Preparation and application of rat myostatin antiserum

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Abstract: Objective To prepare and identify a polyclonal antibody against rat myostatin and investigate myostatin expression in the rat atrophic gastrocnemius muscle after tibial nerve crush. **Methods** The purified fusion protein was used as antigen to immunize rabbits for the preparation of polyclonal antibody. The polyclonal antibody of the protein was measured by enzyme linked immunosorbent assay (ELISA), western-blot and immunohistochemistry. Myostatin protein expression levels in normal and atrophic gastrocnemius muscle were detected by western-blot and immunohistochemistry assays. **Results** The GST-myostatin had a purity of 96% and possessed high titer and specificity. The level of myostatin in gastrocnemius muscle significantly increased one week after tibial nerve crush, reached the peak on day 14, and then returned to normal level on day 28. **Conclusion** We have successfully made antiserum of rat myostatin and found that the expression level of myostatin protein in the gastrocnemius after tibial nerve crush-induced atrophy was time-dependent. This study provides an experimental basis to clarify the possible role of myostatin during skeletal muscle atrophy.

Keywords: myostatin; antibody; tibial nerve crush; western blot; immunohistochemistry

1 Introduction

Myostatin (MSTN or GDF-8), a member of transforming growth factor- β (TGF- β) family^[1], is predominantly expressed in skeletal muscle throughout life, and works as a negative regulator in skeletal muscle development and growth. MSTN null mice^[1,2] showed a dramatic and widespread increase in skeletal muscle mass, with increase in both number (hyperplasia) and thickness (hypertrophy) of muscle fibers. This phenomenon has also been reported in cattle^[3,4], sheep^[5], dogs^[6] and human^[7]. Unlike mammals and birds, fish possess two distinct MSTN genes with differential expressions: MSTN-1 and MSTN-2^[8]. MSTN-1 in *S. aurata* is expressed in muscle as well as in several other tissues, such as brain, eyes, intestine, kidney and heart, while MSTN-2 is expressed mainly in brain, including the olfactory and optic lobes^[9,10].

Some investigators suggest that the expression patterns of MSTN in atrophic skeletal muscles are different. It has been reported that 10 days of hindlimb unloading causes a 16% atrophy in rat plantaris muscle with a 110% and 37% increase in MSTN mRNA and protein level, respectively. However, 30 min daily muscle loading during the unloading period helps prevent significant loss of muscle mass, despite a concomitant 55% increase in myostatin mRNA expression^[11]. We have previously described expression pattern profile of rat MSTN in response to muscle atrophy induced by sciatic nerve injury^[12, 13]. In present study, we extended these results by preparing and identifying a polyclonal antibody against rat MSTN using a GST-myostatin fusion protein and established the model of tibial nerve crush to observe the expression pattern of MSTN in the atrophic gastrocnemius muscle and provide a potential therapeutic target for future clinical applications.

2 Materials and methods

2.1 Animals and main reagents Sprague Dawley (SD) rats (180-220 g, irrespective of gender, $n = 36$) and adult rabbits

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(1–1.5 kg, $n = 2$) were provided by the Experimental Animal Center of Nantong University. All experiments were carried out in accordance with the “NIH Guidelines for the Care and Use of Laboratory Animals” and were approved according to the Animal Care and Use Committee of Nantong University and the Jiangsu Province Animal Care Ethics Committee. GST-myostatin fusion protein (96% purity) was obtained from our laboratory. Freund’s complete adjuvant (FCA) and Freund’s incomplete adjuvant (FIA) were purchased from Sigma (USA). Rabbit anti-rat GST polyclonal antibody and FITC-conjugated goat anti-rabbit polyclonal antibody were obtained from Sigma (USA). IRDye™ 800 conjugated donkey anti-rabbit polyclonal antibody was purchased from ROCKLAND (Shanghai, China), and the HRP-conjugated goat anti-rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Immunization method and preparation of the antiserum

2.2.1 Preparation of antigen The GST-myostatin fusion protein was emulsified with FAC (sample A) or FIC (sample B) to the final concentration of 1 mg/mL, respectively.

