Hematology, Institute of Basic Medical Sciences, University of Tsukuba, Japan*
[5]*Department of Physiology, Jichi Medical School, Tochigi, Japan*
[6]*Abt. f. Mol. u. Med. Virologie, Ruhr-Universit, Bochum, Germany*
[7]*Metabolism Branch, National Cancer Institute, NIH, Bethesda, MD, USA*
[8]*Biomolecular Medical Section, Biomedical Research Center, Sapporo Medical University, Sapporo, Japan*
[9]*PreGentis, Newtown, PA, USA*

*Correspondence to: Takashi Okada, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minami-kawachi, Tochigi 329-0498, Japan. E-mail: tokada@jichi.ac.jp

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Abstract

Background Hybrid adeno-retroviral vector systems utilize the high efficiency of adenovirus transduction to direct the *in situ* production of retroviral progeny. In this study, we show that a single-step transduction of glioma cells with trans-complementing hybrid adeno-retroviral vectors effectively turns these cells into retrovirus vector-producing cells, which in turn facilitates the transduction of adjacent cells.

Methods We have adapted the adeno-retroviral hybrid viral vector system to enhance the ganciclovir (GCV) killing of glioma cells following transfer of the herpes simplex virus thymidine kinase (HSV-*tk*) gene. To assess the effect of the *in situ* production of retroviral vectors on the transduction efficiency of glioma cells, 9L cells were transduced with adeno-retroviral hybrid vectors that separately express a retroviral genome (AVC2.GCEGFP or AVC2.GCTK) and retroviral packaging proteins (AxTetGP and AxTetVSVG). The generation of an integrated HSV-*tk* provirus by trans-complementation of the adeno-retroviral vectors was verified by analysis of the flanking retroviral LTR sequences. Tumors established on *nu/nu* mice were injected with the viruses followed by intraperitoneal injections of either PBS or GCV. We also estimated the copy numbers of the HSV-*tk* transgene present in the tumors of the treated mice. To determine the expression pattern of the HSV-*tk* transcripts within a tumor, *in situ* hybridization analysis was performed using an RNA probe specific for HSV- *tk*.

Results The co-transduction of rat 9L glioma cells with AVC2.GCEGFP together with vectors expressing packaging proteins of retroviruses increased the transduction efficiency. Transduction with AVC2.GCTK together with packaging vectors increased the *in vitro* sensitivity of cells to the pro-drug GCV by one log compared with control cells that were incapable of generating retrovirus. *In vivo*, the injection of established subcutaneous 9L tumors on athymic mice with a combination of AVC2.GCTK and packaging vectors followed by GCV treatment resulted in complete tumor regression in 50% of tumors at day 22, while no tumor regression was observed in control animals. Retroviral sequences diagnostic of 3′ LTR reduplication *in vivo* were detected in genomic DNA extracted from the transduced tumors, indicating pro-viral integration of the retroviral genome derived from the adeno-retroviral hybrid vector. Furthermore, the relative copy number of the HSV-*tk* gene in tumors treated with the adeno-retroviral vectors was up to ~250-fold higher than in control tumors. *In situ* hybridization suggested dispersion of the HSV-*tk*

product across a wider area of the tumor than in control tumors, which indicates the spread of the *in situ* generated retroviruses.

Conclusions Although the efficacy of this system has to be evaluated in orthotopic models, our observations suggest that this hybrid adeno-retroviral vector system could improve the suicide gene therapy of tumors. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords suicide gene therapy; adeno-retroviral hybrid vector; *in situ* generation; glioma; HSV-*tk*

Introduction

When tumor cells that have been transformed with the herpes simplex virus thymidine kinase (HSV-*tk*) gene are treated with the pro-drug ganciclovir (GCV), they regress [1]. However, the clinical benefit of this cancer gene therapy system is limited due to the poor efficiency of gene transfer [2]. To improve the therapeutic potential of this system, it is necessary to enhance the efficiency of the delivery of a therapeutic gene *in vivo* as well as to increase the stability of the expression of the gene.

We and others have previously described hybrid vector systems that use adenoviral vectors to deliver retroviral vector and packaging proteins into cells [3–9]. These systems benefit from the efficient gene transfer characteristics of adenoviral vectors as well as from the stable and long-term gene expression that is typical of retroviral vectors. The initial co-transduction of such adeno-retroviral hybrid vectors results in the transient production of recombinant retrovirus particles that then subsequently transduce neighboring cells. Adenovirus vectors expressing trans-complementing genes for retroviral proteins and retroviral vector RNAs have been successfully used for the *in situ* transduction of tumor cells [3,7].

We describe here an improved adeno-retroviral vector system that efficiently produces pseudotyped retroviral vectors. To simplify the construction of the chimeric vector carrying the retroviral genome, the directional ligation technique with the DNA–protein complex was employed [10]. We show that our adeno-retroviral packaging system can be used to rescue an integrated retroviral provirus in tumor cells and to enhance the therapeutic gene expression in glioma cells. In addition, we demonstrate that an adeno-retroviral HSV-*tk* vector system coupled with GCV enhances GCV-mediated killing of glioma cells both *in vitro* and *in vivo*. This suggests that this strategy produces sufficient levels of vectors *in situ* for the killing of solid tumors.

