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Viral Vector Mediated Gene Therapies

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Abstract

Purpose of review—Gene therapy as a treatment for neuromuscular disease has significantly advanced over the past decade. In the current review the progress of AAV vector mediated gene therapy for Duchenne muscular dystrophy (DMD) during the past year is highlighted.

Recent findings—Modulating the immune response to AAV vector capsid or the transgene has helped to increase stable transduction efficiency. Full-length dystrophin expression via gene editing with targeted nucleases may ultimately be an ideal treatment option. Also genes with homologues function may ameliorate many aspects of the DMD pathophysiology.

Summary—The work during the past year has increased our understanding of AAV vector mediated therapy and has also validated new approaches to treat DMD. The results will aid in the design of both preclinical and clinical trials.

Keywords

Duchenne muscular dystrophy; dystrophin; adeno-associated viruses; Cas9/CRISPR

Introduction

Vectors derived from adeno-associated viruses (AAV) are commonly used in gene therapy studies, primarily because the wild type AAV is not associated with any pathogenicity, the vectors typically have low immunogenicity and can maintain transgene expression over many years in non-dividing cells. Vectors are generated from an AAV virus by removal of all viral genes, including those required for replication, such that the resultant vectors are unable to replicate or integrate at a significant frequency into the host DNA. Therefore, the single stranded vector exists mainly as an episome in transfected cells. Dozens of AAV serotypes have been identified and many novel forms have been generated in the laboratory. Vectors derived from different AAV serotypes have distinct tissue tropism and transfection efficiencies. However all AAV vectors have a relatively small packaging capacity of about 5 kb. In this current review we discuss the development of viral vector mediated gene therapy with a focus on very recent progress relating to Duchenne muscular dystrophy (DMD).

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DMD is characterized by progressive muscle damage and wasting, leading to loss of ambulation in the second decade and death due to respiratory or cardiac failure typically in the third decade (1). DMD is caused by a mutation in the dystrophin gene resulting in the failure to produce a functional dystrophin protein. Approximately 1:3,500 live born boys are affected with DMD or the milder Becker muscular dystrophy (BMD), and one-third of all cases arise from a new mutation. The dystrophin gene is 2.2 megabases in length and contains 7 promoters linked to unique first exons that splice to up to 78 additional exons, making it one of the largest genes in nature (2).

Full-length dystrophin

The full-length ‘muscle’ isoform of dystrophin is 427 kDa and is encoded on a 14 kb mRNA. The large size of the protein and transcript has been a major obstacle to developing methods for gene therapy of DMD. However, numerous studies in both animal models and in the clinic have generated considerable knowledge about the structural domains in dystrophin, and have enabled rational design of highly functional mini- and micro-dystrophins more amenable to gene therapy applications [reviewed in (3)]. In particular, the development of miniaturized “micro-dystrophins” has enabled delivery of therapeutic cassettes to dystrophic muscles in animal models in early phase clinical trials using AAV vectors (4, 5).

Despite development of a variety of functional mini-dystrophins over the past decade by several labs, an ideal form of gene therapy would enable delivery or repair of the full-length dystrophin gene or cDNA. However, to date the only vector shown to be capable of delivering new genes to the muscles of adult mammals in a bodywide or ‘systemic’ manner has been AAV (6). Consequently, efforts to deliver larger or even full-length dystrophin cDNAs have revolved around splitting the full coding sequence into pieces that can be reconstituted into a larger clone in vivo after delivery by multiple AAV vectors. For example, expression of a mini-dystrophin encoded on a 7.3 kb cassette was achieved by co-delivery of two AAV vectors to muscles of *mdx* mice, an animal model for DMD. This approach generated a mini-dystrophin via homologous recombination of smaller dystrophin cassettes in vivo, and led to significant histological and physiological improvements to the mice (7). Utilizing a related approach, the full-length dystrophin cDNA was split onto 3 vectors designed to trans-splice and express full-length dystrophin (8, 9). While this three-vector approach enabled production of some full-length dystrophin in injected muscles, the efficiency was quite low since all three vectors have to transfect the same cell for the trans-splicing to work. Consequently, little functional benefit was observed using this approach. While the double and triple vector approaches demonstrate the potential for such methods to be further developed, the efficiency of micro-dystrophin vector delivery currently holds the most promise for an effective gene therapy in the foreseeable future. Indeed, several groups are currently preparing human clinical trials involving either local or systemic delivery of AAV/micro-dystrophin.

