Muscle regeneration in the prolonged absence of myostatin

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Edited by Louis M. Kunkel, Harvard Medical School, Boston, MA, and approved January 6, 2005 (received for review November 23, 2004)

Myostatin is an endogenous inhibitor of muscle conserved across diverse species. In the absence of myostatin, there is massive muscle growth in mice, cattle, and humans. Previous studies in the *mdx* mouse model of muscular dystrophy demonstrate that inhibiting myostatin attenuates several features of dystrophic muscle. These findings have encouraged the development of human therapies to block myostatin. However, little is known of the long-term effects on muscle of myostatin blockade. To evaluate potential sequelae from the prolonged absence of myostatin, senescent myostatin null (*mstn*^{-/-}) mice were studied. Senescent *mstn*^{-/-} mice continue to have normal muscle with increased mass and strength relative to controls. Muscles of senescent *mstn*^{-/-} mice regenerate robustly from both chronic and acute injury. Early markers of regeneration are enhanced in the absence of myostatin, suggesting a mechanism for the attenuation of dystrophic features found in *mdx* mice lacking myostatin.

aging | muscular dystrophy

Myostatin is a highly conserved TGF- β family member that functions as an endogenous inhibitor of muscle growth in diverse species (1–5). The myostatin gene is expressed almost exclusively in cells of skeletal muscle lineage throughout embryonic development as well as in adult animals (1, 6). In adult animals, myostatin appears to inhibit the activation and differentiation of satellite cells, resident stem cells in skeletal muscle that are critical to muscle regeneration (7, 8). Targeted disruption of the myostatin gene in mice leads to muscle hypertrophy and hyperplasia with an approximate doubling of muscle mass (1). Similarly, natural myostatin mutations in cattle have been shown to be associated with a “double-muscling” phenotype (2, 4, 5). Most recently, the function of myostatin as a negative regulator of muscle growth has been shown to be conserved in humans through the identification of a hypermuscular child with a loss-of-function mutation in the myostatin gene (3).

The potential effects of myostatin inhibition on muscle degenerative disease have been explored in the *mdx* mouse (9, 10). The *mdx* mouse has a nonsense mutation in the gene for dystrophin and serves as a genetic model of Duchenne and Becker muscular dystrophies (11). In this model, skeletal muscle undergoes repeated rounds of degeneration and regeneration with diaphragm muscle most severely affected (12). *Mdx* mice lacking myostatin have greater muscle mass, increased functional strength, and decreased fibrosis and fatty infiltration of muscles than their *mdx* counterparts (9). Postnatal injection of neutralizing mAbs directed against myostatin into either WT or *mdx* mice increases muscle mass and specific force, indicating that myostatin regulates muscle growth in adult animals (10, 13).

These findings have suggested that myostatin inhibition may be a possible therapy for human diseases of muscle weakness and wasting. Recently, clinical trials with an inhibitor of myostatin have been initiated in adult muscular dystrophy. However, little is known about the long-term effects of loss of myostatin. Theoretical concerns have been raised that inhibition of myostatin will lead to a burst of muscle growth, followed by depletion of muscle regenerative capacity in the setting of a chronic muscle

disease. To examine this possibility, we studied muscle regeneration in senescent mice lacking myostatin. In the prolonged absence of myostatin in mice, muscle continues to regenerate robustly.

Materials and Methods

Animals and Muscle Injury. Myostatin null (*mstn*^{-/-}) mice and littermate controls were provided by the laboratory of Se-Jin Lee (The Johns Hopkins University School of Medicine) (1). *Mdx* mice were obtained by Jackson ImmunoResearch and bred to myostatin null mice as described (9). All mice were on a C57BL/6 background. The age range of experimental animals was between 3 weeks and 24 months. Control and disease animals were matched by age and gender. All animal studies were authorized by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

For studies involving muscle injury, mice were anesthetized by metofane inhalation. Tibialis muscle was injected with 0.1 ml of 10 μ M cardiotoxin (Calbiochem), which was diluted in PBS (14, 15). Muscle was harvested at various times after injection (1, 2, 3, 4, 7, 14, and 30 days). Contralateral, uninjected tibialis muscle was used as a control.

