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Review

Adeno-associated virus vector-mediated gene delivery to the vasculature and kidney✪

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Relatively successful elsewhere, gene delivery aimed at the vasculature and kidney has made very little progress. In the kidney, the hurdles are related to the unique structure–function relationships of this organ and in the blood vessels to a variety of, mostly endothelial, factors making the delivery of transgenes very difficult. Among gene-therapeutic approaches, most viral gene delivery systems utilized to date have shown significant practical and safety-related limitations due to the level and duration of recombinant transgene expression as well as their induction of a significant host immune response to vector proteins. Recombinant adeno-associated virus (rAAV) vectors appear to offer a vehicle for safe, long-term transgene expression. rAAV-based vectors are characterized by a relative non-immunogenicity and the absence of viral coding sequences. Furthermore, they allow for establishment of long-term latency without deleterious effects on the host cell. This brief review addresses problems related to transgene-delivery to kidney and vasculature with particular attention given to rAAV vectors. The potential for gene therapy as a strategy for selected renal and vascular diseases is also discussed.

Keywords: gene therapy, viral vectors, recombinant adeno-associated virus, endothelium, vasculature, kidney

GENE THERAPY

In its beginnings the applications for gene therapy were based on the concept that a clinical disorder is caused by closely defined "faulty" gene(s) (e.g., adenosine deaminase (ADA) deficiency, sickle cell anemia, cystic fibrosis) and that it might be prevented, controlled or even cured (Anderson, 1998). Several clinical trails employing such gene replacement therapy have been completed, with a limited degree of progress reported for such single gene disorders as hemophilia, cystic fibrosis, and ADA deficiency. The progress has been disappointing, predominantly due to the inefficiency of the gene transfer vectors. Further impediment for the establishment of gene transfer as a standard therapy is the fact that most diseases are polygenic, involving multiple genetic factors with type 1 diabetes being a prime example where at least 18 separate chromosomal regions have been associated with susceptibility to this disorder (reviewed in Atkinson & Maclaren, 1994).

Faced with this scenario, gene therapy has outgrown the original definition of single gene implantation utilizing a unique vector to one involving transfer of many potential elements, either *in vivo* or *ex vivo*, and not necessarily with the gene thought to cause the clinical disorder. Logically, the number of clinical syndromes subject to treatment *via* gene therapy has expanded and has included complex conditions like transplantation, infectious disease, cancer, and disorders involving immune regulation. This change in conceptual approach along with advances in the vector design has re-established a renewed sense of optimism to the field of gene therapy. While therapeutic benefits of gene therapy may eventually be observed with one of a number of methods for gene delivery (e.g., adenovirus, retrovirus, naked DNA, etc.), this review will focus predominantly on recombinant adeno-associ-

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Abbreviations: AAV, adeno-associated virus; AAV2, AAV serotype 2; ADA, adenosine deaminase; GFP, green fluorescent protein; HSPG, heparan sulfate proteoglycan; rAAV, recombinant adeno-associated virus.

ated viral (rAAV) vectors for gene delivery that has recently seen a marked increase in both interest and enthusiasm.

RECOMBINANT AAV AS DELIVERY VECTOR

The adeno-associated viruses are members of the parvovirus family. There are eleven known serotypes of AAV with the designations AAV1-8. Of these, AAV7 through AAV11 have only recently been described (Gao *et al.*, 2002; 2004; Mori *et al.*, 2004). Thus far AAV serotype 2 (AAV2) has been the most extensively studied serotype. rAAV vectors have been constructed by removing the *rep* and/or *cap* genes and inserting the foreign DNA of interest (Hermonat & Muzyczka, 1984; Tratschin *et al.*, 1984). The early steps of AAV2 infection involve attachment to a variety of cell surface receptors (HSPG, fibroblast growth factor receptor and α_v-β_5 integrin) followed by a clathrin-dependent or independent internalization process (Summerford & Samulski, 1998; Qing *et al.*, 1999; Summerford *et al.*, 1999).

