Expression of Hepatocyte Growth Factor in Rat Skeletal Muscle

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Abstract. The present study examined the localization of hepatocyte growth factor in rat skeletal muscle, and investigated whether levels of hepatocyte growth factor differ between skeletal muscles. Levels of hepatocyte growth factor in soleus and tibialis anterior muscles were measured using enzyme-linked immunosorbent assay. Localization of hepatocyte growth factor and proliferating cell nuclear antigen in the soleus muscle was visualized using immunofluorescence analysis. Level of hepatocyte growth factor was 3.2 ± 1.4 ng/g tissue in the soleus muscle and 3.4 ± 0.4 ng/g tissue in the tibialis anterior muscle. No significant differences were identified between muscles with differential contractile characteristics. Existence of hepatocyte growth factor was observed in cytoplasm of small cells conterminous to muscle fibers. Cells in a similar position displayed reactivity to proliferating cell nuclear antigen, suggesting that they represented activated skeletal muscle satellite cells. Hepatocyte growth factor is produced in normal rat skeletal muscle by activated skeletal muscle satellite cells.

Key words: Skeletal muscle, HGF, PCNA

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INTRODUCTION

Skeletal muscle cells play important roles in muscle regeneration and hyperplasia1). Skeletal muscle satellite cells are usually present in a quiescent state between the plasma membrane and basal lamina2), but become activated following muscular injury or mechanical stretch3–5). Activated satellite cells enter into a cycle of proliferation and division, and differentiate into myoblasts6, 7). These myoblasts undergo coalescence and maturation, finishing with repair and hyperplasia. Growth factors such as hepatocyte growth factor (HGF)5), fibroblast growth factor8, 9) and insulin-like growth factor I10) are associated with the proliferation and differentiation of satellite cells. However, each factor plays a different role. While HGF can cause precocious entry into the cell cycle for satellite cells, the actions of HGF in skeletal muscle in vivo remain unclear. Although the contractile function of skeletal muscles differs between specific muscles, relationships between the contractile properties of skeletal muscle and concentrations of HGF are unknown. Clarification of these mechanisms could prove very useful in determining physical therapy to achieve hypertrophy or hyperplasia. Furthermore,
repair reactions of specific skeletal muscles may differ with function. The present study investigated associations between production of HGF and contractile properties in rat skeletal muscle.

METHODS

Animals and materials
The present study used 6 female, 11-week-old Wistar rats (body weight, 196–220 g). Deep anesthesia was induced in all animals by intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight). For quantitative analysis of HGF levels in tissue, 5 of the rats were exsanguinated. The right soleus and tibialis anterior (TA) muscles were then excised and quick-frozen in liquid nitrogen. For immunofluorescence analysis, right soleus muscle was excised from the other normal rat and oriented for cross-section in embedding medium (Tissue Tek OCT compound; Miles, Elkhart, IN, USA), then quick-frozen in isopentane chilled with liquid nitrogen. Samples were stored at −70°C until use. At the end of the study, all animals were sacrificed. All procedures for animal care and treatment were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Kanazawa University.

Enzyme-linked immunosorbent assay for hepatocyte growth factor
For detection of HGF levels, tissues were completely homogenized in lysis buffer (pH 7.5). Samples containing HGF were separated by centrifugation for 60 min at 16,100 × g and 4°C. Measurement of HGF levels was performed using an enzyme-linked immunosorbent assay (ELISA) kit (Institute of Immunology, Tokyo, Japan).

Histological analysis
Sections (10 µm thick) were cut on a cryostat, then dried for 1 h at room temperature. For morphological observation, cross sections were stained using hematoxylin and eosin.

