Systemic delivery of adeno-associated viral vectors

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Abstract

For diseases like muscular dystrophy, an effective gene therapy requires bodywide correction. Systemic viral vector delivery has been attempted since early 90s. Yet a true success was not achieved until mid-2000 when adeno-associated virus (AAV) serotype-6, 8 and 9 were found to result in global muscle transduction in rodents following intravenous injection. The simplicity of the technique immediately attracts attention. Marvelous whole body amelioration has been achieved in rodent models of many diseases. Scale-up in large mammals also shows promising results. Importantly, the first systemic AAV-9 therapy was initiated in patients in April 2014. Recent studies have now begun to reveal molecular underpinnings of systemic AAV delivery and to engineer new AAV capsids with superior properties for systemic gene therapy.

Highlights

Only intravascular delivery can truly change the course of systemic diseases

AAV has the capacity to escape from the blood and lead to bodywide gene transfer

Systemic AAV therapy has been achieved in rodents and large mammals

Molecular engineering can lead to new AAV capsids for enhanced systemic delivery

Introduction

Many life-threatening diseases affect a number of organs or affect tissues that are widely distributed. A successful gene therapy for these diseases requires a viral vector that can effectively reach all target cells throughout the body. Since our vessels are a built-in and ready-to-use system for bodywide transportation, a convenient strategy to achieve systemic delivery would be infusion of a therapeutic viral vector into the circulation. For this seeming straightforward method to work, a viral vector has to reach the target area, get out from the vasculature and infect the diseased cells.

A report in 1992 claimed to have achieved “widespread long-term gene transfer” to striated muscles in newborn mice using recombinant adenovirus [1]. The authors delivered adenovirus intravenously to 2 to 5-day-old mice and detected some expression in the liver, lung, heart and skeletal muscle. While the adenoviral vector had indeed spread to various tissues and organs, there were only sporadic transduction in skeletal muscle and ~0.2% transduction in the heart. This is far from 20 to 50% gene transfer efficiency required to treat skeletal muscle disease and cardiomyopathy in diseases like Duchenne muscular dystrophy (DMD)[2,3]. Several strategies were developed to overcome the endothelial barrier for systemic adenovirus delivery. These include the application of vessel dilator and permeabilizer, hydrodynamic injection and viral capsid modification [4-6]. Despite improved intravascular transduction of a single limb with pressurized infusion and endothelial permeabilization, adenoviral vectors eventually lost the favor for systemic delivery due to severe immune responses and fatal complications [7].

Over the last two decades, adeno-associated virus (AAV) has emerged and now become the most preferred vector for gene therapy [8-11]. AAV is a single stranded DNA virus discovered in 1965 [12]. It persists mainly as episomal molecules in infected tissues [13-15]. More than 12 different serotypes and hundreds of capsid variants have been isolated from adenoviral stocks and animal tissues or engineered in laboratories. In contrast to adenovirus, intramuscular injection of recombinant AAV serotype-2 (AAV-2) resulted in yearlong robust transduction with nominal cellular immune responses [16,17]. Strategies that have been shown to enhance adenoviral intravascular delivery

(such as pharmacological vessel permeabilization and forced extravasation) also resulted in uniform whole limb muscle transduction by AAV-2[4,18]. However, there remains a significant gap to achieve whole body gene transfer from peripheral vessels. A bona fide breakthrough in systemic gene delivery has to wait until new AAV serotypes are isolated.

