Role of Serum Myostatin during the Lactation Period

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Abstract. Myostatin, also known as GDF-8 (Growth/Differentiation Factor-8), is a member of the TGF-β superfamily that negatively regulates skeletal muscle mass in mammals. Mutation of the myostatin gene in mice, cattle, and humans causes a massively developed skeletal muscle, characterized by muscle hypertrophy and hyperplasia. Although myostatin is predominantly expressed in skeletal muscle tissue, several recent studies have shown the presence of myostatin protein in blood and suggested a possible role for circulating myostatin in the regulation of skeletal muscle mass. In the present study, we examined changes in the levels of active form myostatin (13 kDa) in serum after birth by Western blot analysis to predict the role of serum myostatin in early postnatal muscle growth in the rat. Interestingly, the amount of active form myostatin in serum increased after birth and then decreased along with ageing after weaning. To clarify the role of increased serum myostatin during the postnatal period, we administrated follistatin, an inhibitor of myostatin activity, into postnatal rats intraperitoneally just after birth. Follistatin-administration during the postnatal period caused selective hypertrophy of type II muscle fibers in the soleus muscle. These results demonstrate that myostatin in serum acts on skeletal muscle and negatively regulates early postnatal muscle growth.

Key words: Active form, Lactation period, Myostatin, Serum, Type II muscle fiber

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skeletal muscle [1], it has been reported that myostatin is secreted into the circulation [13–16]. Some investigators have shown that circulating levels of myostatin-like protein are increased in HIV-infected men and elderly persons with weight loss and muscle wasting symptoms [13, 17]. In addition, our previous study using Western blot analysis demonstrated that serum levels of active form myostatin are increased in long term adrenalectomized rats with muscle atrophy [18]. Furthermore, systemic administration of myostatin in nude mice has been shown to induce cachexia-like symptoms with severe weight loss and decreased muscle mass accompanied by skeletal muscle fiber atrophy [16]. These findings suggest a possible association between increased levels of myostatin in circulation and muscle atrophy. On the other hand, the role of serum myostatin in regulation of postnatal muscle growth is still unclear.

In the present study, therefore, we examined the serum levels of myostatin after birth by Western blot analysis to predict the role of serum myostatin in postnatal muscle growth. Interestingly, the active form of myostatin in serum was detected only during the lactation period, indicating the possibility that serum myostatin has some functions during this period. Therefore, we carried out further examinations using follistatin, which is known to inhibit myostatin activity by its binding properties [16, 19, 20], to clarify the role of serum myostatin during the lactation period.

Materials and Methods

Animals and sample preparation

Wistar-Imamichi male rats (neonatal to 4 months old) were used in all experiments. Typically, they were weaned at postnatal day 21. They were housed under temperature-controlled room with a 14 h light and 10 h dark cycle (lights on at 5:00 am). Food and water were provided ad libitum. After the animals were sacrificed by decapitation at the indicated time points described in Results, blood samples and the gastrocnemius (Gast), Tibialis Anterior (TA), Extensor Digitolum Longus (EDL), and Soleus muscles were collected. Skeletal muscle samples were snap-frozen in liquid nitrogen (for protein and RNA extraction) or in liquid nitrogen-cooled isopentane (for sectioning), and stored at –80 C until use. The blood samples were centrifuged to obtain sera at 1,200 g for 15 min at 4 C, and the sera were stored at –20 C until use. Body weight and skeletal muscle wet weight were measured at all time points examined. All experiments using rats were carried out according to the Guidelines for the Care and Use of Laboratory Animals of the University of Tokyo.

RT-PCR

Total RNA was collected from the gastrocnemius muscle tissues (approximately 250 mg) with TRIzol reagent (Invitrogen, CA, USA). Three micrograms of the total RNA were reverse transcribed into cDNA using SuperScript II (Invitrogen, CA, USA) in a reaction volume of 20 µl according to the manufacturer’s protocol. Three microliters of reverse transcribed products (out of 20 µl) were used as a template for semi-quantitative RT-PCR. The primer set for myostatin used consisted of 5’-TTT CAC TTG GCA TTA CTC AAA AG-3’ as the forward primer and 5’-ACA GTG TTT GTG CAA ATC CTG AGA-3’ as the reverse primer (GenBank accession no. U84005, position 28–631). PCR amplification was performed using α-Taq polymerase (Bionex, Seoul, Korea) under the following conditions: 27 cycles with each cycle involving 1 min at 94 C for denaturing, 1 min at 50 C for annealing, and 1 min at 72 C for extension. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control, and the primer set for G3PDH was purchased from Maxim Biotech, Inc. (CA, USA). PCR amplification for G3PDH was performed under the following conditions: 24 cycles with each cycle involving 1 min at 94 C, 1 min at 50 C, and 1 min at 72 C. The appropriate number of PCR cycles was determined so that the numbers versus the intensity of the ethidium bromide-stained PCR product on agarose gel were within a linear range.

