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Myostatin and its precursor protein are increased in the skeletal muscle of patients with Type-II muscle fibre atrophy

S. Wójcik, A. Nogalska, W.K. Engel, V. Askanas

USC Neuromuscular Center, Department of Neurology, University of Southern California Keck School of Medicine, Good Samaritan Hospital, Los Angeles, CA, USA

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Preferential atrophy of Type-II muscle fibres occurs in several clinical situations, including cachexia, muscle disuse, chronic glucocorticoid treatment, remote neoplasia, and sometimes as an aspect of recent-denervation. For the patient, the Type-II atrophy itself might be unfavourable (as a glucocorticoid side--effect) or favourable (survivalistic via the muscle-alanine liver-gluconeogenesis pathway in starvation). The cellular mechanisms underlying Type-II fibre atrophy are unclear. Myostatin (Mstn) is physiologically a negative regulator of muscle mass and strength. In this study we evaluated a possible role of Mstn in Type-II fibre atrophy in human muscle. Mstn and Mstn precursor protein (MstnPP) were studied in 10-muscle biopsies containing Type-II fibre atrophy and in 17 disease and normal control muscle biopsies. When comparison was made with normal control fibres, we found the following: 1) by immunocytochemistry, diffusely increased Mstn/MstnPP in the atrophic Type-II muscle fibres; 2) by immunoblots, Mstn/MstnPP increased individually; 3) by RT-PCR, no increase in MstnPP mRNA. In conclusion, our results a) suggest that Mstn/ /MstnPP might play a role in the pathogenic cascade of Type-II muscle fibre atrophy; b) broaden our previously-described associations of Mstn in human muscle pathology, and c) could possibly lead to clinical prevention when Type-II muscle fibre atrophy is unfavourable, for instance in glucocorticoid therapy. (Folia Morphol 2008; 67: 1–7)

Key words: Type-II muscle fibre atrophy, myostatin, myostatin precursor protein, GDF-8, human muscle

INTRODUCTION

Selective involvement of muscle fibre types can occur in various neuromuscular disorders [10]. Preferential atrophy of the Type-II muscle fibres occurs in cachexia, muscle disuse and chronic treatment with glucocorticoids [10, 27]. Following experimental total denervation, guinea-pig Type-II fibres atrophy more rapidly than Type-I fibres [17]. With a heterogeneous aetiology, the cellular mechanisms underlying pathogenesis of Type-II muscle fibre atrophy remain unclear.

Myostatin (Mstn), also called growth and differentiation factor-8 (GDF-8), is a member of the transforming growth factor-β (TGF-β) superfamily [13, 26]. Myostatin precursor protein (MstnPP) is a 375 amino acid protein translated from a 3.1 kb mRNA [14]. It was suggested that intracellular processing of Mstn from MstnPP occurred through furin [16, 24].

Address for correspondence: V. Askanas, MD, PhD, USC Neuromuscular Center, Good Samaritan Hospital, 637 S. Lucas Ave, Los Angeles, CA 90017–1912, USA, tel: 213 975 9950, fax: 213 975 9955, e-mail: askanas@usc.edu

Mstn is a secreted protein considered a normal negative regulator of muscle growth during development and of muscle mass during adulthood [13]. In mouse models, knocking out the Mstn gene and overexpressing proteins neutralising Mstn caused an increase in muscle mass [13]. In cattle and whippet dogs naturally-occurring myostatin gene mutations led to significantly increased muscle size [26, 28]. A child with a homozygous Mstn gene mutation that results in reduced production of Mstn protein was reported increased muscle bulk and strength [35]. Conversely, mature Mstn protein was reported to have increased in the muscle tissue of patients with HIV-associated muscle wasting [14], and increased MstnPP mRNA was reported in muscle wasting associated with osteoarthritis [31] (these conditions are associated with Type-II fibre atrophy [Engel and Askanas, unpublished]). However, in some studies, Mstn gene-knockout mice or mice carrying Mstn gene mutations had muscle weakness, mitochondria depletion and tubular aggregates, despite their larger muscle mass [1]. Previously, the question was raised as to whether the larger muscle is better [15].

