Introduction

The transforming growth factor-β (TGF-β) superfamily members are structurally related proteins that play important roles during embryonic development and in adult life. This family includes the inhibins, activins, Müllerian inhibiting substance, bone morphogenetic proteins (BMPs), as well as the growth and differentiation factors (GDFs). GDF-8, also known as myostatin, does not fall into any of the known sub-families such as the BMPs or the TGF-β subfamily. Northern blot analysis and in situ hybridization in developing embryos showed that GDF-8 expression was localized to developing somites in early stages, while in later stages of embryogenesis and in adulthood, it is localized predominantly in skeletal muscles. Myostatin expression has also been detected in mammary tissue, adipose tissue, and cardiac muscle using RT-PCR. In keeping with the characteristics of this superfamily, myostatin has a signal peptide, a prodomain, and after cleavage the carboxy-terminal domain forms a homodimer. This dimeric species activates receptor serine-threonine kinases to initiate a signaling cascade that involves the Smad family of proteins.

Myostatin was originally described by McPherron and Lee based on the phenotype of the myostatin gene knockout mice. The skeletal muscles of myostatin null mice have a two to three-fold increase in mass over wild type littermates, with the increase in mass attributed to both an initial hyperplasia and a later hypertrophy of the constituent muscle fibers. A similar, naturally occurring phenotype has been observed in cattle where a 20-25% increase in muscle mass is seen in the Piedmontese and Belgian Blue breeds. This hypermuscled phenotype has been linked to a point mutation or a deletion in exon 3 of the myostatin gene that results in inactive myostatin protein. These data have led to the proposal that myostatin might be a negative regulator of skeletal muscle mass. Further supporting this hypothesis are the findings that transgenic mice carrying a dominant negative form of myostatin display an increased skeletal muscle phenotype; and that serum myostatin levels are elevated in AIDS patients with cachexia and in patients with skeletal muscle atrophy following prolonged bed rest or hip replacement.
in the hind leg muscles consistent with myostatin being a negative regulator of muscle mass14. In contrast, 17 days of space flight that produced a 110% and 37% increase in myostatin mRNA and protein, respectively16. When a daily 30-minute period of muscle loading was superimposed during the unloading period, the loss of muscle mass was totally prevented although the increase in myostatin expression was only blunted by 50%. It was concluded that although increases in myostatin accompany muscle atrophy, significant increases in myostatin do not necessarily produce muscle atrophy16. In another study, a slight elevation of myostatin protein was associated with 31-76% compensatory hypertrophy of the plantaris muscle produced by ablation of the synergistic soleus and gastrocnemius muscles, whereas a marked elevation was associated with denervation-induced atrophy of the soleus and plantaris muscles in the rat17. Similarly, myostatin mRNA level was elevated in the rat femoral muscle at 48 hours after bupivacaine injection18. However, others reported that both myostatin mRNA and protein levels decline in the soleus and extensor digitorum longus muscles of the rat by days 1-3 after bupivacaine injection followed by a return to levels at or above control muscles by day 7. This suggested that myostatin expression was not strongly associated with muscle atrophy19. In contrast, 17 days of space flight that produced 19-24% atrophy of muscles of the lower hind leg in rats was preceded by a 67% elevation of myostatin mRNA levels at day 1 but no increase was observed at day 7. This suggested that myostatin expression was not strongly associated with muscle atrophy20. However, a return to levels at or above control muscles by day 719.

Materials and methods

Materials: All Taqman® reagents were from Applied Biosystems (Foster City, CA). Trizol®, Random Priming kit, SDS-PAGE gels, buffers and blotting membranes were from Invitrogen/Life Technologies (Carlsbad, CA). 32P (α)-dATP, 3000 Ci/mmol, film and ECL® detection kit were from Amersham Pharmacia Biotech (Piscataway, NJ). Nytran® membrane was from Schleicher & Schuell (Keene, NH). All chemicals were from Sigma (St. Louis, MO). Protein detection reagent was from Bio-Rad (Hercules, CA). Secondary antibody was reagent from Jackson Immunoresearch (West Grove, PA).

