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Haploinsufficiency of Utrophin Gene Worsens Skeletal Muscle Inflammation and Fibrosis in *mdx* Mice

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Abstract

To address whether *mdx* mice with haploinsufficiency of utrophin (*mdx/utrn*^{+/-}) develop more severe skeletal muscle inflammation and fibrosis than *mdx* mice, to represent a better model for Duchenne muscular dystrophy (DMD), we performed qualitative and quantitative analysis of skeletal muscle inflammation and fibrosis in *mdx* and *mdx/utrn*^{+/-} littermates. Inflammation was significantly worse in *mdx/utrn*^{+/-} quadriceps at age 3 and 6 months and in *mdx/utrn*^{+/-} diaphragm at age 3 but not 6 months. Fibrosis was more severe in *mdx/utrn*^{+/-} diaphragm at 6 months, and at this age, mild fibrosis was noted in quadriceps of *mdx/utrn*^{+/-} but not *mdx* mice. The findings indicate that utrophin compensates, although insufficiently, for the effects of dystrophin loss with regard to inflammation and fibrosis of both quadriceps and diaphragm muscles in *mdx* mice. With more severe muscle dystrophy than *mdx* mice and a longer life span than utrophin-dystrophin-deficient (*dko*) mice, *mdx/utrn*^{+/-} mice provide a better mouse model for testing potential therapies for muscle inflammation and fibrosis associated with DMD.

Keywords

Duchenne muscular dystrophy; mouse model; *mdx*; *mdx/utrn*^{+/-}; inflammation; fibrosis

1. Introduction

Duchenne muscular dystrophy (DMD) is the most common genetic muscle disease, affecting 1 in 3,500 live male births (1). It is caused by a defect in the dystrophin gene on X-chromosome (2). The disease is characterized by progressive skeletal and cardiac muscle weakness with premature death in the third decade from respiratory and cardiac failure (3). Muscle biopsies

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The authors have nothing to disclose.

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from patients with DMD typically display dystrophic changes, including endomysial inflammation and fibrosis (4). Ameliorating muscle inflammation and fibrosis represents an important therapeutic approach for DMD to improve muscle function and clinical phenotype.

Mdx mice, which harbor a nonsense mutation in exon 23 of the dystrophin gene, have been widely used for studying pathophysiology and for exploring potential therapies for DMD. However, the muscular dystrophy phenotype in this mouse model is mild as compared to human DMD. *Mdx* mice have subtle weakness and a near normal life span. Endomysial inflammation in *mdx* diaphragm and limb muscles starts to develop at age 3 weeks, peaks in limb muscles at 8-16 weeks, and subsides spontaneously in limb muscles but not diaphragm thereafter (5-8). Progressive endomysial fibrosis only develops in diaphragm (8-10). Therefore, this mouse model does not completely recapitulate the human DMD phenotype, which somewhat limits its usefulness for testing potential therapies. To improve this mouse model, mice deficient in both dystrophin gene and its autosomal homolog utrophin gene (*dko* mice) were generated (11,12). *Dko* mice display early onset of muscle dystrophy, severe muscle weakness, joint contracture, growth retardation, and premature death around 10 weeks of age, features which phenotypically mimic human DMD. However, the early death of *dko* mice makes these mice difficult to obtain or maintain. In addition, although the onset of muscle dystrophy is earlier in *dko* mice, the diaphragm pathology, including the number of necrotic fibers (11), the percentage of newly regenerated fibers (11), and endomysial fibrosis (our unpublished data), is similar in *mdx* and *dko* mice at 10 weeks of age before *dko* mice usually die. Therefore, *dko* mice are not ideal for testing therapies. A mouse model intermediate in severity between *mdx* and *dko* mice would carry significant advantages for testing anti-inflammatory and anti-fibrotic therapies. We hypothesize that *mdx/utrn*^{+/-} mice, which survive much longer than *dko* mice, may develop more severe skeletal muscle inflammation and fibrosis than *mdx* mice, and might represent a model with these advantageous characteristics. To address this hypothesis, we quantified and compared the severity of inflammation and fibrosis in quadriceps and diaphragm muscles of *mdx* and *mdx/utrn*^{+/-} male littermates at 3 and 6 months of age.

2. Materials and methods

2.1. Animals and muscles

Six pairs of *mdx* and *mdx/utrn*^{+/-} male littermates were obtained from breeding of *mdx/utrn*^{+/-} mice (C57Bl/10) (11). Three pairs were sacrificed at age 3 months and three pairs at 6 months. Quadriceps and diaphragm muscles of these mice were collected for histopathological and immunohistochemical studies as well as hydroxyproline content measurements. We followed the guide for the care and use of laboratory animals of Cleveland Clinic and Case Western Reserve University.