Tissue specificity of AAV Vectors

Effective gene therapy for DMD will require not only a functional micro dystrophin, but also a vector that can deliver the gene to its target tissues. As noted earlier, vectors derived from AAV appear highly promising for this goal, and are generated by replacement of the viral genes with a micro dystrophin expression cassette. However, since there are so many known and emerging types of AAV, which is the best choice for gene therapy of human muscle disorders? Many of the numerous AAV serotypes exhibit different tissue tropism and transfection efficiencies. For striated muscle AAV1, 6, 7, 8 and 9 have shown high transfection efficiency after vascular infusion in animal models. However the observed tropism can also be species dependent. These intra-species differences make it difficult to predict the optimal vector for clinical application. For example AAV9 was able to transfect rodent hearts well but in neonatal dogs AAV8 achieved a higher transfection rate (10). Similarly, while AAV6 displays better efficiency in rodent striated muscles and in canine cardiac muscle, AAV9 appears to work better in adult canine skeletal muscles (Seto, Ramos et al, in preparation).

While many AAV types are able to target post-mitotic muscle cells, most AAVs tested to date are not able to show a significant transduction of quiescent satellite cells (11). Even if a vector were able to target satellite cells, the episomal AAV genomes would be lost when satellite cells are activated and proliferate to regenerate necrotic myofibers. This inability to target muscle stem cells effectively suggests that AAV gene therapies may need to be repeated at as yet unknown intervals reflective of the half-life of normal adult myofibers. Experiments in large animals models suggest that this interval could be 5–10 years or longer, but it will not be clearly known until the vectors are tested in patients.

In order to improve tissue specificity, efficiency and avoidance of neutralizing antibodies in serum, a variety of new types of AAV are being discovered (12) and existing AAV capsids are being modified using rational and random design (13, 14). Thus, the optimal AAV serotype for human gene therapy of muscle disorders may not yet be known.

Methods for gene delivery using AAV

AAV vectors can be administered via intramuscular (IM) injection to achieve localized, high transduction efficiency. However since DMD affects muscles throughout the body IM injection will not lead to widespread therapy. In mice, systemic transduction of muscles can be achieved via intravascular infusion of several AAV vector serotypes. In larger animals and patients large quantities of vector will be needed and the dilution of vector in the copious blood volume could limit transfection efficiency. Currently different dwell and recirculation methods are being tested for maximal muscle transduction at minimal vector dose. Often ligatures are used to restrict blood flow to a limb before the vector is released into the rest of the body. To transduce cardiac muscles, vector infusions into the jugular vein or the coronary artery using strategically placed catheters has shown some promise, and several groups have explored vector recirculation through isolated hearts in complex surgical procedures (15–17). Whether any of these approaches will be therapeutically relevant remains unclear. However, considerable emerging evidence from (mostly

unpublished) animal and human trials of various disorders using AAV vectors suggest that systemic gene delivery to striated muscles might be achieved using simple infusion of a high dose of vector delivered through single or several veins or arteries [e.g. (10, 18)].

Immune responses to AAV and/or dystrophin

In many large animal studies and clinical trials pre-existing neutralizing antibodies against the AAV capsid were detected that can limit AAV vector transduction efficiency. Many patients, and certain colonies of animal models, display neutralizing antibodies against at least some serotypes of AAV. Preexisting antibodies can bind the AAV vector and prevent it from reaching the target tissue. A recent development that may offer a way around this issue is to remove as many serum immunoglobulins as possible before vector infusion.

Plasmapheresis can eliminate significant percentages of AAV antibodies and enable successful transfection with AAV vectors (19). The AAV vectors themselves can under various conditions also stimulate an immune response to its capsid proteins. Depending on the production method used to make vector, Cap protein can be expressed from cap genes or gene fragments that are incorporated into AAV capsids upon vector assembly during production. The introduction of a large intron into the cap gene can reduce this packaging and significantly reduce the immune response against capsid protein (20).

There are also concerns with any genetic null allele disorder that the therapeutic transgene itself can trigger an immunogenic response that could target successfully transduced cells (21). One strategy to evade an immune response to micro-dystrophin is by intra-amniotic gene delivery, while the immune system is developing. In utero transfection is able to induce tolerance to the transgene and AAV vector in the GRMD model (22). Other emerging strategies are to apply transient immune suppression to prevent B and T-cell activation that could lead to effector cell recognition of dystrophin or AAV proteins, however, these strategies have yet to be tested in the clinic.