Animals: Male Sprague Dawley (SD) rats aged 1.5, 6, 12, or 27 months (n=6 at each time point) were purchased from Harlan (Indianapolis, IN). They were allowed to acclimate to the vivarium for one week, sacrificed by CO2 asphyxiation and the gastrocnemius, tibialis, plantaris, and soleus muscles of the lower hind leg were rapidly dissected out from both legs. Individual muscles were weighed, snap-frozen in isopentane cooled in liquid nitrogen and stored at -80°C until processed.

In a separate study, 6-week-old male SD rats were purchased from Taconic Farms Inc. (Germantown, NY) acclimated to vivarium conditions for one week, weighed and randomized by body weight into 10 groups to undergo unilateral sciatic neurectomy (USN) or sham-USN. Surgical anesthesia was induced with isoflurane, the sciatic nerve was identified and lifted through an incision on the lateral aspect of the mid-thigh of one hind leg. For USN, a 1-cm segment of the sciatic nerve was excised whereas for sham-USN the nerve was not severed. The surgical incisions were closed and the animals were returned to their cages where recovery was uneventful. At day 0 (6-8 hr), 1, 4, 8, and 14 following the USN or sham-USN surgery, 6 rats per time point were sacrificed by CO2 asphyxiation and the gastrocnemius, tibialis, plantaris, and soleus muscles from both the operated and contralateral non-operated limbs were rapidly dissected out. The individual muscles were weighed prior to freezing in isopentane cooled in liquid nitrogen and stored at -80°C until homogenization.

Quantitative PCR and Northern Blotting: A transverse block of sample, taken from the mid-belly of both heads of the gastrocnemius muscle (approximately 250 mg) was homogenized (n=6) in 3 ml Trizol® using a Brinkmann® homogenizer. Total RNA was processed per manufacturer’s instructions plus a second stage purification as previously described20. Transcript abundance was analyzed by quantitative real-time PCR (Taqman®). RNA was reverse-transcribed per manufacturer’s instructions using the Taqman® reverse transcription kit, which employs random hexamers. A control reaction in which the reverse transcriptase was omitted was also run. In addition to primers specific to the gene of interest, this methodology utilizes a quenched fluorescent-labeled probe that is positioned just downstream of the 5’ primer. As DNA synthesis proceeds, the 5’ exonucle-
A.P. Baumann et al.: Myostatin expression during muscle atrophy

ase activity of Taq polymerase degrades the probe, thus freeing the 5' fluorescent label from the quencher linked to the 3' end of the probe. Accumulation of fluorescence with cycle number is thus indicative of the relative transcript abundance. The following primers and probe were generated using Primer Express software (Applied Biosystems, Foster City, CA): 3' primer: CATTGCAGTTTTTCATCATCATT; 5' primer: ACATGCATAATTTTACCTTGGCA; and dual-label probe: FAM-TCAAAAGCAAAAAAGAA-GAAATAAGAACAGGGAAAA-TMRA. Primers were used at 900 nM, probe was used at 50 nM, and 50 ng of cDNA was used as a template for the amplification with Universal Master Mix for Taqman®. Reactions were run in triplicate in optical grade 96 well plates on a Perkin-Elmer 7700 instrument interfaced to Sequencer Detector software. A control reaction lacking the sample template was also included. Reactions using an 18S primer/probe pair were run in parallel. Normalization and differences between samples were calculated using the ΔΔCt (cycle threshold) method where ΔΔCt equals (Ct sample 1 - Ct 18S sample 1) - (Ct sample 2 - Ct 18S sample 2). Percent change was calculated according to the following formulae: For upregulation, % increase = ((2^ΔΔCt - 1) x 100; for downregulation, % decrease = ((1/2^ΔΔCt + 1) - 1) x 100.