Immunofluorescence staining for hepatocyte growth factor and proliferating cell nuclear antigen
Sections (6 µm thick) were cut using a cryostat, and dried for 1 h at room temperature. For detection of HGF, sections were fixed in methanol for 5 min at 4°C. For detection of HGF and proliferating cell nuclear antigen (PCNA; Dako Cytomation Japan, Kyoto, Japan), sections were treated with phosphate-buffered saline (PBS) containing 0.1% TritonX-100 (pH 7.4) for 5 min at room temperature. Non-specific binding sites were blocked using normal swine serum and bovine serum albumin (BSA) in PBS for 10 min. Sections were incubated with each primary antibody, polyclonal anti-rat HGF antibody (Institute of Immunology) diluted 1:10 in PBS and monoclonal anti-mouse PCNA antibody (Dako Cytomation Japan), each for 90 min at 37°C then 30 min at room temperature. Sections were covered with secondary antibody for 20 min at 37°C, using goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) diluted 1:300 in PBS and goat anti-mouse Alexa Fluor 546 (Molecular Probes) diluted 1:500 in PBS. All nuclei were counterstained using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes). Negative controls were incubated with each rabbit serum and mouse IgG. Fluorescein signals in sections were observed and photographed using a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis
Student’s t-test was used for comparisons between HGF levels in soleus and TA muscles. Values of P<0.05 were considered statistically significant.

RESULTS

Tissue HGF level was 3.2 ± 1.4 ng/g of tissue in the soleus muscle and 3.4 ± 0.4 ng/g of tissue in the
TA muscle, with no significant differences noted between muscles.

HGF-positive cells were identified as small cells conterminous to muscle fibers (Fig. 2C, G), and HGF signals were localized to the cytoplasm of these small cells (Fig. 2B, C, F, G). Nuclei displayed no positive staining for HGF. Negative control sections displayed lower background levels of normal rabbit serum (Fig. 2D, H). Some cells in a similar position to HGF-positive cells displayed positive reactivity for PCNA (Fig. 3B, C).
DISCUSSION

C-Met is an HGF receptor, which is expressed in normal adult rat TA muscle satellite cells, and HGF is released from satellite cells by mechanical stretch \textit{in vivo}^{5, 11}. Expression of slow myosin heavy chains represents 78\% of total myosin heavy chain isoforms expressed in the rat soleus, compared to 5\% in TA^{12}. The number of satellite cells proliferating in the soleus muscle is elevated after functional loading^{13}. Thus, in the soleus muscles, which act as “antigravity” postural muscles, HGF concentrations might be assumed to be higher than in TA muscles. However, no significant differences were identified for HGF levels in soleus and TA muscles in the present study. This result suggests that HGF levels in soleus and TA muscles under stationary conditions are around 3.2–3.4 ng/g tissue, with no real difference between muscles displaying differing contractile characteristics.

HGF acted as an activator of quiescent satellite cells \textit{in vivo}^{14}. Cells labeled with PCNA, which can be used to detect entry into the cell cycle, were usable as markers for satellite cell activation^{15}. We therefore assumed that HGF and PCNA were present in activated satellite cells. In the present study, HGF- and PCNA-positive cells were observed in the same region of the soleus muscle. This indicates that HGF is expressed and activated satellite cells are present in normal rat soleus muscles.

Soleus muscle activity can increase to about 3-fold higher than TA muscle activity during exercise^{16}. Muscles of the rat hindlimb were injured by downhill exercise on a treadmill, and the percentage of morphologically altered fibers was 4–8\% in soleus muscles, and 1–2\% in TA muscles^{12, 13}. The number of proliferating satellite cells was then seen to increase within 2 days of injury^{12}. Running exercise might thus account for up-regulation of HGF in the soleus muscle.

The present results reveal that HGF levels in normal rat soleus and TA muscles are 3.2–3.4 ng/g of tissue, with no significant differences between muscles. Furthermore, HGF appears to be present in the normal rat soleus muscle, produced by muscle satellite cells. Further research is required to clarify the mechanisms of hypertrophy, hyperplasia and muscle remodeling.

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REFERENCES