We recently reported that Mstn and MstnPP were increased in the muscle fibres of patients with sporadic-inclusion body myositis, where it was physically associated with amyloid-β precursor protein (AβPP) [37]. Moreover, experimental overexpression of the AβPP gene in cultured human muscle fibres resulted in increased MstnPP in these [39]. Our most recent studies have shown that in ER-stress-induced cultured human muscle fibres MstnPP and its mRNA are increased through an NFκ-B related mechanism [30]. Accordingly, it appears that various pathological mechanisms might be involved in Mstn/MstnPP regulation.

To our knowledge, the association between Type-II muscle fibre atrophy and Mstn expression has not previously been tested directly in human muscle. In the present study we asked whether Mstn is directly associated with Type-II muscle fibre atrophy and thus might be contributing to the pathogenetic cascade.

MATERIAL AND METHODS

Patients

We studied diagnostic muscle biopsies that had been obtained, with the informed consent of the participants, from 27 patients diagnosed with the following conditions: Type-II muscle fibre atrophy [10], peripheral neuropathy [5] and amyotrophic lateral sclerosis (ALS) [2]. The controls consisted of 10 normal age-matched muscle biopsies obtained from patients who, after all the diagnostic studies had been performed, were deemed not to have a muscle disease. All the patients fell within a 30–83 year age range; the median age was 64 for Type-II fibre atrophy patients and 66 for the controls. The diagnoses were based on clinical and laboratory investigations, including 16-reaction diagnostic histochemistry of muscle-biopsies routinely performed in our laboratory. Type-II fibre atrophy was morphologically diagnosed with myofibrillar ATPase staining performed at pH 9.4, with and without pre-incubation at pH 4.35 [8].

Clinically, four of the patients with Type-II fibre atrophy had previously been treated with a glucocorticoid, while in the other six patients the causes could not be determined; none was known to have cancer.

Light-microscopic immunocytochemistry

Immunofluorescence was performed on 10-μm transverse sections of fresh-frozen muscle biopsies as described [3, 4, 37], using a well-characterised rabbit polyclonal antibody against Mstn [14, 30, 32, 37, 39] (Chemicon, Temecula, CA), diluted 1:400. This antibody is developed against Mstn sequence B (NMLYFNGKEQIIYGKI) and recognises on immunoblots a 28 kDa band of Mstn dimer and a 55 kDa band of MstnPP [14, 32, 37]. On immunostaining this antibody recognises both Mstn and MstnPP, here referred to as staining of Mstn/MstnPP.

To block non-specific binding of an antibody to Fc receptors, sections were preincubated with normal goat serum diluted 1:10. The controls for staining specificity were omission of the primary antibody or its replacement with non-immune sera; these were always negative.

Immunoblots

These were performed as described [37, 38]. Briefly, five 20 μm-thick sections of fresh-frozen muscle were collected at –25°C and rapidly homogenised on ice in 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 15 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche Diagnostic GmBH, Mannheim, Germany). The protein concentration was measured by the Bradford method; 20 μg protein in 4X NuPAGE LDS sample buffer and 10X NuPAGE sample reducing agent were heat-denatured for 10 min at 70°C. Subsequently, they were electrophoretically separated using 12%

Bis-Tris gel and 1X NuPAGE MES SDS running buffer. After electrophoresis the samples were transferred to a nitrocellulose membrane for one hour at 30 V in 1X NuPAGE transfer buffer. All reagents were obtained from Invitrogen (Carlsbad, CA). The nitrocellulose membranes were blocked in 5% (wt/vol) blocking reagent (Amersham Pharmacia Biotech, Inc., Piscataway, New Jersey) in 1X TBS plus 0.1% Tween 20, and these were incubated overnight at 4°C with an anti-Mstn antibody diluted 1:1000. After being washed, the membrane was incubated with an appropriate species-specific secondary antibody conjugated to HRP. The blots were developed using the enhanced chemiluminescence system (Amersham). Protein loading was evaluated by actin bands visualised with a mouse monoclonal anti-actin antibody (Santa Cruz, CA) diluted 1:2000. Omission of a primary antibody was the control for reaction specificity. Immunoblots of a recombinant human Mstn peptide (Research Diagnostic Inc., Flanders, NJ) were used as additional controls for the antibody specificity.

