Original Article

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Expression of hepatocyte growth factor is synchronized with expansion of myogenesis in regenerating skeletal muscle

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Abstract

The role of hepatocyte growth factor（HGF）-induced activation（phosphorylation）of its receptor, c-Met, and subsequent downstream signaling during muscle regeneration has not yet been adequately determined. We examined HGF and phospho-c-Met expression and localization during the expansion phase and time course of satellite cell activation, which is critical for muscle regeneration after injury. HGF, c-Met, myogenic differentiation 1（MyoD）, and myogenin mRNA levels were significantly increased during muscle regeneration after injury. Phospho-c-Met was localized on the surface of regenerating muscle cells. HGF was colocalized in the same cells as phospho-c-Met, MyoD, or myogenin. The muscle area occupied by HGF-, phospho-c-Met-, MyoD-, or myogenin-positive cells expanded along with expansion of regenerating muscle. Thus, c-Met is expressed in regenerating muscle cells immediately after injury, and its kinase activity is likely activated by HGF expressed by the same cells, suggesting that HGF and c-Met contribute to myogenesis *in vivo*.

KEY WORDS

skeletal muscle regeneration, hepatocyte growth factor, satellite cell, c-Met

Introduction

Skeletal muscle is capable of regenerating in response to local injury. In this process, skeletal muscle satellite cells, which are immature cells that exist in a quiescent state under the basal lamina of muscle fibers in adult mammals, are activated[1-5] and play an important role in skeletal muscle regeneration through proliferation, differentiation and fusion[6, 7]. During muscle regeneration, satellite cells become mature fibers following the expression of myogenic regulatory factors, including myogenic differentiation 1（MyoD）[3, 8-10] and myogenin[11], which belong to a class of factors that act as transcription factors, thereby inducing myogenesis. Furthermore, recent studies have reported that several growth factors are involved during the process of muscle regeneration[12]. Of these factors, hepatocyte growth factor（HGF）is the only growth factor known to activate quiescent（G0）satellite cells[13, 14], and exogenous HGF can induce satellite cell migration in intact skeletal muscle in vivo[5]. HGF was originally identified as a potent mitogen for hepatocytes[15-17] and is now known to be expressed in various organs including the liver, lungs, kidneys, brain, spleen and skeletal muscle[18, 19]. HGF is secreted into the extracellular space as an inactive single chain and is converted into its active form by specific proteases such as urokinase[17, 18]. Subsequently, active HGF binds to its receptor, c-Met, on target cells[20]. Binding of HGF to the HGF receptor c-Met induces activation of the c-Met tyrosine kinase domain via induction of its autophosphorylation. Activation of c-Met triggers mitogenesis and morphogenesis in many cell types[18]. It is thus likely that c-Met is activated and therefore phosphorylated in dividing satellite cells in regenerating skeletal muscle, leading to muscle regeneration and differentiation. However, activation and phosphorylation of c-Met in regenerating skeletal muscle has not yet been adequately clarified in vivo. Therefore, in

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this study, we aimed to demonstrate HGF expression and c-Met phosphorylation in activated satellite cells in vivo.

Materials and Methods

Animals

Male Wistar rats (nine weeks old; Kiwa Laboratory Animals Co., Ltd., Wakayama, Japan) were used in this study. The rats were housed in a temperature-controlled room (20–24 ℃) with a 12-h light/12-h dark cycle and were provided access to laboratory chow and water ad libitum. The rats were randomly allocated into two groups. All rats were anesthetized by intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight). Muscle injury was induced by softly applying an iron rod 4 mm in diameter, which was previously cooled in liquid nitrogen, to the paracentral area of the right tibialis anterior (TA) muscle for 5 s[21, 22]. In the control group, pressure was applied with a non-cooled iron rod for 5 s. Rats were sacrificed on postoperative days 1, 3 and 5.

All procedures for animal care and treatment were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa University, Kanazawa, Japan, and all protocols were approved by the university's Committee on Animal Experimentation.

Quantitative real-time polymerase chain reaction

Animals were perfused with ice-cold phosphate buffered saline (PBS), pH 7.2, under anesthesia by intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight). Total RNA was then isolated from muscle samples taken from the paracentral area of the TA muscle of the right hind limb using an RNeasy® Fibrous Tissue Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The RNA obtained was reverse-transcribed using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio Inc., Otsu, Shiga, Japan).