Systemic gene delivery with AAV in rodents

In early days of AAV vector development, most studies are focused on AAV-2. Isolation of new serotypes has greatly expanded the repertoire [19-21]. Rutledge et al isolated AAV-6 from an adenovirus stock [22]. Gao et al isolated AAV-8 and AAV-9 from tissues of rhesus monkey and human, respectively [23,24]. These three serotypes open the door to a successful systemic delivery. Gregorevic et al showed efficient whole body striated muscle transduction in mice after tail vein injection of AAV-6 and the vascular endothelium growth factor for transient microvasculature permeabilization [25]. Wang et al achieved widespread saturated transduction of the heart as well as axial and appendicular muscles in mice and hamsters via systemic delivery of AAV-8[26]. Shortly after, successful bodywide systemic gene transfer was established for AAV-9(**Figure 1**) [27-29]. Interestingly, peripheral delivery of AAV-9 resulted in superior myocardial and central nervous system transduction. It is now clear that other AAV serotypes (such as AAV-1 and AAV-7) can also lead to systemic transduction (**Table 1**) [26,30,31]. Nevertheless, AAV-9 remains the most potent serotype for systemic delivery in rodents [30-32].

The establishment of systemic AAV delivery technique immediately raises the possibility for bodywide correction in rodent models of human diseases. Today, impressive results have been reported in neonatal, adult and even aged animals. Some of these examples include AAV-1 mediated gene therapy for Pompe disease, limb-girdle muscular dystrophy (LGMD) and myotonic dystrophy[33-35], AAV-6 mediated gene therapy for DMD and facioscapulohumeral muscular dystrophy[25,36-38], AAV-8-mediated gene therapy for DMD, LGMD and atherosclerosis[26,39-41], and AAV-9 mediated gene therapy for cardiomyopathy, lysosomal storage disorders and neuronal diseases [42-51].

The maximal packaging capacity of an AAV vector is ~5-kb [52-55]. This limits the use of AAV for a number of diseases including DMD and dysferlin-deficient myopathy. Various dual AAV strategies have been developed to overcome this hurdle (reviewed in [56-58]). Optimized dual AAV vectors have reached transduction efficiency of the single AAV vector [59,60]. Ghosh et al provided the first proof-of-principle evidence for efficient systemic dual AAV delivery in normal and diseased mice [61,62]. Subsequent studies from several laboratories showed unequivocal evidence that systemic dual AAV therapy is a viable option for bodywide alleviation for DMD and dysferlin-deficient myopathy [63-65].

Scale-up systemic AAV delivery in large mammals

The remarkable success in rodents and the convenience of the technique have stimulated tremendous interests in adopting systemic AAV delivery to large mammals. The first successful systemic AAV delivery to a large mammal was achieved with AAV-9 in newborn canines (**Figure 1**)[66]. Surprisingly, despite spectacular bodywide skeletal muscle transduction, few cardiomyocytes were transduced [66]. In sharp contrast, AAV-8 yielded robust transduction of both skeletal and cardiac muscles in dog puppies (**Figure 1**)[67,68]. AAV-1 and AAV-6 are two other serotypes that have shown good systemic transduction in rodents [26,30,31]. Recent studies suggest that mutating surface-exposed tyrosine can significantly enhance AAV transduction [69,70]. Hakim et al tested tyrosine modified AAV-1 and AAV-6 in neonatal dogs [71]. Interestingly, AAV-1 showed high efficient whole body striated muscle transduction but AAV-6 resulted in little muscle transduction (**Figure 1**). Two groups explored systemic AAV-9 delivery in newborn DMD puppies [72,73]. Gene transfer was observed in multiple muscles up to 4 months of age. However, Kornegay et al encountered a catastrophic inflammatory response potentially linked to the transgene product [73]. Contrary to Kornegay et al, Hinderer et al reasoned that neonatal period could be a window to induce immune tolerance to the transgene product [74]. Indeed, they were able to achieve this goal by systemic delivery of low-dose AAV-8 (30-fold lower than used by Kornegay et al) in rhesus monkeys and type I mucopolysaccharidosis dogs [74].

