Effects of continuous passive motion on the expression of membrane type 1-matrix metalloproteinase in rat immobilized muscles

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Abstract We examined the effects of continuous passive motion (CPM) on membrane type 1-matrix metalloproteinase (MT1-MMP) expression in rat immobilized muscles. Eight-week-old male Wister rats were used for each of two trials, one with 2 weeks, and another one with 4 weeks of immobilization with/without CPM. In each trial, rats were immobilized (immobilization group), and immobilized and simultaneously given CPM (CPM group). The soleus muscle of each rat was evaluated by gelatin zymography, western blotting and reverse transcription-polymerase chain reaction (RT-PCR). Gelatin zymography revealed a greater level of gelatinase activity in the extract of the muscles of the immobilization group than in those of the control and CPM group. The expressions of matrix metalloproteinase 2 (MMP-2) and MT1-MMP mRNA in the muscle extract of the immobilization group were also greater than those in the control and CPM group. Our results suggested that joint immobilization induces expression of MT1-MMP, a cleavage enzyme of MMP-2 in muscles, resulting in muscular degeneration, and that CPM can prevent these changes.

Health Science Research 23(2): 1-6, 2011

Key Words : Membrane type 1 matrix metalloproteinase (MT1-MMP), matrix metalloproteinase 2 (MMP-2), rat, muscle, continuous passive motion (CPM)

Received 3 April 2011
Accepted 30 June 2011

Introduction

Immobilization is the most common orthopaedic treatment for musculoskeletal injury, deformity, and inflammation. After prolonged immobilization, however, complications such as joint contracture and atrophy of muscle and bone may occur[1]. Physical therapy can prevent such joint contracture and disuse muscle atrophy, although the mechanisms responsible for this prevention remain uncertain.

Previous studies have evidenced that experimental immobilization results in morphological degeneration in the soft tissues around articular joints[1),2)]. These changes seem to be related to joint contracture and muscle atrophy. However, little is known about how morphological changes are induced after immobilization in muscle.

Recently, several reports have suggested that loss of skeletal muscles can be attributed to multiple and complex proteolytic systems[3-5)]. The connective tissues surrounding the muscle fibers are broken down primarily by extracellular proteolytic systems such as matrix metalloproteinases (MMPs)[6)] and lysosomal proteases of invading macrophages[4),7)]. There also exist intracellular proteolytic systems, such as the ubiquitin-proteasome system[8,9)], intracellular lysosomal proteases, and calcium-dependent proteases (calpains)[4)].

In addition, the effects of physical therapy have been studied in mouse models of joint contracture and muscle atrophy in recent years[10-12)]. However, most of these studies have focused on morphological changes of skeletal muscles. Hurst et al. and Sasa et al. reported that continuous passive motion (CPM) was useful for the restoration of atrophied muscle in rats[13,14)]. These reports suggested that CPM might be would be useful for preventing the disuse atrophy of muscle tissues

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after during immobilization.

In this study, we focused on the MMP activation network of the degradative process in muscle fibers of immobilized rats, and clarified the biochemical or molecular effect of CPM on disuse atrophy in immobilized rats.

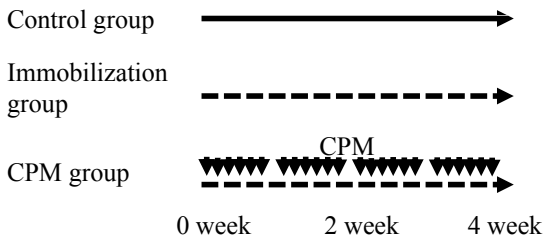

Figure 1. Study protocol

Materials and Methods

Animals

Eight-week-old, male SPF Wister rats weighing 200-300g were obtained from CLEA (Tokyo, Japan). For each of the 2 immobilization trials, 20 of the rats were randomly divided into an immobilization group (n=10) and a CPM group (n=10). After the each period, rats were anaesthetized (pentobarbital sodium 40 $mgkg^{-1}$) and soleous muscles were excised, snap frozen in liquid nitrogen and then stored at -80°C for subsequent analysis. All animals were maintained according the principles of laboratory animal care formulated by Nagasaki University (Japan) and the experimental procedures received approval from the Ethics Review Committee for Animal Experimentation of Nagasaki University.

