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Molecular Pathogenesis of Genetic and Inherited Diseases

Elimination of Myostatin Does Not Combat Muscular Dystrophy in dy Mice but Increases Postnatal Lethality

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Myostatin is a TGF-β family member and a negative regulator of skeletal muscle growth. It has been proposed that reduction or elimination of myostatin could be a treatment for degenerative muscle diseases such as muscular dystrophy. Laminin-deficient congenital muscular dystrophy is one of the most severe forms of muscular dystrophy. To test the possibility of ameliorating the dystrophic phenotype in laminin deficiency by eliminating myostatin, we crossed *dy*^{W} laminin α2-deficient and myostatin null mice. The resulting double-deficient *dy*^{W}/*dy*^{W};*Mstn*$^{-/-}$ mice had a severe clinical phenotype similar to that of *dy*^{W}/*dy*^{W} mice, even though muscle regeneration was increased. Degeneration and inflammation of muscle were not alleviated. The pre-weaning mortality of *dy*^{W}/*dy*^{W};*Mstn*$^{-/-}$ mice was increased compared to *dy*^{W}/*dy*^{W}, most likely due to significantly less brown and white fat in the absence of myostatin, and post-weaning mortality was not significantly improved. These results show that eliminating myostatin in laminin-deficiency promotes muscle formation, but at the expense of fat formation, and does not reduce muscle pathology. Any future therapy based on myostatin may have undesirable side effects. *(Am J Pathol 2005, 166:491–497)*

Myostatin is a TGF-β family member that has been identified as a negative muscle regulator.[1] Myostatin null mutant mice and "double-muscled" cattle with spontaneous mutations in the myostatin gene have significantly greater muscle mass due to both muscle hypertrophy and hyperplasia.[2–4] *In vitro* studies showed that myostatin inhibits cell proliferation and protein synthesis in myoblasts.[5,6] Recently, it was reported that blockade of myostatin by treatment with antibodies or elimination of the myostatin by gene knock out significantly increased muscle mass, decreased muscle degeneration, and reduced fibrosis in *mdx*, dystrophin-deficient mice.[7,8] Other agents may be used in the future to eliminate or reduce myostatin at selected times, such as the myostatin antagonists follistatin, myostatin propeptide, and growth and differentiation factor-associated serum protein-1, GASP-1.[9–13] In the present study, we wanted to test if elimination of myostatin may be of benefit, not only in dystrophin-deficiency, but also in other forms of muscular dystrophy such as laminin-deficient congenital muscular dystrophy.

Congenital muscular dystrophy is a group of severe forms of muscular dystrophy, often leading to early death in humans.[14–18] The majority of cases are caused by mutations in the major laminin in the muscle basement membrane, laminin containing the α2 chain (Laminin-2/Merosin). This disease has been termed merosin-deficient congenital muscular dystrophy (MCMD) or more recently MDC1A.[19] Several different mutations that result in lack of laminin α2 or in the presence of a truncated form of laminin α2 have been identified in human patients.[19] Several mouse models for this disease are available,[20–29] including the *dy*^{W}/*dy*^{W} mouse generated by gene targeting in our laboratory.[27,28,30] The lack of functional laminin α2 in the muscle basement membrane of *dy*^{W}/*dy*^{W} mice leads to severe degeneration in skeletal muscle fibers, and most of the mice die at 3 to 6 weeks of age.[28,30] The deterioration in this disease is thought to be caused by the failure to form the primary laminin scaffold, which is necessary for basement membrane structure and interaction with the dystrophin-glycoprotein complex (DGC) and the integrins.[31] Transgenic expression of a functional human *LAMA2* gene[30] or of an agrin minigene with related activities[32] prevented the muscle degeneration in *dy*^{W}/*dy*^{W} mice. Normal muscle has a significant capacity for regeneration, and effective regeneration

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would be expected to improve longevity in muscular dystrophy. While the *mdx*, dystrophin-deficient mice have excellent muscle regeneration,[33,34] the dy^W/dy^W mice have poor regeneration,[28] which may be a factor in the poor prognosis for laminin-deficient mice. We hypothesized that elimination of myostatin may improve the regeneration in dy^W/dy^W mice. Indeed, dy^W/dy^W mice lacking myostatin showed increased muscle regeneration and had increased muscle mass. However, lack of myostatin did not improve the well-being of the mice or the pathological changes in muscle; instead, lack of myostatin had a negative effect on fat tissue and increased the postnatal mortality of the mice.