2.2.2 Immunization method Briefly, 1.5 mL blood sample was collected on the day 1 from the rabbit’s marginal ear vein and was used to prepare a pre-immunized-sera as a negative control, after which, 1 mL of sample A was injected intradermally on the back and proximal limbs, followed by a 0.5 mL booster on the day 3 for immunization strengthening. On the day 28 and day 56, 0.5 mL of sample B was injected in the same way. Then, on the day 63, 1.5 mL blood sample was collected from the marginal ear vein and was used to check antibody titer by ELISA. After that, the rabbits were anesthetized, total blood was collected via cardiac puncture, and antiserum was divided into aliquots and stored at -70°C ^[14].

2.2.3 Detection of antiserum titer by ELISA GST-myostatin fusion protein was diluted to a final concentration of 1 mg/mL and was added to 96 well plates (100 μL /well) and incubated at 4°C overnight. Wells were washed with ddH_2O once and 0.01 mol/L PBST (10 min $\times$ 3), and then they were blocked with 5% BSA overnight at 4°C . After being washed, wells were incubated with our rabbit anti MSTN antiserum in 9 dilutions (1:1 000, 1:2 000, 1:5 000, 1:10 000, 1:20 000, 1:40 000, 1:80 000, 1:160 000, 1:320 000) for 2 h at 37°C . Meanwhile,

PBS and pre-immunized-sera were also used as the blank and negative control, respectively. Wells were washed and further incubated with a 1:5 000 dilution of goat anti-rabbit IgG antibody, conjugated with horseradish peroxidase (HRP) for 2 h at 37°C . After the plate wells were washed, fresh substrate was added to each well and 30 min later stop solution was added. The optical density of each well was determined within 30 min.

2.3 Detection of the antiserum specificity

2.3.1 Western blot analysis Protein was extracted from the normal gastrocnemius muscle tissue with lysis buffer (1% SDS, 100 mmol/L Tris-HCl, 1 mmol/L PMSF, and 0.1 β -mercaptoethanol). Protein concentration of each specimen was detected by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) to maintain the same loads. Protein extracts were heat denatured at 95°C for 5 min. The samples were then resolved by 12% SDS-PAGE and transferred to PVDF membrane. After blocking in TBS (50 mmol/L Tris-HCl, 100 mmol/L NaCl, pH 7.4) containing 5% non-fat milk, the membranes were washed with TBST and incubated with 1:200 and 1:500 dilutions of our rabbit anti-rat MSTN antiserum in 5% non-fat dry milk in TBST (TBS containing 0.1% Tween-20) at 4°C overnight. The membranes were washed with TBST (5 min $\times$ 3), and further incubated with a 1:10 000 dilution of IRDye™ 800 conjugated donkey anti-rabbit polyclonal antibody at room temperature for 2 h. After washing the membranes, the fluorescence signals were captured by Odyssey (IL-CDR, England). Data were analyzed using PDQuest 7.2.0 software (Bio-Rad). Integrated density values were calculated, and GAPDH served as an internal control.

2.3.2 Immunohistochemistry analysis Specimens of normal gastrocnemius muscle were obtained from the mid-belly (about $1.0 \times 0.5 \times 0.5$ cm), and were fixed in 4% paraformaldehyde, followed by cryoprotection in graded sucrose solutions. The sections (10 μm) were blocked with 3% BSA at 37°C for 1 h, and incubated with a 1:100 dilution of our rabbit anti-rat myostatin antiserum at 4°C overnight. Then, sections were washed and incubated with a 1:400 dilution of FITC-conjugated goat anti-rabbit polyclonal antibody at 4°C overnight. After being washed, sections were observed under the light microscopy and photomicrographs were taken with the aid

of a DC 300F color digital camera. Data were analyzed using Q550 IW image analysis system (Leica, Imaging System Ltd., Cambridge, England).

