Muscle Atrophy and Weakness After Anterior Cruciate Ligament Tear

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ABSTRACT: Anterior cruciate ligament (ACL) tears are among the most frequent knee injuries in sports medicine, with tear rates in the US up to 250,000 per year. Many patients who suffer from ACL tears have persistent atrophy and weakness even after considerable rehabilitation. Myostatin is a cytokine that directly induces muscle atrophy, and previous studies in rodent models and patients have demonstrated an upregulation of myostatin after ACL tear. Using a preclinical rat model, our objective was to determine if the use of a bioneutralizing antibody against myostatin could prevent muscle atrophy and weakness after ACL tear. Rats underwent a surgically induced ACL tear and were treated with either a bioneutralizing antibody against myostatin (10B3, GlaxoSmithKline) or a sham antibody (E1-82.15, GlaxoSmithKline). Muscles were harvested at either 7 or 21 days after induction of a tear to measure changes in contractile function, fiber size, and genes involved in muscle atrophy and hypertrophy. These time points were selected to evaluate early and later changes in muscle structure and function. Compared to the sham antibody group, 7 days after ACL tear, myostatin inhibition reduced the expression of proteolytic genes and induced the expression of hypertrophy genes. These early changes in gene expression lead to a 22% increase in muscle fiber cross-sectional area and a 10% improvement in maximum isometric force production that were observed 21 days after ACL tear. Overall, myostatin inhibition lead to several favorable, although modest, changes in molecular biomarkers of muscle regeneration and reduced muscle atrophy and weakness following ACL tear. © 2017 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

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With rates of up to 250,000 per year, anterior cruciate ligament (ACL) ruptures are one of the most common knee injuries to physically active individuals, often interfering with their athletic career.1 Many patients who suffer ACL tears develop persistent atrophy and weakness of their quadriceps, even after ACL reconstruction (ACL-R) and completion of a rehabilitation program with good compliance. Several studies demonstrate that quadriceps strength deficits can exceed 30% at 6 months post ACL-R, and can persist for longer periods of time.2,3 A reduction in satellite cell abundance and whole muscle physiological cross-sectional area is also observed in the quadriceps after ACL tear.4 Other muscle groups in the hip, thigh, and lower leg, such as the hip extensors, hip adductors, knee flexors, and ankle dorsiflexors and plantarflexors also experience varying degrees of atrophy and weakness following ACL tear (ACLT).2,5–7 Persistent weakness can lead to poor physical performance, increase the risk for recurrent injury, and alter knee kinematics in a way that promotes early onset osteoarthritis.8–11 While the majority of rehabilitation interventions targeted at preventing muscle atrophy after ACL-R are focused on neurogenic-mediated pathways of muscle inhibition,9 almost no studies have directly addressed the cellular and molecular mechanisms of atrophy and the loss of intrinsic force production in muscle fibers themselves.

Myostatin (GDF-8) is a member of the transforming growth factor-β (TGF-β) superfamily of cytokines and functions to induce muscle fiber atrophy and weakness.12 At the cellular level, myostatin induces muscle atrophy by directly activating the ubiquitin-proteosome and autophagy pathways, and by blocking protein synthesis pathways activated by IGF-1 signaling.13–16 The mature myostatin protein sequence is conserved across humans and rodents, and the therapeutic inhibition of myostatin has been shown to protect against atrophy, weakness, and fibrosis in several different models of cancer cachexia and neuromuscular diseases.17,18 Patients with ACL tears have up to a 50% increase in circulating levels of myostatin,9 and data from a rat model of ACL tears demonstrated a six-fold increase in myostatin expression in quadriceps muscles.19 Since myostatin plays a central role in directing muscle atrophy and is elevated in quadriceps muscle after ACLT, it is a potentially attractive therapeutic target in protecting against weakness in patients with torn ACLs.

Given our limited ability to prevent muscle atrophy in patients following ACLT and the high economic cost and burdens associated with both the immediate and long-term consequences of this atrophy, our objective was to evaluate the ability of a bioneutralizing monoclonal antibody against myostatin (10B3, GlaxoSmithKline) to prevent muscle weakness after ACL
tear. Using a preclinical rat model, we tested the hypothesis that blocking myostatin activity after an ACL tear will prevent atrophy of lower limb muscles and also protect against the loss in muscle maximum isometric force production.