AAV generally requires a helper virus (usually an adenovirus or herpes virus) or a physical or chemical insult to undergo second strand synthesis and productive replication *in vitro* and *in vivo* (Ferrari *et al.*, 1997). Infection with wild-type AAV leads to the establishment of a long-term latency which is due primarily to site-specific integration in the AAVS1 site on human chromosome 19, although some forms can persist as episomal forms or are integrated at other sites (Giraud *et al.*, 1994; Berns & Linden, 1995). On the other hand, rAAV vectors persist in cells as a combination of episomal forms and random site integrants (Afione *et al.*, 1996; Ponnazhagan *et al.*, 1997). Importantly, rAAV retains the propensity of the parental virus to establish long-term latency without any observed ill effects in the host cell. It is this unique capacity of the virus to establish stable latency without toxicity that gives rAAV the properties required to serve as a useful vector for long-term gene transfer. rAAV vectors have been shown to be highly efficient for gene transfer and expression at a number of different sites *in vitro* and *in vivo*.

A drawback of rAAV vectors is the size limitation for packaging (<5 kb) which restricts the use of these vectors to the insertion of small genes. However, recent advances in vector engineering of AAV may be able to overcome this barrier. For smaller transgenes, a successful concomitant transfer of two genes using a bicistronic rAAV vector has been recently demonstrated (Kapturczak *et al.*, 2002). Stable expression and safety has been demonstrated in studies performed in the respiratory tract (Flotte *et al.*, 1993; Conrad *et al.*, 1996; Wagner *et al.*, 1998; 1999), the central nervous system (Kaplitt *et al.*, 1994; McCown *et al.*, 1996), skeletal muscle (Kessler *et al.*, 1996; Xiao *et al.*, 1996; Fisher *et al.*, 1997; Song *et al.*, 1998), and several other sites. Recently, the efficiency of rAAV-mediated transduction has increased as the titer and purity of rAAV preparations has improved (Kapturczak *et al.*, 2001). In particular rAAV transduction of the muscle, brain, and retina, has been shown to be consistently very efficient and very long-lived, essentially lasting for the life span of the animal in numerous rodent studies. Also, rAAV appears to be relatively non-immunogenic (Jooss *et al.*, 1998), although recent studies suggest that the immune response in genetically predisposed species/strains may be underestimated (Zhang *et al.*, 2004). Due to its growing success, rAAV has been used to deliver genes of potential therapeutic relevance in human clinical trials of gene therapy in hemophilia B, cystic fibrosis and α-1-antitrypsin deficiency (Kay *et al.*, 2000; Flotte *et al.*, 2003; 2004).

Although the AAV2 has been so far the most utilized vector, recent attention has been given to the other serotypes. Several studies have demonstrated that alternative serotypes of rAAV, particularly AAV1 and AAV5, have higher transduction efficiencies in tissues such as skeletal muscle, retina, pancreatic islets, liver and neurons (Chiorini *et al.*, 1999; Alisky *et al.*, 2000; Chao *et al.*, 2000; Davidson *et al.*, 2000; Zabner *et al.*, 2000; Flotte *et al.*, 2001; Walters *et al.*, 2001; Auricchio *et al.*, 2002; Rabinowitz *et al.*, 2002). Each AAV serotype interacts with specific receptors and may account for the differential tissue tropism (Summerford & Samulski, 1998; Qing *et al.*, 1999; Kaludov *et al.*, 2001; Walters *et al.*, 2001; Hauck & Xiao, 2003).

rAAV AND VASCULATURE

The vascular endothelium participates in many physiological and pathophysiological processes including vascular permeability, angiogenesis, inflammation, tumor progression and rejection of transplanted organs (Gonzalez & Selwyn, 2003). Damage to the endothelium has been implicated in the pathogenesis of many diseases including atherosclerosis, hypertension, ischemic heart disease, diabetic microangiopathy, vasculitis, thromboembolic disorders and cancer. Thus, there is growing interest in targeting endothelial cells using gene therapy based techniques (Katusic, 2002; Crook & Akyurek, 2003; Work *et al.*, 2003). Products of transduced genes can not only affect the endothelium *per se* but also may influence the local and systemic milieu through paracrine or endocrine effects. However, limited progress has thus far been made in gene therapy strategies for endothelial cells. Transcytosis, regular replacement of endothelial cells and an abundance of extracellular matrix are but a few factors that

limit the efficacy of transduction in endothelial cells (Pajusola *et al.*, 2002; Pascariu *et al.*, 2004).