Other therapeutic genes

An alternative approach to preventing elicitation of an immune response against dystrophin would be to deliver a surrogate gene to patient muscles. DMD severity can be alleviated by delivery of several genes other than dystrophin. While these other approaches do not fix the primary cause of the disease, the missing dystrophin, they can instead target secondary pathologies or proteins with homologous function to dystrophin.

The up-regulation of utrophin, a dystrophin paralog, has been demonstrated to be beneficial in several animal models of DMD. However like dystrophin, full-length utrophin is larger than the AAV packaging capacity, and several groups have been engineering micro-utrophin expression cassettes and proteins (23, 24). Utrophin is expressed along the entire sarcolemma during muscle development, yet in adult muscles utrophin only localizes to the neuromuscular and myotendinous junctions (NMJ/MTJ). Since there is no mutation in utrophin in DMD patients, upregulation of endogenous utrophin such that it is also expressed outside the NMJ can effectively ameliorate dystrophic pathophysiology. To that end, an engineered zinc finger-based transcription factor, called 'Jazz', leads to muscle-specific robust utrophin upregulation. Jazz fits within AAV8 vectors and has been used to

treat dystrophic animals (25). One shortcoming of the strategy is that recent studies have revealed that utrophin is not able to fully substitute for dystrophin, as utrophin does not bind nNOS or restore microtubule binding (26). Therefore utrophin can only partially improve the phenotype. Nonetheless, efforts are underway in some labs to generate hybrid dystrophin/utrophin clones that might maximize functionality while minimizing immune responses (Guy Odom et al, in preparation).

Underscoring the importance of proper nNOS signaling in dystrophic muscle, viral vector overexpression of a truncated nNOS can ameliorate cardiac dysfunction in dystrophic hearts of mice (27). Mutations in the dystrophin gene can lead to DMD or the milder Becker muscular dystrophy (BMD). There is a wide range of BMD disease severity including patients with almost no motor impairment to those patients that show impairments closer to that of DMD patients. While many therapeutic approaches are aimed at treating DMD, a recent clinical trial was focused on BMD patients, utilizing AAV vectors to overexpress the myostatin inhibitor follistatin (28). Of 6 patients enrolled, 4 had improved 6MWT (six minute walk test) distances after intramuscular injection; the two patients that did not show significant improvement appeared to have more advanced muscle fibroses, perhaps limiting vector dissemination. For DMD it may be more beneficial to combine delivery of micro-dystrophin with follistatin or other modulators of muscle mass (29, 30).

Additional surrogate genes have also been tested in a variety of DMD animal models, and many show some propensity to ameliorate dystrophic phenotypes without eliciting an immune response against dystrophin. These include $\alpha 7$ -integrin, myostatin peptides and antibody fragments against myostatin, Igf1, mini-agrins and various anti-inflammatory molecules designed to reduce fibrosis and alleviate inhibition of muscle regeneration. One of the most recent studies to be published explored delivery of GALGT2 to skeletal muscles of macaques. This enzyme is able to modulate glycosylation of dystroglycan, leading to upregulation of several members of the dystrophin complex, including utrophin and dystrophin itself (18). AAV-delivery of GALGT2 thus represents an alternative approach to treating numerous forms of muscular dystrophy.

Gene editing of dystrophin

While micro-dystrophin delivery can halt muscle necrosis and significantly improve muscle strength, it does not restore full function to dystrophic muscles. Consequently, the ability to repair a mutant dystrophin gene and allow expression of a full or nearly full-length dystrophin could be the most effective therapy for DMD/BMD, depending on the underlying mutation. Current strategies to induce expression of larger dystrophins include stop codon read through and antisense oligonucleotide-mediated exon skipping. With the exon skipping approach the need for continuous readministration of the oligonucleotide drug is a challenge, and current approaches do not work in cardiac muscle. To avoid continued readministration, an AAV vector expressing an antisense sequence could be helpful. Administration of AAV8-U7snRNS-E6/E8, expressing an antisense oligonucleotide (AO) to induce exon 6–8 skipping, led to significant dystrophin expression in GRMD dogs (31). Using AAV to deliver AOs allows for less frequent administration, as with micro-dystrophin.