Muscle Histology and Morphometric Analysis. Muscle for histological analysis was flash frozen in isopentane. Cryostat cross sections (10 μ m) were stained with hematoxylin and eosin. Muscle fiber diameters were determined by using IPLAB software (Scanalytics, Fairfax, VA) as described (9). We measured 300–500 consecutive fibers in each of three tibialis muscles per genotype per time point. Percentage of central nucleation was determined by counting the total number of central nucleated fibers and total myofibers in cross section of the triceps midbelly.

Evans Blue Dye (EBD). We injected into the peritoneal cavity of 3-week-old animals 1% volume per body mass of 1% EBD, an autofluorescent dye that is excluded by normal sarcolemma but is internalized during membrane permeability and myofiber necrosis (16). Animals were returned to their cages and killed 24 h after injection. EBD-positive fibers and total myofiber number were counted in three different cross sections of each

This paper was submitted directly (Track II) to the PNAS office.

Freely available online through the PNAS open access option.

Abbreviations: EBD, Evans blue dye; mrf, myogenic regulatory factor.

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[‡]Under a licensing agreement between MetaMorphix, Inc., and The Johns Hopkins University, The Johns Hopkins University is entitled to royalty payments on sales of the growth factor, myostatin, described in this article. Also, The Johns Hopkins University is entitled to a share of sublicensing income from arrangements between MetaMorphix and Wyeth. The Johns Hopkins University owns MetaMorphix, Inc., stock, which is subject to certain restrictions under The Johns Hopkins University policy. The terms of this arrangement are being managed by The Johns Hopkins University in accordance with its conflict of interest policies.

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triceps including the midbelly, and the average number of EBD-positive fibers per 1,000 fibers was calculated.

EBD-positive fibers were also quantified in 20- to 24-month-old animals after prolonged exercise. At 1 h after injection with EBD, mice were placed on a motorized treadmill (Eco 3/6, Columbus Instruments, Columbus, Ohio) and run at a 15° downward angle at a rate of 10 m/min for 10 min. Mice were killed 3 h after exercise, and EBD-positive fibers in triceps muscles were quantified as described above.

Functional Muscle Strength. Forearm grip strength was assessed by using an automated grip strength meter (Columbus Instruments). Total peak force (in N) was determined by an electronic strain gauge as described (17). Five measurements within 2 min were taken from each animal. Maximum values were used for statistical analysis (Student's paired *t* test).

Myogenic Regulatory Factor (mrf) Expression. Tibialis muscle from senescent, 20- to 24-month-old *mstn*^{-/-} and WT littermates was injured with cardiotoxin, as described above. Muscle was harvested before injury and at 1, 2, 3, 4, and 7 days after injury, and RNA was prepared by using TRIzol reagent according to the manufacturer's instructions (Invitrogen). We separated 10 μ g of total RNA on a denaturing agarose gel and transferred to GeneScreen Plus hybridization transfer membrane (PerkinElmer). Membranes were hybridized with myogenin, myoD, and mrf4 cDNA probes provided by Se-Jin Lee. Hybridization was quantified by using a phosphorimager (Fuji). Membranes were then stripped and reprobed with *phospho*-oligo(dT), and phosphorimager analysis was repeated. The relative abundance of mrf transcripts per lane was expressed in arbitrary units as mrf transcript per polyA transcript $\times 10^{-3}$.

Satellite Cell Isolation and Cell Culture. Satellite cells were isolated from 6- to 7-month-old and 18-month-old WT (C57BL/6) and *mstn*^{-/-} mice as described (18). Satellite cells were plated on polylysine and fibronectin-coated 48-well culture plates at a density equivalent to 0.2 g of muscle tissue per well. The cells were maintained in growth medium consisting of DMEM (HyClone), 10% horse serum (Gemini Biological Products, Calabasas, CA), 1% antibiotic/antimycotic, and 1% gentamicin. For evaluating the effect of myostatin on proliferation and differentiation of WT and *mstn*^{-/-} satellite cells, cultures were maintained in growth medium from the time of plating to 48 h. The cells were then maintained in growth medium with or without 300 ng/ml recombinant mouse myostatin (R & D Systems) until 120 h after plating.