Immobilization

Animals of both experimental groups were subjected to immobilization. After anesthetization with pentobarbital sodium (40mg/kg), their right ankle joints were fixed in full planter flexion by plaster casts, with the soleus muscles immobilized in a shortened position. The plaster cast, which ran from above the knee joint to the distal foot, was changed weekly because of loosening due to muscle atrophy. The ankle joint was immobilized for 2, or 4 weeks, with 10 rats being used in each immobilization period. In addition, the left ankles of the rats in the immobilization group were used as controls.

CPM

Rats in the CPM group received CPM during the immobilization period. We performed CPM 6 times each week (Fig.1). Briefly, the right hind foot was stretched maximally by dorsiflexing the ankle joints with angular velocities of 10 degrees/second using an ankle stretcher (Sakai Iryo Co., Ltd., Osaka, Japan) under anesthesia for 30min once a day. The range of the stretching was adjusted individually. We fixed the hip and knee joints of the rat and dorsiflexed the ankle joint. When resistance was felt, we determined the angle to the maximal dorsiflexing range of the ankle joint. Measurement of the range was defined as the angle (0-180 degrees) of a straight line connecting the 5th metatarsal and malleolus lateralis of the fibula to a line connecting the malleolus lateralis of the fibula and the center of the knee joint when the ankle was passively dorsiflexed maximally. Both ankle joints of rats of the CPM group were fixed in full planter flexion by plaster cast except when CPM was performed.

Gelatin zymography

At 2 and 4 weeks after immobilization, rats were anesthetized with pentobarbital sodium (40mg/kg). The right soleus muscle was excised and muscle samples were randomly selected. Muscle samples were homogenized (1:7 v/w) in extraction buffer (100mM Trizma-HCl, pH7.6, 200mM $MgCl_2$, 100mM $CaCl_2$, and 0.1% TritonX-100) at 4°C. After centrifugation (15,000g), the supernatant was divided into aliquots, and the protein concentration was determined by Bradford assay against a bovine albumin serum standard curve. Equal amounts of total protein (50μg/lane) were electrophoresed on 8% polyacrylamide gels containing 1mg/ml gelatin at 4°C. After electrophoresis, the gels were washed in 2.5% Triton X-100 and incubated with 50mM Tris-HCl buffer (pH7.5) containing 0.15M NaCl, 10mM $CaCl_2$ and 0.02% NaN_3 for 16h at 37°C and stained with 0.1% Coomassie Brilliant Blue R250.

Immunoblot analysis

An identical amount of proteins (100μg/lane) for each muscle extraction was subjected to 8% SDS-PAGE. Proteins were transferred to nitrocellulose membranes. The filters were blocked for 1.5h using 5% non-fat dried milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T), washed with TBS-T, and incubated at room temperature for 2h in a 1:200 dilution of rabbit anti-MT1-MMP monoclonal antibody or a 1:200 dilution of rabbit anti-Tissue inhibitor of metalloproteinases (TIMP)-1 monoclonal antibody. Filters were later washed with TBS-T and incubated with a 1:2000 dilution of donkey anti-rabbit

IgG antibody, coupled with horseradish peroxidase. An enhanced chemiluminescence (ECL) system (Amersham) was used for detection. Filters were subsequently exposed to film for 15seconds and processed.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from each muscle using Isogen (Nippon Gene, Co., Ltd. Tokyo, Japan). First-strand cDNA was synthesized by reverse transcription at 37°C for 90min in a 20μl reaction mixture containing oligo dT primer by MuLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) from 1μg of total RNA. After heating at 95°C for 5min to denature the proteins and cooling to 4°C, cDNA was amplified in a total volume of 20μl with 1U Taq DNA polymerase (Gibco BRL), 1mM of both primers, and Taq polymerase buffer containing 1.5mM $MgCl_2$ with 1.5mM of dNTP. PCR was carried out on a thermal cycler (Perkin Elmer Cetus) using a program of 30cycles (1min denaturation at 94°C, 1min annealing at 55°C, 1min elongation at 72°C and a final extension at 72°C for 10 min). The specific primers were as follows: for MMP-2, 5'-GCTGATACTGACACTGGTACTG-3' (sense) and 5'-CAATCTTTTCTGGGAGCTC-3' (antisense), with a predicted fragment size of 217 bp; for MT1-MMP, 5'-AGAGGGTCATTCATGGGCAG-3' (sense) and 5'-TCCCATGGCGTCTGAAGAAG-3' (antisense), with a predicted fragment size of 314 bp. After amplification, 6μl of each PCR reaction mixture was subjected to electrophoresis on 1.5% (w/v) agarose gels with ethidium bromide (0.5mg/ml).

