Improvement of the mdx mouse dystrophic phenotype by systemic in utero AAV8 delivery of a minidystrophin gene

BM Koppanati¹, J Li², DP Reay¹, B Wang³, M Daood⁴, H Zheng¹, X Xiao², JF Watchko⁴ and PR Clemens¹,⁵

¹Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA; ²Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA; ³Department of Orthopedic Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA; ⁴Department of Pediatrics, Magee-Women’s Research Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA and ⁵Neurology Service, Department of Veterans Affairs Medical Center, Pittsburgh, PA, USA

Duchenne muscular dystrophy (DMD) is a devastating primary muscle disease with pathological changes in skeletal muscle that are ongoing at the time of birth. Progressive deterioration in striated muscle function in affected individuals ultimately results in early death due to cardiopulmonary failure. As affected individuals can be identified before birth by prenatal genetic testing for DMD, gene replacement treatment can be started in utero. This approach offers the possibility of preventing pathological changes in muscle that begin early in life. To test in utero gene transfer in the mdx mouse model of DMD, a minidystrophin gene driven by the human cytomegalovirus promoter was delivered systemically by an intraperitoneal injection to the fetus at embryonic day 16. Treated mdx mice studied at 9 weeks after birth showed widespread expression of recombinant dystrophin in skeletal muscle, restoration of the dystrophin-associated glycoprotein complex in dystrophin-expressing muscle fibers, improved muscle pathology, and functional benefit to the transduced diaphragm compared with untreated littermate controls. These results support the potential of the AAV8 vector to efficiently cross the blood vessel barrier to achieve systemic gene transfer to skeletal muscle in utero in a mouse model of muscular dystrophy, to significantly improve the dystrophic phenotype and to ameliorate the processes that lead to exhaustion of the skeletal muscle regenerative capacity.

Key words: Muscle; Skeletal Dependovirus Fetal Research Muscular Dystrophy; Duchenne Gene Therapy

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive muscle disorder affecting 1 in 3500 live male births.¹ The disease is characterized by progressive loss of muscle function leading to premature death, most commonly caused by cardiac and respiratory failure.² Although improvements in medical management and treatment with glucocorticoids have significantly improved life expectancy and the quality of life of DMD patients, a specific treatment addressing the genetic defect causing DMD is not yet clinically available. A crucial challenge of DMD therapeutic research is to develop approaches with functional benefit to widespread muscle tissues. Preclinical gene replacement therapy has shown promising results in postnatal dystrophic mice³–⁶ and dogs⁷,⁸ although widespread gene delivery often requires large vector doses and issues of immunity induced by viral vectors remain.

Among gene delivery vectors, adeno-associated viral (AAV) vectors hold great promise for gene therapy because of their safety, low toxicity and stable transgene expression.⁹ In particular, AAV serotype 8 (AAV8) vectors show robust muscle transduction after systemic delivery to postnatal mice and hamsters.¹⁰,¹¹ However, there are very few studies testing gene delivery and functional efficacy of therapeutic transgenes carried by AAV8 vectors in dystrophic animal models.¹²

In utero gene delivery for muscle gene replacement therapy offers several advantages. Compared to gene delivery later in life, gene transfer to the fetus provides an opportunity to target a higher percentage of cells when the tissue mass is small. In humans, myotube formation is observed between gestation weeks 7 and 14 and by week 20, muscle fibers are arranged in discrete bundles.¹³ In mice, secondary myotubes begin to form on the scaffold of the primary myotubes from embryonic day 14 (E14) to E17.¹⁴–¹⁹ Therefore, most murine in utero muscle gene delivery studies have been performed at E15 or E16 when secondary myotubes are forming.