Myosin Heavy Chain Detection. Cultured cells were fixed with 2% paraformaldehyde in PBS, incubated overnight at 4°C in a 1:5 dilution of anti-myosin heavy chain mAb MF20 (Developmental Studies Hybridoma Bank, Iowa City, IA), and incubated for 2 h at room temperature in a 1:100 dilution of peroxidase-conjugated goat anti-mouse IgG (Sigma). After incubation with 1 mg/ml 3,3'-diaminobenzidine (DAB; plus 0.02% H₂O₂), the total number of nuclei and nuclei in MF20-positive myotubes and myocytes per field were counted in 10 random microscope fields, and the total number of nuclei per square millimeter and percentage of nuclei MF20-positive cells were calculated.

DNA Fluorometric Assay for Cell Proliferation. DNA fluorometric assay for cell proliferation was adapted from Rago *et al.* (19). Plates were rinsed twice with sterile PBS, stored at -70°C, and thawed, and 100 μ l of Hoechst 33258 dye (20 μ g/ml in 10 mM Tris/1 mM EDTA/2 M NaCl, pH 7.4; Sigma) was added to each well. Plates were incubated at room temperature for 1 h in the dark with gentle shaking, and fluorescence was measured with excitation at $\lambda = 350$ nm and emission at $\lambda = 460$

Fig. 1. Increased muscle mass of senescent (24-month-old) mice lacking myostatin. Triceps (tric), pectoralis (pect), quadriceps (quad), and gastrocnemius (gast) muscle isolated from C57BL/6 WT (gray, *n* = 5) and *mstn*^{-/-}/C57BL/6 (black, *n* = 3) mice. **, *P* < 0.01; ***, *P* < 0.001.

nm. Serial diluted double-stranded calf thymus DNA (Sigma) samples from 20–1,000 μ g of DNA per well were used for a standard curve.

Creatine Kinase Activity Assay for Cell Differentiation. The creatine kinase assay for cell differentiation was adapted from Florini (20). We added 100 μ l of 0.05 M glycylglycine (pH 6.75) to each well, and the plates were frozen at -70°C for 1 h and thawed, and 300 μ l of reaction mixture (20 mM creatine phosphate/20 mM glucose/10 mM magnesium acetate/1 mM ADP/10 mM AMP/0.4 mM thionicotinamide-adenine dinucleotide/10 mM DTT/0.5 unit/ml hexokinase/1 unit/ml glucose-6-phosphate dehydrogenase in 0.1 M glycylglycine, pH 6.75) was added to each well. Plates were incubated at room temperature for 15 min, and absorbance was measured at 405 nm.

Satellite Cell Activation. Mouse satellite cells were isolated and cultured in growth medium as mentioned above. Cultures were pulse-labeled from 40 to 42 h after plating with 10 μ M BrdUrd. After labeling, cells were rinsed with PBS, fixed in 100% methanol and prepared for immunolocalization of BrdUrd as described (21). The percentage of BrdUrd-labeled nuclei was determined in 10 random microscope fields per culture.

Results

To investigate whether muscle structure and growth remains normal in the prolonged absence of myostatin, senescent, 24-month-old *mstn*^{-/-} were analyzed and compared with their

Fig. 2. Muscle histology of senescent (24-month-old) WT and *mstn*^{-/-} mice. (A) Uninjured tibialis muscle. (B) Tibialis muscle at 1 month after cardiotoxin injury. (Scale bar, 50 μ m.)

Fig. 3. Average myofiber diameters in uninjured and regenerating 24-month-old WT (circles, dashed line) and myostatin null (squares, solid line) muscle. Diameters at days 7 and 15 after injury were significantly different ($P < 0.04$; $n = 3$ for each group).

WT littermates. Similar to young animals lacking myostatin as described (1), muscle weights of senescent *mstn*^{-/-} animals remain elevated by >2-fold compared with their WT counterparts (Fig. 1). Histology of multiple limb muscles is normal with no evidence of myopathy including no significant split fibers, central nucleated fibers, or fibrosis, compared with WT senescent animals (Fig. 2A). Myofibers of senescent *mstn*^{-/-} animals are not prone to eccentric contraction-induced injury. Prolonged exercise on a declined treadmill produces negligible (mean, ≤ 2 per 1,000 fibers), scattered pre-necrotic fibers, as identified by EBD in both senescent *mstn*^{-/-} ($n = 5$) and WT ($n = 7$) animals.