RNA isolation and RT-PCR

Total RNA from muscle biopsies was isolated as described [29]. Next, 1 μg of RNA was used in an RT-PCR reaction, utilising OneStep RT-PCR kit (Qiagen, MA) and primers for MstnPP (Fw-GTGG-TACCTCATGCAAAAACTGCAACTCTGT, Rv-ATGGATCCAATCTCATGAGCACCCACAGC) [30, 39], or GAPDH (glyceraldehade-3-phosphate dehydrogenase) [20]. The optimised conditions for MstnPP were 30 min at 50°C and 15 min at 95°C followed by 31 cycles of amplification (94°C for 30 s, 60°C for 30 s, 72°C for 90 s) and a final incubation for 10 min at 72°C. The PCR products were separated on a 1.6% agarose gel and stained with SYBR Safe DNA gel stain (Invitrogen, CA). The conditions of the reactions were experimentally checked to ensure that the signals were in the linear range of the PCR. The identity of the products was confirmed by sequencing. The amount of MstnPP mRNA was quantified by the NIH Image J 1.310 software and was normalised to the expression of GAPDH in the same sample to account for differences in loading between samples.

Statistical analysis

The statistical significance of differences between groups was determined by Student's t-test. The level of significance was set at $p < 0.05$. All data are presented as means ± SEM.

RESULTS

In the biopsies with Type-II muscle fibre atrophy, 70–80% of the small fibres were Type-II, which was revealed by the stainings for two myofibrillar ATPases (Fig. 1A). The degree and extent of the Type-II fibre atrophy varied between patients from moderate to prominent. There was no necrosis or phagocytosis and no increase of endomysial connective tissue. There was no recent denervation.

By immunofluorescence atrophic Type-II muscle fibres were strongly and diffusely immunostained with the antibody against-Mstn/MstnPP as compared to the normal-sized muscle fibres within the same section, which were very weakly stained (Fig. 1B, C). Immunohistochemically, Type-II and Type-I fibres appeared to have the same amount of Mstn/MstnPP in the normal human muscle. Denervated small angulated muscle fibres in ALS and peripheral neuropathies did not have increased staining with that antibody, and some appeared to have decreased immunoreactivity (not shown).

Immunoblots of muscle biopsies obtained from control and Type-II fibre atrophy patients revealed two specific bands of 28 and 55 kDa; the lower band corresponding to the Mstn dimer and the upper to MstnPP [32, 37]. Both of those bands were increased in biopsies with Type-II fibre atrophy (Fig. 2A). Omission of the primary antibody resulted in no bands being present (data not shown). Densitometric analysis (Fig. 2B) revealed that in patients with Type-II fibre atrophy the amount of Mstn protein was increased 2.2-fold ($p < 0.05$), while the MstnPP was increased 3.3-fold ($p < 0.01$) in comparison with normal muscle biopsies.

By semi-quantitative RT-PCR the amount of MstnPP mRNA did not differ significantly between Type-II fibre atrophy and control biopsies (Fig. 3).