Quantitative real-time RT-PCR was performed using the LightCycler® ST300 (Roche Diagnostics K. K., Tokyo, Japan), the SYBR® Premix Ex Taq™ (Takara Bio Inc.) intercalation method and the following primer sets: HGF; (5'-CTTAAACATTTCCCAGCTAGTC-3', 5'-CTCGTAATAAACCATCTGCGT-3'), c-Met; (5'-TAGGATTCGGTCTTCAAGTAG-3', 5'-AAATCAGCAACCTTGACAGT-3'), MyoD; (5'-ACTACAGCGGCGACTCAGAC-3', 5'-ACTGTAGTAGGCGGCGTCGT-3'), myogenin; (5'-TGAATGCAACTCCCACAGC-3', 5'-CAGACATATCCTCCACCGTG-3'), and glyceraldehyde-3-phosphate dehydrogenase; (GAPDH; 5'-AACGGGAAACCCATCACCA-3', 5'-CGGAGATGATGACCCTTTTG-3'). Quantitative real-time RT-PCR involved an initial denaturation step of 10 s at 95 ℃. PCR then proceeded for 40 cycles using a denaturation step of 5 s at 95 ℃ and an annealing and extension step of 20 s at 60 ℃, followed by melting curve analysis. Reaction conditions were optimized to provide efficiencies for standard curve analysis. For each primer set, PCR specificity was judged determined by a single peak on melting curve analysis at the product melting temperature. Expression of HGF, c-Met, MyoD and myogenin mRNA was normalized against that of GAPDH mRNA. The relative content of each mRNA was compared in injured and uninjured muscles.

Immunohistochemistry

For immunofluorescent localization of HGF, MyoD and myogenin in muscles, animals were perfused with ice-cold PBS, pH 7.2, under anesthesia by intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) and the paracentral area of the TA muscle of the right hind limb was removed. The samples were placed in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek Japan, Tokyo, Japan) and frozen in liquid nitrogen-cooled isopentane. The frozen muscle tissues were cut into10-μm thick transverse sections for hematoxylin and eosin staining and 20-μm thick sections for immunostaining, using a cryostat. Transverse sections were fixed in 70% ethanol for 30 min at 4℃. Non-specific binding sites were blocked using 10% normal goat serum in PBS for 30 min at room temperature. For immunofluorescent localization of phospho-c-Met and HGF, animals were perfused with ice-cold 4% paraformaldehyde (PFA) in PBS under anesthesia by intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight) and the resected muscle tissues were soaked in 4% PFA for 1.5 h at 4 ℃. The 20-μm thick frozen sections were air-dried for 2 h and then treated with 10% normal goat serum/0.2% Triton X-100/PBS for 30 min at room temperature. The sections were incubated overnight at 4℃ with rabbit polyclonal anti-HGFα (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) antibody; with mouse monoclonal anti-MyoD or anti-myogenin antibodies (both from Dako Japan Inc., Tokyo, Japan), or with rabbit monoclonal anti-phospho-c-Met antibody (phospho-Tyr$^{1234/1235}$; Cell Signaling Technology Inc., Tokyo, Japan). Antibody dilutions used were: anti-HGF α; 1:20, anti-MyoD, -myogenin and -phospho-c-Met; 1:100 each. The sections were subsequently incubated with 1:600 dilution of Alexa Fluor® 488-conjugated anti-rabbit IgG or Alexa Fluor® 546-conjugated anti-mouse IgG (both from Molecular Probes, Eugene, OR, USA) for 20 min at room temperature, and were mounted with ProLong® Gold Antifade Reagent with 4'6-diamidino-2-phenylindole (DAPI; Molecular Probes). Fluorescent signals in sections were captured using a fluorescence microscope (Biozero BZ-8000; Keyence Corporation, Osaka, Japan) and a confocal laser scanning microscope (FV10i-LIV; Olympus, Tokyo, Japan). Three-dimensional images were constructed using image data captured by a confocal laser scanning microscope (FV10i-LIV).

For immunoperoxidase staining, the tissue sections were fixed with 4% PFA. The sections were then incubated overnight at 4 ℃ with rabbit monoclonal anti-phospho-c-Met antibody diluted 1:20 in PBS-T and 10% normal goat serum. The sections were incubated with Histofine Simple Stain Rat MAX PO (Nichirei Biosciences, Tokyo, Japan) for 30 min at room temperature and were then stained using a Histofine DAB-3S kit (Nichirei Biosciences) for 5 min at room temperature. Nuclei were counterstained with hematoxylin.