Fig. 4. mrf expression after injury. Northern blot analysis of mrf expression (Left) and relative abundance of transcripts (Right) in WT (circles, dashed line) and *mstn*^{-/-} (squares, solid line) mice after cardiotoxin injury. Myogenin (A), myoD (B), and myf4 (C) expression at days 0, 1, 2, 3, 4, and 7 after injury displayed as mrf transcript per total polyA transcripts, times 10^{-3} . Similar results were obtained in duplicate experiments.

Fig. 5. Comparison of proliferation and differentiation of satellite cells isolated from the skeletal muscle tissue of WT (gray bars) and *mstn*^{-/-} mice (black bars). Cells were cultured in DMEM plus 10% horse serum in 48-well plates up to 96 h. (A) Satellite cell proliferation measured by nuclei number per square millimeter. (B) Satellite cell differentiation measured by percentage of nuclei in sarcomeric myosin heavy chain-positive cells. (C) Satellite cell differentiation measured by creatine kinase activity at OD = 405 μ m per mg of DNA. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

When acutely injured by cardiotoxin, tibialis muscle from senescent 24-month-old *mstn*^{-/-} animals retain the ability to regenerate. Histological abnormalities of fully regenerated muscle are limited to variability of fiber size, centralized nuclei, and rare split fibers similar to regenerated WT muscle (Fig. 2B). However in contrast to WT muscle, *mstn*^{-/-} muscle more rapidly attains larger diameter myofibers in the first week after injury (Fig. 3). For example, at 7 days after injury, *mstn*^{-/-} myofibers have diameters 43% larger than WT myofibers ($23.5 \pm 2.7 \mu$ m versus $13.4 \pm 3.5 \mu$ m, $P = 0.04$, $n = 3$ for each group). These diameters correspond to 55% of the respective uninjured myofiber size in the *mstn*^{-/-} animals and 36% of uninjured myofiber size in the WT animals. WT myofibers do not attain a comparable fiber size ($22 \pm 1.6 \mu$ m) for another week at 15 days after injury. By 30 days after injury, myofibers from both genotypes have reached 85–89% of the myofiber diameter from their respective uninjured counterparts.

Satellite cells are induced to proliferate and differentiate in growth and regeneration. This requires the initiation of a muscle specific gene program involving the expression of basic helix-loop-helix mrf. The expression of these mrf at various time points after injury by cardiotoxin injection was evaluated in *mstn*^{-/-} and WT counterparts. As shown in Fig. 4, where the relative abundance of transcripts in *mstn*^{-/-} and control animals is normalized to total polyA⁺ RNA, the expression of myogenin (4A) is similar in *mstn*^{-/-} and control animals. However, myoD expression (4B) is more robust in *mstn*^{-/-} than control animals

fibers, was almost identical between *mdx* ($59 \pm 7\%$, $n = 16$) and *mstn*^{-/-}/*mdx* animals ($58 \pm 7\%$, $n = 17$).

To evaluate further the regenerative potential after chronic injury in the prolonged absence of myostatin, aged *mstn*^{-/-}/*mdx* mice were analyzed. Muscle weights of 18-month-old *mstn*^{-/-}/*mdx* animals remain elevated compared with *mdx* controls (Fig. 7). Functional strength, as measured by forearm grip strength, was significantly ($P = 1.5 \times 10^{-5}$) greater in 18-month-old *mstn*^{-/-}/*mdx* (mean, 1.51 ± 0.13 N; $n = 8$) compared with *mdx* controls (mean, 1.02 ± 0.18 N; $n = 9$). Diaphragm is the most severely affected muscle in the *mdx* model and by 18 months frequently consists of only a thin strip of fibrotic tissue (12, 25). In the prolonged absence of myostatin, diaphragm muscle demonstrated some fibrosis and fatty infiltration but consistently contained abundant myofibers (Fig. 8A). Regenerative capacity in the prolonged absence of myostatin was further challenged by acutely injuring aged *mdx* muscle. Tibialis muscle from 18-month-old *mstn*^{-/-}/*mdx* animals injured with cardiotoxin injection was able to regenerate with pathology indistinguishable from regenerated *mdx* muscle (Fig. 8B and C).