Western blotting

Serum samples partially fractionated by anion-exchange chromatography as described in our previous study [18] were diluted with an equal volume of 2 × Laemmli sample buffer [21]. The protein concentrations of the muscle and serum samples were measured by the Bradford method [22] using a Protein assay reagent (Bio-Rad, CA, USA). An aliquot of 50 µg of protein was boiled for 5 min and separated on 15% polyacrylamide-SDS
gel by electrophoresis. Following electrophoresis, the proteins were transferred to a PVDF membrane (Immobilon-P, Millipore, MA, USA). The membrane was soaked in 5% skim milk in phosphate-buffered saline (PBS) (pH 7.4) at room temperature (RT) for 1 h, and then it was incubated with goat anti-mouse GDF8 polyclonal antibody (R&D Systems, MN, USA) diluted with PBS (1:500) at 4 C overnight. The anti-GDF8 polyclonal antibody recognizes the C-terminal region of myostatin protein, which is the terminal region of myostatin protein, and therefore both form of myostatin protein can be detected [18]. Following washing with PBS (5 min × 3), the membrane was incubated with biotin-labeled anti-goat IgG (Chemicon, CA, USA) diluted with 8% skim milk/PBS (1:4,000) at RT for 1 h. After incubation with horseradish peroxidase-labeled streptavidin-biotin complex (Vector Laboratories, CA, USA) at RT for 30 min, the membrane was washed with PBS (5 min × 3) and the signal was detected using an ECL (Amersham Bioscience, Uppsala, Sweden) according to manufacturer's protocol. ECL signals were visualized by exposing the membrane to X-ray film (BioMax, Eastman Kodak, NY, USA). Quantification of myostatin protein on each lane was done by scanning the X-ray film using an image scanner (model GT-9700F, EPSON, Nagano, Japan), and the intensities of the signals were obtained as pixel units.

Follistatin administration
Wistar-Imamichi male rats were intraperitoneally injected with 100 µg/kg body weight of bovine follistatin (kindly provided by Dr. Hasegawa, Kitasato University) diluted with PBS. Follistatin administration was carried out every three days after birth until weaning (postnatal days 0, 3, 6, 9, 12, 15, 18, and 21). On postnatal day 22, the rats were sacrificed and the hindlimb muscles in each leg and blood samples were collected. The skeletal muscles were weighed, and the type II muscle fiber diameter of soleus muscle was measured as described below. Age-matched control rats were injected with PBS.

Immunohistochemical analysis of type II myosin heavy chain (MHC)
Frozen tissue sections of soleus muscle (7-µm thick) that were prepared from follistatin-injected rats and the age-matched controls after weaning (postnatal day 22) were air-dried and fixed with acetone at −80 C for 15 min. After washing in PBS, the sections were soaked in 5% normal goat serum (NGS) in PBS containing 0.3% H₂O₂ at RT for 30 min. The anti-fast MHC (MY-32)-specific monoclonal antibody (Sigma, MO, USA) (1:500; diluted with 1% BSA/PBS) was applied and incubated at RT for 60 min. Following by washing in PBS, the sections were incubated with simple stain MAX-PO (Nichirei, Tokyo, Japan), which is a horseradish peroxidase labeled-secondary antibody, at RT for 60 min. The sections were washed in PBS and then color reaction was conducted using diaminobenzidine (DAB).

Stained sections from three rats per experimental group were photographed, and the diameters of type II muscle fibers were analyzed from 3–6 randomly chosen field. The total numbers of fibers examined were 1301 (control) and 1062 (follistatin-administration).

Statistical analysis
Results are expressed as means ± SEM. Statistical analysis for comparison between ages was conducted using one-way ANOVA. When a significant interaction was identified (p<0.01), statistically significant levels of less than 0.01 were followed up with a post hoc comparison test using Bonferroni/Dunn correction.

Results
Changes of myostatin expression levels and its form in serum with advancing age
Rats were sacrificed at postnatal day 1, day 20, 1 month, 2 months, 3 months, and 4 months, and skeletal muscle (gastrocnemius) was collected at each time point. Both body and muscle weights were temporally increased with advancing age (Table 1). Myostatin gene was expressed in postnatal rat skeletal muscle (day 20, 1 month, and 4 months after birth), and its expression level at 1 month after birth was lower than that on day 20 and 4 months (Fig. 1A).

