Original Paper

Adeno-Associated Virus-Mediated Gene Transfer to Renal Tubule Cells via a Retrograde Ureteral Approach

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Adeno-associated virus · Gene therapy · Kidney injury

Abstract
Background/Aims: Gene therapy involves delivery of exogenous DNA to provide a therapeutic protein. Ideally, a gene therapy vector should be non-toxic, non-immunogenic, easy to produce, and efficient in protecting and delivering DNA into target cells. ***Methods:*** Adeno-associated virus (AAV) offers these advantages and few, if any, disadvantages, and over 100 isolates exist. We previously showed that AAV-mediated gene therapy can be used to restore vision to patients with Leber's congenital amaurosis, a disease of childhood blindness. ***Results:*** Here we show that novel recombinant AAV2/8 and AAV2/9 transduce kidney tubule cells with high efficiency both in vitro in cell culture and in vivo in mice. In addition, we adapted and modified a retrograde approach to allow for optimal transgene delivery to renal tubular cells that further minimizes the risk of an immunogenic reaction. ***Conclusions:*** We believe that recombinant AAV2, especially AAV2/8, gene delivery to renal tubule cells via a retrograde approach represents a viable method for gene therapy for a multitude of renal disorders ranging from autosomal dominant polycystic kidney disease to acute kidney injury.

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Introduction

Gene therapy involves delivery and targeting of exogenous DNA into cells to provide a therapeutic protein. Though no renal disease has yet successfully been treated in animals or patients using gene therapy, this modality holds great promise [1]. Ideally, a gene therapy vector should be non-toxic, non-immunogenic, easy to produce, and efficient in protecting and delivering DNA into the target cells. Exogenous DNA can be delivered with non-viral or viral vectors, each offering advantages and disadvantages. For example, although non-viral vectors have low toxicity, their entrapment in endosomes with subsequent destruction of the cargo DNA, low efficiency, and transient expression currently limits their use [2].

The use of viruses ensures highly efficient gene transfer to mammalian cells, as viruses have evolved specialized mechanisms for cell binding and intracellular delivery of DNA. Some viruses, however, such as adenoviruses, can evoke severe and even fatal immunogenic responses [3]. Retroviruses and lentiviruses have a tendency to integrate near transcriptionally active genes resulting in leukemia-like syndromes in recipients [4]. On the other hand, adeno-associated virus (AAV) has many properties of an ideal gene therapy vector:

(1) AAV is non-toxic and AAV particles are endemic to human populations (up to 40% of adult humans in the Philadelphia area are seropositive), with no known clinical sequelae [5].

(2) AAV particles have been selected by millions to billions of years of evolution for the delivery of DNA to cells, with features selected for endosomal entry, internal trafficking/tropism for the cell nucleus, sizing to enter nuclear pores, payload delivery and degradation.

(3) The AAV 'part list' is complete, with the AAV capsid being composed of 60 subunits, comprising three closely related viral proteins (VP1, 90 kDa; VP2, 72 kDa, and VP3, 60 kDa), held together in a weakly bonded network [6].

(4) The receptor interaction is understood, with FGF and $\alpha v\beta 5$ being facilitators of host cell endocytosis, at least for AAV2 [7, 8].

(5) The cell entry mechanism and path taken inside the cell are understood. For example, the mechanisms governing AAV2 entry (endosomal pathway) and internal trafficking are known [9, 10].

(6) AAV is engineerable by means of molecular biology, making it possible to optimize these particles for cell-specific delivery of genetic material, for minimizing immunogenicity, for tuning stability and particle lifetime, for efficient degradation, and for accurate delivery to the nucleus, for example.

(7) Nine natural serotypes and >100 additional isolates are available. AAV capsids can be exchanged using molecular engineering to generate novel recombinant AAVs, thus greatly increasing the chances of identifying an AAV that will optimally transduce a tissue of interest, in our case kidney tubule cells.

Importantly, we showed that gene therapy with AAV can be used to restore vision to patients with Leber's congenital amaurosis, a disease of childhood blindness [11, 12].

The kidney is accessible to gene delivery by different routes, including via the renal artery, injection into the parenchyma, and retrograde injection via the ureter. Retrograde injection is an attractive route for treating a variety of diseases and disorders affecting renal tubule cells, as these cells are readily accessible by retrograde ureteral injection. Equally important, retrograde ureteral injection further minimizes the potential for a toxic immunologic reaction. We demonstrate here the feasibility of transducing renal tubule cells via the retrograde route and obtaining rapid transgene expression with recombinant AAV serotypes, especially AAV2/8.