Materials and methods

Plasmid construction

The hybrid adeno-retroviral vector plasmid pAVC2.GCE-GFP was constructed as follows. The 3′ long terminal repeat (LTR) (*Cla* I-*Nde* I fragment) of the Molony murine leukemia virus (MMLV)-based retroviral vector pGCsap was replaced with the 3′ LTR (*Cla* I-*Nde* I fragment) of the myeloproliferative sarcoma virus (MPSV) to form pGCsapM. To generate pGCsapMEGFP, the *Nco* I-*Not* I fragment from pEGFP-N1 (Clontech Laboratories, Palo Alto, CA, USA) containing cDNA encoding the enhanced green fluorescent protein (EGFP) was cloned into *Nco* I-*Not* I-digested pGCsapM. This ensured that the EGFP translational initiation site was precisely located at the *env* translational start site found in the wild-type MMLV retrovirus. The retroviral vector GCsapMEGFP cassette was then cloned as an *Asc* I–*Xba* I-fragment into *Asc* I–*Xba* I-digested pAVC2.LXSN [5], from which the LXSN retroviral vector genome had been removed, to generate pAVC2.GCEGFP (Figure 1A).

The hybrid adeno-retroviral vector plasmid pAVC2.GC-TK was derived from pGCsam [11]. The full length coding region of the HSV-*tk* cDNA contained in the *Spe* I-*Cla* I fragment from pAVS6TK [12] was subcloned into pBluescript SK (+) and then cloned as a *Not* I–*Cla* I fragment into *Not* I–*Cla* I-digested pGCsam, which generated pGCsamTK. To construct pAVC2.GCTK (Figure 1A), the retroviral vector GCsamTK cassette was cloned as an *Asc* I–*Xba* I fragment into *Asc* I–*Xba* I-digested AVC2.LXSN.

Generation of adenovirus vectors

The recombinant hybrid adeno-retroviruses AVC2.GCE-GFP and AVC2.GCTK were generated using the mutant adenovirus type 5, dl327 [13]. A DNA–protein complex of dl327 was prepared as described previously [10]. The dl327 DNA–protein complex was digested with *Cla* I and *Bst* Z17 and treated with bacterial alkaline phosphatase (LTI, Grand Island, NY, USA) at 37 °C for 30 min. The enzymes were removed by three cycles of dilution with TE (pH 8.0) followed by concentration by using a Centricon YM-100 column according to the manufacturer's instructions (Amicon, Beverly, MA, USA). The AVC2.GCEGFP and AVC2.GCTK cassettes were excised from their respective plasmids (pAVC2.GCEGF and pAVC2.GCTK) by *Rca* I and *Bst* Z17 digestion and purified by agarose gel electrophoresis (QIAquick gel extraction kit; Qiagen, Hilden, Germany). The purified

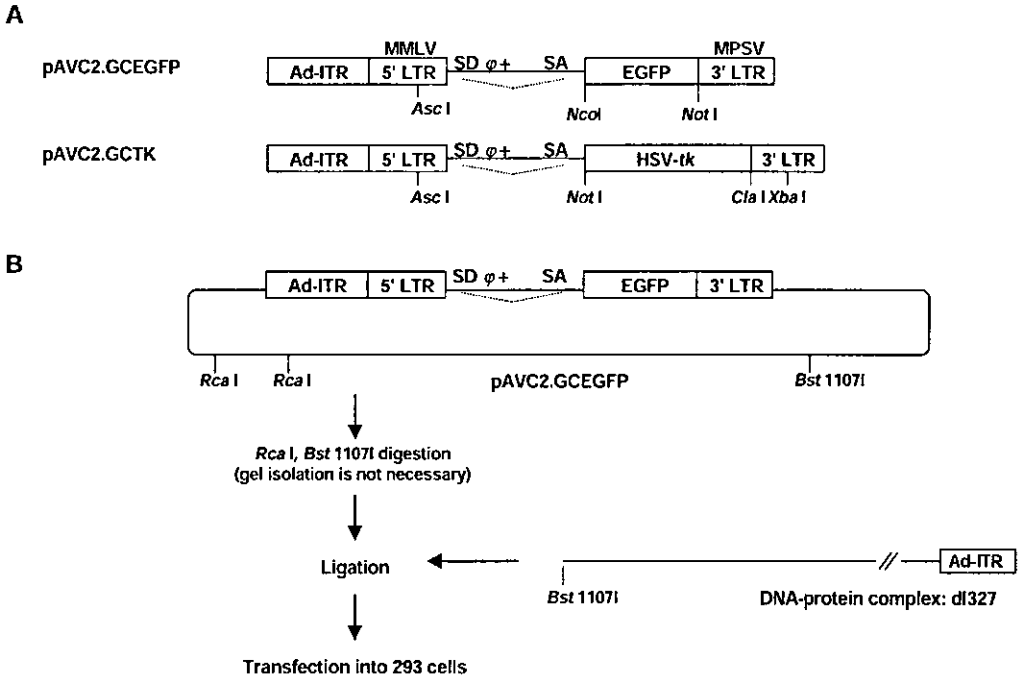

Figure 1. Schematic representation of the adeno-retroviral hybrid vectors used in this study. (A) The adeno-retroviral hybrid vector plasmids containing retroviral vector genomes expressing either GFP (AVC2-GCEGFP) or HSV-*tk* (AVC2-GCTK). SD, splice donor; SA, splice acceptor. (B) Cloning of recombinant adeno-retroviral hybrid vectors using directed ligation of a linearized vector plasmid into a DNA–protein complex in a single step without homologous recombination