Statistical analysis

Differences between regenerating and uninjured muscles were analyzed using Welch's t-test, followed by the F-test. For all tests, $p < 0.05$ was considered statistically significant.

Results

In this experiment, dehiscence of surgical sutures, limping, nor conspicuous behavior changes have not been observed in all rats.

In all pictures, the upper part of picture shows the surface of the tibialis muscle and the lower part shows the deeper part of the muscle (Figure 1, 3, 4, 5, 6 and 7).

Histological changes after muscle injury

TA muscles were examined histologically over five days after muscle injury (Figure 1). On Day 1 after freeze injury, the TA muscles were necrotic. Edema and sparse inflammatory cell infiltration were observed around coagulation necrosis (Figure 1A, Day 1). By Day 3, mono- and multi-nucleated regenerating muscle cells with scant cytoplasm had accumulated around necrotic muscle tissue (Figure 1B, Day 3). By Day 5, centrally located nuclei were visible in regenerating muscle cells and multi-nucleated regenerating muscle cells showed distinct differentiation into muscle cells, exhibiting distended eosinophilic cytoplasm (Figure 1C, Day 5).

mRNA expression during regeneration

Subsequent quantitative analysis of mRNA expression using real-time RT-PCR showed that HGF, c-Met, MyoD, and myogenin mRNA expression was significantly higher in injured than in uninjured muscles on all days during regeneration (Figure 2).

Localization of hepatocyte growth factor, myogenic differentiation 1, myogenin and phospho-c-Met in regenerating tissue

The cellular localization of these factors in regenerating muscle was analyzed using immunohistochemistry

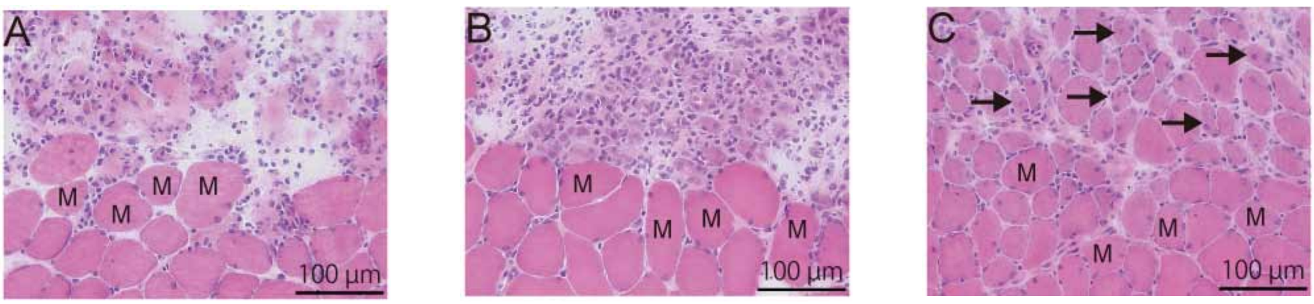

Figure 1 Histological changes in regenerating muscle over five days of regeneration

The process of regeneration in the region of the tibialis anterior muscle injured by freezing was visualized in hematoxylin and eosin-stained transversely cut tissue sections on Days 1, 3, and 5 following regeneration. Multi-nucleated regenerating muscle cells are indicated by black arrows. M indicates uninjured muscle fibers.

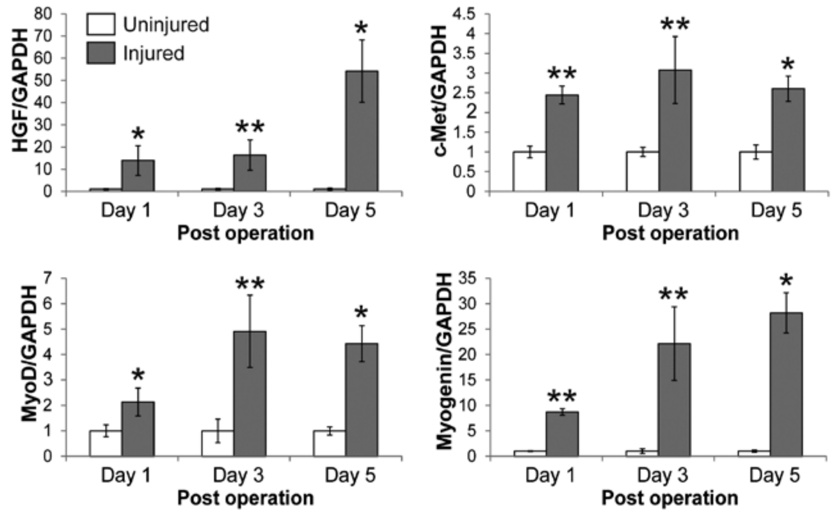

Figure 2 Quantitative reverse transcription-polymerase chain reaction analyses of gene expression in regenerating muscle over five days of regeneration