Materials and Methods

Cell Culture

Low passage type I Madin-Darby canine kidney (MDCK) cells were obtained from Keith Mostov (University of California, San Francisco) and used between passages 3 and 10.

Western Blot

Media from MDCK cells transduced with recombinant AAV encoding FLAG-tagged EGF-containing fibrillin-like extracellular matrix protein 1 (EFEMP1) were collected. Using antibody against the FLAG tag of EFEMP1, immunoprecipitation was performed and the protein solubilized in radio-immunoprecipitation assay buffer. Total protein content of the homogenate was determined using a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, Ill., USA) and EFEMP1 levels were determined using Western blot as described in the Results section.

Animals

Adult (4-week-old) C57BL/6 mice were used for all studies. All experiments were performed in compliance with institutional regulations and were approved by the Institutional Animal Care and Use Committee. Under anesthesia, the kidney was surgically exposed and AAV virus was injected as described in the Results section.

Histology

Kidneys were removed following carbon dioxide-induced euthanasia and immediately fixed in 4% paraformaldehyde/PBS. They were cryoprotected in 30% sucrose/PBS overnight and frozen in optimal cutting temperature compound (Fisher Scientific, Pittsburgh, Pa., USA). For each kidney, serial sections were cut with a cryostat (Reichert Jung model 822; Leica Microsystems, Wetzlar, Germany) and counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Vectashield mounting medium; Vector Laboratories, Burlingame, Calif., USA). All were mounted and analyzed with a Leica DME microscope (Leica Microsystems) equipped with epifluorescence, and images were captured with a Hamamatsu digital camera and Openlab 2.2 image analysis software (Improvision, Boston, Mass., USA), as previously described [13].

Production of AAV Vectors

Since the capsid protein of AAV is responsible for its tropism (and thus efficacy), a pseudotyping strategy was used enabling the packaging of an AAV genome into the capsid of another serotype [13]. For production of recombinant AAVs (e.g. of the AAV2 genome), the AAV2 genome was packaged into an AAV1, 2, 5, 6, 7, 8 or 9 capsid using a triple transfection method, as described previously [13, 14]. Viruses were concentrated via a heparin column or with 3× CsCl banding on a density gradient [15]. Titers were determined by real-time polymerase chain reaction [16]. The vectors carried transgene cassettes under the control of a cytomegalovirus (CMV) promoter.