fragments were mixed with 0.2 μg of *Cla* I- and *Bst* Z17-digested dl327 DNA–protein complex (Figure 1B) at a molar ratio of 3 : 1 and ligated with 5 Weiss U of T4 DNA ligase (New England Biolabs, Beverly, MA, USA) in a volume of 10 μl overnight at 9 °C. The ligated samples were desalted by using a Centricon YM-100 column and transfected into 293 cells by calcium phosphate co-precipitation, after which the cells were overlaid with agar as previously described [14]. The infectivity of the ligated DNA–protein complex was approximately 10 plaque-forming units/μg of DNA–protein complex for both vectors. Virus plaques were isolated and expanded in 293 cells. All viruses screened by PCR for the presence of the inserted retrovirus vector genome were positive. A control adenovirus vector AVC2.null with no expression gene cassette was constructed and propagated as described before [10]. The adenoviruses AxTetGP and AxTetVSVG expressing the MMLV gag-pol and the G protein of vesicular stomatitis virus (VSV-G) envelope protein, respectively, have been described previously [15]. The expression of the gag-pol and VSV-G proteins is under the control of the transcriptional regulator reverse Tet-controlled transactivator (rtTA) that is supplied by the adenoviral vector AV-rtTA [7]. These adenoviral vectors were propagated and purified as described previously [14].

Cell lines

The amphotropic retroviral vector producer cell line FLYA13 [16], the rat glioma cell line 9L [17], the human glioma cell line D54 [18], and the human embryonic kidney cell line 293 [19] were cultured in Dulbecco's modified Eagle's medium (DMEM high glucose; 4.5 g/l, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37 °C, 10% CO_2. To express the transgene from AxTetGP and AxTetVSVG, the cell culture medium was supplemented with 1 μg/ml doxycycline (Sigma, St. Louis, MO, USA). The GFP reporter cell line D54GFPneo was established by transfecting the retroviral expression plasmid pGC-GFP-loxP-EN [20] into D54 cells by calcium phosphate co-precipitation [21] followed by selection of independent GFP-positive colonies with 0.5 mg/ml G418 (Life Technologies).

Viral transductions

The cells were transduced with adenoviruses for 3 h at a variety of multiplicities as described for each experiment. The cells were washed with growth medium supplemented with 1 μg/ml of human serum γ-globulin (Miles Research Products Division, Elkhart, IN, USA) as a source of neutralizing antibody against adenovirus (these washes significantly reduce the carryover of residual adenoviral vectors in newly synthesized retroviral supernatants [5]). Finally, growth medium supplemented with human serum γ-globulin (1 μg/ml) and doxycycline (1 μg/ml) was added to each flask.

Putative retroviral-containing supernatants were harvested at the indicated time and centrifuged at 700 *g*, 4 °C,

filtered (0.45 μm), aliquotted and stored at −80 °C. For retroviral transduction, target cells were plated in 6-well plates at 5×10^4 cells per well and incubated overnight at 37 °C in 5% CO_2. Duplicate retroviral transductions were conducted at 37 °C by plating 2 ml of undiluted viral supernatant supplemented with polybrene (8 μg/ml) for 48 h.

FACS analysis

Fluorescence-activated cell sorting (FACS) analysis was performed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) equipped with an argon gas laser. A standard band-pass filter was used to determine green fluorescence intensity. Approximately 5×10^4 cells from each sample were analyzed. The FL1 signal was acquired in logarithmic mode, and the data were analyzed with CellQuest software (Becton Dickinson).

RNA dot blot analysis

To estimate the retroviral supernatant titers, RNA dot blot analysis was performed as described previously [22]. Briefly, nylon membranes (Hybond N^+; Amersham Life Sciences, Arlington Heights, IL, USA) were soaked for 10 min in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) and placed onto a manifold dot blot apparatus (Schleicher & Schuell Inc., Keene, NH, USA). To avoid detection of the adenovirus DNA, supernatant (180 μl) without denaturing procedure was transferred onto nylon membranes using the manifold dot blot apparatus and vacuum suction. The membranes were cross-linked by UV and hybridized with a MMLV *psi* cDNA probe that had been randomly labeled with [α^{32}P] dCTP (Megaprime DNA labeling systems, Amersham Life Sciences). The membranes were subjected to phosphoimager analysis, and dot blot densities were measured with a Bio imaging analyzer (BAS1500; Fuji Photo Film Co., Ltd., Tokyo, Japan). Dot densities were expressed as relative pixel intensity and retroviral titers were determined by comparison with the known expression titer in a supernatant of the retrovirus vector LASN [23].

Adenovirus detection in the retroviral supernatants

To assay for the presence of biologically active adenovirus in the retroviral supernatants, 293 cells (1×10^4) were incubated with 200 μl of samples for 72 h. The presence of adenovirus was assessed by monitoring for evidence of cytopathic effects and the detachment of the cells 2 weeks later.