Quantitative analysis by real-time Reverse transcription-polymerase chain reaction (RT-PCR) of chronological change in the expression of hepatocyte growth factor (HGF), HGF receptor (c-Met), myogenic differentiation 1 (MyoD) and myogenin mRNA, relative to that of control Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, in regenerating injured tissues on Days 1, 3, and 5 after injury and in uninjured tissues in control animals. The number of animals in the injured groups was 4, 8 and 4, and the number in the control groups was 3, 7 and 3, on Days 1, 3, and 5 respectively. A single asterisk (*) indicates $p < 0.05$, and double asterisks (**) indicate $p < 0.01$ vs. control.

(Figure 3-7). HGF, MyoD or myogenin protein expression was not detected on Day 1 in necrotic areas of muscle regeneration and in the uninjured muscle (Figure 3A and 4A). On Days 3 and 5 after injury, HGF was strongly immunostained in the cytoplasm of regenerating muscle cells and was weakly stained in the extracellular matrix (Figure 3 and 4). Three dimensional images of double staining for HGF and either MyoD or myogenin clearly demonstrated that HGF was found in the same cells that displayed immunoreactivity for MyoD and myogenin in their nuclei in regenerating muscle cell on Day 3 (Figure 3B and 4B). In these cells, staining for MyoD and myogenin was restricted to within the area of the nucleus. In images of wider views of the muscle, areas of muscle that were occupied by cells displaying double staining for HGF and MyoD or myogenin were larger on Day 5 than on Day 3 (Figure 5).

Phospho-c-Met was not detected on Day 1 of muscle regeneration (Figure 6). On Day 3, it was strongly immunostained in the cytoplasm of regenerating muscle cells (Figure 6). On Day 5, diffuse immunopositive staining within the distended cytoplasm, and staining on the surface of regenerating muscle cells, was observed (Figure 6). No immunostaining for phospho-c-Met was detected in the uninjured muscle (Figure 6).

Immunostaining for HGF or phospho-c-Met in serial sections of regenerating muscle on Day 3 demonstrated that HGF and phospho-c-Met were co-localized in the same regenerating muscle cells (Figure 7).

Discussion

We examined the expansion and time-course of satellite cell activation and its association with HGF expression and c-Met activation (phosphorylation) in regenerating muscle in rats.

MyoD and myogenin are expressed in the nuclei of activated satellite cells and in newly fused myotubes in regenerating muscle[3, 8, 9, 23, 24]. Their expression is therefore a useful indicator of muscle differentiation[11]. Our data are consistent with these findings.

A previous real-time PCR analysis study reported that levels of HGF, MyoD, and myogenin mRNA expression were significantly increased after 24 hr, and from 48 hr to 72 hr, in regenerating plantaris muscle induced by bupivacaine injection[25]. The present study also showed that the levels of HGF, c-Met, MyoD and myogenin mRNA expression were significantly increased in regenerating muscle on Days 1, 3 and 5 after injury. These results suggest that HGF and c-Met may contribute to myogenesis in vivo.