Results

Efficient Transduction of Polarized Kidney Tubule Cells

To determine which AAV would best transduce renal tubular epithelial cells, MDCK strain I cells were cultured as confluent epithelial monolayers on Transwell filters and exposed to a panel of AAVs. The AAV2 serotype had the best transduction efficiency in this
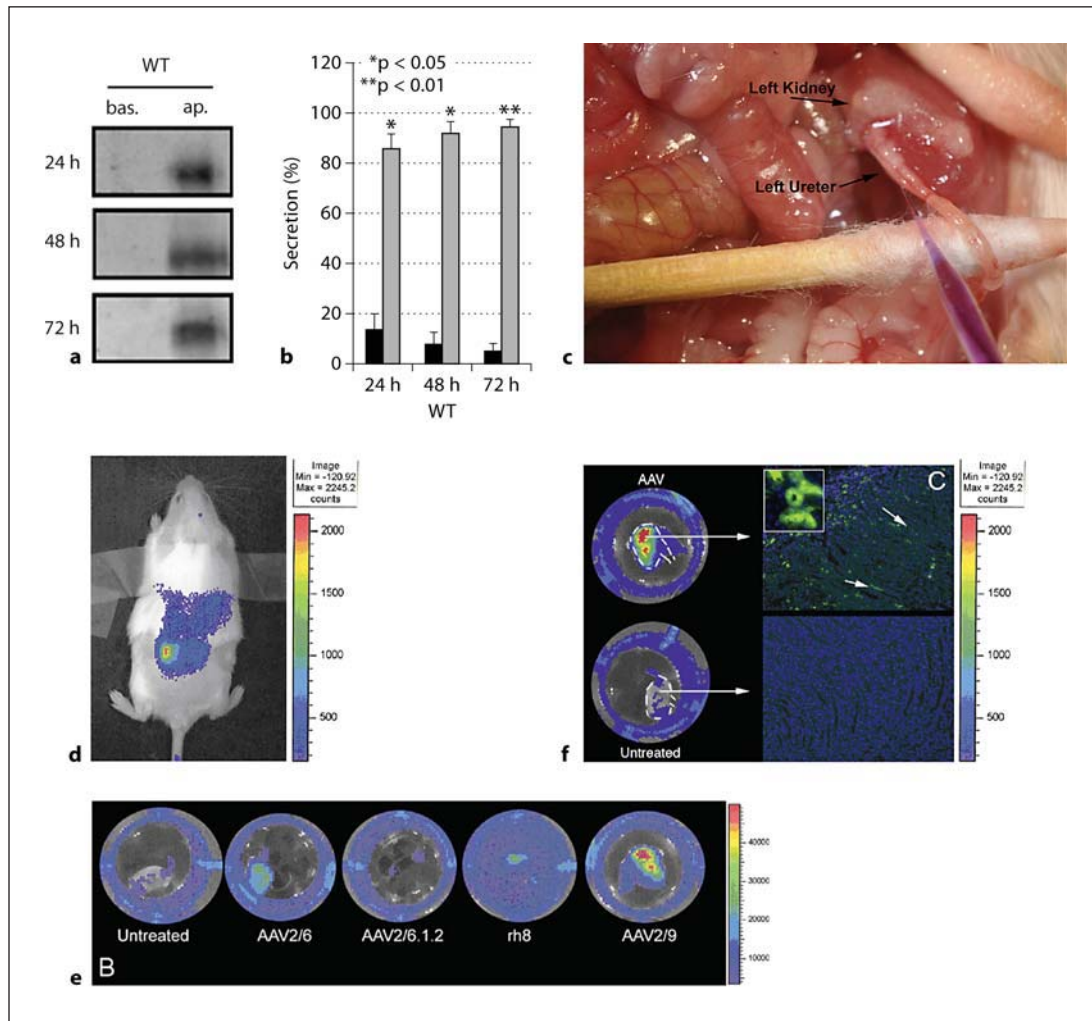
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Fig. 1. a Efficient transduction of polarized MDCK strain I cells is seen with AAV2/5.EFEMP1-FLAG. MDCK strain I cells were cultured as confluent epithelial monolayers on Transwell-Clear membranes and were exposed to AAV2/5 carrying WT FLAG-tagged EFEMP1: AAV2/5.EFEMP1-WT. Directional (apical; ap.) secretion of EFEMP1-WT was observed through immunoprecipitation of basal (bas.) and apical media. **b** Apical secretion persisted through 72 h after infection (the latest time point evaluated). Apical = Gray; basal = black bar. **c** Retrograde intra-ureter injection targeting renal tubule cells. Four-week-old WT mice were anesthetized and a 2-cm flank incision was made to expose the left kidney. The ureter was clamped below the renal pelvis to prevent the solution containing AAV from flowing in the opposite direction of the kidney. Using a 30-gauge glass needle pipette and a microinjection apparatus, 50 μl of PBS containing 1×10^9 genome copies of recombinant AAV2 virus were injected into the pelvis of the kidney. The viral medium contained a colored dye to help visualize the injection. After 5 min, the ureter clamp was released to allow urine flow to resume and to prevent obstruction of the kidney. **d–f** Reporter gene expression in the kidney after in vivo administration of novel AAVs carrying CMV.Luciferase (**d**) or CMV.EGFP (**e**, **f**). **d** Non-invasive imaging of luciferase bioluminescence using the Xenogen IVIS system 2 weeks after injection of AAV2/9.CMV.Luciferase to the left ureter. **e** EGFP fluorescence in kidneys dissected from representative animals 3 weeks after delivery of the designated AAV to the ureter. Scale bars (**d–f**) show intensity of transgene expression, with the red color indicating the highest levels. **f** Histological studies reveal high levels of EGFP in renal tubular epithelial cells in the medulla and in the cortex (not shown) after retro-ureteral delivery of AAV.EGFP, but not in the contralateral untreated kidney. Dashed lines on the left side of the panel delineate the kidney and ureter. Arrows indicate strongly positive EGFP-expressing tubules (inset ×120), while diffuse lower level EGFP expression is also seen. Nuclei are stained with DAPI (blue).