GCV sensitivity assay

The killing effect of GCV (Syntex, Palo Alto, CA, USA) was measured by ^{3}H-thymidine incorporation [24]. Briefly, 5×10^4 cells were cultured in 96-well flat-bottomed microtiter plates in the presence of varying concentrations (0–50 μM) of GCV. After 42 h the cells were pulsed with 0.25 μCi per well of ^{3}H-thymidine (Amersham International, Bucks, UK) and harvested 6 h later. The effect of GCV on tumor cell proliferation is expressed as a percentage of the thymidine incorporation found in the identical cultures not treated with GCV.

Molecular analysis

To confirm the integration of a retrovirus vector genome rescued by trans-complementation of the hybrid vectors, PCR analysis was conducted with high molecular weight DNA extracted from the target cells 14 days post-transduction (DNA extraction kit, Qiagen Inc.). A PCR-amplified DNA product (560 bp) was extended from the 5′ LTR or the 3′ LTR of the retroviral vector (sense primer 5′ AGGGCCAAGAACAGATGAGACAGC 3′) to a region downstream of the 5′ LTR (antisense primer: 5′ GTACAGACGCAGGCGCATAACATC 3′). Conversion of the 3′ LTR to the 5′ LTR after provirus integration was confirmed by digestion with *Xba* I. The PCR product was transferred to a nylon membrane (Hybond N^+; Amersham Life Sciences) by the capillary transfer method.

Vector treatment *in vivo*

Athymic female BALB/c *nu/nu* mice (Clea Japan, Tokyo, Japan) were inoculated subcutaneously with 9L cells (3×10^6 cells) in 100 μl Hank's balanced salt solution containing 25% (v/v) basement membrane matrix (Matrigel; BD Biosciences, Franklin Lakes, NJ, USA). The tumors were allowed to grow *in vivo* to an average volume of 70 mm^3 (tumor volumes were calculated as $a \times b^2 \times 0.5$ where a is the length and b is the width of the tumor in millimeters). The established subcutaneous tumors were injected with a control vector (group 1, AVC2.null), a therapeutic vector (group 2, AVC2.GCTK), a combination of vectors without the *gag-pol* expressing vector (group 3, AVC2.GCTK, AVC2.null, AxTetVSVG, and AV-rtTA), or the complete set of trans-complementing adenoviral vectors that rescues the retrovirus (group 4, AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA). In each group, 1.0×10^9 plaque-forming units (pfu) of each virus were administered. Viruses were injected at four sites in each tumor with 100 μl of the virus solution in total. The animals received doxycycline as a 10 mg/ml solution in 5% sucrose via their water bottles for a period of 3 days starting 24 h after virus administration. When the tumor volume reached an average volume of 100 mm^3, the tumor-bearing animals were treated with an intraperitoneal injection of GCV at 30 mg/kg or phosphate-buffered saline (PBS) twice a day for 14 consecutive days. Tumors with an average volume of 100 mm^3 require more than 2.0×10^9 pfu of HSV-*tk* expression adenovirus vector for their complete

elimination [12]. Tumor growth was monitored two to three times a week by measuring two perpendicular tumor diameters using calipers. The total observation time was 2 months after the virus injection. Animals with tumors larger than 1 cm in diameter were euthanized. Tumor elimination that continued for more than 4 weeks was considered to indicate complete regression.

Determination of HSV-*tk* transgene copy number

To estimate the amplified copy number of the transgene caused by the production of retroviral progeny, PCR analysis was conducted on high molecular weight DNA extracted from tumors (DNA extraction kit, Qiagen Inc.). Using pAVS6TK [12] as a standard, an HSV-*tk*-specific nucleotide sequence (nucleotides +418 to +737) was amplified by PCR. Primers were chosen with the assistance of the computer program Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). We performed BLAST searches against dbEST and nr (the non-redundant set of GenBank, EMBL, and DDBJ database sequences) to confirm the total gene specificity of the nucleotide sequences chosen as primers. The specificity of the primer sets was confirmed by sequencing the PCR amplification products cloned into the pGEM-T vector (Promega, Madison, WI, USA). Quantitative values were obtained from the threshold cycle (*Ct*) number that indicated exponential amplification of the PCR product (ABI PRISM 7700 sequence detection system; Applied Biosystems, Foster City, CA, USA). To normalize each sample, we also quantified the copy number of the *GAPDH* gene. The relative target gene copy number was also normalized with a calibrator (tumors treated with AVC2GCTK alone). The final result, expressed as *N*-fold differences in target gene copy number relative to the GAPDH gene and the calibrator, was determined by the following formula: $N\text{target} = 2^{\text{correctedΔCt(GAPDH−TK)}}$. *Ct* values of the sample were determined by subtracting the average *Ct* value of the target gene from the average *Ct* value of the *GAPDH* gene.