**METHODS**

**Animals and Surgical Procedure**

This study was approved by the University of Michigan IACUC, and followed PHS guidelines for the ethical treatment of animals. Three month old male Fischer 344 rats were obtained Charles River (Wilmington, MA) and housed under specific pathogen free conditions. A total of 36 rats were used in the study. Four rats served as non-operative controls. The remaining 32 rats were randomly distributed into four equal groups of eight, defined by their treatment (sham antibody or anti-myostatin antibody) and time since surgery (7 or 21 days), as described below. These time points were selected based upon the work of Delfino and colleagues,3,16,19,20 our experience with other rat models of muscle atrophy, and our clinical experience in treating patients with ACL tears. The study was powered based on maximum isometric force values of extensor digitorum longus (EDL) muscles from a previous study.21 To detect a 30% difference between groups with a power of 0.80 required a sample size of six per group. We included two additional animals in each group. One rat was lost in each group except for the 7 day myostatin antibody group, providing a N = 7 for the 7 day sham antibody group, N = 8 for the 7 day myostatin antibody group, N = 7 for the 21 day sham antibody group, and N = 7 for the 21 day myostatin antibody group.

Surgical transection of the left hindlimb ACL was performed, modified from Delfino and colleagues,19 to induce mechanical instability and an acute arthropathy. Animals were deeply anesthetized with 2% isoflurane and placed in a supine position. The left knee was shaved and prepared with Chloraprep (CareFusion, El Paso, TX). A 1 cm midline skin incision was made over the anterior left knee. The tibiofemoral joint was exposed using a lateral parapatellar approach. With the knee in full flexion, the ACL and intermeniscal ligament were identified and transected under direct visualization, and instability was confirmed with a positive anterior drawer test. The intermeniscal ligament was transected to provide additional knee instability to better model the human injury condition. The capsule was closed with 4-0 Vicryl (Johnson & Johnson, New Brunswick, NJ) sutures, the skin was closed using a subcutaneous running suture of 4-0 Vicryl, and GLUture (Abbott Laboratories, Abbott Park, IL) was applied over the incision. Subcutaneous buprenorphine at a dose of 0.05 mg/kg was administered for post-operative analgesia. Immediate weightbearing and cage activity was allowed postoperatively and rats were monitored for signs of distress or infection. All rats were ambulatory and demonstrated signs of adequate food and water intake.

At the time of surgery, rats received a single intraperitoneal injection of a bioneutralizing murine anti-myostatin monoclonal IgG antibody (10B3, GlaxoSmithKline, King of Prussia, PA)22 or a sham murine anti-human papillomavirus E1 helicase monoclonal IgG antibody (E1-82.15, GlaxoSmithKline)23 that does not bind to any endogenous rat proteins. The dose of antibodies was 30 mg/kg, with apparent half lives greater than 7 days.22,23 This approach was selected to block the acute effects of myostatin in the immediate post-ACL tear period. Additional pharmacokinetic data about the MSTN mAb is available through US Patent WO2010070094A1.

At either 7 or 21 days after tear, rats were anesthetized with sodium pentobarbital. EDL muscles were removed to be used for in vitro muscle contractility measurements. The EDL was selected as a model of muscle function as several lower limb muscles experience muscle atrophy after ACL tear, and because of the frequent use of this muscle in studies of rodent muscle function. The distal 1.5 cm of the vastus lateralis was removed and fixed in 10% neutral buffered formalin (NBF) to be used for histology. The distal 1.5 cm of the rectus femoris muscle group was removed, finely minced and snap frozen at −80°C for gene expression analysis. Following removal of tissues, rats were euthanized by overdose of sodium pentobarbital followed by induction of bilateral pneumothorax.