system, and recombinant AAV2-based viruses (AAV2/2, 2/1, 2/5, 2/6, 2/7, 2/8 and 2/9) were further studied. For example, MDCK strain I cells growing as confluent monolayers on Transwell filters were exposed to recombinant AAV2/5 virus encoding wild-type (WT) FLAG-tagged EFEMP1: AAV2/5.EFEMP1-WT. Directional secretion of EFEMP1-WT was observed through immunoprecipitation of basal and apical media (fig. 1a). Apical secretion persisted through 72 h after infection (the latest time point evaluated; fig. 1b). As anticipated, EGFP (enhanced green fluorescent protein), delivered by transduction of additional aliquots of cells via AAV2/5.EGFP, was not observed in the media as EGFP, an intracellular protein, and is not secreted (data not shown).

Recombinant AAV Serotypes Delivering Reporters by Retrograde Injection

AAV-mediated gene transfer was then tested in vivo by delivering 10^9 genome copies of the candidate AAV2 serotypes carrying CMV.EGFP or CMV.Luciferase via retrograde injection into the kidneys of WT mice. Retrograde injection involved a 2-cm left lateral incision under inhaled isoflurane anesthesia to expose the left kidney. The left ureter was isolated and clamped to prevent the vector solution containing AAV from flowing in the opposite direction of the kidney. PBS (50 μl) containing 1×10^9 genome copies of AAV virus was injected into the ureter, just distal to the pelvis of the kidney, with a fine bore glass pipette attached to a nitrogen-powered microinjection/infusion apparatus. The viral medium also contained 0.5 μl of 0.25% fast green dye to help visualize the injection (fig. 1c).

Two to 3 weeks after injection, animals were imaged for luciferase activity using a heat probe in sedated living mice (fig. 1d), or mice were euthanized, the kidneys harvested, cryopreserved, sectioned, and evaluated for EGFP expression (fig. 1e, f). AAV2/8 and AAV2/9 serotypes resulted in high levels of reporter gene expression specific to the targeted kidney; lower levels of transgene expression were detected after injection of AAV2/6, AAV2/6.1.2, rh8 (fig. 1e) and other recombinant AAV2 (not shown).

Histological evaluation revealed that EGFP was efficiently and specifically expressed in renal tubule cells in the kidney region exposed to virus (fig. 1f). There was no evidence of inflammation or an immune response relating to the presence of AAV capsid antigens or reporter proteins, and, by immunofluorescence, there was no evidence of hematological spread of the vector (data not shown).

Discussion

We report two principal findings. First, we show excellent transduction efficiency of renal tubule cells using recombinant AAV2 serotypes, such as AAV2/8, in vitro in cell culture and in vivo in mice using two different reporters, luciferase and GFP. While a number of viruses, including lentivirus, adenovirus, and AAV serotypes 1–5 have been tested in vivo in the kidney, none have resulted in as efficient or as stable transduction of tubule cells as we found with the recombinant virus AAV2/8 [2, 17–19]. AAV-WT has been used to deliver transgenes to renal tubule epithelial cells [20]; however, recombinant AAV2/8 offers superior delivery. In addition, a problem with AAV-WT, which does not apply to recombinant AAVs, such as AAV2/8, is that transgene expression after transduction is delayed [14].

Our second principal finding is that we can specifically and efficiently deliver AAV2/8 to renal tubule epithelial cells via a modified retrograde approach. While retrograde injection of the ureter has been used to introduce reporter genes [18], it has not, to our knowledge, been used with AAV, and transduction efficiencies have been lower. An obvious advantage to a retrograde approach is that, if successful in animal trials of renal tubular disease, it could be scaled up to humans, and AAV2/8 encoding the appropriate cargo could be injected in a

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minimally invasive manner by urologists using ureteroscopy. Since many kidney diseases are bilaterally symmetrical, it is possible to perform controlled experiments targeting renal disease by using one kidney as the experimental kidney and the contralateral kidney as the control. Candidate diseases for treatment include autosomal dominant polycystic kidney disease and acute kidney injury.

If successful in humans, gene delivery using AAV2/8 injected in a retrograde manner would represent a novel treatment for the many disorders that manifest in renal tubule epithelial cells. This is especially important given that there are currently no approved treatments other than supportive care for many renal tubular disorders, including autosomal dominant polycystic kidney disease and acute kidney injury.

Acknowledgments

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