2.3.3 Antibody-absorption test The antigen was diluted to 1 mg/mL with a 1:500 dilution of antiserum, and incubated at 4 °C overnight, then centrifugated at 12,000 g×10 min. Supernatant was used for immunochemistry detection as described above.

2.4 Tibial nerve crush

2.4.1 Surgical procedures Thirty-six SD rats were randomized into six groups (6/group) for a tibial nerve crush (4 operated groups), a sham operation and a control group (non-operated). The rats were anesthetized by a mixture of injection (10 mg/kg xylazine, 95 mg/kg ketamine, 0.7 mg/kg acepromazine), and both tibial nerves were identified and lifted through an incision on the lateral aspect of the mid-thigh hind limb. The left tibial nerve was crushed for 5 mm using a 14 cm microsurgical haemostatic forceps (No. JZ07CR; Shanghai Medical Instrument Group, Shanghai, China) three times (10 s each time), with 10 s intervals, whereas the contralateral nerve was intact. All nerve-crush was done by one operator to ensure consistency. This led to complete but reversible denervation, resulting in morphological and histochemical changes similar to those occurred after nerve injury.

Animals in each group were anesthetized and killed 1, 2, 3, and 4 weeks later. Gastrocnemius muscles from the operated and sham-operated limbs were obtained, frozen in liquid N₂ and stored at -70 °C. Before freezing, blocks of muscle (1.0×0.5×0.5 cm) from the mid-belly of the heads of both gastrocnemius muscles, were fixed in 4% paraformaldehyde for subsequent measurements.

2.4.2 Morphometric analysis of gastrocnemius For each specimen, six sections were included in immunochemistry detection, and six random fields in each section were recorded by a DC 300F color digital camera. Data were analyzed using a Q550 IW image analysis system to measure the cross-sectional area (CSA) of muscle fibers.

2.5 Statistical analysis Experiments were repeated three times. All data were expressed as mean ± SD (*n* = a minimum of 3 experiments). One-way analysis of variance (ANOVA)

was used to compare differences between the operated, sham and control groups. All statistical analysis was conducted using STATA 7.0 software package (Stata Corp., College Station, TX, USA). *P* < 0.05 was considered as statistically significant.

3 Results

3.1 Purification of GST-myostatin GST-myostatin protein was purified and characterized by 12% SDS-PAGE (Fig. 1A). Results showed it was about 36 kDa with a purity of 96%. Western blot using rabbit anti rat GST polyclonal antibody further confirmed the results (Fig. 1B).

3.2 Antibody titer detection by ELISA In this assay, PBS was used as a control blank and the pre-immunized-sera was used as a negative control. Data showed that the anti-myostatin serum could be detected within dilutions of up to about 1:20 000 (Fig. 2).

3.3 Western blot and immunochemistry analysis Western blot analysis showed that there were two bands at about 26 kDa (maturation protein) and 52 kDa (precursor protein, Fig. 3). These bands were also present at higher dilutions

Fig. 1 Purification of GST-myostatin. A: 12% SDS-PAGE gel stained with coomassie brilliant blue shows the size of the purified protein in lane 2. B: Western blot of GST-myostatin visualized with a rabbit anti rat GST polyclonal antibody.

Fig. 2 The titer of anti-myostatin antiserum. The optical density was normalized as the ratio of the optical density of the positive reaction to the optical density of negative reaction (P/N), $*P/N > 2.1$. B, blank; Neg, negative serum.

Fig. 3 Western blot analysis of anti-myostatin antiserum against normal gastrocnemius muscle protein. Both bands are present at the higher dilution of anti-myostatin serum (1:500).

(e.g. 1:500, Fig. 3).

Immunohistochemistry confirmed the above results and showed that the pre-immunized serum and the supernatant of the antibody-absorption test did not label the rat gastrocnemius muscle (Fig. 4A and 4B). Sections of normal rat gastrocnemius muscle were sporadically labeled with the anti-myostatin antiserum, showing that very low level of MSTN was present in the normal muscle (Fig. 4C).