DISCUSSION

For the patient, the Type-II atrophy itself might be unfavourable (as a glucocorticoid treatment side-effect) or favourable (survivalistic via the muscle-alanine liver-gluconeogenesis pathway in starvation). Muscle wasting and decrease in muscle strength accompany various clinical conditions. Fibre type distribution is one of the factors determining muscle strength, and prevalence of type Type-II fibres has been reported to correlate positively with muscle strength [15]. Type-II fibre atrophy is the typical and sometimes prominent manifestation associated with muscle wasting of various causes, such as

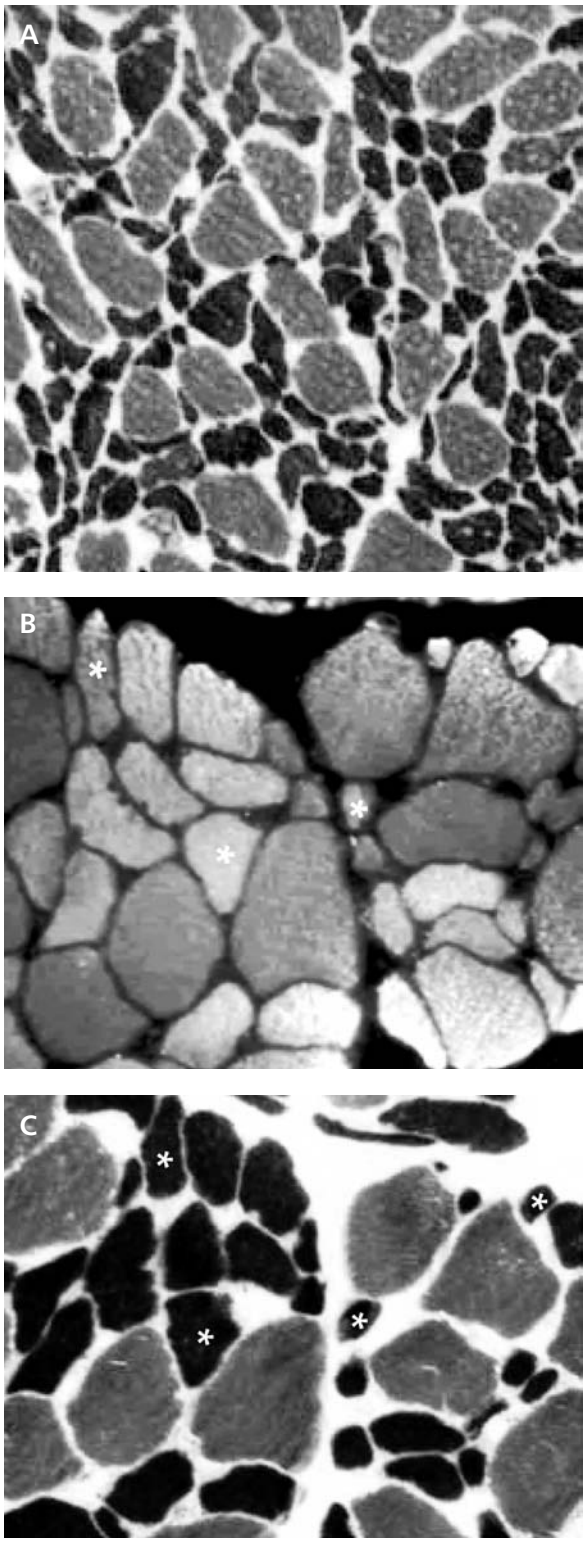

Figure 1. A. Representative muscle biopsy, transverse section, from a patient with Type-II muscle fibre atrophy. In this biopsy 70–90% of the small fibres were Type-II as shown by myofibrillar ATPase staining (pH 9.4, without acid pre-incubation). ×700; **B.** Staining with antibody against Mstn/MstnPP showed a diffusely increased immunoreactivity in the atrophic muscle fibres; **C.** ATPase staining (pH 9.4, without acid pre-incubation) on a parallel, but not closely adjacent, section to B confirmed that those atrophic fibres were Type-II; for clarity some of the corresponding fibres on B and C have been marked with asterisks. B, C ×1250.

cachexia, muscle disuse, glucocorticoid treatment, and remote neoplasia [9, 10, 27]. Knowledge of the mechanisms leading to Type-II fibre atrophy could therefore be of general importance. For example, many patients with cancer are severely incapacitated by generalised cachectic muscle weakness. The various causes may operate in the same way or in different ways, leading to the increased Mstn/MstnPP.

