Molecular Therapy
Review

Progress toward Gene Therapy for Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) has been a major target for gene therapy development for nearly 30 years. DMD is among the most common genetic diseases, and isolation of the defective gene (DMD, or dystrophin) was a landmark discovery, as it was the first time a human disease gene had been cloned without knowledge of the protein product. Despite tremendous obstacles, including the enormous size of the gene and the large volume of muscle tissue in the human body, efforts to devise a treatment based on gene replacement have advanced steadily through the combined efforts of dozens of labs and patient advocacy groups. Progress in the development of DMD gene therapy has been well documented in *Molecular Therapy* over the past 20 years and will be reviewed here to highlight prospects for success in the imminent human clinical trials planned by several groups.

Background

Duchenne muscular dystrophy (DMD) was identified as a genetic disorder by several groups in the mid-19th century.[1] The disease is inherited in an X-linked recessive pattern, and in-line with Haldane's hypothesis, one-third of all cases arise from spontaneous, new mutations. Accordingly, genetic counseling or even curing all current cases will not greatly reduce the incidence. Individuals with DMD display a progressive loss of skeletal muscle mass, increasing weakness, and a later-onset cardiomyopathy. Approximately one-third of patients display varying degrees of cognitive dysfunction, and in some cases, smooth muscle manifestations lead to gastrointestinal issues.[1] A milder and more slowly progressing variant of the disorder is termed Becker muscular dystrophy (BMD). While DMD typically arises from genetic null allele mutations, BMD generally results from mutations that allow production of lower levels of, or partially functional, dystrophin protein. Patients from families without a prior history of the disorder are typically diagnosed between the ages of 2 and 6 years, but a family history enables early diagnosis, with the possibility for carrier testing and prenatal diagnosis. Increasing use of respiratory and cardiac support has extended lifespans over the past 20 years from the late teens up to the mid-30s, but these interventions do not by themselves significantly improve muscle function. The one treatment to date that has slowed muscle loss and extended ambulation is the use of corticosteroids, such as prednisone and deflazacort.[2]

DMD is among the most common single-gene disorders in humans, affecting ~1 in 5,000 newborn males.[3] Despite this relatively low incidence in the general population, it is one of the most well-known genetic disorders and has attracted enormous interest in the scientific and patient advocate communities. Much of this interest grew from early work by the Muscular Dystrophy Association (USA) (MDA) and a high-profile telethon (the first of its kind) that raised money under the leadership of the entertainer Jerry Lewis. The interest in DMD led the MDA to direct significant funding in the 1980s to finding the gene responsible for DMD. Funding efforts were an enormous success and, in a seminal series of publications by the laboratory of Louis Kunkel, resulted in the identification of the gene in 1986.[4] That work enabled highly accurate prenatal diagnosis and carrier detection, an understanding of the tissue-specific effect of mutations, and delineated the differences between DMD and BMD. Cloning of the DMD gene arguably represents the beginning of the human genome project, as the gene was isolated based on genetic studies that identified its chromosomal location on Xp21. The availability of the gene and the cDNA for the muscle isoform made DMD an early candidate for gene therapy.[5]

The DMD Gene

Despite early enthusiasm for the development of genetic therapies, many features of DMD presented important obstacles to development of a therapy. The gene is 2.2 Mb in size, and numerous isoforms are expressed in muscle and non-muscle tissues from seven different promoters and via alternative splicing. The enormous size of the locus is likely a major reason that the gene displays the highest known spontaneous mutation frequency of any human gene. Fortunately, a number of discoveries suggested approaches to gene therapy that were simpler than initially envisioned. One was the identification of rare patients with large deletions within the gene, in one case encompassing almost half the gene, that were associated with extremely mild cases of BMD.[6] A second came from isolation of the muscle cDNA, which was 14 kb but had an 11.2-kb open reading frame. These initial observations led to a series of studies establishing transgenic mouse lines on the *mdx* background, a model for DMD. In one transgenic line it was found that expression of the full-length dystrophin cDNA in a muscle-specific manner eliminated virtually all known muscle aspects of the disorder.[7] From these studies, it became clear that an effective therapy could be developed if a synthetic gene