For Northern blotting, 20 μg of total RNA was electrophoresed into a 1% agarose-formaldehyde gel, blotted onto a Nytran® membrane and hybridized as described previously22,23 to a random primed 32P (α) dATP 272 bp fragment (nt 1-272 of the coding region) of the murine myostatin gene (U84005). This region was chosen based on less sequence similarity to a related molecule, GDF-11. The low GC content of the probe necessitated using dATP for labeling. Washes were done as follows: 3 washes at room temperature in 1xSSC/0.1% SDS plus a final high stringency wash in 0.1xSSC/0.1% SDS at 55 °C. The blot was exposed to film (Amersham). The purified carboxy-terminal domain of murine myostatin expressed in E. coli was used as a control. To ensure selectivity of the polyclonal anti-myostatin antibody, a BLAST search was done with the peptide sequence above. The only molecule with significant similarity was a related Growth and Differentiation Factor, GDF-11, which when aligned with myostatin differs only by two amino acid residues. Therefore, the murine GDF-11 peptide, NMLYFNDQKQIIYGKI (differing amino acids underlined), was used to verify the specificity of the myostatin antibody used for Western blotting.

Statistical Analyses: Data are presented as mean ± SEM. One-way analysis of variance and the Scheffe test were used to compare differences in body and muscle weights and myostatin mRNA expression in the gastrocnemius muscle between the 1.5, 6, 12 and 27-month-old rats. For the USN study, the denervated limb was compared by paired t-test against the contralateral non-denervated limb that served as control, and values at the day 0 time-point were taken as baseline. P < 0.05 was considered to indicate statistically significant difference.

Results

Atrophy and Expression of Myostatin in Aged Gastrocnemius

In this study, we examined the expression of myostatin mRNA as a function of age. The ages of the rats studied (1.5, 6, 12 and 27 months) were chosen to represent growing, young adult, middle age and aged rats, respectively. Body weight and muscle mass peaked at about 6 months of age, and while body weight was maintained through 27 months of age, muscle mass declined to approximately 50% of the values in the 6 and 12-month-old animals (Figure 1). Because the myostatin mRNA levels were normalized to 18S, no further normalization to muscle mass was done. The amount of myostatin mRNA as quantified by real-time PCR using the Taqman assay and normalized to 18S rRNA expression declined progressively with age with levels at 6, 12 and 27 months being 9, 34, and 56% lower than levels at 1.5 months of age (Figure 2). Northern blot analysis of the gastrocnemius muscle also yielded similar results (Figure 3).

Western blot analysis of the gastrocnemius muscle revealed two isoforms of the active molecule (the carboxy-terminus that has undergone cleavage from the prodomain) of myostatin protein, one at 17 kDa and the other at 15 kDa. Two other species were also detected at 42 and 30 kDa (data not shown). Performing PAGE under more stringent reduc-
ing and denaturing conditions did not alter the migration of these species (data not shown) suggesting that the 30 and 42 kDa species represent incompletely reduced and unprocessed monomeric species of the protein, respectively. In contrast to the transcript, the expression of both the 15 and 17 kDa isoforms of myostatin protein was elevated at 27 months when compared with 1.5 months of age (Figure 4(A)).

The carboxy-terminal domain of the murine myostatin that was expressed in *E. coli* and used as a positive control migrated predominantly at 17 kDa with a weak band at 30 kDa (data not shown). To rule out cross-reactivity with GDF-11, the GDF-11 peptide was subjected to SDS-PAGE and Western blot analysis alongside the GDF-8 peptide from which it differs by only two amino acids (see Materials and Methods). As shown in Figure 4(B), the antibody reacted with only the myostatin peptide. Taken together with the facts that the observed bands corresponded to the predicted molecular mass of the aforementioned species, and that the recombinant protein migrated similarly, this finding supports our conclusion that the visualized bands are indeed myostatin.

The level of expression of myostatin protein was also investigated in other muscles of the hind leg, namely the plantaris and tibialis anterior. In these muscles, only the
higher molecular mass species of the myostatin protein (30 and 42 kDa) were observed and the expression of these molecular species was increased with age (Figure 5).

Muscle Atrophy and Expression of Myostatin after Denervation

To further explore the regulation of myostatin during muscle atrophy, we examined myostatin expression during denervation-induced atrophy in young growing rats. Unilateral sciatic neurectomy (USN) in these rats did not affect their body weight gain but resulted in progressive atrophy of all leg muscles (irrespective of fiber type composition) in the USN leg relative to sham-USN or non-operated litter mate controls (Figure 6). Thus, by day 14 of USN all muscles, both fast and slow-twitch, had lost approximately 50% of their mass relative to their contralateral or sham-USN counterparts. USN and sham-USN did not alter the weight of muscles in the contralateral non-operated legs relative to age- and weight-matched controls (data not shown).