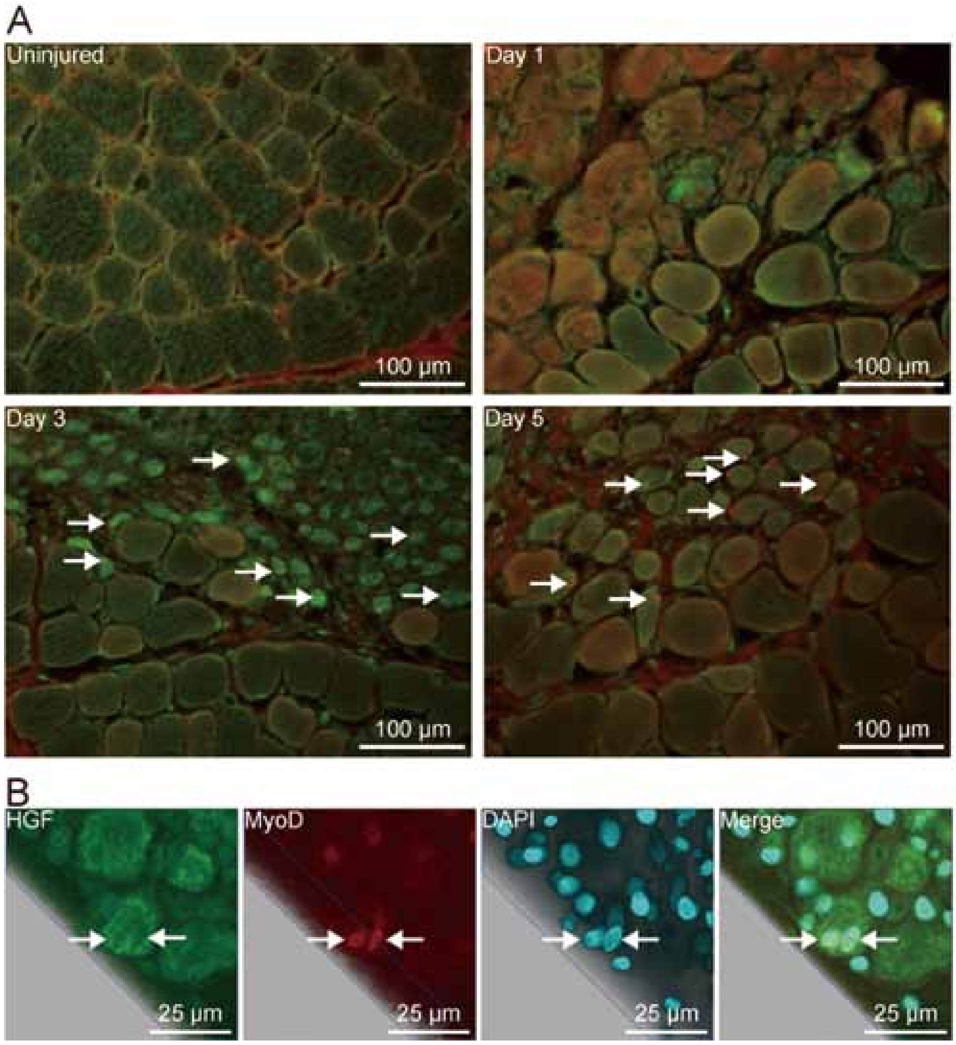

Figure 3 Immunofluorescent analysis of the localization of hepatocyte growth factor and myogenic differentiation 1 in regenerating muscle over five days of regeneration

Sections of regenerating muscle on Day 1, 3 and 5 after injury and uninjured muscle were stained for hepatocyte growth factor (HGF; green) and myogenic differentiation 1 (MyoD; red). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). (A) Merged images for HGF and MyoD are also shown. (B) Three dimensional images of a stained section of regenerating muscle on Day 3 were constructed using confocal laser scanning microscope. Merged images for HGF, MyoD and DAPI are also shown. In (A) and (B), the MyoD positive nuclei of regenerating muscle cells are indicated by arrows.

A previous immunohistochemical study showed immunopositive HGF protein staining of myocytes during the early stages of muscle regeneration[14, 25, 26]. Furthermore, although HGF mRNA was not expressed in macrophages but it was detected in regenerating muscle cells by in situ hybridization[27]. In addition, many previous studies have reported that HGF stimulates satellite cell proliferation[5, 22, 28], and that the source of the HGF in injured tissues appears to be endogenous, i.e., HGF is secreted by regenerating muscle cells[19, 20]. In this study, we confirmed that HGF is secreted by regenerating muscle cells, based on our findings that HGF expression increases after freeze injury and is detected in the cytoplasm of MyoD- and myogenin-positive cells. Moreover, we demonstrated that the area occupied by HGF-, MyoD- and myogenin positive cells extended with