Very few studies have evaluated systemic AAV delivery to adult large mammals. An early work in cynomolgus monkeys suggests that even very low-dose AAV ($\leq 5 \times 10^{10}$ particles/kg) can result in vector genome accumulation in the spleen and expression in the lymph nodes [75]. Intriguingly, regional intravascular delivery of AAV to nonhuman primates is devoid of cellular immunotoxicity [76-78]. Several groups have shown successful regional limb perfusion with AAV-1 and 8 in normal and diseased dogs [79-82]. A breakthrough of systemic AAV transfer in a diseased adult large mammal has not been achieved until recently. Yue et al injected tyrosine mutant AAV-9 to young adult DMD dogs from a peripheral vein and observed efficient global skeletal and cardiac muscle transduction without serious complications (**Figure 1**)[83]. Hakim et al further extended this result demonstrating systemic delivery of an AAV-9 micro-dystrophin vector can lead to near saturated expression for at least 12 months without any toxicity in adult affected dogs [84].

The most exciting progress is the ongoing clinical trial in neonatal spinal muscular atrophy (SMA) patients using AAV-9 by Drs. Mendel, Kaspar and colleagues (clinical trial ID: NCT02122952)[85]. SMA is caused by mutations in the survival motor neuron 1 (SMN1) gene. Earlier studies from several groups have revealed remarkable therapeutic benefits in newborn SMA mice with AAV-9 mediated systemic SMN1 gene therapy [86-89]. In this game-changing clinical trial, fifteen 1 to 8-m-old patients received intravenous injection of up to 2×10^{14} particles/kg of the AAV-9 SMN1 vector. Some patients have been treated for almost two years. There was no major safety concerns. Importantly, high-dose group patients showed clinical improvement[85].

Mechanistic insights of systemic AAV delivery

A better understanding on the mechanisms of systemic AAV transduction is essential to further improve this important gene therapy technology. The major rate-limiting steps may include interaction with serum proteins, blood clearance, vessel escape, attachment, endocytosis, intracellular processing, nuclear entry and vector genome conversion (**Figure 2, Table 1**). The last five steps have been extensively reviewed elsewhere and will not be discussed here [90,91].

Interaction of AAV with circulating proteins greatly influences the outcome of systemic delivery. Inactivation by pre-existing neutralizing antibodies has been well documented. Recently, Denard et al found that some blood proteins bind to certain AAV serotypes in a species-specific manner [92,93]. In particular, AAV-6 interacts with the galectin 3 binding protein in human and dog sera but not macaque and mouse sera. This interaction aggregates AAV particles and hampers systemic delivery [92]. On the other side, AAV-6 interacts with the C-reactive protein in mouse but not human sera. Instead of inhibition, this interaction boosts systemic delivery [93].

Blood clearance varies dramatically among AAV serotypes [31,94]. While rapid clearance may not necessarily abort systemic delivery, prolongation of the circulation time certainly enhances it [31,94-96]. In this regard, delayed clearance has been suggested as a primary reason underlying pronounced systemic delivery of AAV-9 [94]. Shen et al investigated the underlying mechanisms for extended persistence of AAV-9 in blood and found that it is due to the low abundance of the AAV-9 receptor, hence reduced tissue binding [95]. It is very likely that the blood clearance of AAV is regulated by many factors. Additional studies may reveal these yet unknown factors. On the other side, future studies are also needed to explain why fast blood clearance of some AAV serotypes (such as AAV-8) has minimal impact on systemic delivery (**Table 1**).

Depending on the architecture of capillary, AAV may get out from blood via two different pathways, paracellular or transcellular (**Figure 2**). Paracellular transport refers to the escape of virus from the circulation through the space between adjacent endothelial cells. Capillaries in the liver and spleen are fenestrated and discontinuous (**Figure 2**). In these tissues, AAV can readily diffuse out through large gaps between endothelial cells. This paracellular mechanism contributes to the accumulation of the AAV genome in the liver following systemic delivery [25,27,30,31,83,97].