Discussion

Blockade or loss of myostatin causes increased muscle growth by disinhibition of muscle progenitor cells (reviewed in ref. 26). The effects of long-term myostatin loss are unknown and have been cause for concern, particularly with the current introduction of myostatin inhibitors as potential human therapies. The results presented in this article suggest that there are not long-term deleterious effects to mouse muscle in the prolonged absence of myostatin. Senescent myostatin null mice maintain increased muscle mass and strength relative to controls. No myopathic features or increased susceptibility to contraction-induced injury were observed. Aged *mdx* mice lacking myostatin that have undergone rounds of degeneration and regeneration also continue to maintain increased muscle mass and strength relative to *mdx* controls.

These results suggested that the regenerative capacity of mouse muscle was not exhausted in the prolonged absence of myostatin. The ability of muscle to regenerate in the prolonged absence of myostatin was challenged by cardiotoxin injury from which senescent myostatin null mice readily regenerated. Also, aged *mdx* mice lacking myostatin also readily regenerated from cardiotoxin injury.

Contrary to an exhaustion of regenerative capacity, several lines of evidence suggest that in the absence of myostatin, muscle regenerates more robustly. First, as has been shown (9), *mdx* mice lacking myostatin have decreased fibrosis and fatty infiltration in diaphragm muscles. This was evident, as shown in this article, even in aged 18-month-old *mdx* animals that have more myofibers and less fibrosis in diaphragm muscle than *mdx* controls. Second, after injury, muscle lacking myo-

statin regenerate large diameter myofibers earlier than controls and express myoD and mrf4 at least 24 h earlier than controls. Third, *in vitro* experiments reported in this study are consistent with the enhanced regenerative capacity of myostatin null muscle in demonstrating greater proliferation and earlier differentiation of *mstn*^{-/-} satellite cells when compared with WT cells.

Although it has been postulated that lack of myostatin decreases myofiber degeneration in *mdx* mice, our studies do not support an effect on degeneration (10, 27). Previously, we had not observed a significant difference in creatine kinase, a serum marker of muscle breakdown, in the *mdx* versus *mstn*^{-/-}/*mdx* populations (9). In this study, there was no significant difference between large numbers of *mdx* and *mstn*^{-/-}/*mdx* animals in EBD-positive fibers during the major period of muscle degeneration at 3 weeks of age, and there was a nearly identical number of regenerated fibers at 6 weeks of age. Therefore, a plausible interpretation of studies (9, 10) showing amelioration of the *mdx* phenotype with blockade or loss of myostatin is that muscle continues to degenerate because of the loss of dystrophin, but that regeneration is enhanced without the endogenous inhibitor myostatin.

The results in this article suggest that mice are not at risk of depleting the pool of muscle progenitor cells and exhausting their regenerative reserve in the prolonged absence of myostatin. This observation can be understood if myostatin normally inhibits satellite cell proliferation as well as differentiation, as shown here and in refs. 8, 23, and 24. In the absence of myostatin, satellite cells are disinhibited to proliferate, replenishing the stem cell pool and subsequently differentiate into new myofibers or fuse with preexisting fibers. In the prolonged absence of myostatin, satellite cells from 18-month-old myostatin null mice are activated to divide at least as well as those from control animals.

These results are encouraging for the human application of myostatin inhibitors, suggesting that myostatin inhibition may provide a sustained benefit of improved muscle regeneration. However, most cells do not replicate indefinitely, potentially because of the loss of telomeric DNA via the end-replication problem. There are substantial differences in the telomerase activity in humans and rodents, and shortening of telomere length has been found in some (28) but not all (29) studies of human dystrophic muscle. Similar experiments of regeneration in the prolonged absence of myostatin need to be conducted in larger mammals or telomerase-deficient mouse models that more closely resemble the replicative aging seen in humans.

We thank Se-Jin Lee for supporting this work and Alexandra McPherron for offering helpful suggestions on the manuscript. This work was supported by National Institutes of Health Grant K08NS02212 (to K.R.W.), grants from the Muscular Dystrophy Association (to K.R.W. and R.E.A.), and a grant from the U.S. Department of Agriculture National Research Initiative Competitive Grants Program (to R.E.A.).

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