Myostatin mRNA levels in the gastrocnemius muscle as measured by Taqman PCR displayed a fluctuating pattern during the 14 day period (Figure 7). Initially, USN samples had 31% less message than their sham counterparts. It must be emphasized that the day-0 samples were taken approximately 6-8 hours after surgery or sham operation. Subsequently, the levels of myostatin message in operated animals compared with sham animals ranged from insignificant changes on days 1-8 to a 34% increase on day 14 post-surgery.

Western blotting of the gastrocnemius muscle revealed
the expression of the 42, 30, 15 and 17 kDa species of myostatin in the gastrocnemius, the levels of which paralleled those of the transcript. That is, the levels of myostatin protein in the gastrocnemius muscle were decreased at day 0 and elevated by day 14 following denervation compared with sham-operated controls (Figure 8).

Discussion

The hypothesis that myostatin is a negative regulator of skeletal muscle mass\(^5\) derives almost solely from studies in which myostatin activity was manipulated during embryonic development. Only recently has it been shown more directly that myostatin regulates muscle mass postnatally\(^13\) further supporting the potential for myostatin as a target for therapeutic intervention to prevent or treat sarcopenia. For the latter to hold true, however, elevated levels of myostatin protein would be expected in various conditions of sarcopenia, whether due to normal aging, denervation or cachectic states, irrespective of the initiating etiology. To test this hypothesis, this study investigated the levels of expression of myostatin mRNA and protein in age-related sarcopenia as well as in denervation-induced atrophy in young adult rats, in both of which conditions there was approximately 50% muscle atrophy.

In this study, the level of expression of myostatin was examined at both the transcript (mRNA) and protein levels. Myostatin mRNA expression was evaluated by Taqman PCR and confirmed by Northern blot using primers and probes, respectively, that discriminate between myostatin and GDF-11. It should be mentioned that the quantitative PCR technique employed is highly specific and it would be extremely unlikely that the amplicon is anything other than myostatin. The level of myostatin protein expression was estimated by Western blotting using a polyclonal antibody directed against a peptide in the carboxy-terminus. This antibody recognized four immunoreactive species in the gastrocnemius muscle under reducing conditions, sized 15, 17, 30 and 42 kDa. These sizes correspond to expected molecular masses of a monomer (two isoforms), dimer and unprocessed protein, consistent with reports on other members of the TGF-β superfamily\(^24\). The phenomenon of two low molecular mass species has been reported for osteogenic protein-1 (OP-1, BMP-7) and was attributed to either differential glycosylation or the existence of dynamic intrachain disulfide bonding in the “cysteine knot” that could result from the close proximity of all cysteine residues in the fold-protein\(^24\). Additionally, in teleost fishes, two different
genes have recently been identified for myostatin. The carboxy-terminal domain of murine myostatin expressed in E. coli that was used as control ran predominantly at 17 kDa and a weaker band which represents incompletely reduced dimer was visible at 30 kDa. Previous studies had reported a 28-30 kDa band as well as a 49 kDa band in rat tibialis muscles with no traces of the 14-17 kDa bands. In the rat soleus and extensor digitorum longus muscles, an 18-19 kDa band of myostatin was identified. Also in the rat, a single polypeptide at approximately 35 kDa and 37 kDa were identified, respectively, in the plantaris and soleus muscles. On the other hand, a 26-kDa myostatin-immunoreactive species was identified in the quadriceps muscle of HIV-infected men with wasting as well as in the diaphragm, soleus, tibialis and extensor digitorum longus muscles of the rat. The reasons for the discrepancies in the reported sizes of the myostatin protein are not known.

