

*Original Article***Changes muscle and plasma hepatocyte growth factor levels under casting immobilization****Hideto Okazaki, MD, PhD,¹ Hidehiko Beppu, MT, PhD,² Kenmei Mizutani, MT, PhD,² Shigeru Sonoda, MD, PhD^{1,2}**¹Department of Rehabilitation Medicine II, School of Medicine, Fujita Health University, Mie, Japan.²Fujita Memorial Nanakuri Institute, Fujita Health University, Mie, Japan.**ABSTRACT**

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Purpose: Hepatocyte growth factor (HGF) contributes to the proliferation of muscle cells, however, the relationship between the level of HGF in muscle and that in the blood is unknown. In this study, we examined the influence of exercise on HGF levels in muscle and blood using a rat model of hindlimb muscle atrophy.

Methods: Thirty female Sprague-Dawley rats were used. Fifteen of the rats were cast-immobilized for 14 days (CAS); the remaining fifteen rats were allowed normal activity as the control group (CNT). After removal of the cast, rats were divided into three groups (n=5 per group) as follows: a pre-exercise group (PE), a normal activity group (normal activity for seven days) (NA) and a treadmill training group (20° slope, 15 m per min, 20 min per day for seven days; TR). The CNT group was also divided into the same three groups. At the end of the study period, the wet weight of the left soleus muscle per body weight, plasma HGF level and left soleus muscle HGF level were measured and multiple comparisons were performed.

Results: Muscle weight was significantly lower in CAS-PE than in all CNT groups. Muscle HGF level was significantly higher in CAS-TR than in either CAS-PE or CAS-NA. There were no statistically

significant differences between any of the other groups.

Conclusion: Muscle HGF is expressed in response to relatively high intensity exercise but the dynamics of HGF are limited to intramuscular distribution.

Key words: atrophy, exercise, HGF

Introduction

Evaluation during treatment provides important information for decision-making. During rehabilitation, muscle strength and daily activity are generally used for evaluation; however, deciding on the appropriate amount of exercise based on these evaluations can sometimes be difficult. A factor that can be evaluated objectively and that relates to decisions about exercise would be very useful for evaluation in clinical situations.

A recent study reported the participation of several cytokines in the hypertrophy of skeletal muscle [1]. Moreover, satellite cells are attracting attention as the target of these cytokines [2]. Satellite cells are the stem cells that are present in the extracellular matrix. They exist predominantly in a quiescent state, but after activation, satellite cells migrate to the injured site, where they differentiate, proliferate, and induce hypertrophy of skeletal muscle after fusing with the muscle fiber [3]. Neither insulin-like growth factors nor fibroblast growth factors activate satellite cells [4]; it is believed that only the hepatocyte growth factor (HGF) is capable of triggering their activation [3]. It has been reported that the expression of HGF is increased by muscle injury and exercise [5], that mechanical stimulation of satellite cells causes the release of HGF mediated by nitric oxide, and that HGF activates satellite cells, the activation of which is pH dependent [6]. Satellite cells express mRNA for HGF, and treatment with an HGF-neutralizing antibody has been shown to reduce the proliferation of satellite cells in culture [7]. Consequently, the satellite cell is believed to be an autocrine cell that releases HGF and causes its own activation. There have been few studies

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that measured the changes in muscle HGF levels under immobilization or exercise. Tanaka et al. subjected rats to two-week immobilization to induce atrophy of the soleus muscle and found that loading of the atrophic muscle increased muscle HGF levels [8]. They concluded that the activity of a muscle may affect the level of muscle HGF. Regarding the HGF level in blood, O'Reilly reported changes in the serum HGF level in healthy subjects 4 h after isokinetic knee extension exercise compared to pre-exercise levels [9]. Measurement of muscle HGF requires resection of the muscle. Therefore, if changes in the muscle HGF level are reflected in the blood HGF level, the blood HGF level could be used as an indicator for exercise; however, we have not found any reports on this. In the present study, we examined how exercise after immobilization-induced muscle atrophy affects HGF levels in muscle and blood.

Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee of Fujita Health University. Thirty female Sprague-Dawley rats (mean body weight 275 ± 25.6 g, age 15 weeks) were used for this experiment. Fifteen of the rats were used for the control group (CNT) and the remaining 15 rats were used for the casting group (CAS). Control rats were housed in individual cages and were provided with food and water. Rats in the casting group were anesthetized with Nembutal and the left hindlimb was wrapped in a thermoplastic cast. The rats were then housed in the same environment as the CNT group. The limbs were immobilized in a position of 90° knee flexion and 0° ankle flexion. Fourteen days after casting, the casts were removed under anesthesia and 5 rats in the CNT group and 5 rats in the CAS group were designated as the pre exercise group (PE). Whole blood was drawn from the heart using heparin vacuum blood collection tubes, and the soleus muscle, gastrocnemius muscle and plantaris muscles were resected. As the gastrocnemius and plantaris muscle were damaged during resection, the soleus muscle was used for this study. After measuring the wet weight of the soleus muscle, it was placed in a microtube, frozen in liquid nitrogen and then preserved at -80°C until analysis. Blood samples were centrifuged for 20 min at 3,000 rpm. The plasma was collected and frozen at -80°C until analysis. The remaining rats in the CNT and CAS groups were divided into two groups: the normal activity group (NA) and the treadmill training group (TR). The TR rats ran on 20° sloped treadmills for 20 min per day at a velocity of 15 m per min. This setting was decided after several trials of different settings with rats of the same age. Seven days after removing the casts, blood samples were drawn from all rats and the soleus muscle was resected. One animal

in each of the CNT-NA, CNT-TR and CAS-NA groups was excluded due to wounding of the hindlimb.

Measurement of HGF

Five hundred microliters of protease inhibitor cocktail was added to 25 mL of rat HGF organic extraction buffer. Soleus muscle samples were homogenized in this buffer using a micro homogenizer (Phycotron NS-310E-NS4, NITI-ON, Chiba, Japan). The samples were then centrifuged for 30 min at 4.4°C , 15,000 rpm. After centrifuging, the middle layer was carefully collected. The HGF level of the plasma and the muscle extract was measured using a rat HGF ELISA system (Institute of Immunology, Tokyo, Japan).

Statistical analysis

The soleus wet weight was divided by the body weight and the ratio was used for analysis. Differences in muscle weight and HGF level as a result of casting and training were analyzed using the Bonferroni multiple comparison test. To investigate the relationship between plasma and muscle HGF, analysis of the correlation coefficient was performed, and a scatter plot was created. The level of significance was set at $p < 0.05$ for all analyses.

Results

Measurement of the wet weight of the soleus muscle revealed that it was significantly lower in the CAS-PE group than in the CNT-PE, CNT-NA and CNT-TR groups. It was also lower in the CAS-NA group than in the CNT-NA group, and in the CAS-TR group compared to the CNT-TR group; however, there were no significant differences between these groups (Fig. 1). Levels of HGF in the soleus muscle were significantly higher in the CAS-TR group than in the CAS-PE or CAS-NA groups. CAS-PE was lower than CNT-PE; however, this difference was not significant.

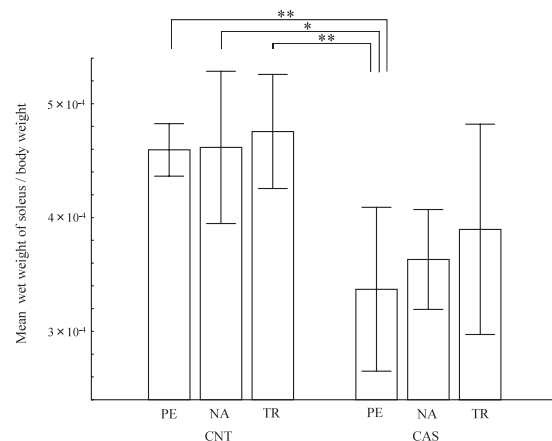


Figure 1. Mean wet weight of soleus muscle / body weight. Error bars indicate 95% confidence interval * $p < 0.05$, ** $p < 0.01$.