3.4 Western blot and immunohistochemical analysis after nerve injury As shown in Fig. 5, in the gastrocnemius muscle, expression of the maturation MSTN was significantly increased between 1 and 3 weeks following tibial nerve crush-induced

Fig. 4 Immunolabeling using anti-myostatin serum against normal rat gastrocnemius muscle protein. A: negative control serum showed no labeling (scale bar = 50 μ m) of the normal muscle tissue. B: anti-body-absorption test showed no labeling (scale bar = 50 μ m) of the normal muscle tissue. C: in normal gastrocnemius muscle, very sporadic and light labeling was evident after incubation with anti-myostatin serum (scale bar = 50 μ m). D: higher magnification micrograph of labeling in C (scale bar = 20 μ m).

Fig. 5 Western blot analysis of myostatin protein level in the gastrocnemius muscle at different time points after tibial nerve crush. A: representative series of myostatin protein expression for the two proteins seen in Fig. 3. GAPDH was used as an internal control to quantify the results. B: Histograms show the changes in the expression levels of the precursor protein and maturation protein. NOR: control group. $*P < 0.05$.

atrophy, which reached a peak at 2 weeks, and had returned to normal level by 4 weeks. In contrast, the precursor protein of MSTN was only significantly higher 2 weeks after the nerve crush (Fig. 5A). There was no significant difference in either maturation MSTN or precursor protein of MSTN between sham and normal groups (data not shown).

Immunofluorescent analysis showed that anti-rat MSTN labeling was present throughout the gastrocnemius muscle fibers between 1 and 4 weeks (Fig. 6A-D) compared to the very low staining level in the normal muscle (Figs. 4 and 6E). Quantification of the fluorescent signal was consistent with the data in Fig. 5B and showed that the fluorescence signal

was significantly higher after the nerve crush compared to the control group (Fig. 7). Fluorescent intensity reached a peak at 2 weeks following nerve crush, and declined at 4 weeks. In contrast to the protein expression shown in Fig. 5B, fluorescent labeling intensity remained significantly higher even at 4 weeks post crush compared to the normal animals. There was no significant difference between sham and normal groups (data not shown).

The muscle-fiber CSA decreased from 1 week to 2 weeks, but recovered from 2 to 4 weeks, which represented an obvious time-dependent change after nerve crush (Fig. 8). There was still no significant difference between sham and normal

Fig.6 Immunofluorescence detection of myostatin protein levels in the rat gastrocnemius muscle at different time points after tibial nerve crush. A-D: anti-MSTN fluorescent staining 1, 2, 3, 4 weeks, respectively, after tibial nerve crush. E: anti-MSTN fluorescent staining of normal gastrocnemius. a-e: higher magnification micrographs of that in A-E. Scale bars = 50 μ m in A-E, 20 μ m in a-e.

Fig.7 Quantification of anti-rat MSTN. The fluorescence intensity was quantified and was consistent with the changes in protein levels shown in Fig. 4. Animals with tibial nerve crush vs. control group (NOP). * $P < 0.01$.

Fig.8 CSA (μ m²) of the rat gastrocnemius muscle. The CSA of muscle fibers was consistent with the changes in protein levels shown in Fig. 4. Animals with tibial nerve crush vs. control group (NOP). * $P < 0.05$.

groups (data not shown).