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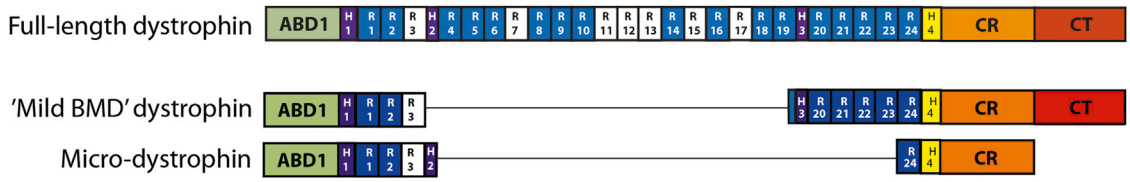

Figure 1. Domain Structure of Dystrophin

Comparative domain structures of full-length dystrophin (top), the mini-dystrophin expressed in a very mildly affected Becker muscular dystrophy (BMD) patient carrying a genomic deletion that removed exons 17–48[6] (middle) and the structure of a micro-dystrophin protein[10] (bottom). Domains within dystrophin are abbreviated as follows: ABD, actin-binding domain; R, spectrin-like repeats; H, hinge domains; CR, cysteine-rich domain; CT, carboxy-terminal domain. Note that the exon 17–48 genomic deletion removes approximately two-thirds of the spectrin-like repeat 19 coding region. Numerous variants of micro-dystrophin structures have been described by different labs.

derived from the muscle cDNA could be delivered to striated muscle, thus avoiding the need to deliver the entire gene, multiple isoforms, or to target tissues that expressed many of the smaller dystrophin isoforms. Truncated versions of the cDNA (~6 kb in size) based on genetic deletions in mildly affected BMD patients were subsequently shown to almost completely prevent disease in the *mdx* mouse models, which was accompanied by intensive efforts to understand the overall structure and function of the dystrophin protein.[8,9] Such studies enabled the design of smaller but highly functional mini- and micro-dystrophin cDNAs as short as 3.6 kb (Figure 1).[10–14] The next and enormously challenging task was finding a means to deliver the synthetic gene to the striated muscles that make up nearly 40% of human body mass.

Development of Vectors for Dystrophin Gene Delivery

While transgenic animal studies led to important insights into dystrophin protein structure and function that informed the design of dystrophin expression cassettes needed for therapy, such technology is not directly applicable to human use without a way to administer the cassettes to patients. How could dystrophin mini-genes be delivered bodywide such that all muscles are rescued? The advent of mini-genes with a size of less than 7 kb allowed early studies of direct intramuscular gene transfer using retroviral and adenoviral vectors.[14–17] However, this route of administration resulted in localized, not systemic, gene delivery and did not appear amenable to whole-body therapy. Development of improved, helper-dependent adenoviral vectors overcame many immune-system-related barriers to vector delivery and allowed for delivery of cassettes expressing the full-length dystrophin protein, but these studies were also largely limited to administration via intramuscular injection.[18–21] A major advance came in 1997, when the lab of Hansell Stedman showed that the musculature of an entire limb could be transduced by infusing large quantities of adenoviral vectors into hindlimb blood vessels under elevated pressure.[22] While this approach did not target muscles outside of the limb, such as the heart and respiratory muscles, it suggested that vasculature delivery of vectors might be adapted for bodywide gene delivery. Further testing of adenoviral vectors revealed numerous disadvantages for muscle gene transfer, including residual induction of an immune response, slow loss of gene expression, and difficulties targeting widespread muscles due to the large vector size and its high tropism for liver.[18,23] Many labs thus embarked on studies to identify more effective vectors, and several studies showed that recombinant vectors derived from adeno-associated virus (AAV) could lead to long-term expression following intramuscular injection.[13,24,25]

Adapting AAV vector technology for DMD still required significant work. AAV vectors have a carrying capacity of ~5 kb, and the early studies all used vectors derived from serotype 2, which poorly transduced striated muscles and could only be administered by intramuscular injection. However, studies in transgenic mice had revealed that highly functional "micro-dystrophin" cassettes could be generated with a size less than 4 kb.[10–12,26] A major breakthrough occurred when it was discovered that improved vectors could be generated from newly discovered AAV serotypes (such as AAV6, 8 and 9) which, when injected into the vasculature at high dose (in the range of 10^{14} vector genomes [vg] per kilogram) could transduce all the striated muscles in adult mice.[27] This led to the demonstration that dystrophy could be almost entirely halted and largely reversed in an adult mammal via systemic deliver of AAV/micro-dystrophin vectors (Figure 2).[27,28] Refinement of the gene delivery cassette through miniaturization of muscle-restricted gene regulatory cassettes provided greater vector functionality.[29,30]