Results

Detection of gelatinase activity

We analyzed the gelatin-degrading activities in the muscles of three groups: the control (Fig.2a), immobilization (Fig.2b), and CPM (Fig.2c) groups.

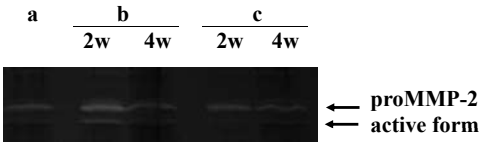

Figure 2. Effect of immobilization and CPM on gelatinolytic activity in soleus muscle

We analyzed the gelatin-degrading activities in the muscles of the (a) control, (b) immobilization (2 weeks and 4 weeks), and (c) CPM (2 weeks and 4 weeks) groups. ProMMP-2 was indicated as a 66kDa band. The active form of MMP-2 was detected under this band.

Gelatin zymography revealed the induction of gelatinase activity corresponding to an electrophoretic mobility of 66kDa. The 66kDa band was strongly induced in the immobilization group. In addition, gelatinolytic bands were detected beneath the 66kDa band in this lane. However, the induction of 66kDa and 62kDa bands was inhibited by CPM.

The expression of MMP-2 mRNA

To examine the regulation of MMP-2 expression, we extracted the mRNA and evaluated the expressions of MMP-2 by RT-PCR. The expression of MMP-2 mRNA was induced in the immobilization group (Fig.3b). However, the induction of MMP-2 expression was not detected in the control and CPM groups (Fig. 3c).

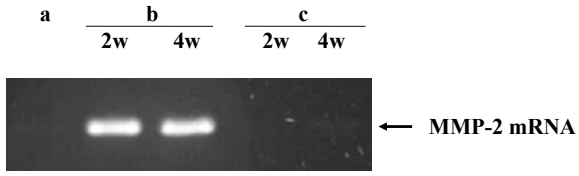

Figure 3. Effect of immobilization and CPM on MMP-2 mRNA expression of soleus muscle

We analyzed the expression of MMP-2 mRNA using the RT-PCR technique in the muscles of the (a) control, (b) immobilization (2 weeks and 4 weeks), and (c) CPM (2 weeks and 4 weeks) groups.

The expression of MT1-MMP

It has been reported that MT1-MMP induced the activation of MMP-2[15-17]. In this study, we examined the expression of MT1-MMP by western blotting. MT1-MMP was induced in the immobilization group, but not in the CPM group (Fig.4b and c).

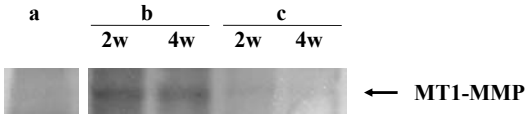

Figure 4. Effect of immobilization and CPM on MT1-MMP expression in soleus muscle

We analyzed the expression of MT1-MMP using western blot analysis in muscles of the (a) control, (b) immobilization (2 weeks and 4 weeks), and (c) CPM (2 weeks and 4 weeks) groups.

The expression of MT1-MMP mRNA

To examine the regulation of MT1-MMP expression, we extracted the mRNA and evaluated the expressions of MT1-MMP by RT-PCR. The expression of MT1-MMP mRNA was induced in the immobilization group (Fig.5b). However, the induction of MT1-MMP

expression was not detected in the control and CPM groups (Fig.5a and c).

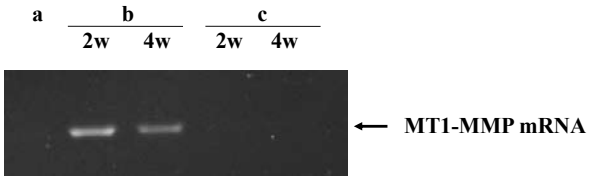

Figure 5. Effect of immobilization and CPM on MT1-MMP mRNA expression in soleus muscle

We analyzed the expression of MT1-MMP mRNA using RT-PCR technique in muscles of the (a) control, (b) immobilization (2 weeks and 4 weeks), and (c) CPM (2 weeks and 4 weeks) groups.