In situ hybridization

Tumors from mice subjected to intratumoral injection with adenovirus along with intraperitoneal PBS treatment were removed 14 days after the injection and *in situ* hybridization was performed using an antisense RNA probe specific for the HSV-*tk* transcripts. Tissue specimens from subcutaneous tumors were fixed for 12 h in 4% paraformaldehyde at 4 °C, cut into 30-μm sections and mounted on APS-coated slides. Using pAVS6TK [12] as a template, an HSV-*tk*-specific nucleotide sequence (nucleotides +418 to +737) was amplified by PCR and cloned into pGEM-T vector (Promega). The HSV-*tk* RNA probes were synthesized and labeled with digoxigenin (DIG)-labeled deoxyuridine triphosphate by using a DIG RNA-labeling kit (F. Hoffmann-La Roche Ltd, Basel, Switzerland) according to the manufacturer's instructions. A DIG-labeled antisense RNA probe was obtained by using DIG RNA labeling mixture with a *Spe* I-cut linearized template and T7 RNA polymerase. Similarly, a sense probe was prepared for negative control experiments by using an *Nco* I-digested template and SP6 RNA polymerase with the DIG RNA labeling mixture.

The 30-μm tumor sections were pretreated with 100 μg/ml proteinase K at 37 °C for 20 min. These sections were rinsed with 2 mg/ml glycine and then incubated with hybridization buffer containing 100 ng of labeled RNA probe in a moist chamber at 42 °C overnight. After hybridization, the sections were washed in 1 × SSC for 10 min at room temperature, 0.2 × SSC for 20 min at 42 °C, 2 × SSC for 2 min at room temperature, and digested with 10 μg/ml RNase A at 37 °C for 20 min. The sections were incubated in 0.5% blocking reagent for 60 min and then in a 1 : 100 dilution of alkaline phosphatase conjugated anti-DIG antibody for 60 min in a moist chamber, followed by rinsing with Tris-buffered saline. The alkaline phosphatase reaction was visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. The sections were counterstained with methylgreen and coverslipped for light microscopy.

Results

Adeno-retroviral hybrid vectors can rescue an integrated retroviral genome from human glioma cells

To determine if an integrated retroviral genome could be rescued from glioma cells by use of our adeno-retroviral hybrid system, a human glioma cell line harboring a retroviral vector expressing GFP (D54GFPneo) was infected with a combination of adenoviral vectors that express proteins required for retroviral packaging proteins. Two trans-complementing adeno-retroviruses were used. AxTetGP expresses the MMLV gag-pol and AxTetVSVG expresses the VSV-G, which acts as the retrovirus envelope protein. In addition, a third adenovirus (AV-rtTA) was employed to regulate the expression of these viruses. As a control system in which retrovirus production does not occur, the AxTetGP virus was replaced by the non-expressing adenovirus vector AVC2null. Cells were infected with the appropriate adenovirus vectors for 3 h at various multiplicities of infection (MOIs). Free adenovirus was removed by washing cells with medium supplemented with human γ-globulin. Putative retrovirus-containing supernatants were harvested 48 h post-adenoviral transduction and used to transduce rat glioma 9L cells (Figure 2). Analysis of the 9L cells showed significant levels of GFP 2 days after the initiation of transduction (Figure 2A). In contrast, target 9L cells exposed to supernatants obtained from D54GFPneo cells transduced with the null adenoviral