Large Animal Studies

Since the murine studies showed significant promise, AAV/micro-dystrophin studies moved to testing in the larger canine model for DMD (*CXMD*). The dystrophic dog models not only allowed for evaluating scalability in a larger animal, but also enabled more sensitive assessment of potential immune responses against the vector. Several early studies using AAV6 suggested that T cell immune responses against the vector were limiting in this model, but other studies with AAV8 and 9 revealed minimal immune reactivity.[31–35] It remains unclear whether this reflected differences in vector properties, vector formulation, or lab protocols. However, following extensive testing using various AAV vectors carrying reporter genes or variants of microdystrophin, systemic delivery protocols have been established in canine models that support the potential for whole-body gene delivery to human muscle.[35–41]

These encouraging results have led to considerable interest in developing clinical gene therapy protocols involving administration of AAV/micro-dystrophin vectors for DMD. To date only one clinical trial has been completed, involving intramuscular injection of an

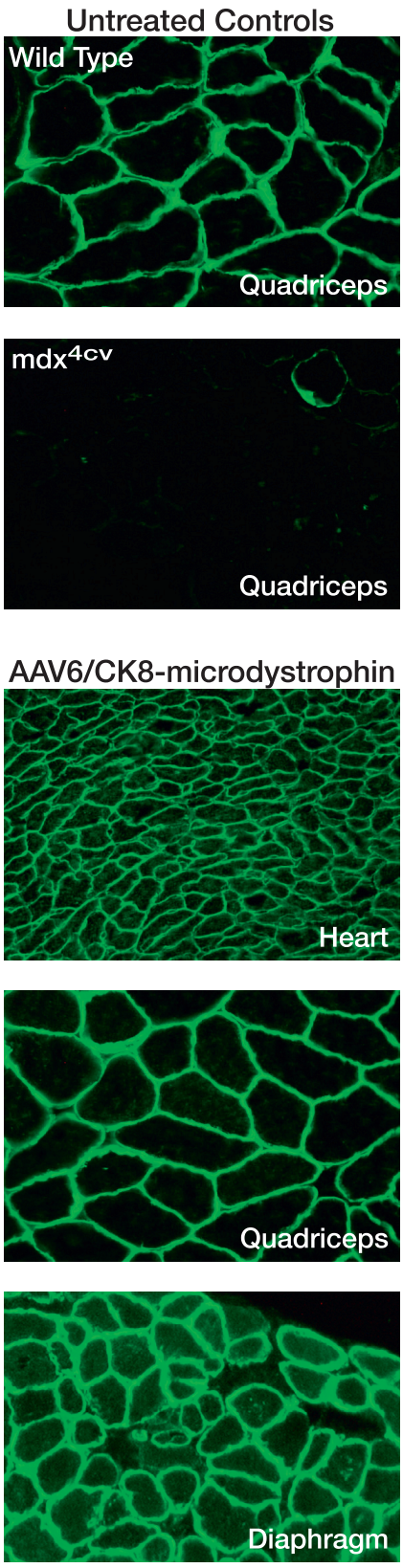

Figure 2. Systemic Delivery of AAV/Micro-dystrophin to Adult Mouse Muscles

Shown are images of muscle cryosections immunostained using an N-terminal antibody against dystrophin. Control quadriceps cryosections from wild-type (C57BL/6) or *mdx*^{4cv} mice (top). Representative cryosections (heart, quadriceps, and diaphragm muscle) from *mdx*^{4cv} mice infused with 4×10^{14} vector genomes per kilogram of AAV6/CK8-microdystrophin (bottom). Vector was administered via retro-orbital injection at ~2 months of age, and mice were analyzed at 6 months.

AAV2.5 vector. This study, performed collaboratively between the laboratories of Jerry Mendell and R. Jude Samulski, involved six patients, and while the vector injection proved safe, none of the patients expressed significant levels of micro-dystrophin (only two had any detectable exogenous dystrophin).[42] Two of the six patients displayed a low-level T cell immune response (assayed by ELISpot) against dystrophin, and one other patient displayed a clear T cell response against the AAV vector (defined as greater than 3× background on the ELISpot assay).[42,43] These results indicated that immune responses against dystrophin and/or the AAV capsid were responsible for the poor transduction, but given the limited data available and the relative insensitivity of ELISpot assays, it is difficult to make firm conclusions. All the patients developed high-titer neutralizing antibodies against the vector, and two had pre-existing neutralizing antibodies.[43] This, combined with a potential suboptimal vector serotype could have led to poor vector uptake by the injected muscle. In this study dystrophin expression was regulated by the ubiquitously active cytomegalovirus (CMV) immediate early enhancer plus promoter, which could have facilitated an immune response against dystrophin (through expression in immune effector cells) and/or resulted in loss of expression due to promoter shutdown. Finally, intramuscular injection tends to induce more inflammation and is better able to elicit a T cell immune response than is a vascular delivery method. These results suggested that better vectors (especially alternate capsid serotypes) coupled with a vascular delivery system and a gene regulatory cassette that is inactive in immune cells might lead to improved expression. Consequently, most current efforts to develop human clinical trials revolve around the use of AAV8 or 9 and a muscle-specific promoter/enhancer.