In the central nerve system, the tight junctions between neighboring endothelial cells form the highly selective blood-brain barrier (**Figure 2**). In this case, the only way to escape from the circulation is transcellular transcytosis (**Figure 2**). In this process, AAV is taken into endothelial cells in specialized vesicles [98]. These vesicles traffic to the other side of the cell and release virus into the interstitium. AAV transcytosis has

been documented *in vitro*[99-101]. Interestingly, AAV-8 and 9 show efficient transcytosis but AAV-6 does not [100-102]. Recent studies have revealed two distinctive mechanisms of transcytosis, dependent or independent of caveolae [103]. Kotchey et al found that systemic AAV-9 transduction is not compromised in caveolin-1 knockout mice. Since AAV-9 displays unparalleled superior neuronal tissue transduction when delivered through the peripheral vein [104], it is very likely that transvascular transport of AAV-9 is through caveolae-independent transcytosis [94].

Re-engineering AAV for improved systemic delivery

Despite the great promise of systemic delivery, the current technology remains limited. For example, a large proportion of humans are seropositive for known AAV serotypes [105,106]. Further, systemic delivery often leads to gene transfer in non-target tissues and organs. AAV transduction is largely determined by the viral capsid, especially variable loops on the surface[107-109]. Several strategies have been used to develop novel capsids for improved systemic delivery. These include (1) isolation and reconstruction from existing or ancestral species[110,111], and (2) modification by rational design and directed evolution[112,113].

Many new AAV isolates have been tested for systemic delivery recently. These studies have revealed some unique organism, organ, tissue, or cell-type specific transduction pattern after intravascular delivery. For example, AAV-3B showed superior hepatotropism in primate but not rodent liver [114-116]. AAV-4 showed selective cardiopulmonary tropism [117]. AAV-rh8 and rh10 are as efficient as AAV-9 in crossing the blood-brain barrier [118].

A hurdle to systemic AAV delivery is the high prevalence (40-80%) of pre-existing immunity in human populations (reviewed in [119]). While some successes have been achieved with the application of immune suppressive drugs (such as anti-CD20 antibody rituximab) and plasmapheresis [120-122], modification of the antigenic epitope on the capsid may yield neutralizing-resistant “designer” AAV variants. Several approaches have been used to map the neutralizing antibody binding epitopes for different AAV serotypes (reviewed in [123]). These studies suggest that protrusions around the 3-fold axis and 2/5-fold wall participate in interactions with neutralizing

antibodies [123]. Targeted mutagenesis of these residues may circumvent preexisting immunity[124,125]. An alternative and highly effective method to known epitope mutagenesis is forced evolution in the presence of high amounts of neutralizing antibodies (such as pooled immunoglobulins from human donors) (reviewed in [112,126]). This approach has allowed isolation of neutralizing antibody escaping AAV variants AAV-r2.15 by Maheshri et al and AAV-DJ by Grimm et al [127,128]. More recently, Li et al found that capsid variants isolated following in vitro selection in human serum had poor in vivo transduction strength although they were able to escape neutralization[129]. In vivo selection in the presence of the patient serum may yield escaping-capsids with better in vivo performance[129].

Blood clearance is a rate-limiting barrier in systemic delivery. The determinants for AAV-9 blood clearance were reported recently [94,95,130]. These mainly consist of surface-exposed amino acids and overlap with the receptor footprint. Mutations in these residues substantially shorten circulation half-life and reduce systemic transduction[94,95,130].

Delivery of a viral vector through the bloodstream will likely spread the virus to untoward tissues/organs. This raises safety concerns. Sequestration of AAV in these non-target locations also reduces the amount of vectors that can be delivered to the targets and hence has significant implications on the effective vector dose needed for systemic therapy. Tissue/cell-specific AAV will help resolve this problem. A series of tropism-modified AAV capsid variants have been developed using either in vivo evolution or educated engineering. These capsid chimeras are highly desirable for systemic gene therapy of various diseases, for example, liver-detargeted vectors for muscular dystrophy [96,130-132], liver-enhanced vectors for hemophilia [128,133], myocardium tropic vectors for cardiomyopathy [134], central nerve system-enhanced vectors for neurodegenerative diseases[135].