**EDL Contractility**

Contractile properties of EDL muscles were performed as previously described.21,24 The EDL was selected due to its uniform architecture, its frequent use in rodent models of muscle injury, and because patients with ACL tears have ankle dorsiflexion strength deficits of 20% or more after ACLT.6 A 4-0 silk suture was tied to the proximal and distal tendons of intact EDL muscles, just distal to the aponeuroses. Following suture placement, muscles were then removed from the animal and immediately placed in a bath that contained Krebs mammalian Ringer solution supplemented with 11 mM glucose and 0.3 mM tubocurarine chloride. The bath was maintained at 25°C, and bubbled with a mixture of 95% O2 and 5% CO2 to maintain a stable pH of 7.4. The distal tendon of the EDL was tied to a dual-mode servomotor/force transducer (Aurora Scientific, Aurora, ON) and the proximal tendon tied to a fixed hook. Using square wave pulses delivered from platinum electrodes connected to a stimulator (Aurora Scientific), muscles were stimulated to contract. Custom designed software (LabVIEW, National Instruments, Austin, TX) controlled pulse properties and servomotor activity, and recorded data from the force transducer at 20 kHz. The voltage of pulses was serially increased, and the muscle length was increased or decreased to provide the length (L0) that resulted in maximum twitch force. Muscles were held at L0 and stimulated with pulse trains of 300 ms to generate isometric contractions. Stimulus frequency was increased until maximum isometric force was achieved. To calculate specific force, maximum isometric force was divided by the physiological cross-sectional area (PCSA), which was determined by dividing muscle mass by the product of fiber length (L0) and 1.056 g/cm3, the density of mammalian skeletal muscle. Following measurement of contractile properties, muscles were fixed in 10% neutral buffered formalin and prepared for histology.

**Histology**

Histology was performed as described.21,25 EDL and vastus lateralis muscles were fixed in 10% NBF for 2 days, dehydrated in 70% ethanol and then embedded in paraffin. Muscles were sectioned at the midbelly in a microtome at a thickness of 10 μm, rehydrated and stained with wheat germ agglutinin (WGA) lectin conjugated to AlexaFluor 488 (WGA-488, Life Technologies, Carlsbad, CA) to identify extracellular matrix. Slides were imaged using a Zeiss Axiosvert 200M microscope (Carl Zeiss, Thornwood, NY), and ImageJ software (NIH, Bethesda, MD) was used to perform quantitative fiber
cross-sectional area (CSA) measurements. All fibers in cross-section were counted by two independent assessors who were blinded to the study treatment groups.

Gene Expression
Gene expression was performed as described. Total RNA was isolated from homogenized rectus femoris muscles using a miRNeasy kit (Qiagen, Valencia, CA). After treatment with DNase I (Qiagen), RNA was reverse transcribed into cDNA with iScript supermix (Bio-Rad, Hercules, CA), and quantitative PCR (qPCR) was conducted in a CFX96 real time thermal cycler using iTaq SYBR green supermix reagents (Bio-Rad). The 2^(-ΔΔCt) technique was used to normalize the expression of RNA transcripts to the stable housekeeping gene β-actin, and each of the treatment groups was further normalized to control, non-operated muscles. A listing of RNA transcripts and primer sequences is provided in Supplementary Table S1.

Statistics.
Data are presented as mean ± SD. Differences between treatment groups were tested with a two-way ANOVA (α = 0.05) followed by Fisher’s LSD post-hoc sorting in GraphPad Prism 6.0 (La Jolla, CA).

RESULTS
No difference in body mass was observed between the sham mAb or MSTN mAb group at either 7 days (254 ± 7 g for sham mAb, 261 ± 9 g for MSTN mAb) or 21 days (300 ± 12 g for sham mAb, 304 ± 11 g for MSTN mAb). For EDL muscle fiber cross-sectional area (CSA) (Fig. 1A), no differences were observed between the 7 day groups. The 21 day sham mAb group was approximately 40% greater than the 7 day groups, but the 21 day MSTN mAb group had approximately 54% greater CSA values than the 7 day groups, and was also 22% greater than the 21 day sham mAb group. Similar trends were observed for vastus lateralis muscles (Fig. 1B).