Systemic Delivery Clinical Trials for DMD

With considerable advances in the types of AAV vectors available, muscle-specific gene regulatory cassettes, and production/purification protocols, several groups are now planning human clinical trials involving vascular delivery of AAV/micro-dystrophin to patients. These plans are supported by extensive new data involving large-scale vector delivery to *CXMD* dogs and, for safety studies, to wild-type non-human primates.[44–46] While most of these new data remain unpublished or proprietary, several trends are emerging that are being used to support upcoming clinical trial applications to the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA). Planned trials have many similarities but differ in details including vector serotype, micro-dystrophin design, gene regulatory cassette usage, and the age of the patients. Depending on regulatory agency approval, some of these trials could begin within the next year.

These impending phase 1 clinical trials will answer several critical questions about the long-term feasibility of AAV/micro-dystrophin gene therapy for DMD. While safety will be the primary focus, the systemic gene delivery approaches will also enable important data to be gathered on efficacy. Outcome measurements should provide initial data on the functional capacity of the different micro-dystrophin designs. For example, will such miniaturized proteins improve muscle physiology similarly to what has been observed in mice and dogs? If improvement is obtained, how soon can this be observed and will the effect be sustained? Will DMD patients tolerate high-dose vector delivery as has been observed in canine and non-human primate studies? Will age and disease progression be a factor in transduction efficiency? Will T and/or B cell immune responses be observed against either dystrophin or the vector capsid proteins? Finally, can these immune responses be avoided or controlled, such that vector could be readministered years later if dystrophin expression drops below the threshold needed for therapy (see below)?

A difficulty with conducting these studies involves measuring vector distribution and dystrophin expression in widespread muscles throughout the body. The only direct method to measure dystrophin expression requires analysis of a muscle biopsy, and due to the invasive nature of obtaining biopsy specimens, very few can be obtained from any patient, limiting information on expression in multiple muscles or in one muscle over extended periods of time. Consequently, there is enormous interest in developing serum biomarkers that can be monitored non-invasively, and using imaging techniques, such as magnetic resonance imaging (MRI), to follow muscle structure over time.[47–50] The clearest indication of benefit will come from functional measurements, many of which have been developed for other DMD trials such as the 6-min walk test or newer, more informative tests.[51]

The potential for vector readministration is an important issue. Without some form of transient immune suppression, high-dose administration of AAV vectors can elicit production of neutralizing antibodies that preclude the ability to administer a second dose of vector.[52] If the results from animal studies translate well to humans, it may be possible to obtain sufficient levels of dystrophin expression from a single dose to stabilize all striated muscles. However, the half-life of this expression is unknown in human muscles. Studies in dogs and non-human primates suggest that the episomal AAV vector genome is fairly stable over a period of at least 5 years in normal skeletal muscle, but a shorter half-life is likely in treated dystrophic muscles that might display a partially mosaic pattern of micro-dystrophin expression.[53] Even moderate exercise can cause focal damage that would presumably be accompanied by partial vector loss. If immune responses against the vector can be avoided, such as with a transient immune suppression protocol, then readministration might be possible.[37,54,55]

Future Prospects

It has been 31 years since the dystrophin gene was cloned, 24 years since muscle-specific dystrophin expression was shown to eliminate muscular dystrophy in transgenic mice, and 13 years since systemic delivery of AAV/micro-dystrophin vectors was demonstrated.[4,7,27] Progress in therapeutics for DMD has been accelerating, and the field is now at a critical juncture where the feasibility and efficacy of AAV-mediated systemic therapies for DMD will be tested in the clinic. Upcoming clinical trials should provide data on whether current technology can be adapted for widespread use or whether refinements are needed. Such refinements could include testing alternate AAV serotypes; using different regulatory cassettes; varying the route of delivery, dose, or age at treatment; and testing immune-suppression strategies. AAV-mediated delivery of dystrophin genes appears to have enormous potential for therapy, as it enables correction of the fundamental cause of DMD and BMD: failure to produce functional levels of the dystrophin protein. If successful, this approach could be applicable to any patient with DMD or BMD.