2.2. Histopathological analysis

Muscle tissue was fresh-frozen in liquid nitrogen-cooled isopentane, sectioned at 8 micron (μm), stained with hematoxylin and eosin, and viewed under a bright field microscope.

2.3. Immunostaining

Eight μm cryostat sections of fresh frozen muscle tissue were blocked in 5% normal rabbit (for CD45 immunostaining) or donkey (for collagen III immunostaining) serum at room temperature for 1 hour followed by incubation in primary antibodies against mouse CD45 (Serotec, UK) or collagen III (Southern Biotechnology) overnight at 4 °C. For CD45 immunostaining, the sections were then incubated in biotinylated rabbit anti-rat secondary antibody (Vector Laboratories, Burlingame, CA) at room temperature for 1 hour followed by incubation with ABC (Vector Laboratory, Burlingame, CA) at room temperature for 30 minutes. Antibody binding was visualized with DAB substrate (Vector Laboratories,

Burlingame, CA) and viewed under a light microscope. For collagen III immunostaining, following the primary antibody incubation, the sections were incubated in FITC-conjugated donkey anti-goat secondary antibody (Sigma) at room temperature for 1 hour. Antibody binding was visualized under a fluorescent microscope.

2.4. Quantification of inflammation

Quantitative analysis of inflammation was performed by determining CD45 immunoreactivity as previously described (13). CD45 immunoreactivity, defined as area occupied by reaction product except for normal endomysial capillaries and expressed as a percentage of total muscle cross section area, was calculated on digitized images using an integrated image analysis system attached to a microscope (Olympus BX41). Serial sections were obtained from quadriceps and diaphragm muscles. Eight sections, representing one out of every five sections, of each sample were used for CD45 immunostaining. Immunostained muscle sections, with each section containing five nonoverlapping fields of view, were digitized under a 10× objective, using a 3-CCD color video camera interfaced with Sport Image analysis software. Digitized images were analyzed using the National Institute of Health Image J1.34 software. An area measurement analysis procedure was established to determine the proportion of immunoreactivity area within each fixed field of view. These parameters were then held constant for each set of images obtained at equal objectives and light sensitivities on slides that were processed at one session. The data represented the mean area occupied by immunoreactivity and were expressed as a percentage of total quadriceps or diaphragm cross section area.

2.5. Hydroxyproline assay

Hydroxyproline content was measured according to the protocol described previously (14). Briefly, muscle samples were homogenized thoroughly in distilled water with a final tissue concentration of 20 mg/ml. Duplicates of each sample and standard hydroxyproline solution were used for the assay. Twenty-five µl of each sample and standard hydroxyproline solution (2-20 µg/25 µl) were mixed with equal volume of sodium hydroxide (2N final concentration) and were then hydrolyzed by autoclave at 120°C for 20 minutes. Oxidation was taken at room temperature for 25 minutes by adding 450 µl of chloramine-T to each sample and mixing gently. This was followed by adding 500 µl of Ehrlich's aldehyde reagent to each sample and incubating the samples at 65°C for 20 minutes to develop the chromophore. Absorbance of each sample was read at 550 nm using a spectrophotometer. Hydroxyproline content of each sample was calculated using the standard hydroxyproline curve.

2.6. Statistical analysis

Differences in percentage of inflammation area and mean hydroxyproline content between different groups were evaluated by Student *t*-test. A *p*-value of <0.05 was considered significant.

3. Results

3.1. Endomysial inflammation was more severe in *mdx/utrn*^{+/-} quadriceps and diaphragm

At age 3 months, *mdx* quadriceps showed multiple endomysial foci of mononuclear inflammatory cells (Fig. 1A) which were stained positive for CD45 (Fig. 1B); the inflammatory foci were larger in *mdx/utrn*^{+/-} quadriceps (Fig. 1E, F) than in *mdx* quadriceps (Fig. 1A, B). The area occupied by inflammatory cells was higher in *mdx/utrn*^{+/-} (Mean±SD: 35.2%±4.4) than in *mdx* mice (6.1%±1.1, *p*<0.01) (Fig. 3). At 6 months, endomysial inflammation was reduced in *mdx* quadriceps, and only scattered endomysial inflammatory cells were present. Some small central nucleated regenerated muscle fibers were observed, but there was no

myofiber degeneration or necrosis (Fig. 1C, D). In contrast, *mdx/utrn*^{+/-} quadriceps at this stage showed marked fiber size variation and multiple small-to-medium foci of endomysial inflammation (Fig. G, H). The area occupied by inflammatory cells was significantly increased in *mdx/utrn*^{+/-} quadriceps ($15.7\% \pm 7.4$) as compared to *mdx* quadriceps ($4.4\% \pm 1.0$, $p=0.02$) (Fig. 3).