Despite the attractive properties of rAAV vectors, the success in using them as a means of vascular transgene delivery has been limited. Previous studies have reported varied and usually relatively low transduction of the vasculature using rAAV serotype 2 vectors especially as far as the endothelial cells are concerned (Gnatenko *et al.*, 1997; Maeda *et al.*, 1997; Richter *et al.*, 2000; Nicklin *et al.*, 2001; Dishart *et al.*, 2003; Vassalli *et al.*, 2003, Gruchala *et al.*, 2004) and are consistent with our recent experience (Chen *et al.*, 2005). Endosomal acidification and proteasomal activity may, at least in part, account for the limited transduction efficiency of rAAV2 vectors (Duan *et al.*, 2000; Nicklin *et al.*, 2001). On the other hand, recent studies by Pajusola and coworkers suggest that the extracellular matrix compartment of endothelial cells is a major barrier to efficient cellular transduction (Pajusola *et al.*, 2002). They have analyzed the expression pattern of HSPG, the primary receptor for AAV2, and demonstrated significant deposition of HSPG in the extracellular matrix of endothelial cells. Transduction with rAAV2 resulted in binding of the vector to the extracellular matrix rather than the cell surface thereby contributing to the low efficiency. Removal of the matrix component by infecting cells in suspension increased rAAV2-mediated transduction by 11-fold, suggesting that matrix associated HSPG is an important barrier to successful endothelial cell transduction (Pajusola *et al.*, 2002).

Recent studies have incorporated peptides identified by phage display into rAAV2 vectors and have reported modestly improved transduction in vascular cells and isolated arteries (Nicklin *et al.*, 2001; Muller *et al.*, 2003; White *et al.*, 2004; Work *et al.*, 2004). Nicklin *et al*. (2001) engineered a retargeted rAAV vector (AAVsig) by the incorporation of SIGYPLP, a heptamer peptide isolated from a linear phage display, into position I-587 of the AAV2 capsid. Transduction with AAVsig increased gene expression about 5.9-fold in human umbilical vein endothelial cells and 28-fold in human saphenous vein endothelial cells, while no transduction was observed in human saphenous vein-derived smooth muscle cells (Nicklin *et al.*, 2001). In separate studies these authors have developed rAAV2 vectors with a smooth muscle specific ligand (EYHHYNK) and have reported significantly higher transduction of venous and arterial smooth muscle cells, but not in endothelial cells (Work *et al.*, 2004). Muller *et al.* (2003) screened AAV serotype 2 libraries on human coronary artery endothelial cells and identified several specific peptide motifs. Intravenous injection of a vector carrying one such motif, NSSRDLG, in the AAV capsid, resulted in a five-fold higher selective transduction in the hearts of mice as compared to animals injected with wild-type vector (Muller *et al.*, 2003). Whether the coronary arteries were specifically transduced *in vivo* was not reported. White *et al.* (2004) incorporated human venous endothelial cell targeted peptides identified by phage display into AAV serotype 2 capsids after position 587 and reported higher transduction of venous endothelial cells, but not arterial endothelial cells.