In addition to AAV/micro-dystrophin, several other gene therapies approaches are being contemplated for DMD. Many of these were summarized in a recent review, but a few comments are relevant here.[56] Due to the possibility of an immune response against dystrophin, various groups are testing delivery of a surrogate gene that could partially substitute for dystrophin. Candidate genes include the paralog utrophin, *GALGT2*, or alpha7-integrin.[57–59] Another emerging technology that might be tested in coming years involves the use of gene editing using the CRISPR/Cas9 system.[60]

Gene editing is attractive as a therapy as it has the potential to directly modify the mutant *DMD* gene to enable production of the dystrophin protein. In cases where the mutation is small, such as a point mutation or small deletion, this approach could lead to production of a nearly full-length protein.[61] The potential for this strategy was demonstrated using AAV vectors to deliver CMV-Cas9 and guide RNA cassettes to bypass the premature stop codon in the genomes of *mdx* mice,[62–64] and more recently, multiple strategies were shown to have potential for muscle-specific editing in the more complex mutational context in *mdx*^{4cv} mice.[56,65] However, with large deletions, editing would only enable production of smaller dystrophins, similar to approaches using antisense oligonucleotides (below). A method to circumvent this issue by using editing to introduce a portion of the dystrophin cDNA into the mutant gene to restore production of larger and more functional dystrophins has recently been suggested.[66] Overall, multiple strategies will be needed for treating the wide variety of mutations found in DMD patients.

A number of issues need to be resolved before gene editing can be tested in the clinic. One is the obvious problem of low efficiency observed to date. A second is the issue of expressing the bacterial Cas9 enzyme for extended periods in muscle. As noted above for micro-dystrophin, the studies that expressed Cas9 from the CMV enhancer/promoter are likely to lead to an immune response against the bacterial Cas9.[23,62–64,67] Muscle-specific expression could reduce this concern but would still lead to long-term nuclease expression, albeit only in post-mitotic cells.[61] Safety issues related to off-target editing also need to be addressed. While gene editing has been suggested

as a method that could lead to permanent correction of dystrophin deficiency, such a goal will require targeting the gene in myogenic stem cells. This latter issue arises because even normal muscles cells display a low rate of turnover. At present, it is unclear whether AAV vectors are capable of transducing myogenic stem cells in vivo at an efficiency needed for significant gene editing, but future studies may clarify this issue.[63,68]

Non-viral genetic approaches include systemic administration of morpholino antisense oligonucleotides that can induce skipping of mutant exons from pre-mRNAs to restore dystrophin production in muscles. Such a strategy recently resulted in FDA approval for *exondys 51* (tradename of eteplirsen).[69,70] The EMA has given conditional approval for the use of *ataluren*,[71] a small molecule designed to suppress use of premature stop codon mutations found in ~10% of DMD patients. Other strategies are aimed at testing small molecules that could target aspects of muscle pathology resulting from dystrophin deficiency. These include drugs designed to upregulate expression of utrophin, reduce muscle inflammation, reduce fibrosis, enhance regeneration by stimulating myogenic stem cell function, or even induce new muscle cell formation via myogenic stem cell transplantation. Ultimately, an optimal treatment might result from the use of several approaches in combination. For example, one can envision gene therapy to restore dystrophin expression coupled with small-molecule therapies to reduce fibrosis.

Conclusions

The prospects of gene therapy using systemic delivery of AAV/microdystrophin vectors appears increasingly feasible and will soon be tested in clinical trials. The method as originally developed in *mdx* mice was shown to be safe and largely eliminates dystrophic pathophysiology for the lifespan of the mice. Recent and ongoing studies suggest that similar results are observed in canine models for DMD, and various types of AAV vectors have been shown to be safe in non-human primate studies and clinical trials for other genetic disorders. The general approach is amenable to significant modification if initial human studies do not achieve satisfactory results. While DMD was once viewed as an incurable disease, progress in the field suggests that successful gene therapies may soon be available.

CONFLICTS OF INTEREST

J.S.C. is a member of the scientific advisory boards for Solid GT and Akashi.

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