Studies have shown that fetal gene delivery can induce immune tolerance²⁰–²² compared to postnatal gene delivery. In addition, the immaturity of the basal lamina has an important role²³ in widespread gene delivery to fetal muscle.²⁴ Furthermore, for genetic diseases such as DMD where the clinical signs and symptoms are often not detected until affected children are 2–5 years old, fetal DNA diagnostics and gene...
transfer in utero provide a unique opportunity to treat at the earliest stage of the disease. Finally, the minimal transduction of liver by AAV8 vectors when delivered systemically in utero is a desirable outcome of muscle-targeted gene delivery and limits the possibility of liver toxicity from gene delivery.

Since most primary muscle disorders affect multiple muscle groups including the diaphragm, an important challenge for muscle gene therapy is to achieve transgene expression in widespread muscle tissues. In our previous study of the biodistribution of an AAV8 vector carrying the lacZ gene after systemic administration in utero, we demonstrated successful widespread gene expression in various muscle tissues including high levels of expression in diaphragm and intercostal muscles. This biodistribution study suggested that AAV8 could be an ideal vector for the treatment of primary muscle disorders in utero. Therefore, we explored the therapeutic potential of an AAV8 vector carrying a minidystrophin gene when delivered systemically in utero to the dystrophic mdx mouse model of DMD.

Results

Expression of recombinant dystrophin and improvement in muscle morphology after intraperitoneal administration of AAV8 minidystrophin in utero

A dose of $6.4 \times 10^{11}$ vector genomes of an AAV8 vector carrying a canine mini-dystrophin cDNA driven by the human cytomegalovirus promoter was injected intraperitoneally per fetus on E16. Pups were delivered naturally at full term. At 9 weeks of age, experimental mice were killed and diaphragm, upper limb and lower limb muscles were collected from the injected pups and the un.injected litters. To assess the gene transfer efficiency, cryosections of these tissues were analyzed for expression of dystrophin (Figure 1). The treated diaphragm showed the highest level of transgene expression compared to limb muscles, whereas in the control mdx muscles we observed only rare revertant dystrophin positive fibers. The dystrophic change of mdx muscle tissue at 9 weeks of age is characterized by necrotic and regenerating muscle fibers, a mononuclear cell infiltrate and increased fibrous connective tissue. To evaluate the histology of muscle tissues, cryosections of diaphragm, upper limb and lower limb muscles collected from the injected pups at 9 weeks of age were stained with hematoxylin and eosin (H & E). The treated muscles showed decreased fiber size variability, less mononuclear cellular infiltration, and reduced fibrosis compared with untreated mdx controls (Figure 1). Together, the morphological findings suggest skeletal muscle benefit from expression of recombinant dystrophin delivered by an AAV8 vector in utero to mdx mice.

Restoration of dystrophin-associated glycoprotein (DAG) complex in muscle after intraperitoneal administration of AAV8 minidystrophin in utero

The absence of dystrophin expression in mdx muscle disrupts the structural link between cytoplasmic actin filaments and the sarcolemmal DAG complex leading to the dysfunction and/or loss of the DAG complex from the sarcolemma. Dystrophin protein functions in conjunction with the DAG complex (reviewed in Blake and Rando). Hence, we studied whether in utero gene transfer of an AAV8 vector carrying minidystrophin can restore the DAG complex. At 9 weeks of age the diaphragm, the upper limb, and the lower limb muscles from the injected pups, and the un injected litters were analyzed for z-sarcoglycan and $\beta$-dystroglycan expression. Expression of z-sarcoglycan and $\beta$-dystroglycan was restored in fibers expressing recombinant dystrophin (Figure 2) suggesting functional benefit of AAV8 vector-mediated minidystrophin gene delivery in utero to mdx mice.