The present study addressed the issue of specificity of the antibody used for the Western blot analysis and showed that the bands visualized were myostatin and not GDF-11. Thus, the results indicate that in rat skeletal muscle the levels of myostatin protein increases while the level of myostatin mRNA decreases with aging, indicating possible accumulation in the extracellular matrix. These age-related changes in the expression of myostatin transcript and protein occurred with atrophy of the gastrocnemius muscle. In contrast, denervation-induced atrophy of the gastrocnemius muscle in young rats which was apparent by days 4, 8 and 14 after sciatic neurectomy, was not associated with elevation of myostatin mRNA or protein until day 14. Moreover, unlike in aging where the increase in levels of myostatin protein and transcripts varied inversely, in denervation-induced atrophy the changes in myostatin protein and transcripts varied in parallel. These discrepancies between age- and denervation-induced atrophy may be reflective of the rates of onset and progression of muscle atrophy in these conditions. They may also suggest that myostatin might be playing different roles or be differentially regulated in age-induced versus denervation-induced sarcopenia.

The sarcopenia of aging is insidious in onset, usually beginning some time after the fifth and seventh decade of life and progressing until death. It is often accompanied by changes in the expression and function of several genes and proteins including those of growth factors such as insulin and insulin-like growth factor, those involved in excitation-contraction coupling such as the ryanodine and dihydropyridine receptors, and myogenic regulatory factors such as myogenin. However, although myostatin is hypothesized to negatively regulate skeletal muscle mass and to be a potential mediator of the sarcopenia of aging, there is a dearth of reports on the expression of myostatin transcript or protein in age-related sarcopenia. The present finding of increased levels of myostatin protein appears consistent with myostatin being a negative regulator of muscle mass despite the paradoxical decline in the expression of myostatin mRNA with aging. The decline in myostatin mRNA may reflect a negative feedback on transcription arising from the accumulation of latent myostatin protein in the extracellular matrix. Similar accumulations of protein in the extracellular matrix and binding to matrix components have been reported for other members of the TGF-β superfamily of secreted proteins. Thus, a similar pattern of expression of the genes and protein of myostatin would be expected in other conditions associated with slowly progressive muscle wasting. Indeed, although the level of expression of myostatin mRNA was not reported, myostatin-immunoreactive proteins were elevated in both the quadriceps muscle and blood of humans with HIV-induced cachexia.

In contrast with aging, denervation-induced atrophy is rapid in onset. In the rat gastrocnemius muscle, significant atrophy was present as early as four days after denervation at a time when the level of expression of the transcript or protein of myostatin was suppressed or unchanged relative to sham-operated muscles. These findings are in partial agreement with a previous report that the level of myostatin protein increased slightly at days 4 and 28 after denervation-induced atrophy of the gastrocnemius muscle in the rat. Although the expression of both myostatin mRNA and protein became elevated by day 14 after denervation, the lack of increase in their expression earlier, as well as the small magnitude of the increase, would appear to argue against a major role for myostatin in the etiology of the atrophy. However, it is possible that rapid processing and usage of stored precursor myostatin resulted in its depletion without adequate time for increasing either its mRNA or protein synthesis/accumulation. Detailed studies of the rate of synthesis, storage, processing, usage and degradation of myostatin following denervation will be necessary to address this.
possibility. Alternatively, it is possible that myostatin acts as the chalone that regulates muscle mass. In this scenario, myostatin message, protein and activity would increase in response to hypertrophy and decrease in response to atrophy. In partial support of this hypothesis, mechanical overloading that produced over 30-76% hypertrophy of the plantaris muscle was associated with elevated levels of myostatin protein. These conflicting reports confound elucidation of the role of myostatin in regulating muscle mass postnatally and may well reflect the difficulties in separating the effects of myostatin from the complex interplay that it shares with other growth factors such as IGF-I and growth hormone.

In summary, the present study showed for the first time that the expression of myostatin protein is elevated in age-related sarcopenia, and that the mRNA and protein are differentially expressed in two models of atrophy that have varying rates of onset. This finding provides support for the prospect of using inhibitors of myostatin to prevent or reverse the slowly progressive sarcopenia that occurs in aging, cancer and HIV-induced cachexia.

Acknowledgments

We thank Mohammed Morsey and Craig Findly for antisera against myostatin, Peter LeMotte, Doug Tan, Boris Chrunyk, and Michele Rosner for purified recombinant E. coli myostatin, and Kim Johnson for helpful input about Taqman.

References