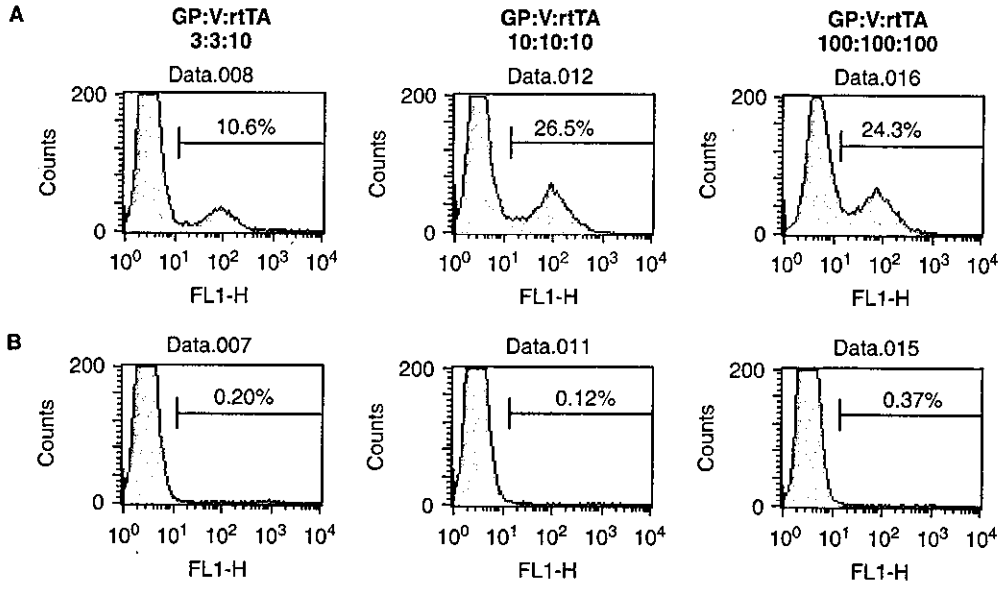

Figure 2. Rescue of an integrated GFP-expressing retroviral vector in 9L glioma cells by transduction with trans-complementing adeno-retroviral hybrid vectors. Putative retrovirus-containing supernatants were obtained from a human glioma cell line carrying an integrated retrovirus (D54GFPneo) following transduction with trans-complementing adeno-retroviral hybrid vectors at various MOIs. The putative retroviral supernatants were used to transduce 9L glioma cells so that the production of progeny retroviruses carrying the integrated GFP gene could be detected. (A) FACS analysis of 9L cells exposed to supernatants from D54GFPneo cells that had been transduced with all of the adeno-retroviral vectors required for the generation of a retrovirus, namely, AxTetGP (GP), AxTetVSVG (V), and AV-rtTA (rtTA). (B) FACS analysis of 9L cells exposed to supernatants from D54GFPneo cells transduced with all of the adeno-retroviral vectors except for the retroviral gag-pol-expressing vector, which was replaced by the control AVC2.null vector

vector plus the adenoviral vectors expressing the VSV-G envelope protein and the rtTA showed little or no GFP expression (Figure 2B). The percentage of target cells that were transduced reached a plateau when an MOI of 10 was used for each of the input adenoviruses. No biologically active adenovirus was detected in any of the supernatants.

In situ transduction efficiency of the adeno-retroviral hybrid vector system in glioma cells

To assess the effect of the *in situ* production of retroviral vector on the transduction efficiency of glioma cells, 9L cells were transduced with adeno-retroviral hybrid vectors expressing a retroviral genome (AVC2.GCEGFP) together with all of the retroviral packaging proteins (AxTetGP, AxTetVSVG, and AV-rtTA). Four days after the adenoviral transduction, a higher percentage of the cells were GFP-positive when they had been exposed to all of the trans-complementing adeno-retroviral vectors compared with cells exposed to AVC2.GCEGFP alone or when AxTetGP was replaced by the non-expressing adenoviral vector AVC2.null (Figure 3A). To confirm the long-term transgene expression from the retrovirus progeny generated by the adeno-retroviral vectors, we compared 9L cells transduced by all of the required adeno-retroviral hybrid viruses with those transduced by the control vector combination (AVC2.GCEGFP, AVC2.null, AxTetVSVG, and AV-rtTA). These cells were mixed with non-infected cells at a ratio of 10% transduced cells to 90% non-transduced cells and passaged every 7 days. Two weeks after the transduction, GFP reporter gene persistence and expression were analyzed by fluorescent microscopy. The GFP-positive cells from the group infected with AVC2.GCEGFP, AxTetGP, AxTetVSVG, and AV-rtTA were present in a clustered outgrowth after long-term culture, suggesting local retroviral spread and clonal origin (data not shown). This was in contrast to the cells infected with the control combination of adenoviruses (AVC2.GCEGFP, AVC2null, AxTetVSVG, and AV-rtTA), which showed attenuated GFP expression.

To determine how long cells can produce retrovirus after being infected with the vectors, a time course experiment was performed. The 9L cells were infected with all of the required adeno-retroviral hybrid viruses (AVC2.EGFP, AxTetGP, AxTetVSVG, and AV-rtTA) at an MOI of 3 in the presence of doxycycline. The titers increased and reached nearly 4×10^5 TU/ml by day 2, were maintained at similar levels until day 4, and then decreased (Figure 3B). At day 6, retrovirus production was still observed at a titer of 9×10^4 TU/ml. Thus, infected 9L cells continued to sustain retrovirus production for at least 6 days post-infection. The retention of transgene expression over time in infected cells was determined by evaluating the percentage of EGFP-positive cells by FACS analysis. The percentage of EGFP-positive cells increased over time, while the control group reached plateau at day 4 (Figure 3C).