Decreased percentage of fibers harboring centrally placed nuclei in muscle after intraperitoneal administration of AAV8 minidystrophin in utero

Cycles of degeneration and regeneration of muscle fibers result in the accumulation of muscle fibers harboring centrally placed nuclei. Characterize the degree of this pathological process we calculated the percentage of muscle fibers with centrally placed nuclei in mdx untreated fibers, AAV8 minidystrophin-treated dystrophin-positive fibers, AAV8 minidystrophin-treated dystrophin-negative fibers and C57BL/10 fibers in diaphragm and hindlimb muscles of 9-week-old mice (Figure 3). We observed a high percentage of fibers with centrally placed nuclei in untreated mdx diaphragm (62.6% ± 4.7) and hindlimb muscle (71.8% ± 4.6), whereas C57BL/10 muscle showed very few fibers with centrally placed nuclei in the diaphragm and hindlimb (1.1% ± 0.7 and 1.3% ± 0.5, respectively). The percentage of fibers with centrally placed nuclei in dystrophin-expressing fibers of AAV8 minidystrophin-treated mdx mice was significantly less ($P < 0.05$) in diaphragm and hindlimb (21.7% ± 2.4 and 26.7% ± 2.7, respectively) compared to untreated control mdx mice. The improvements in mdx muscle pathology observed after AAV8 minidystrophin treatment in utero are indications of its potential benefit in a muscular dystrophy animal model.

Improvement of ex vivo functional force generation properties of costal diaphragm after intraperitoneal administration of AAV8 minidystrophin in utero

To test whether widespread expression of minidystrophin in costal diaphragm could provide functional improvement, the force properties of treated mdx diaphragm, untreated mdx diaphragm and C57BL/10 control diaphragm were tested and compared. Nine weeks after birth, costal diaphragm was collected for ex vivo force measurements. Diaphragm-specific force (peak isometric tetanic force normalized for muscle cross-sectional area) and force generation during repetitive isovelocity lengthening activations were performed as previously described. AAV8 minidystrophin-treated diaphragm showed a statistically significant ($P < 0.05$) 32.6% increase in specific force compared to paired littermate untreated mdx diaphragm (mdx untreated (n = 11 mice): 14.7 ± 3.0 N/cm², mdx treated (n = 6 mice): 19.5 ± 1.5 N/cm², C57BL/10 control (n = 5 mice) 21.1 ± 2.7) (Figure 4a). Specific force of AAV8 minidystrophin-treated diaphragm approximated (statistically insignificant difference) age-matched C57BL/10 wild-type controls. After determining peak
tetanic force the diaphragm was subjected to repetitive lengthening activations, a paradigm of mechanical stress.31 Residual diaphragm force following 10 repetitive lengthening activations, expressed as a percent of the initial, was significantly greater in AAV8 minidystrophin-treated diaphragm than untreated littermate paired mdx diaphragm (mdx untreated (n = 11 mice): 84.5 ± 5, mdx treated (n = 6 mice): 99 ± 7.6, C57BL/10 control (n = 5 mice): 101 ± 7.1) (Figure 4b). These data show that AAV8 minidystrophin delivered systemically in utero provides significant functional improvements in the dystrophic mdx diaphragm as shown by specific force generation and the ability to withstand eccentric muscle contraction.

**Figure 1** Restoration of dystrophin and amelioration of dystrophic pathology in AAV8 minidystrophin-treated mdx diaphragm, upper limb and lower limb muscles. Tissues were collected at 9 weeks after birth following an intraperitoneal injection of AAV8 minidystrophin into E-16 pups of pregnant mdx mice and analyzed for dystrophin expression. Dystrophin immunohistochemistry (upper panels) and H & E staining (lower panels) were evaluated in (a) diaphragm, (b) upper limb and (c) lower limb muscles. Uninjected mdx littermates and C57BL/10 tissues were used as negative and positive controls, respectively, for immunohistochemistry and histology. Dys, dystrophin; α-SG, α-sarcoglycan; β-DG, β-dystroglycan. H&E, Hematoxylin & Eosin. Bar = 100 μm.