There was a time-dependent effect on EDL muscle mass and PCSA, with both groups at 21 days having an approximately 15% increase in mass and a 13% increase in PCSA compared to 7 day groups (Fig. 2A and B). For maximum isometric force (Fig. 2C), the 21 day MSTN mAb group had the highest values, which were approximately 21% greater than both 7 day treatment groups and 10% greater than the 21 day sham mAb group. No differences in specific force were observed between groups, suggesting no changes in the overall density of myofibrils in muscle fibers (Fig. 2D).

We next measured the expression of several genes involved in muscle atrophy and hypertrophy (Fig. 3). Nearly all genes at all time points were elevated compared to control, uninjured muscles. For the ubiquitin ligases atrogin-1, MuRF-1, and MUSA-1, which are important rate limiting steps in muscle protein degradation, myostatin mAb treatment resulted in a downregulation in the expression of these genes at 7 days compared to the sham mAb group, although no differences between groups were present at 21 days. Similar observations were made for Beclin-1, which plays a central role in autophagy. IGF-1Ea and IGF1-Eb which play important roles in activating signaling cascades that promote muscle protein synthesis, as well as the 18S ribosomal rRNA subunit which directly translates mRNAs into proteins, were induced by treatment with myostatin mAb at 7 days.

Finally we measured the expression of genes involved in extracellular matrix (ECM) synthesis and fibrosis (Fig. 4). Similar to transcripts involved in muscle atrophy and hypertrophy, nearly all measured genes were upregulated compared to control, uninjured muscles. Type I and III collagen, which are the major ECM proteins in skeletal muscle, were both downregulated by myostatin mAb treatment at 7 days compared to the sham mAb group, which was FSP-1, which is a marker of muscle fibroblasts. No difference was observed for the major muscle gelatinase MMP-2 between groups at different time points, but myostatin mAb treatment decreased the expression of the major muscle collagenase MMP-8 at 7 days. Similar observations were made for inhibitors of MMPs, TIMP-1, and...
TIMP-2, although myostatin inhibition at 21 days also resulted in a downregulation in the expression of these genes compared to the 21 day sham group.

**DISCUSSION**

Many patients with ACL tears have persistent muscle atrophy and weakness of 30% or greater despite compliance with intense postoperative rehabilitation programs. Myostatin is a potent muscle atrophy-inducing cytokine that is elevated after ACL tear. We hypothesized that blocking myostatin activity with a bioneutralizing monoclonal antibody would prevent the atrophy of lower limb muscles and also protect against the loss in muscle maximum isometric force.

**Figure 2.** Extensor digitorum longus (EDL) muscle mass and in vitro contractility measurements. (A) Muscle mass, (B) physiological cross-sectional area (PCSA), (C) Maximum isometric force, (D) Specific force (maximum isometric force normalized to PCSA) of EDL muscles from sham mAb and anti-myostatin mAb treated animals 7 or 21 days after inducing ACL tear. Values are mean ± SD, N ≥ 7 for each group. Dashed line indicates mean values from control, uninjured animals. Differences tested with a two-way ANOVA (α = 0.05) followed by Fisher’s LSD post-hoc sorting. Letters above bar graphs indicate post-hoc sorting differences.

**Figure 3.** Atrophy and hypertrophy-related gene expression. Gene expression, measured by quantitative PCR, from rectus femoris muscles. Target genes were normalized to the expression of the stable housekeeping gene β-actin, and then further normalized to the relative expression of that gene in control muscle from an uninjured knee. Any expression value greater than 1 indicates an upregulation compared to control muscle, and values less than 1 indicates a downregulation compared to control muscle. Values are mean ± SD, N ≥ 7 for each group. Differences tested with a two-way ANOVA (α = 0.05) followed by Fisher’s LSD post-hoc sorting. Letters above bar graphs indicate post-hoc sorting differences.
production. The outcomes from the current study indicate that myostatin inhibition was able to reduce the expression of proteolytic genes, leading to improvements in muscle fiber size and strength. Reductions in the expression of genes involved in ECM accumulation and fibrosis were also observed. The combined results of this preclinical study indicate that blocking myostatin is able to protect against some of the muscle atrophy and weakness that occur following an ACL tear.