Thus far several cell lines and tissues including muscle, liver, lung, retina, pancreatic islets and brain have been successfully transduced with rAAV1 and 5 with variable degrees of transduction efficiencies (Chiorini *et al.*, 1999; Alisky *et al.*, 2000; Chao *et al.*, 2000; Davidson *et al.*, 2000; Zabner *et al.*, 2000; Flotte *et al.*, 2001; Walters *et al.*, 2001; Auricchio *et al.*, 2002; Rabinowitz *et al.*, 2002). Studies by Zabner *et al*. (2000) have shown that rAAV5 was more efficient in transducing both human and murine airway epithelia compared to rAAV2 or rAAV4 and utilized 2,3-linked sialic acid residues as potential receptors (Walters *et al.*, 2001). In rat retina, serotypes 5 and 4 were shown to be the most efficient (Rabinowitz *et al.*, 2002). We have also observed that in comparison to rAAV2, the transduction efficiency using rAAV1 and 5 is significantly higher in pancreatic islets (Flotte *et al.*, 2001; Loiler *et al.*, 2003). In our most recent study (Chen *et al.*, 2005), we have demonstrated the superior efficacy of rAAV serotype 1 and 5 vectors to rAAV2 in transduction of primary vascular endothelial and smooth muscle cells *in vitro*, *ex vivo* and *in vivo* in rat aortic segments. It has been demonstrated that rAAV7-based vectors show transduction efficiencies similar to rAAV1 in skeletal muscle, and rAAV8 is far superior to the other serotypes in the liver (Gao *et al.*, 2002). An evaluation of rAAV serotype 7 and 8 in vascular cell transduction would be of great interest.

The differences in tissue tropism and transduction efficiencies of the various AAV serotype vectors suggest the requirement for specific receptors for virus uptake. The requirement of HSPG in conjunction with fibroblast growth factor-1 or α_v-β_5 integrin for AAV2-mediated transduction has been described above. Comparisons of transduction efficiencies in a variety of cells, lack of inhibition by soluble heparin, as well as co-transduction experiments have suggested that AAV5 utilizes a mechanism of uptake distinct from that of AAV2, leading to the identification of 2,3-linked sialic acid as a high affinity receptor for this vector (Walters *et al.*, 2001). This has been confirmed by the observation that binding of AAV5 is blocked by genetic or enzymatic removal of sialic acid from the cell surface. Our studies have also demonstrated the requirement of sialic acid residues in rAAV5-mediated transduction of endothelial cells (Chen *et al.*, 2005). The high transduction efficiency of rAAV5 in endothelial cells has prompted us to investigate the role

of sialic acid residues in rAAV1-mediated uptake in endothelial cells. Our studies based on enzymatic removal of sialic acid with neuraminidase (sialidase) on endothelial cells, suggest that sialic acid residues may be part of the receptor complex required for rAAV1 uptake (Chen *et al.*, 2005). Specifically, sialidase treatment blocks rAAV1-mediated transduction while heparin has no effect. These latter findings are consistent with the report of Hauck and Xiao showing that the heparin binding domain is not required for AAV1-mediated transduction in muscle (Hauck & Xiao, 2003). Further studies using re-sialylation experiments in endothelial cells will be necessary to confirm these observations.

In summary, although a significant progress has been made in the design of rAAV vectors, it is obvious that further studies and developments are needed. Depending on intended applications, choice of a particular AAV serotype and/or creation of specific capsid mutations allowing for selective endothelial receptor binding offer thus far the most promise for the use of rAAV vectors.

rAAV AND KIDNEY

As the vasculature creates seemingly enough difficulties to overcome, the hurdles for rAAV-based gene delivery are only multiplied in the kidney. The kidney is a highly vascularized organ receiving about 25% of the cardiac output. While this would seem to be a favorable factor for renal gene delivery *via* the vasculature, the anatomical architecture of the kidney consisting of over a million filtering units (in the human kidney) and a multitude of cell types, each with a specific function, has precluded significant advances in this area of investigation. The functional unit of the kidney is the nephron, which consists of a glomerulus and a tubular compartment enveloped by an intricate peritubular capillary network. The tubular compartment is made up of the proximal tubule, loop of Henle and the distal tubule. Although developmentally not a part of the nephron, the collecting duct that continues on from the distal tubule is an important component of the renal parenchyma and a potential target for gene therapy. Each of these compartments contains specialized cells that have specific function(s) and distinct transporters, channels and receptors on their apical or basolateral surfaces. Most kidney diseases result from dysfunction in specific regions and cell types in the nephron as a consequence of a local and/or systemic condition. For example, obstruction of the urinary tract (renal pelvis, ureter, bladder or urethra) resulting from a local condition (e.g. stone, fibrosis, cancer) leads to hydronephrosis and eventual scarring of the kidney and loss of function. Diabetes mellitus is an example of a systemic condition in which the initial kidney damage occurs in the microvasculature, specifically in the glomerulus. Unlike the heart, liver or the brain, the fact that the kidney is a paired organ is an additional barrier to gene therapy, particularly since most systemic diseases affect both kidneys. Thus, delivery of genes for most kidney diseases would require either a systemic approach or local gene delivery into both kidneys. An exception to this requirement is the transplant setting, where a single kidney is available *ex vivo* for potential genetic manipulation prior to transplantation.