Changes in relative amount of serum myostatin protein (both the precursor and active forms) were examined by Western blot analysis and the results are shown in Fig. 1B. The amount of precursor forms increased from postnatal day 1 to day 20, and then decreased at 1 month to a level similar to that
seen on day 1. This change coincided with the amount of active forms. However, although the amount of precursor forms increased again at 2 months, and this level was maintained for up to 4 months, the amount of active forms remained at low levels after 1 month. When changes in the total amount of serum myostatin proteins were expressed as the sum of the precursor and active forms (Fig. 1C), the profiles were similar to those of gene expression, indicating that the serum levels of myostatin are correlated with those of gene expression in skeletal muscle (Fig. 1A).

**Follistatin administration during the lactation period**

To verify the role of the increase in active form myostatin in serum during the early postnatal period, we administered follistatin protein to early postnatal rats. The body weight of the follistatin-injected rats was significantly increased at postnatal day 21 compared with the age-matched controls \((p<0.01)\), while there were no differences in the weights of the TA, EDL, soleus, and gastrocnemius muscles (Fig. 2A and B).

**Hypertrophy of the type II fibers of the soleus muscle after follistatin administration**

Since the type II fiber selective action of myostatin has been reported previously [23], the sections of soleus muscle obtained from follistatin-injected and control rats were stained with anti-fast type myosin heavy chain (MHC) antibody, and the diameter of the type II fibers was measured (Fig. 3A).

### Table 1. Body and muscle weights after birth

<table>
<thead>
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<th>Day 1</th>
<th>Day 20</th>
<th>1 month</th>
<th>2 month</th>
<th>3 month</th>
<th>4 month</th>
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<tr>
<td>Body weight (g)</td>
<td>6.0 ± 0.78</td>
<td>47.3 ± 1.3</td>
<td>154.4 ± 18.56</td>
<td>352.1 ± 13.1</td>
<td>425.3 ± 13.1</td>
<td>505.9 ± 13.1</td>
</tr>
<tr>
<td>Muscle weight (g)</td>
<td>0.02 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.66 ± 0.09</td>
<td>1.79 ± 0.07</td>
<td>2.26 ± 0.03</td>
<td>2.53 ± 0.09</td>
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(Gastrocnemius)

Values are expressed as means ± SE \((n=4\) rats per time point).
and B). Intriguingly, the diameter of the type II fibers of the follistatin-injected rats were much larger than in the controls (Fig. 3B), indicating that hypertrophy of type II fibers was caused by follistatin administration. There were no differences in total muscle fiber numbers for soleus muscle between the follistatin-injected and control rats (data not shown).
Myostatin gene expression and serum myostatin level were unaffected by follistatin administration

To examine whether follistatin administration affects either myostatin gene expression or protein levels, we analyzed the expression of the myostatin gene in skeletal muscle and myostatin protein levels in serum. Both myostatin gene expression in skeletal muscle and the serum level of myostatin protein (both the precursor and active forms) were unaltered in the follistatin-injected rats (Fig. 4A and B), indicating that follistatin-administration did not affect myostatin gene expression and cleavage. This, therefore, suggested that follistatin might have indeed neutralized myostatin activity.

Discussion

Yarasheski et al. [17] demonstrated by radioimmunoassay that serum levels of myostatin are increased in elderly persons with muscle atrophy, although they did not mention the forms of myostatin they detected in their assay system. In the present study, the changes in the relative amounts of precursor form myostatin and those of active form myostatin in serum did not correlate in 1-month-old rats. This clearly indicated that mere measurement of total myostatin in serum does not necessarily represent the amount of active form myostatin present in serum. To support this, our previous study showed that atrophy of skeletal muscle fibers in bilateral adrenalectomized (ADX) rats was correlated only with the increase of active form, not precursor form, myostatin in serum [18].

Several previous studies have demonstrated that myostatin acts, not only during embryogenesis, but that it also acts on skeletal muscle development in adult animals [5, 16, 24]. In this regard, a transient increase of active form myostatin in serum during the early postnatal period (between postnatal day 1 and day 20) was unexpected. Immature muscle fibers, formed during the fetal developmental stage, terminally differentiate into mature muscle fibers after birth. It is known that terminal differentiation of muscle fibers is complete by 3–4 weeks postnatal, namely, the time of sexual maturity in mice [25]. Therefore, possible involvement of myostatin is suspected in the terminal differentiation of postnatal muscle fibers. Follistatin is capable of binding to myostatin, and
this leads to suppression of myostatin activity [16, 20]. Indeed, administration of follistatin during the early postnatal period in the present study resulted in hypertrophy of type II muscle fibers in the soleus muscle. Although it is currently whether follistatin-administration also affects type I fibers during the early postnatal period, the fact that type II fiber-selective hypertrophy has been observed in double-muscle Belgian blue cattle [26] and that proteomic analysis of the skeletal muscle of double-muscled cattle has identified an alteration in protein expression associated with fast-twitch glycolytic muscle fiber [27] strongly suggests that myostatin predominantly acts on type II fibers during the early postnatal period.