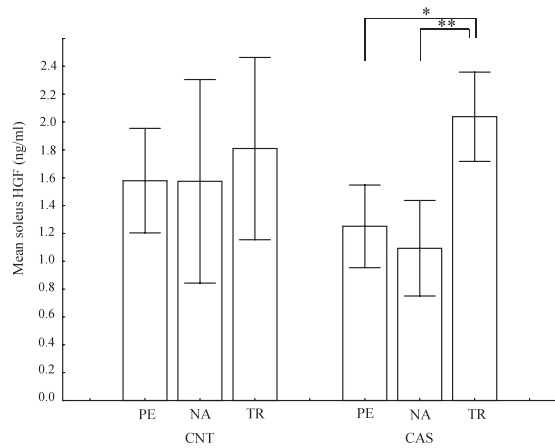


Figure 2. Mean level of HGF in soleus muscle. Error bars indicate 95% confidence interval. * $p < 0.05$, ** $p < 0.01$.

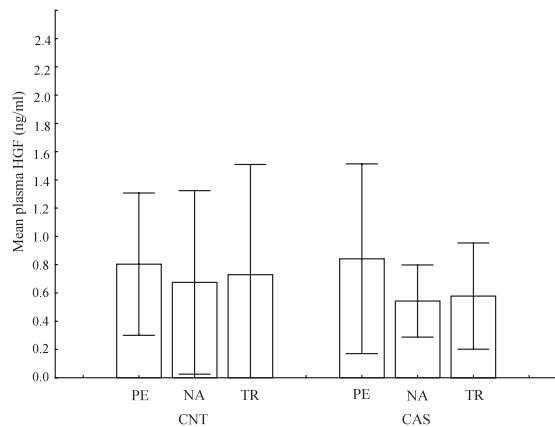


Figure 3. Mean level of HGF in plasma. Error bars indicate 95% confidence interval.

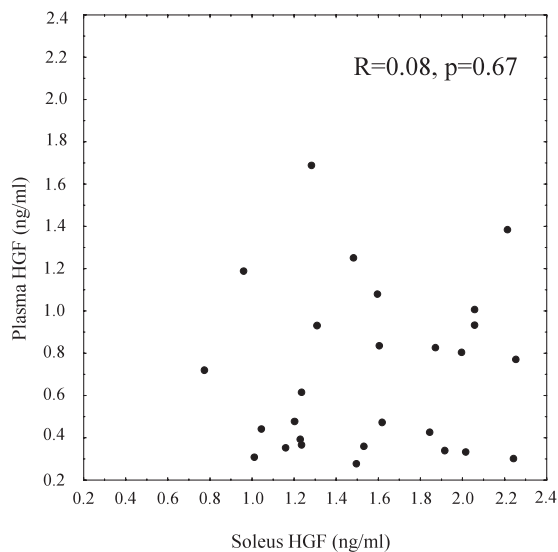


Figure 4. Scatter plot and correlation coefficient between plasma HGF and soleus HGF.

The remaining groups did not show any significant differences (Fig. 2). Regarding the levels of HGF in the plasma, there were no significant differences among any of the groups (Fig. 3). The correlation coefficient between plasma HGF level and muscle HGF level did not show a significant correlation and the scatter plot did not show a significant relationship (Fig. 4).

Discussion

In this study, a significant change in muscle HGF level was observed only in the atrophied group and we found no relationship between the level of muscle HGF and that of plasma HGF. This result can be interpreted as indicating that, given the same load, atrophic muscle and non-atrophic muscle show different reactions, and that only a small amount of the HGF in the muscle enters the bloodstream.