Only a limited experience exists with the use of rAAV in the kidney. In one of the earlier reports, rAAV carrying green fluorescent protein (GFP) and/or β-galactosidase has been injected directly into the renal parenchyma of mouse kidneys and resulted in a transduction of renal tubular cells only along the needle track (Lipkowitz *et al.*, 1999). While encouraging, these results showed the limitations of the direct intra-parenchymal approach. It became obvious that other delivery strategies will be necessary for a gene delivery route to be of practical value in the kidney. In our studies, an intra-renal arterial delivery of a rAAV2-GFP vector resulted in a successful transduction of renal tubular epithelial cells (Chen *et al.*, 2003). Proximal tubule cells, specifically in the S_3 segment, as well as intercalated cells in the medullary collecting duct were transduced. No transduction was observed in blood vessels, glomeruli or the interstitium. The ischemia associated with the procedure of gene delivery caused mild changes of tubular injury and interstitial mononuclear cell infiltration and was observed in both saline and vector-treated animals, suggesting that these morphological changes were not due to an adverse immune response to the viral vector or the transgene.

The reasons for the preferential transduction of the proximal tubule and intercalated cells observed in our studies are not entirely clear. Several possible mechanisms may be implicated. It is possible that ischemic cell injury results in increased surface expression of receptors that facilitate AAV uptake in the proximal tubule, particularly in the S_3 segment, a region that is more susceptible to ischemic injury (Lieberthal & Nigam, 1998). Selective transduction of intercalated cells in the collecting duct was also observed. Because these cells are capable of apical endocytosis (Brown *et al.*, 1987) it is tempting to speculate that the vector might have gained access to the tubule lumen and subsequently been absorbed by endocytosis. Takeda and coworkers have confirmed our observations regarding the *in* vivo utility of rAAV2 to transduce epithelial cells (Takeda *et al.*, 2004). In addition to AAV2, they have also used four other serotypes of AAV and have demonstrated that, *in vitro*, the AAV serotype 1, 2, and 5 vectors have lead to transgene expression in

rat kidney epithelial NRK52E cells, whereas AAV serotype 3 and 4 vectors showed no effect. However *in vivo*, following a selective injection into the renal arteries of rats and mice, only AAV2 vectors resulted in transgene expression in the proximal tubule (Takeda *et al.*, 2004).

In summary, due to its tremendously complicated structure, the kidney remains a challenge for gene therapy as far as the development of appropriate techniques of vector delivery is concerned. Furthermore, the extensive heterogeneity of renal cellular components will require very specific targeting strategies in order to become a practical tool in treatment of specific renal disorders. Our laboratory is currently evaluating expression of transgenes using the different AAV serotypes and capsid mutants as well as specific promoter systems to optimize cell-specific gene delivery in the kidney.

SUMMARY AND FUTURE DIRECTIONS

Due to the multitude of diseases affecting the vasculature and the kidneys and health care impact, gene delivery-based therapeutic approaches will continue to be developed. Many vectors are currently being subjected to investigation in an attempt to improve gene delivery into these tissues. Viral based vectors have shown the ability to target and enter host cells with a high degree of efficiency and therefore form the subject of most such efforts, with recent improvements in the propagation and delivery of rAAV awakening major enthusiasm. Although the vasculature and kidney continue to pose major hurdles to rAAV-based vectors, the availability of different serotypes with varying tissue tropisms as well as the possibility of designing specific capsid mutations for selective cell targeting continue to provide reasons for optimism.

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