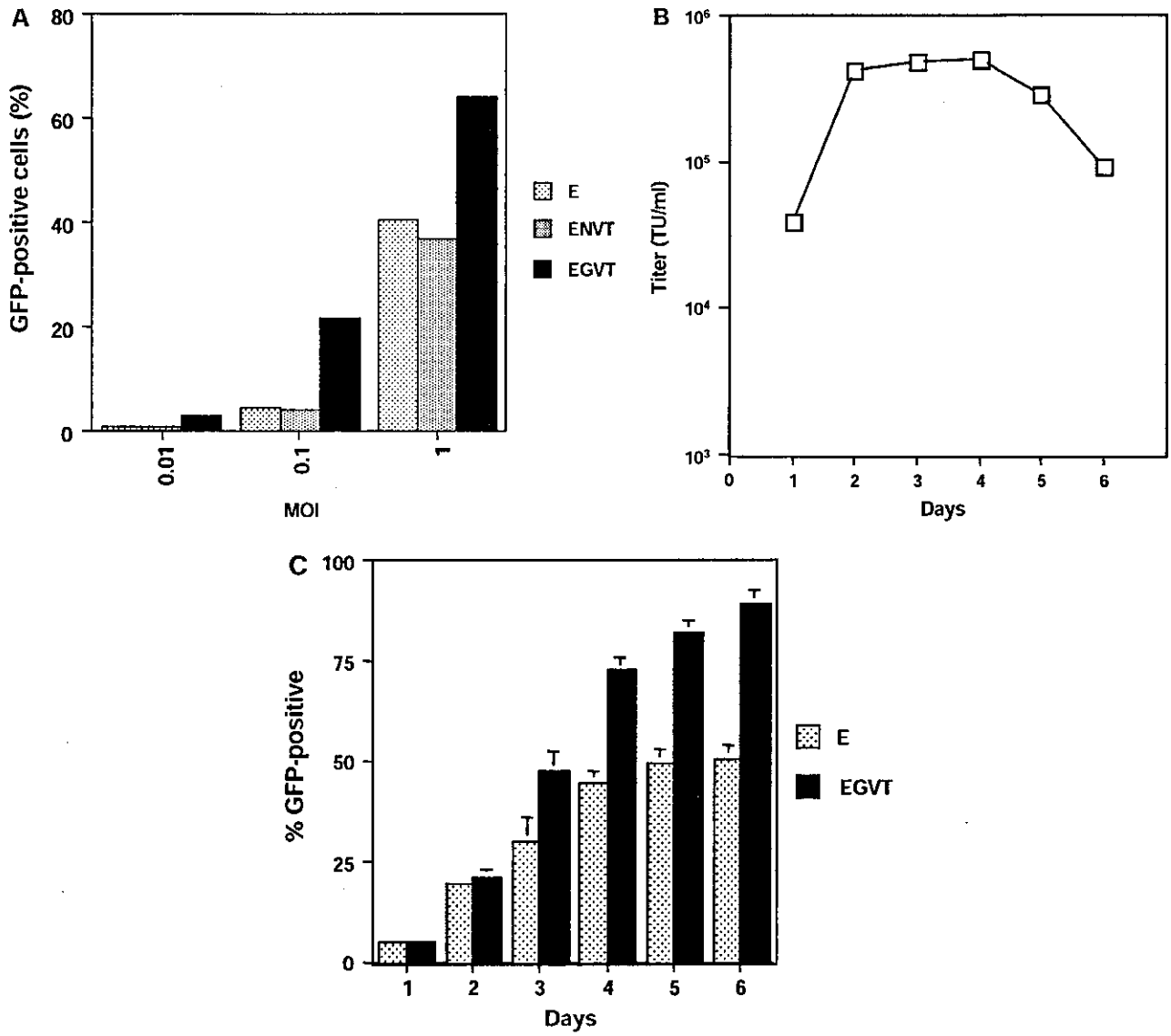

Figure 3. Transduction efficiency of the adeno-retroviral hybrid vector system in 9L cells. (A) Rat 9L glioma cells were infected with the trans-complementing adeno-retroviral vectors at a variety of MOIs (0.01, 0.1, and 1 of each virus) in the presence of 1 μg/ml doxycycline. The hybrid adeno-retroviruses used were as follows: AVC2.GCEGFP alone (E), AVC2.GCEGFP, AVC2null, AxTetVSVG, and AV-rtTA (ENVT), or AVC2.GCEGFP, AxTetGP, AxTetVSVG, and AV-rtTA (EGVT). Cells were harvested for FACS analysis of GFP expression 4 days after transduction. (B) Time course of retroviral production by 9L cells infected with the hybrid vectors. Cells were infected with AVC2.GCEGFP, AxTetGP, AxTetVSVG, and AV-rtTA at an MOI of 3 of each virus in the presence of doxycycline. At different time points, the medium was replaced and the supernatant was titrated for EGFP expression on 293 cells. Data shown represent means from three independent experiments. (C) The percentage of EGFP-positive cells transduced with the hybrid vectors was determined at various time points by FACS. Cells were infected with AVC2.GCEGFP (E) or with AVC2.GCEGFP, AxTetGP, AxTetVSVG, and AV-rtTA (EGVT) at an MOI of 3 of each virus in the presence of doxycycline. Data shown represent means and standard deviations from three independent experiments

Characterization of an adeno-retroviral vector carrying the HSV-*tk* gene

To demonstrate that the retroviral vector cassette within AVC2.GCTK could be used as a retroviral genome template, the retroviral producer cell line FLYA13 was transduced with the adeno-retroviral hybrid vector AVC2.GCTK at an MOI of 10. The retroviral supernatants harvested 48 h post-transduction gave a titer of 1×10^7 TU/ml, which is consistent with titers obtained using this cell line for the conventional production of retrovirus [5].

To examine the generation of the progeny retroviruses in glioma cells using this AVC2.GCTK adeno-retroviral vector system, we transduced rat glioma 9L cells with this vector and the trans-complementing adenovirus carrying the retroviral gag-pol and VSV-G proteins at an MOI of 10 for each, followed by doxycycline treatment. The retroviral titer of supernatants harvested 4 days after transduction, as measured by RNA dot blot analysis, was estimated to be 4×10^5 TU/ml. This experiment was performed several times using different pools of supernatants and produced similar results in all cases.