Quantification of AAV vector genomes in muscle tissues after intraperitoneal administration of AAV8 minidystrophin in utero
To determine viral vector gene transfer efficiency and compare with the previous marker gene study in normal
mice, we quantified the number of viral particles in individual tissues by real-time PCR. The highest levels of viral vector particles were observed in the diaphragm. The diaphragm had 227.77 ± 85.07 viral particles per 1000 nuclei. Upper and lower limbs had 38.70 ± 22.59 and 33.90 ± 13.82 viral particles per 1000 nuclei, respectively. These findings are similar to and confirm our previous study in normal mice in whom a similar dose of AAV8 carrying a lacZ gene was delivered by the same route in utero.25 The results thus show the efficacy of AAV8 minidystrophin to successfully deliver the therapeutic gene into dystrophic muscle tissues that were comparable with gene delivery to normal muscle tissue.

Discussion
This study demonstrates the potential of fetal gene therapy to correct a genetic defect that is lethal in humans, in a preclinical model. Other disorders where preclinical fetal gene transfer studies have shown partial
correction of a genetic defect include mucopolysaccharidosis type VII, 12,23 Crigler-Najjar Syndrome Type 1, 34 z-thalassemia, 35 hemophilia B, 36 cystic fibrosis 36 and Pompe disease. 37 In this study, the improvements in muscle pathology, which are also reflected in functional benefit in the diaphragm, suggest that dystrophin gene transfer in utero for the treatment of dystrophin deficiency has the potential to preserve muscle regenerative capacity by gene replacement at this very early stage.

This study demonstrates the ability of AAV8 to systemically transduce widespread muscles, including the diaphragm, to provide therapeutic benefit in a DMD model in utero. Although we also achieved in utero dystrophin gene delivery in mdx mice with an adenoviral vector, 34 the AAV8 vector provides markedly more evidence of morphological and functional benefit. One limitation of the AAV8 vector for the treatment of DMD in utero, however, is a low level of cardiac transduction. 29 Another serotype of AAV, AAV9, offers higher levels of cardiac transduction when delivered postnatal, 10 but has not been tested for in utero gene delivery.

Most previous muscle gene transfer studies with AAV8 have been performed in postnatal animals and showed that the AAV8 vector efficiently transduces muscle tissues of neonatal and adult animals. In a study of multiple AAV vector serotypes, Wang et al., 11 showed that AAV8 was systemically delivered efficiently to muscle in neonatal and adult mice. Similarly, Inagaki et al. 10 showed robust transduction of muscle tissue following systemic delivery of various doses of AAV8 vector in adult mice. Zincarelli et al. 46 observed that tail vein injection of an AAV8 luciferase vector into 6- to 8-week-old mice not only showed persistent transgene expression for at least 100 days, but also showed uniform expression of luciferase in hindlimb, abdominal and thoracic regions.

The biodistribution of AAV8 when delivered in utero was shown in our previous study of AAV8 gene delivery in utero. Intraperitoneal delivery of an AAV8 vector carrying a lacZ gene to fetal mice in utero resulted in widespread postnatal gene expression in multiple muscle tissues, including diaphragm, intercostal muscles, forelimb and hindlimb muscles with the highest expression seen in the diaphragm and intercostal muscles.

Encouraged by these results with gene delivery of a marker gene in utero using an AAV8 vector, we performed the present in utero gene transfer study in a muscular dystrophy model with a therapeutic transgene and showed for the first time that an AAV8 vector carrying a minidystrophin gene injected systemically in utero could restore muscle structure and function. The AAV8 vector carrying a minidystrophin gene was injected intraperitoneally into E16 mdx pups and muscle was analyzed 9 weeks after birth. We observed efficient transduction and restoration of dystrophin in diaphragm, forelimb and hindlimb muscles. In addition, immunostaining of the transduced muscles showed restoration of the DAG complex, evidenced by expression of α-sarcoglycan and β-dystroglycan at the sarcolemma of those fibers expressing recombinant dystrophin.