Both *mdx* and *mdx/utrn*^{+/-} diaphragm showed multiple small-to-medium foci of endomysial inflammation at 3 months as shown by H&E staining (Fig 2A, E) and highlighted by CD45 immunostaining (Fig. 2B, F). At 3 months, diaphragmatic inflammation in *mdx/utrn*^{+/-} mice was more severe than in *mdx* animals, with a greater area occupied by inflammatory cells ($25.7\% \pm 1.9$) as compared with *mdx* mice ($10.6\% \pm 2.4$, $p<0.01$) (Fig. 3). At 6 months, a moderate degree of inflammation persisted in diaphragm, unlike quadriceps, of both *mdx* (Fig. 2C, D) and *mdx/utrn*^{+/-} mice (Fig. 2G, H). At this time point, an equivalent area within diaphragm was occupied by inflammatory cells in diaphragms of *mdx/utrn*^{+/-} mice ($26.9\% \pm 9.6$) as compared to *mdx* ($18.0\% \pm 4.3$) mice (Fig. 3).

3.2. *Mdx/utrn*^{+/-} quadriceps developed mild endomysial fibrosis at 6 months

Fibrosis entails excessive collagen deposition. Collagen contains Gly-X-Y repeats and Y is usually hydroxyproline. Therefore, we used collagen III immunostaining and hydroxyproline content measurements to qualitatively and quantitatively assess endomysial fibrosis.

Mdx quadriceps showed a normal pattern of collagen III immunostaining at 3 (Fig. 4A) and 6 (Fig. 4C) months. *Mdx/utrn*^{+/-} quadriceps at 3 months also showed normal collagen deposition without endomysial fibrosis (Fig. 4B). However, endomysial fibrosis was evident in *mdx/utrn*^{+/-} quadriceps at 6 months with increased collagen III deposition (Fig. 4D) and a significantly higher mean hydroxyproline content ($\mu\text{g}/\text{mg}$ muscle tissue) (1.16) than that of *mdx* quadriceps (0.53, $p=0.02$) (Fig. 5).

3.3. Endomysial fibrosis was more severe in *mdx/utrn*^{+/-} diaphragm

Both *mdx* and *mdx/utrn*^{+/-} diaphragm developed progressive endomysial fibrosis with increased collagen III deposition (Fig. 4). The degree of fibrosis was comparable in the two mouse strains at 3 months (Fig. 4E, F), but was more severe in *mdx/utrn*^{+/-} mice at 6 months, with markedly increased collagen III deposition (Fig. 4H) as compared to *mdx* mice (Fig. 4G). Consistent with the morphological findings at 6 months, the mean hydroxyproline content of *mdx/utrn*^{+/-} diaphragm ($3.27 \mu\text{g}/\text{mg}$ muscle tissue) was significantly higher than that of *mdx* diaphragm (1.90 , $p=0.03$) (Fig. 5).

4. Discussion

This study shows that haploinsufficiency of utrophin gene worsens inflammation and fibrosis in quadriceps and diaphragm muscles of *mdx* mice. The finding supports a compensatory role of utrophin for the effects of dystrophin loss on skeletal muscle inflammation and fibrosis. The finding also suggests that *mdx/utrn*^{+/-} mice will provide advantages, as a mouse model of DMD, as compared with either *mdx* or *dco* mice, for testing anti-inflammatory and anti-fibrotic therapies.

Consistent with a compensatory role for utrophin, the onset of muscle dystrophic changes in *mdx* mice corresponds to the time when utrophin disappears from sarcolemma and localizes at the neuromuscular junction. Overexpression of utrophin in skeletal muscle restored dystrophin protein complex (DPC) and rescued the muscular dystrophy phenotype in a dose-dependent manner in *mdx* mice (15)(16). These findings suggest that dystrophin and utrophin can exert similar functions once distributed to the sarcolemma, and the mild muscular dystrophy

phenotype in *mdx* mice is probably due to the compensation by endogenous utrophin. Consistent with this hypothesis, *dko* mice, lacking both dystrophin and utrophin, show many features of human DMD, including premature death. Although *dko* mice do show more severe functional abnormalities of muscle than *mdx* mice (17,18), the cause of death in *dko* mice remains elusive. Myofiber necrosis, regeneration, and fibrosis in diaphragm muscles are not significantly different between *mdx* and *dko* mice (11 and our unpublished data) at 10 weeks of age when *dko* mice usually die. Because of this early death of *dko* mice, it is not entirely clear to what extent utrophin compensates for the effects of dystrophin loss on diaphragm muscle necrosis and fibrosis.