AAV uses cell surface carbohydrates as its binding receptors [136]. The nature and abundance of these extracellular glycans vary dramatically between different species and at different developmental stages. Since AAV attachment is a determining factor in systemic delivery, cautions should be taken to extrapolate re-engineered capsids for different applications [102,115,116,137].

Conclusion

The non-invasive nature and the convenience of peripheral vascular delivery promise a straightforward approach to treating a number of diseases. Many barriers have been overcome. However, there remain significant hurdles to translate the promise of systemic delivery into clinical benefits in human patients. Further, new challenges will surface as we learn more about AAV (such as the discovery of the universal AAV receptor) and begin to apply systemic delivery to new technologies (such as gene editing with the CRISPR technology) [138,139]. The study on systemic viral vector delivery has just reached its prime time and the best is yet to come.

Tables

Table 1. Comparison of AAV-2 with AAV-1, 6, 7, 8 and 9 for systemic delivery

Figure legends

Figure 1. Systemic AAV delivery results in bodywide gene transfer in rodents and large mammals. Peripheral vascular delivery provides a method that allows an AAV vector to reach most, if not every, part of the body. **A**, Bodywide muscle transduction in mice following tail vein delivery of an alkaline phosphatase (AP) reporter gene AAV-9 vector. **B**, Robust and persistent (up to one year) skeletal muscle and myocardial transduction after jugular vein injection of an AAV-8 AP vector in a neonatal dog. **C**, Tyrosine mutant AAV-9 results in whole body striated muscle transduction in young adult dystrophic dogs. Top panel, representative full-view images from selected skeletal muscles; middle panel, representative high-power images from selected skeletal muscles, heart and internal organs; bottom panel, quantification of the AAV genome and AP expression in selective tissues. BB, biceps brachii; Bra, brachialis; Dia, diaphragm; CT, cranial tibialis; ECR, extensor carpi radialis; FCU, flexor carpi ulnaris; FD, flexor digitorum; Gas, gastrocnemius; IS, interstitial septum; LV, left ventricle; LVa, left ventricle anterior portion; LVx, left ventricle apex; PM, papillary muscle; Sep, septum; TB, triceps brachii; Ter, teres; Ton, tongue; VM, vastus medialis.

Figure 2. Rate-limiting steps in systemic AAV delivery. The major rate-limiting steps include interaction with serum proteins (such as neutralizing antibodies), blood clearance, vessel escape, attachment, endocytosis, intracellular processing, nuclear entry and vector genome conversion. Capsid engineering can yield new AAV variants with enhanced systemic delivery properties. Numerical numbers highlight five rate-limiting barriers. Capillaries in the central nerve system (CNS) are sealed by the blood-brain barrier. Transcytosis is the only way for AAV to exit the vasculature in CNS. Capillaries in the liver and spleen are fenestrated and discontinuous. This allows for efficient paracellular diffusion of AAV into the parenchyma. Capillaries in muscles may allow for limited paracellular transport of AAV. However, transcytosis may likely be the primary pathway for AAV to get to muscle.

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Table 1. Comparison of AAV-2 with AAV-1, 6, 7, 8 and 9 for systemic delivery

	AAV-1	AAV-2	AAV-6	AAV-7	AAV-8	AAV-9	References
In vitro capsid stability	Moderate	Low	?	?	Moderate	?	140
Blood clearance	Fast	Fast	Fast	Fast	Fast	Slow	31, 94
Transcytosis	?	Poor	Poor	?	High	High	99-101
Direct muscle transduction efficiency	High	Moderate	Very high	High	Moderate	High~Very high	23, 129,
Systemic transduction efficiency	High	Low	High	High	High~Very high	Very high	26, 30-32
Unique features		Very efficient in cultured cells			Low immunity ^a	Cardiotropic in rodents ^b ; Cross BBB ^c	

a, See references 40, 82, and 141-147.

b, See references 27-29 and 30-32.

c, See references 85-89. BBB, blood-brain-barrier

Figure 1AB

Figure 1C

Figure 2 revised