MdX muscle tissues undergo degeneration evidenced pathologically by necrosis and regeneration. The degree of regeneration is proportional to the percentage of fibers harboring centrally placed nuclei. In this study, we observed that the dystrophin-expressing fibers in treated muscle had significantly fewer centrally placed nuclei compared with muscle fibers of untreated mdx mice. This finding suggested that recombinant dystrophin provided by systemic gene delivery in utero partially protected transduced muscle fibers from cycles of degeneration and regeneration. Furthermore, in treated mdx muscle, even among the fibers where recombinant dystrophin was not detected, there was a non-significant decrease in the number of fibers with centrally placed nuclei compared with muscle of untreated mdx mice. It is possible that despite absence of immunohistochemical detection of dystrophin expressed by these fibers, dystrophin expression may have been present at a level below the detection threshold and may have provided functional benefit. Alternatively, there may be a ‘bystander’ benefit to non-transduced fibers or non-transduced regions of fibers from being in a treated muscle in close proximity to fibers and fiber segments that express recombinant dystrophin. Therefore, a significant potential benefit of in utero muscle gene transfer for DMD may be to reduce the degree of exhaustion of the satellite cell pool by achieving gene transfer early.

Figure 4 Improvement in force generation properties in diaphragm after intraperitoneal administration of AAV8 minidystrophin vector in utero. The diaphragm muscles were collected at 9 weeks of age from mice treated in utero and were analyzed ex vivo. Diaphragms of control C57BL/10 (n= 5 mice), AAV8 minidystrophin vector-treated mdx (n= 6 mice) and untreated mdx (n= 11 mice) mice were analyzed for (a) specific force (N/cm²) and (b) residual force following 10 repetitive lengthening activations divided by initial force and expressed as a percentage. The data is shown as mean ± s.e. Significant differences from untreated mdx mice are shown (P<0.05).
To date, AAV8 has successfully provided therapeutic benefit to skeletal muscle in two disease models in previous postnatal studies. Ziegler et al. showed that systemic administration of an AAV8 vector carrying the human acid α-glucosidase gene to 2-month-old presumptomatic α-glucosidase-deficient mice that model Pompe disease resulted in nearly complete correction of the lysosomal storage of glycogen in all the affected muscles. Another study also showed correction of α-glucosidase deficiency in muscle tissue of immunodeficient GSD-II mice by treatment with an AAV8 vector. In another muscle disease model, systemic delivery of an AAV8 vector carrying a myostatin inhibitor in adult mdx mice enhanced muscle growth and also ameliorated the dystrophic phenotype.

Only one previous study reported on AAV gene transfer in utero for treatment of a muscle disease. In a mouse model of Pompe disease, an AAV2 vector carrying the α-glucosidase gene was delivered intraperitoneally in utero and resulted in improvement in diaphragmatic in vitro isometric force-frequency studies 6 months after birth. However, AAV8 has not been previously reported in therapeutic gene delivery studies in utero in a preclinical model of a muscle disease.

In postnatal gene delivery studies, restoration of dystrophin expression has been correlated with muscle functional benefit after AAV8 gene delivery. In a study involving AAV8 carrying microdystrophin delivered systemically into the femoral artery in 3- to 4-week-old mdx mice and non human primates, significant improvement in tetanic force and protection against eccentric contraction in the EDL muscle was shown. Dystrophic patients usually die due to respiratory and cardiac failure; restoring dystrophin expression to the diaphragm, which has a critical role in respiration and survival, will be highly important to DMD patients. In the study reported here, the diaphragm was collected 9 weeks following birth for ex vivo force measurements. AAV8 minidystrophin-treated diaphragm showed a 32% improvement in specific force compared with diaphragms from untreated mdx littermates. In normal muscle, dystrophin provides resistance against contraction-induced injury. We observed that the residual force generated following 10 repetitive lengthening activations was significantly improved by AAV8 minidystrophin gene delivery in utero compared with untreated mdx diaphragm.

In summary, systemic delivery of minidystrophin with an AAV8 vector in utero provides efficient transduction of diaphragm and limb muscles of the mdx mouse when studied after birth. The functional benefit demonstrated in transduced diaphragm muscle encourages further studies to test the persistence of vector expression and in utero gene delivery in large animal models of DMD.