The expression of TIMP-1

We also examined the expression of TIMP-1 by western blotting. The expression of TIMP-1 was induced in the immobilization group (Fig.6b). However, the induction of TIMP-1 expression in the CPM group was almost the same as that in the immobilization group (Fig.6c).

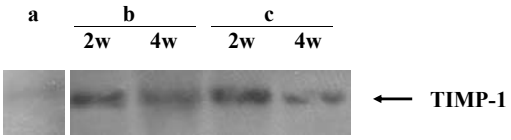

Figure 6. Effect of immobilization and CPM on TIMP-1 expression of soleus muscle

We analyzed the expressi]n of TIMP-1 using western blot in muscles of the (a) control, (b) immobilization (2 weeks and 4 weeks), and (c) CPM (2 weeks and 4 weeks) groups.

Discussion

MMP-2 is one of proteolytic enzymes that play important roles in maintaining the structure and the activity of the basal lamina[18]. MMP-2 is activated under various conditions such as chronic vasodilatation, innervation, and exercise[19-23]. Our zymographic data show that rat MMP-2 was upregulated during the second week of limb immobilization in all three groups. MMP-2 is secreted by a wide range of connective tissue cells and is able to degrade many interstitial collagens (I-V). Giannelli et al. identified the localization of MMP-2 in the endomysium and sarcolemma, and suggested that MMP-2 may act as interstitial collagenases in endomysial fibrosis and may be involved in muscle tissue remodeling or regeneration[24].

We also examined the expression of MT1-MMP to clarify the activation process of MMP-2 in rat soleus muscles by immobilization. MT1-MMP is a 63kDa metalloproteinase, which has been shown to specifically induce the activation of pro-MMP-2[15-17].

It has also been reported that treatment with inflammatory cytokines induced MT1-MMP in human osteoarthritic cartilage[25,26]. Our results suggested that a novel role for the MMP-2 activation was possibly mediated by MT1-MMP in soleus muscles during immobilization. The activation of MMP-2 was correlated with the muscle degradation. We propose a potential mechanism for the pro-MMP-2 activation in muscle degeneration of disuse atrophy. In these muscles, pro-MMP-2 may be converted to the active form by expressing the MMP-2 activator, MT1-MMP, on the cell surface.

The activities of MMPs are also under the control of enzyme tissue inhibitors of matrix metalloproteinases (TIMPs)[27]. Western blot analysis of protein extracts demonstrated the expression of TIMP-1 in immobilized groups. A strong positive correlation between MMP-2 and TIMP-1 has recently been reported in bovine skeletal muscle[27]. Also, intense TIMP-1 staining surrounding individual myofibers and in the adjacent connective tissue and surrounding blood vessels has recently been described in abnormal human masseter muscle.[21]

Our results showed that CPM inhibited the activation of MMP-2, together with the inhibition of MT1-MMP activation at both the protein level and mRNA level. Although CPM prevented the expression of both MMP-2 and MT1-MMP, it did not influence the expression of TIMP-1. It is likely that CPM corrected the increased expression of MMPs while maintaining the balance with TIMP, and this slowed the progression of proteolysis. Giannelli et al. suggested that muscle disuse induced a proteolytic imbalance of MMPs, which could be responsible for the breakdown of basal lamina structures, and that this led to an altered permeability, which could explain the reduced diameter of the soleus fibers and the reduced protein concentrations[18].

The nutrient and oxygen supply of muscles was promoted by adequate exercise, which improved the flow in the muscle microenvironment, and led to a decline in MMP-2 activation and other degeneration processes. Moreover, the inhibition of the activation of MMP-2 would prevent the degradation of ECM. In this way the viability of the capillaries was protected, and the microenvironment was maintained, which led to prevention of the degeneration process in the muscles and surrounding ECM.

Conclusion

We concluded that joint immobilization induced the activation of matrix metalloproteinases, including MT1-MMP, and thereby resulted in degeneration and atrophy of muscle fibrils, and that CPM as a useful technique for preventing these changes.

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Acknowledgments

This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.