4 Discussion

Myostatin was first identified by McPherron and Lee^[1] in Johns Hopkins University in 1997. MSTN protein is a member of transforming growth factor beta (TGF- β) superfamily. It is also known as growth and differentiation factor-8 (GDF-8). MSTN-null mice were reported to develop two to three times more muscle than mice that contained the intact gene^[2]. MSTN is a negative regulator in skeletal muscle development and growth. Recent studies showed that fish possess two distinct MSTN genes with differential expressions: MSTN-1 and MSTN-2^[8-10]. In transgenic Coho Salmon that over-expressed growth hormone (GH), MSTN-1 transcription level was not altered in muscle or brain, compared with wild-type adult Coho Salmon. However, in transgenic fish over-expressing GH, expression of the MSTN-2 transcript was greater in red muscle than in white muscle, compared with wild-type fish in the same size^[15]. The differential expressions of MSTN in atrophic skeletal muscle induced by various causes suggest that MSTN may play different roles in response to different kinds of skeletal atrophy.

Skeletal muscle atrophy is a kind of malnutrition and disuse atrophy. The denervation of skeletal muscle may result in irreversible denaturalization before re-innervation has occurred. Decrease in protein synthesis and increase in protein degradation contribute to skeletal muscle atrophy. However, despite the existence of several robust candidate pathways, the molecular mechanisms underlying skeletal muscle atrophy and the subsequent restoration of muscle mass remain unknown. In this respect, studies have been focused on the MSTN's influence on skeletal muscle^[16]. It has been suggested that MSTN could inhibit myogenesis and promote atrophy of adult skeletal muscle by suppressing proliferation of satellite cells^[17]. However, other studies supported that the role of MSTN was to inhibit hypertrophy of skeletal muscle rather than to induce atrophy^[11, 18, 19]. Our previous studies showed that there was an apparent association between MSTN expression and rat sciatic nerve injury^[12, 13]. In accordance with our previous studies using a sciatic nerve crush, tibial nerve crush caused an increase in both the pre-

cursor MSTN and the maturation MSTN expressions from the first week, which reached a peak two weeks after the injury and then returned to normal level. Although they had the same change tendency, the quantity of the precursor protein was less than the maturation protein. This difference might be correlated with the MSTN forming process. MSTN protein is synthesized first as a precursor with 376 amino acids, which then undergoes proteolysis between the propeptide domain and the C-terminal domain, producing a N-terminal propeptide and the mature form of myostatin. The maturation protein flows to the target tissue/organ through blood circulation. Thus, the precursor protein of MSTN in the muscle was less than the maturation protein.

Our results may provide a suitable tool and rationale to investigate the molecular mechanisms of skeletal muscle atrophy, and raise the potential possibility of developing myostatin as a therapeutic target for future clinical applications. If myostatin protein expression level could be manipulated to control the time course and the degree of denervated skeletal muscle atrophy, clinical therapy in the repair of injured peripheral nerves would be promising.

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大鼠 myostatin 蛋白的抗体制备及其应用

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摘要: 目的 采用 GST-myostatin 融合蛋白进行多克隆抗体的制备、鉴定并进一步阐明神经损伤后腓肠肌肌萎缩过程中 myostatin 蛋白表达变化。方法 将纯化好的 GST-myostatin 融合蛋白免疫家兔, 获得抗血清, 经 Western blot、免疫荧光组织化学及 ELISA 检测抗体特异性及效价; 建立大鼠胫神经夹伤模型, 采用 western blot 及免疫组织化学法检测胫神经夹伤后不同时间段腓肠肌中 myostatin 蛋白表达变化。结果 得到较高纯度的 GST-myostatin 融合蛋白, 获得兔源的抗 myostatin 血清, 经 western blot、免疫组织化学及 ELISA 实验证实所得抗体具有较高的特异性及效价; 证实在胫神经夹伤后, 腓肠肌 myostatin 蛋白水平迅速上升, 在第 14 天达到高峰, 随后逐渐下降, 至第 28 天降至接近正常水平。结论 成功制备了兔抗大鼠 myostatin 抗血清, 进一步检测表明了胫神经损伤后腓肠肌中 myostatin 蛋白的表达具有时间依赖性。本研究为阐明 myostatin 在骨骼肌萎缩过程中的作用提供了实验依据。

关键词: myostatin; 抗体; 胫神经夹伤; western blot; 免疫组织化学