Myostatin is widely regarded as a key regulator of muscle protein degradation. Inactive myostatin is sequestered to its inhibitory propeptide and other binding proteins in the ECM of muscle. Following injury and immobilization, active myostatin can be released from the matrix by proteolytic degradation of the propeptide and binding proteins by various proteases. Once activated, myostatin can bind to the ACVR2A and ACVR2B receptors, as well as the ALK4 and ALK5, to initiate activation of the Smad2/3 and p38 MAPK pathways in skeletal muscle cells. Among the downstream targets of these pathways are the E3-ubiquitin ligases atrogin-1 and MuRF-1 which direct proteins for breakdown in the 26S proteasome. Although the molecular mechanisms are not fully understood, myostatin also appears to be able to activate autophagy in muscle cells, which is a parallel protein breakdown pathway to the ubiquitin proteasome system. IGF-1 is one of the major muscle growth factors and works primarily by increasing muscle protein synthesis, and in addition to turning on protein degradation, myostatin can also block muscle growth by inhibit IGF-1 signaling.

During physiological growth, such as muscle hypertrophy that occurs as a result of resistance exercise training, myostatin plays an important role in turning on proteolytic pathways to breakdown proteins that are damaged during eccentric contractions. However in severe muscle or joint injuries, or following immobilization, myostatin levels are dramatically elevated which leads to profound muscle atrophy and persistent weakness. In the current study, 7 days after ACL tear the inhibition of myostatin lead to a decrease in atrogin-1, MuRF-1, and the related ubiquitin ligase MUSA-1, as well as Beclin-1 which is a key factor in the initiation of phagophore nucleation in autophagy. Myostatin inhibition also increased the expression of the two IGF-1 isoforms as well as 18S rRNA, which is a core subunit of ribosome organelles that directly synthesize proteins in cells. However, by 21 days there were no differences between myostatin antibody and sham antibody treated groups in molecular regulators of muscle size. Interestingly, while dramatic effects were observed 7 days after tear at the molecular level, no functional changes in muscle size or contractility occurred at the same time point, but by 21 days an increase in muscle fiber size and maximum isometric force production were observed in the myostatin inhibitor treatment group. These results suggest that the therapeutic inhibition of myostatin in the acute post-ACL tear injury phase leads to early, modest positive changes in the expression of genes that regulate muscle atrophy and growth, which then lead to more rapid recovery of muscle fiber size and force production.

Muscle ECM accumulation and fibrosis can occur after injury, joint trauma and in chronic degenerative myopathies. In general, excessive ECM accumulation can interfere with lateral force transmission between muscle fibers, leading to increased susceptibility to eccentric injuries. While less is known about muscle fibrosis and ECM accumulation in patients with ACL tears, collagen accumulation was reported in the quadriceps muscles of rats with ACL tears. In addition to inducing protein degradation, myostatin signaling can also induce the expression of type I

Figure 4. Extracellular matrix-related gene expression. Gene expression, measured by quantitative PCR, from rectus femoris muscles. Target genes were normalized to the expression of the stable housekeeping gene β-actin, and then further normalized to the relative expression of that gene in control muscle from an uninjured knee. Any expression value greater than 1 indicates an upregulation compared to control muscle, and values less than 1 indicates a downregulation compared to control muscle. Values are mean ± SD, N ≥ 7 for each group. Differences tested with a two-way ANOVA (α = 0.05) followed by Fisher’s LSD post-hoc sorting. Letters above bar graphs indicate post-hoc sorting differences.
collagen and several genes that regulate ECM structure and function.\textsuperscript{21,32,33} In the current study, therapeutic inhibition of myostatin lead to several encouraging changes in the expression of ECM genes, including a reduction in type I and III collagen and FSP-1, which is a marker of muscle fibroblasts. No difference between groups at each time point was observed for MMP-2, and modest reductions in the major muscle collagenase MMP-8, as well as a downregulation in the MMP inhibitors TIMP-1 and -2 were present. Although the extent of fibrosis and its contribution to contractile dysfunction in patients with ACL tears has not been well documented, if the same effects are observed in other muscle injuries and disorders, the changes in collagen, MMP and TIMP genes observed in this study likely suggest an overall favorable effect of myostatin inhibition on muscle ECM composition after ACL tear.