Materials and Methods

Generation of Mice

Myostatin-deficient mice of mixed C57BL and 129SV/J background were obtained from Dr. Sejin Lee (Johns Hopkins University, Baltimore, MD).[1] The dy^W/dy^W mice[30] used have been back-crossed to C57BL/6 for at least nine generations. To generate dy^W/dy^W, laminin-deficient mice that lack myostatin, heterozygous dy^W ($Lama2^{+/-}$) were bred to heterozygous ($Mstn^{+/-}$) or homozygous ($Mstn^{-/-}$) myostatin mutant mice. The first generation produced double-heterozygous mice ($Lama2^{+/-}$/$Mstn^{+/-}$), which were then used to produce laminin α2-deficient mice with or without myostatin at an expected frequency of 1/16.

PCR Genotyping

Tails were biopsied from 3-week-old mice, and genomic DNA was extracted. Four pairs of PCR primers were designed according to wild-type *Lama2* (forward 5′-ACTGCCCTTTCTCACCCACCCTT-3′ and reverse 5′-GTTGATGCGCTTGGGACTG-3′); *lacZ* knock-in and *Lama2* chimeric sequence (forward same as Lama2 forward and reverse 5′- GTCGACGACGACAGTATCGGCCTCAG-3′);[30] Mstn (forward 5′-CAGCCATGGTAGTAGACCG-3′ and reverse 5′- GATGTGCTCTCACTTCCTTG-3′); neomycin knock-in and *Mstn* chimeric sequence (forward 5′-TCTATCGCCTTCTTGACGAG-3′and reverse same as myostatin reverse)[1] to detect all four alleles. PCR conditions were 94°C for 3 minutes, 40 cycles at 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 1 minute, and 72°C for 10 minutes. PCR products were separated on an agarose gel and visualized by ethidium bromide.

Muscle Histology and Morphometric Study

Four-week-old mice were weighed and then sacrificed. Quadriceps, gastrocnemius, and triceps muscles were embedded in OCT, snap-frozen in isopentane pre-cooled in liquid nitrogen, and stored at −80°C until further processed. Ten-μm sections were cut from the mid-belly of the muscles and stained with hematoxylin and eosin (H&E). The cross-sectional area (CSA), cross-sectional fiber number (CSFN), and single fiber area (SFA) were measured and calculated using NIH image and Scion Image programs. At least four mice of each genotype were used for analysis.

Immunofluorescence

Ten-μm sections of rectus femoris muscle at mid-belly were cut on a cryostat and air-dried. Regenerated muscle fibers were detected by staining with mouse anti-embryonic myosin heavy chain (eMHC, 1:10; Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by the University of Iowa, Department of Biological Science, Iowa City, IA) and slow fibers by mouse anti-MHC Type I (1:60; Sigma, St Louis, MO) using the M.O.M. kit from Vector. Myoblasts were detected by staining with rabbit anti-MyoD (1:100; Santa Cruz, Santa Cruz, CA) and macrophages with rat antibody F4/80 (1:100; Caltag, Burlingame, CA) followed by FITC-labeled goat anti-rabbit or rabbit anti-rat IgG. Leukocytes were detected with PE-Cy5-labeled anti-CD45 (1:100; eBiosciences, San Diego, CA) and mature T cells with FITC-labeled anti-CD3e (1:100; eBioscience). After washing, sections were mounted with VECTASHIELD and observed under a fluorescence microscope.

Fat Tissue Analysis

Gonadal white fat pads and interscapular brown fat pads were dissected from 4-, 8-, and 12-week-old mice according to Johnson and Hirsch.[35] Fat pads from the left side were weighed and the weight expressed as percentage of total body weight. Fat pads from the right side were quick frozen, embedded in OTC, sectioned at 10 μm, and stained with H&E.

Statistics

Paired student's *t*-test was used in all analyses to determine significance.

Results

dy^W/dy^W;$Mstn^{-/-}$ Mice Have a Severe Clinical Phenotype Similar to dy^W/dy^W Mice

Mice were genotyped at 3 weeks and sacrificed at 4 weeks. At 4 weeks of age, the body weight of dy^W/dy^W; $Mstn^{-/-}$ was not significantly different from that of dy^W/dy^W mice (Figure 1A). As the body weight of the mice is a measure of muscle mass as well as of overall well-being, this result indicates that lack of myostatin did not have the same effect in the laminin-deficient mouse as it had in the dystrophin-deficient mdx mouse.[7] Necrosis and inflammation, as evaluated by staining of muscle sections with H&E, were not decreased in dy^W/dy^W; $Mstn^{-/-}$ mice compared to dy^W/dy^W mice (Figure 1D). There was also no difference in the number of infiltrating