The major energy metabolic systems of slow and fast muscle fibers are the β-oxidation of fatty acids and the glycolytic system, respectively [28]. Since pups are only supplied with nutrition from their mother’s milk, which is a high-fat and low-carbohydrate diet, during the lactation period, it is possible that pups may have an insufficient supply of glucose in their bodies. Indeed, in our preliminary study, blood glucose levels in pups during the lactation period were significantly lower than in weaned pups (data not shown). Therefore, considering the physiological significance of transiently increased active form myostatin before weaning, it is possible that serum myostatin during the lactation period negatively regulates the size of type II muscle fibers, thus preventing exhaustion of internal glycogen in skeletal muscle. This may allow preferential supply of glucose to other organs that are highly dependent on it, such as the brain.

Although the precise mechanisms of serum myostatin in regulation of muscle fiber size during the lactation period are unknown, myostatin may act on muscle fibers to regulate fiber size through inhibition of myoblast proliferation and/or differentiation. In addition, we believe that there is possibility that myostatin may regulate muscle fiber diameter to a suitable fiber size through induction of apoptosis, since apoptosis has been reported to naturally occur in rat muscle fibers during the lactation period via unknown mechanisms [29]. It is also possible that myostatin may induce apoptosis-related gene expression [26]. These are points of interest that should be examined in the future.

The regulatory mechanism of myostatin expression in skeletal muscle is unknown. It has been shown that the promoter region of the human myostatin gene has multiple putative protein binding sites, such as the glucocorticoid response elements (GREs) [30], and administration of a synthetic glucocorticoid, dexamethasone, increases myostatin expression in skeletal muscle and cultured myogenic cells [31–33]. In addition, it has been also shown that recombinant human growth hormone (GH) inhibits myostatin mRNA and protein expression in cultured myogenic cells, and long term administration of GH to GH-deficient patients reduced myostatin mRNA expression in skeletal muscle [34, 35]. It is thought that GH is a major regulatory factor of growth in neonatal animals and that the plasma GH levels of rats are high during the neonatal period and then decline with advancing age [36–38]. Furthermore, some reports have shown that the basal level of plasma glucocorticoid increases with advancing age [39, 40]. Therefore, it is possible that myostatin expression levels are regulated, at least in part, by several hormones, such as glucocorticoid and GH, during the early postnatal period.

Myostatin is believed to be proteolytically processed by furin protease or other proteases to generate the active form protein [1, 41–44]. In the present study, the presence of the active form of myostatin was mostly limited to the lactation period. This indicates the presence of a mechanism that regulates proteolytical processing of myostatin during this period. Further study is required to examine whether the levels of protease involved in myostatin activation change according to age.

Muscle atrophy and myostatin biological activity were inhibited in vitro by follistatin in transgenic mouse overexpressing follistatin in skeletal muscle [16, 19]. Based these findings, we carried out in vivo follistatin administration during the neonatal period in an attempt to neutralize myostatin activity. Kocamis et al. [45] showed that myostatin gene expression in C2C12 cells was acutely increased by the addition of recombinant human follistatin, indicating the possibility that follistatin modulates myostatin gene expression in skeletal muscle. However, the results obtained in the present experiment revealed that both myostatin gene expression in skeletal muscle and the protein level in serum were not altered by follistatin when administrated in vivo, indicating that the hypertrophic effect of follistatin on skeletal muscle fiber was not due to alteration of myostatin
expression. In addition, it could be argued that the effect of follistatin administration might have been due to its neutralizing activity on activin, since activin has been shown to inhibit myogenic differentiation in the chicken [46]. However, in our preliminary study, we did not see any inhibitory effect of activin on myogenic differentiation of satellite cells obtained from rats (data not shown). Thus, it is not plausible that the effect of follistatin is due to a neutralizing activin action.

In summary, the results presented herein demonstrate that myostatin acts on skeletal muscle during the early postnatal period. We therefore conclude that circulating myostatin may be associated with postnatal muscle development during the lactation period.

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References