As a model of muscle atrophy, rats were immobilized in a soleus extended position. It was previously reported that the reduction in the number of sarcomeres in the rat soleus was almost the same between immobilization in a shortened position and that in an extended position [10]. In our experiments the weight of the atrophied muscle showed a significant decrease. Consequently we have no concerns about our method of casting. In the casting groups, there were no differences between the levels of exercise tolerance; however, the muscle weight of the TR group was increased compared to the PE group and there were no significant differences between the control and the casting TR groups. Hence, these atrophic muscles might have been in the recovery stage.

The HGF level in skeletal muscle is increased by muscle injury and by mechanical stress on satellite cells, and HGF also activates satellite cells [5,6]. Satellite cells are present in the extracellular matrix, consequently, they would be stressed by muscle activity. The control and casting groups in this study were subjected to two different exercise tolerances and only the casting group showed any significant difference. This can be attributed to the fact that atrophic muscle is easily injured by exercise, and consequently, the change in the level of HGF was significant. Muscle injuries are known to be caused by exercise [11]. It has been reported that uphill running causes soleus muscle injury in rats [12]. In that study, the treadmill setting was 90 min running at a velocity of 17 m/min, and so the exercise tolerance was higher than that in our study. Hence, the control group might have sustained less or no muscle injury. On the other hand, the atrophic soleus muscle in the casting group might have experienced a relatively higher load than the non-atrophic soleus muscle in the control group, and so the atrophic soleus muscle in the casting group might have been more prone to muscle injury than the non-atrophic soleus muscle in the control group. As a

result, there were no differences between the control groups, and the atrophic soleus muscle in the casting group experienced muscle injury and a relatively high load on the muscle fibers, which led to a significant increase in the level of HGF.

Muscle injury due to exercise may have contributed to the change in muscle HGF level in the present study. Intense muscle exercise can cause muscle weakness and limitation of the range of joint motion [13]. This extreme exercise would be a risky type of exercise. If a large number of rats in the casting group had been unable to complete the exercise in our study, we would have suspected that the rats had muscle weakness due to the intense exercise. However, all rats completed the exercise during the 7 day study period. Therefore, we concluded that the exercise caused muscle injury of the atrophied muscle; however, this exercise need not be considered intensive.

On the other hand, the HGF level of the atrophic soleus muscle did not show any significant decrease. A previous report also did not show a significant decrease under atrophy, nevertheless, both our study and the previous study showed a decrease [8]. In rats, immobilization of the hindlimb induced a decline in the number of satellite cells [14]. Hence, this decrease in the number of satellite cells and decreasing activity may have contributed to the reduction in the HGF level.

We found no significant relationship between plasma HGF level and muscle HGF level. It is known that myofiber proteins such as lactate dehydrogenase (LDH) in the blood are increased as a result of muscle injury [15]. The molecular mass of LDH is about 148 kDa and that of HGF is about 90 kDa [3], and so it cannot be the case that HGF does not enter the bloodstream because it has a large molecular mass. Most HGF exists on the surface of satellite cells in the absence of mechanical stress [6]. Thus, HGF that is not bound to a receptor binds to the cell surface and is pooled. According to O'Reilly's report, the HGF level 4h after exercise was significantly increased; however, at 24 and 72 h after training the levels were decreased, thus the change was temporary [9]. Therefore, this result suggests that exercise promotes the release of HGF, and that a portion of the HGF enters the bloodstream; however, most of the HGF is bound to its receptor or to the cell surface and consequently, exercise does not affect the plasma HGF level.

The limitation of this study is the small number of subjects and the lack of any histological examination. In a further study, we will collect more data and include different intensive exercise regimes with histological examination.

In conclusion, HGF is expressed in muscle in response to relatively intensive load and muscle injury,

however, changes in expression cannot be detected in the blood, since most of the movement of muscle HGF is limited to the intramuscular environment and therefore plasma HGF cannot be used for decision-making on the amount of exercise.

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