There are several limitations to this study. Rats are frequently used as a preclinical model to study ACL tears, but the open surgical method of ACL tear used in this study differs substantially from the mechanical disruption of the ligament that occurs with closed cutting or pivoting knee injury in patients. We measured the expression of several genes, and while we do not anticipate substantial posttranslational regulation of these transcripts, it is possible that changes in RNA levels would not predict subsequent changes in protein abundance. Three month old rats were selected for this study, and modest increases in body mass would be expected in these rats at this age. Whole animal functional performance measures like stability and balance were not performed. We evaluated the contractility of the EDL as the simple architecture of the muscle provides a convenient way to measure force production in vitro, and because this is one of the most common muscles used to assess function in studies of muscle atrophy in rodents, but did not measure the function of the quadriceps group which tend to be most severely affected muscle group after ACLT. Improvements in muscle size and function were seen in both sham and MSTN mAb groups over time. However, this is similar to what is frequently observed in patients, who are typically non-ambulatory immediately after ACLT. By several weeks after the injury they are able to walk and even run, albeit with an unstable knee, and objective deficits in muscle strength in the affected limb.\textsuperscript{2,3} This is also likely why numerous atrophy and fibrosis associated genes were downregulated in both groups at 21 days compared to 7 days. A single dose of myostatin inhibitor was used and the changes were evaluated at only two time points. Performing multiple doses over a longer period of time would provide additional insight into the mechanism of action of the drug. The antibodies used in this study were generated in mouse, and it is likely that the rats in this study generated antibodies against these mouse proteins. This phenomenon is frequently encountered in testing antibodies that have potential pharmaceutical use in preclinical models.\textsuperscript{34} While this is a known limitation and not unique to this study, it generally takes some time for an organism to generate sufficient antibodies to decrease the efficacy of therapeutic antibody being tested, in some cases up to two weeks.\textsuperscript{34} Therefore it is likely that the ability of the antibody to block myostatin in the acute post-tear phase was preserved. To further ensure this was the case, we administered a relatively high dose of the antibody (30 mg/kg). Despite these limitations, this study provided important insight into the biology of muscle changes after ACLT and also identified a potentially promising pharmacological therapy for the treatment of muscle atrophy in patients with an ACLT.

Muscle atrophy and persistent weakness commonly occur after ACLT, and this atrophy limits the return to play of patients and may contribute to the development of early OA. In the current study, inhibition of myostatin immediately following ACLT lead to modest improvements in markers of muscle growth, and functional changes in muscle fiber size and force production. However, there are some concerns within the sports medicine community as to the potential use of myostatin inhibitors as doping agents. While we report functional improvements in muscle size and contractility in this paper, there was no effect of myostatin inhibition on muscle specific force values. This parameter, which normalizes muscle force by cross-sectional area, can be increased through the use of anabolic agents,\textsuperscript{25} but studies in animals have shown that inhibiting myostatin in otherwise healthy muscle is unable to increase muscle fiber specific force production.\textsuperscript{14,21} These papers, along with the results from the current study, suggest that myostatin inhibition is able to preserve muscle size and strength, to some extent, after injury and disease by blocking protein catabolism and not necessarily by increasing protein anabolism. While further research is necessary, given the encouraging results from this preclinical study, the targeted inhibition of myostatin along with accelerated rehabilitation programs may be able to improve the long-term functional outcomes of patients with ACL tears.

AUTHORS’ CONTRIBUTIONS
CNWW, JPG, AJR, AB, and CLM conceived and designed the study. CNWW, JPG, JAG, RKK, and CSD completed the experiments. CNWW, JPG, JAG, and CLM analyzed the data. AJR provided reagents/materials. CNWW and CLM wrote the paper. The authors declare that they have read and approved submitting the manuscript.

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