Studies performed on C2C12 mouse and L6 rat myoblasts in culture, and in rats *in vivo*, reported that a glucocorticoid (dexamethasone) increases MstnPP mRNA, MstnPP and Mstn levels in muscle [6, 18, 22, 23]. It was suggested that the glucocorticoids induced Mstn gene transcription, possibly through a glucocorticoid receptor-mediated pathway [23]. In humans, the Mstn promoter sequence contains, among other putative muscle growth-factor response elements, response elements for glucocorticoid [22]. It has also been reported recently that dexamethasone decreased the mean muscle-fibre cross-section area by approximately 15% in the gastrocnemius and tibialis anterior muscles of wild mice, while in myostatin knock-out mice it did not exert this effect [12].

In the present study we have shown that, as compared to controls, biopsies with Type-II muscle fibre atrophy had increased MstnPP and mature Mstn dimer but did not have increased MstnPP mRNA. The unchanged MstnPP mRNA might be reflecting a negative feedback on MstnPP transcription caused by the increased Mstn and MstnPP protein [11, 24, 40]. Mstn is known to auto-regulate its own synthesis and processing by stimulating production of a transcription factor, Smad 7, via the interaction between proteins Smad 2, 3, 4 and the Smad binding-element in the Smad 7 promoter. The inducible overexpression of Smad 7 by Mstn inhibits the MstnPP--promoter activity [11], resulting in decreased Mst--nPP transcription [40]. Similar to our results was the finding that in muscle atrophy of older rats, as compared to young rats, Mstn protein was increased, while the MstnPP mRNA was decreased [5]. It is also possible that increased Mstn and MstnPP at the protein level we observed was influenced by translational and/or post-translational modulation of these.

The cause of the increased Mstn/MstnPP in Type-II atrophic muscle fibres observed by us is not known. In rodents Mstn is predominantly present in muscle fibres that express myosin heavy chain isoform IIb (which are, histochemically, Type-II fibres), as evident both during development and in mature states [2, 7, 34]. Our study of normal adult human muscle

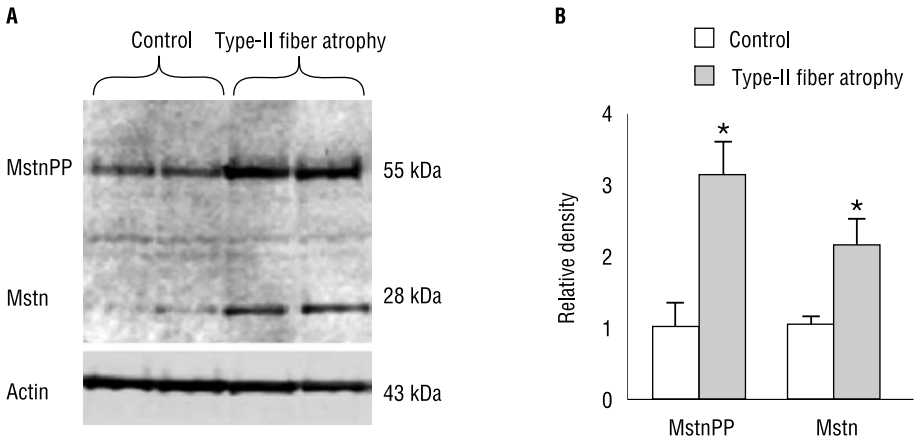

Figure 2. A. Representative immunoblots of homogenates of two biopsies having Type-II muscle fibre atrophy demonstrate stronger expression of Mstn dimer (28 kDa) and MstnPP (55 kDa) as compared to two normal-control muscle biopsies. The actin band shows the protein loading in each specimen; **B.** Densitometric analysis of results from 6 patients versus 6 normal controls, as in A, showed increase of MstnPP and Mstn (3.3-fold and 2.2-fold, respectively) in Type-II muscle fibre atrophy as normalised to the actin band. Data are indicated as mean ± SEM. Significance was set at *$p < 0.05$.