Other emerging gene editing techniques utilizing targeted nucleases, for example CRISPR/Cas9, TALEN or Zinc fingers appear to be promising methods for restoring dystrophin expression by repairing or removing mutations from patient DNA. This approach has recently been shown capable of allowing dystrophin expression in cells derived from DMD patients in culture (32–34) and in *mdx* mouse zygotes (35). With all targeted nucleases a recognition sequence assists in localizing the nucleases to the DNA sequence of interest and induces a double stranded break. The double stranded break is then fixed either by non-homologous end joining (NHEJ) or homologous recombination. For NHEJ two double stranded breaks in the dystrophin gene can restore an open reading frame and result in a slightly truncated dystrophin protein, depending on the size of the genomic region that must be excised and the presence of any genomic deletion mutations in the dystrophin gene. Besides the nucleases a template can be provided that allows for homologous recombination, in which case a wild type full-length dystrophin could be generated from the targeted allele. However, it remains unclear whether homologous recombination can occur efficiently in post-mitotic cells. As a way to achieve systemic delivery of the targeted nucleases and templates AAV vectors could be used as a vehicle to deliver the nuclease and the template sequence. While not yet published, several groups (including our own) have presented encouraging data at recent meetings suggesting that AAV vectors may prove to be a promising approach for delivering CRISPR and Cas9 expression cassettes to muscles of dystrophic mice.

CRISPR/Cas9 and TALEN could also be used to create new animal models to study DMD or other neuromuscular disorders. Independent of each other, two dystrophic rats were created by targeting the dystrophin gene (36, 37). Since rats are larger than mice they may better recapitulate the DMD pathology than in mouse models. Macaques are often used in preclinical studies since they closely model humans physiology and immunology, and they may better model human clinical trials since most non-human primates used in research are not inbred. However, there had not been a non-human primate model for DMD, precluding functional studies of therapy. However, this limitation may have been eliminated due to application of CRISPR/Cas9 technology, which was recently used to induce a mutation in the dystrophin gene of macaques. These animals display multiple DMD manifestations, but will need more thorough characterization before becoming widely used (38).

Conclusions

Not long ago the development of effective therapies for DMD seemed a far away goal. However, technological breakthroughs over the past decade have stimulated far-reaching research in multiple areas suggesting that highly effective treatments are coming closer to reality. In our opinion, gene therapy represents one of the most powerful approaches to treating DMD/BMD due to the ability to correct the fundamental cause of the disorder, the lack of functional dystrophin expression. Whether by micro-, mini- or full-length dystrophin expression, antisense oligonucleotide delivery or genetic correction of mutations, AAV vectors are showing tremendous promise for use in patients (Table 1). The dystrophin replacement methods in particular have the potential for being applicable to all patients, regardless of their causative mutation. Recent, multiple successes in clinical trials using AAV, coupled with the approval of the AAV drug *Glybera* in the European Union add

credence to the hope that AAV therapies may become the method of choice for treating DMD and BMD.

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Key points

- Currently micro-dystrophins are the most promising strategy for gene therapy to treat Duchenne muscular dystrophy.
- Determining the most effective AAV vector stereotypic for clinical trials can be challenging due to intra-species differences.
- Multiple strategies are being evaluated to prevent an immune response to either the vector or the transgene.

Table 1

Summary of studies using AAV gene transfer during the review period

Study	Species in study	Transgene product	Serotype (route of injection)
Lostal et al. (8)	mdx4cv mice	full-length dystrophin	Y445F AAV6 (im.)
Koo et al. (9)	mdx mice	full-length dystrophin	AAV2/5 (im.)
Pan et al. (10)	DMD carrier dog (neonatal)	alkaline phosphatase	AAV8 and AAV9 (iv.)
Arnett et al. (11)	WT and mdx mice	eGFP and mCherry	AAV6,8 and 9 (im./iv.)
Chicoine et al. (18)	WT NHP	GALGT2	AAVrh74 (iv.)
Chicoine et al. (19)	WT NHP	micro-dystrophin	AAVrh74 (iv.)
Wang et al. (20)	WT dogs, DMD dogs	alkaline phosphatase, micro-dystrophin	AAV6 (im.)
Hayashita-Kinoh et al. (22)	CXMD dogs (fetal)	micro-dystrophin	AAV9 (i.u.)
Strimpakos et al. (25)	mdx mice	Jazz (utrophin gene regulator)	AAV8 (ip.)
Lai et al. (27)	mdx mice	PDZ nNOS	Y731F AAV9
Mendell et al. (28)	BMD patients	follistatin	AAV1 (im.)
Le Guiner et al. (31)	GMRD dogs	antisense oligonucleotide	AAV8 (iv.)

BMD, Backer muscular dystrophy; DMD Duchenne muscular dystrophy; GALGT2, β 1-4-N-acetyl-D-galactosamine glycosyltransferase gene; im., intramuscular; iv., Intravascular; i.u. in utero; ip., intraperitoneal; NHP, non-human primate; WT, wild type