**Materials and methods**

**Production of AAV8 minidystrophin vector**

The cloning and construction of the canine minidystrophin cDNA has been previously described. Briefly, for the construction of the vector, AAV8 vector carrying a canine minidystrophin expression cassette driven by the human cytomegalovirus promoter (AAV8 minidystrophin) vector stocks were generated by the triple-plasmid transfection method using the three plasmids, the AAV–CMV–minidystrophin vector plasmid, the mini-adeno helper plasmid, and the AAV8-packaging plasmid containing the AAV2 Rep gene and AAV8 Cap gene, as described previously. AAV8 minidystrophin viral particles were purified by double CsCl gradient centrifugation and dialyzed three times against PBS containing 5% Sorbitol. The titer of vector genomes (vg) was determined by a standard DNA dot-blot assay.

**In utero administration of AAV8 minidystrophin vector**

AAV8 minidystrophin was administered intraperitoneally in utero into E16 pups of timed pregnant mdx female mice as described previously according to a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee. The vector was injected at a dose of $6.4 \times 10^{11}$ vg per pup. To identify the injected pup, a fluorescent marker, 2% orange fluorescent FluoSpheres (Invitrogen, Carlsbad, CA, USA), was injected into one of the limbs permitting identification of injected pups several days after birth by observation under a fluorescent microscope. The vector-treated mice were analyzed at 9 weeks of age in parallel with age-matched untreated mdx littermate and C57BL/10 controls.

**Ex vivo functional analysis of diaphragm**

Ex vivo functional analysis was performed on diaphragm 9 weeks after birth following in utero treatment with AAV8 minidystrophin vector. Diaphragm-specific force (peak isometric tetanic force normalized for muscle cross-sectional area) and force generation during repetitive isovelocity lengthening activations were performed as previously described. Analysis of variance with Tukey’s post hoc test for multiple comparisons was used to identify statistical differences between groups with respect to specific force and residual force following lengthening activations ($P < 0.05$).

**Immunohistochemistry**

Muscle samples were snap frozen and sectioned using a cryostat. Immunostaining for α-sarcoglycan and β-dystroglycan was performed as described previously. For dystrophin staining, sections were incubated with a rabbit anti-dystrophin antibody (Invitrogen, Eugene, OR, USA) at a dilution of 1:800, followed by AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Inc., West Grove, PA, USA) at a dilution of 1:1000. Cell nuclei were stained with Hoechst dye. The sections were blocked with 2% bovine serum albumin and washed with $1 \times$ phosphate-buffered saline 3 × after each step. Uninjected mdx littermate and C57BL/10 normal muscles were used as negative and positive controls, respectively, for immunostaining.

**Quantification of fibers with centrally placed nuclei**

Muscle sections stained for dystrophin and Hoechst dye were counted to score the number of fibers with centrally placed nuclei in dystrophin positive and dystrophin negative fibers in muscle sections from treated mice. A similar analysis of uninjected mdx littermates and C57BL/10 mice was performed for disease and normal controls, respectively. The percentage of fibers with centrally placed nuclei was than calculated from analysis of approximately 300 muscle fibers in randomly selected...
fields. Analysis of variance with Tukey’s post hoc test for multiple comparisons was used to identify statistical differences between groups \((P<0.05)\).

**Analysis of vector genomes by real time PCR**

Total DNA was isolated from the muscle tissues of treated and untreated mdx and C57BL/10 mice by ethanol precipitation (Wizard genomic DNA purification kit, Promega, Madison, WI, USA) and the genomes calculated as described previously.\(^{25}\) PCR primers for the minidystrophin gene had the following sequence: forward primer: CAACCATAGGAAAAGGCT TCTAGAAA and reverse primer: GAGATCTTGCAATTTG TTATCAG. The TaqMan probe sequence was: ATTC CAAGGGAGTAAGAGA. The results were presented as copies of vector particles per 1000 nuclei.

**Conflict of interest**

The authors declare no conflict of interest.

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