The HSV-*tk* adeno-retrovirus enhances GCV pro-drug killing of glioma cells

To assess if the killing effect of HSV-*tk* and GCV is enhanced as a result of the use of our adeno-retroviral hybrid vector system, a GCV-sensitivity assay was performed using a mixture of transduced and non-transduced 9L cells at a ratio of 1:19 or 1:9. Three different groups of transduced 9L cells were studied: (1) cells transduced with AVC2.null alone (MOI 40); (2) cells transduced with AVC2.GCTK, AVC2.null, AxTetVSVG, and AV-rtTA (MOI of 10 each); and (3) cells transduced with AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA) (MOI of 10 each). The cells were analyzed for GCV sensitivity 48 h post-transduction. A dose range of GCV was used (0.01–100 μM). Representative data are shown in Figure 4. In the presence of GCV, infected cells mixed with non-infected cells at a ratio of 5% to 95% that were transduced with all the adeno-retroviral vectors except that AxTetGP, which is required for the generation of retrovirus, had an IC50 value of 46.8 μM (Figure 4A). In contrast, 9L transduced with the progeny-producing combination of adeno-retroviruses had an IC50 value of 1.1 μM. The increase in sensitivity to GCV that the ability to produce progeny conferred to the 9L glioma cells is thus more than one log. Similar results were obtained when infected cells were mixed with non-infected cells at a ratio of 10% to 90% (Figure 4B).

Proviral integration

The generation of an integrated HSV-*tk* provirus by trans-complementation of the adeno-retroviral vectors was verified by analysis of the flanking retroviral LTR sequences. Typical retrovirus reverse transcription and integration resulted in the re-duplication of the 3′ LTR of the retrovirus to form the 5′ LTR (Figure 5A). The 5′ and 3′ LTR sequences of the retroviral vector in the original adeno-retroviral vector differed slightly as the 5′ LTR was derived from MMLV and the 3′ LTR was from MPSV. Re-duplication of the 3′ LTR as the result of retroviral transduction, reverse transcription, and integration should result in the generation of two LTRs with the same sequence. To assess if the expected re-duplication of the 3′ LTR occurred following the transduction of 9L cells with the trans-complementing adenoviruses and the AVC2.GCTK adeno-retroviral vector, we extracted high molecular weight cellular DNA from the cells transduced with AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA. A PCR product of the expected size was amplified from the DNA sequences within the LTR to a region downstream of 5′ LTR. *Xba* I digestion of the PCR product resulted in the generation of fragments of the expected size, which suggests that the 3′ LTR had been converted into the 5′ LTR following the generation of progeny retrovirus (Figure 5B).

Therapeutic effect of the HSV-*tk* adeno-retroviral vector system *in vivo*

To assess if intratumoral administration of the trans-complementing HSV-*tk* adeno-retroviral vectors leads to the *in situ* production of a retrovirus expressing HSV-*tk* and thus enhanced sensitivity to GCV killing, 9L tumors established in *nu/nu* mice were injected with either AVC2.null alone (n = 20, group 1), AVC2.GCTK alone (n = 20, group 2), AVC2.GCTK, AVC2.null, AxTetVSVG, and AV-rtTA (n = 20, group 3), or AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA (n = 16, group 4). Subsequently, half the animals in each group (n = 10 or 8 in each group) received intraperitoneal injections of either PBS or GCV. Figure 6 shows the tumor size in *nu/nu* mice after vector administration. Compared with treatment with combinations that are insufficient for retrovirus production, treatment with the ideal combination of vectors significantly inhibited the growth of the established tumors (group 4 vs. group 3 plus GCV, $p < 0.05$ by the

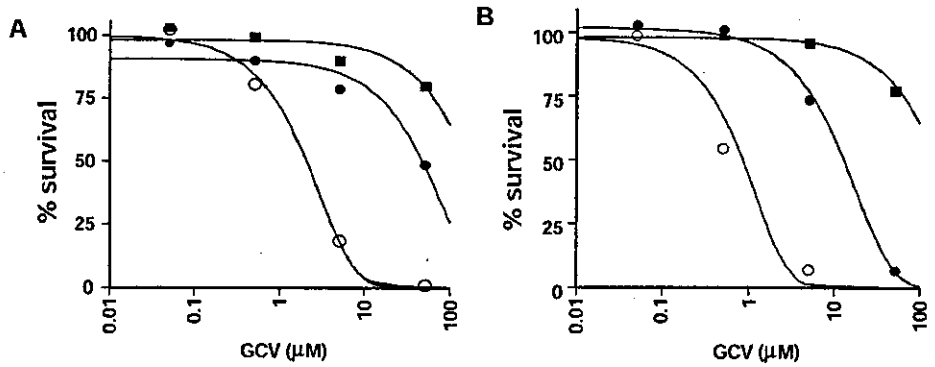

Figure 4. Enhanced killing of rat 9L glioma cells following transduction with adeno-retroviral vectors. GCV-sensitivity assays were performed using mixtures of transduced and non-transduced 9L cells at a ratio of 1 : 19 (A) or 1 : 9 (B). Three different groups of transduced cells were used, namely, (1) AVC2.null at an MOI of 40 (■); (2) AVC2.GCTK, AVC2null, AxTetVSVG, and AV-rtTA (MOI of 10 for each) (●); and (3) AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA (MOI of 10 for each) (○). The transduced 9L cells were harvested 48 h post-transduction and the killing effect of GCV was measured by ^{3}H-thymidine incorporation. The effect on tumor cell proliferation is expressed as a percentage of the thymidine incorporation found in identical cultures that had not been treated with GCV