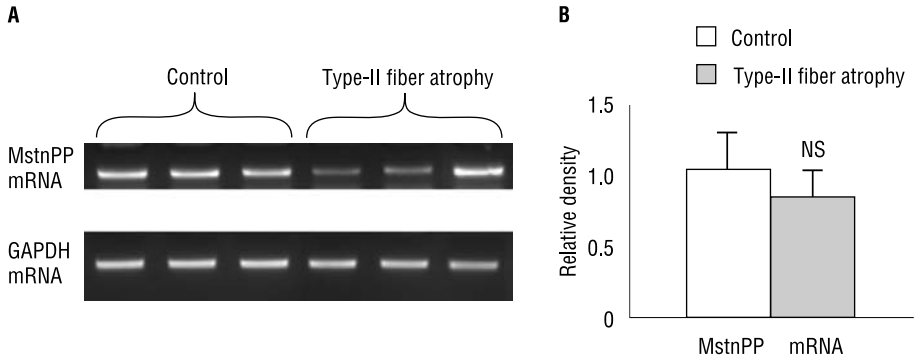

Figure 3. A. Representative agarose gel electrophoresis of products corresponding to MstnPP mRNA and GAPDH mRNA, amplified by the RT-PCR method; **B**. Densitometric analysis of results from 5 patients versus 5 controls, as in A, showed that levels of MstnPP mRNA, normalised to the GAPDH mRNA, were similar in control and patients with Type-II muscle fibre atrophy. All data are indicated as mean ± SEM. Significance was set at $p < 0.05$.

shows approximately equal amounts of immunoreactive Mstn/MstnPP in Type-II and Type-I fibres.

Mstn is considered to be a protein secreted by the muscle fibre, which exerts its influence fairly locally through extracellular autocrine/paracrine signalling. This is considered to be achieved by its interaction with the activin/TGFβ ActIIb receptor on the muscle fibre [13, 25, 33]. It is suggested that outside the muscle fibre Mstn is inactivated by being bound to follistatin, which inhibits Mstn binding to its receptors on muscle fibres [13, 25]. Within the muscle fibre, the role of Mstn and that of its intracellular binding partners remains to be studied.

Our previous data demonstrating increased expression of Mstn and MstnPP and the co-localisation and physical association of MstnPP with AβPP within s-IBM muscle fibres suggested that Mstn/MstnPP may exert a novel intracellular influence within human muscle fibres [37]. Studies by others reporting that Mstn is present in the nuclei of myotubes derived from the mouse C2C12 cell line suggest that Mstn may play an intracellular role in regulating transcription [2].

CONCLUSION

Our results a) suggest that myostatin and the myostatin precursor protein may play a novel role in the pathogenetic cascade leading to Type-II muscle fibre atrophy; b) broaden our previously-described associations of myostatin in human

muscle pathology and c) could possibly lead to clinical prevention when Type-II atrophy is unfavourable, for instance in glucocorticoid therapy.

Therapeutic methods intended to decrease or eliminate myostatin, which might possibly be considered for patients with Type-II fibre atrophy, are very limited. A previous study reported that treatment with growth hormone significantly decreased muscle myostatin precursor protein mRNA level in growth hormone-deficient patients, and in mouse C2C12 muscle it decreased both myostatin precursor protein mRNA and protein [21]. Whether growth hormone treatment would benefit Type-II atrophy patients is not known. Other potentially therapeutic anti-myostatin compounds, such as a) myostatin-binding proteins, such as follistatin, and those binding competitively to activin/TGFβ ActIIb receptors, are not yet available for human use [19, 36]. Studies of animal models from various laboratories differ greatly as to whether the inhibition of myostatin is beneficial or detrimental [1, 15, 36]. Accordingly, the intention to inhibit myostatin therapeutically in human muscle disorders must be considered circumspectly and with regard to whether it is appropriate or not.

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