In the current study, we compared limb and diaphragm muscle inflammation and fibrosis in *mdx/utrn*^{+/-} and *mdx* mice at 3 and 6 months of age. *Mdx/utrn*^{+/-} mice are indistinguishable from *mdx* mice by size and weight; they do not show the same behavioral signs as *dko* mice. However, both skeletal muscle inflammation and fibrosis are more severe in *mdx/utrn*^{+/-} mice than in *mdx* mice. The findings support the hypothesis that utrophin compensates for the effects of dystrophin loss on skeletal muscle inflammation and fibrosis, probably through interactions with other protein components involved in the DPC. Haploinsufficiency of utrophin may compromise its compensatory function, which leads to more impairment of sarcolemmal integrity and subsequent more severe skeletal muscle inflammation and fibrosis in *mdx/utrn*^{+/-} mice. However, functional compensation provided by utrophin is incomplete, as *mdx* mice do develop mild muscular dystrophy.

We propose that *mdx/utrn*^{+/-} mice may represent a better mouse model than *mdx* and *dko* mice for testing chronic anti-inflammatory and anti-fibrotic therapies. Inflammation and fibrosis are prominent pathological features in muscle tissue of patients with DMD. Ameliorating these pathological changes represents an important therapeutic strategy to improve muscle function in DMD. The widely used mouse model, *mdx* mice, displays a mild phenotype, in which inflammation spontaneously subsides in the limb muscles and slowly progressive fibrosis only occurs in the diaphragm muscle. Although *dko* mice display all clinical features of human DMD, they die too early to develop significant diaphragm fibrosis (11,12). These models are not ideal for testing therapies. Our results indicate that *mdx/utrn*^{+/-} mice may well represent a DMD mouse model which carries significant advantages for testing chronic anti-inflammatory and anti-fibrotic therapies. First, *mdx/utrn*^{+/-} mice develop more severe skeletal muscle inflammation and fibrosis, and the progression of diaphragm fibrosis is faster than *mdx* mice. These attributes are advantageous for testing anti-inflammatory strategies to prevent or reduce fibrosis. Second, *mdx/utrn*^{+/-} mice survive much longer than *dko* mice, which allows significant diaphragm fibrosis to develop. Based on our observations, *mdx/utrn*^{+/-} mice can live beyond 1 year of age, which is permissive for testing chronic anti-fibrotic therapies. Third, it is easier to obtain and maintain *mdx/utrn*^{+/-} mice than *dko* mice, due to early death of the latter strain. For these reasons, although the genetic deficit in *mdx/utrn*^{+/-} mice is not identical to human DMD, this mouse model is potentially more useful than *mdx* and *dko* mice for testing therapeutic interventions to preclude muscle inflammation and fibrosis associated with DMD.

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Figure 1.

H&E staining (A, E, C, G) and CD45 immunostaining (B, F, D, H) of quadriceps of *mdx* (A, B, C, D) and *mdx/utrn*^{+/-} (E, F, G, H) mice showed larger inflammatory foci in *mdx/utrn*^{+/-} mice (E, F) than in *mdx* mice (A, B) at 3 months and persistent moderate degree of endomysial inflammation in *mdx/utrn*^{+/-} mice at 6 months (G, H) in contrast to largely resolved inflammation in *mdx* littermates (C, D). Bar=25 μ m.

Figure 2.

H&E staining (A, E, C, G) and CD45 immunostaining (B, F, D, H) of diaphragm showed larger inflammatory foci in *mdx/utrn*^{+/-} mice (E, F, G, H) than in *mdx* mice (A, B, C, D) at 3 months (A, B, E, F) and 6 months (C, D, G, H). Bar=25 μ m.

Figure 3.

Bar graph showing significantly higher percentages of CD45 positive inflammation area in *mdx/utr+/-* quadriceps at 3 and 6 months and *mdx/utr+/-* diaphragm at 3 months than in *mdx* mice. * $p < 0.05$

Figure 4.

Collagen III immunostaining of *mdx* (A, C, E, G) and *mdx/utrn*^{+/-} (B, D, F, H) quadriceps (A, B, C, D) and diaphragm (E, F, G, H) showed mild fibrosis with increased collagen deposition in *mdx/utrn*^{+/-} quadriceps at 6 months (D) but not 3 months (B) or in *mdx* quadriceps at 3 (A) or 6 months (C). Fibrosis was also present and comparable in *mdx* (E) and *mdx/utrn*^{+/-} (F) diaphragm at 3 months, and worse at 6 months with more collagen deposition in *mdx/utrn*^{+/-} (H) than in *mdx* mice (G). Bar=25 μ m.

Figure 5.

Bar graph showing significantly higher hydroxyproline contents in *mdx/utr*^{+/-} quadriceps and diaphragm at 6 months than in *mdx* mice. *p<0.05