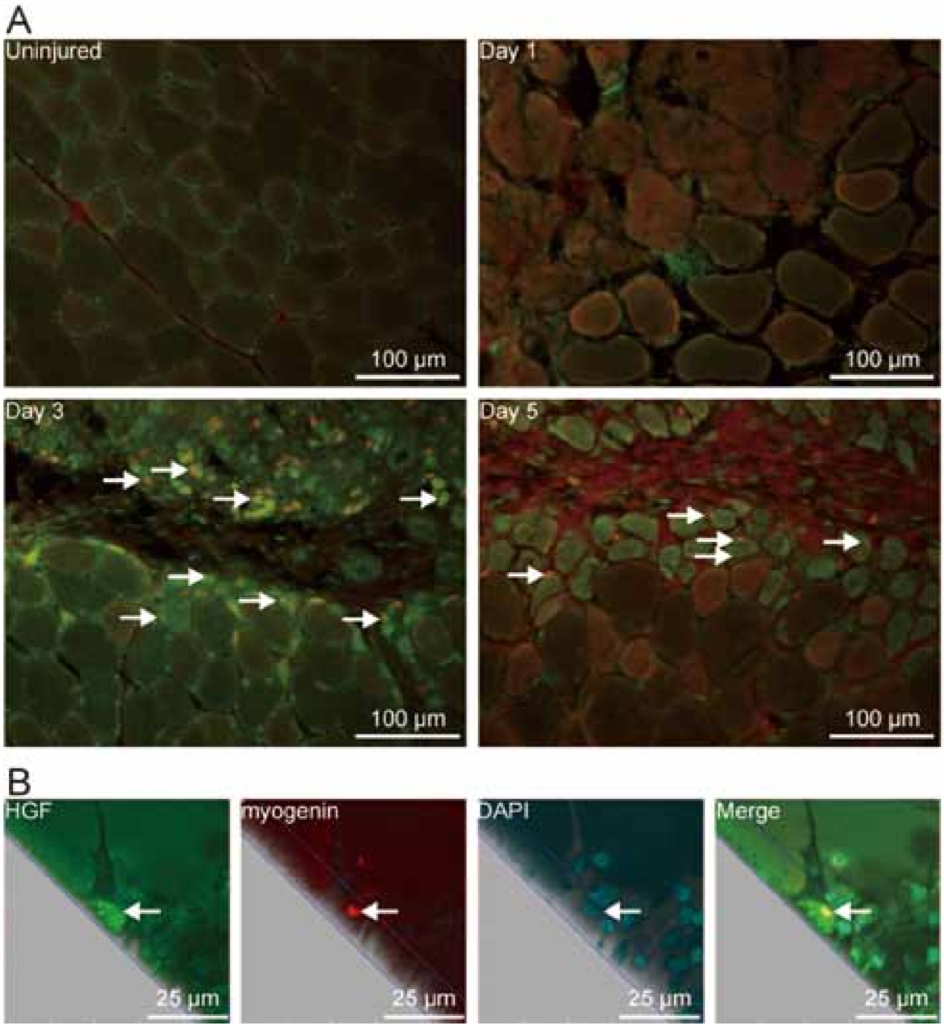

Figure 4 Immunofluorescent analysis of the localization of hepatocyte growth factor and myogenin in regenerating muscle over five days of regeneration

Sections of regenerating muscle on Day 1, 3 and 5 after injury and uninjured muscle were stained for HGF (green) and myogenin (red). Nuclei were counterstained with DAPI (blue). (A) Merged images for HGF and myogenin are also shown. (B) Three dimensional images of a stained section of regenerating muscle on Day 3 were constructed using a confocal laser scanning microscope. Merged images for HGF, myogenin and DAPI are also shown. In (A) and (B), the myogenin positive nuclei of regenerating muscle cells are indicated by arrows.

expansion of the regenerating area from the superficial to the deeper parts of the injured muscle.

Following HGF binding, the kinase activity of the c-Met receptor is switched on by receptor dimerization and trans-phosphorylation at tyrosines (Tyr) 1234/1235 in the c-Met catalytic tyrosine residues[29]. Subsequent downstream signaling through multiple pathways is induced by this event[30]. Indeed, Cornelison and Wold[23] found that satellite cells that respond to regeneration activity were positive for c-Met. Furthermore, Sisson et al.[31], using Western blotting analysis, reported that phospho-c-Met increased in regenerating muscle tissue. Additionally, Webster et al.[30] demonstrated that muscle regeneration was deficient in a c-Met phospho Tyr 1234/1235 mutant mouse that was injured by injection of cardiotoxin. However, in that study, it was unclear in

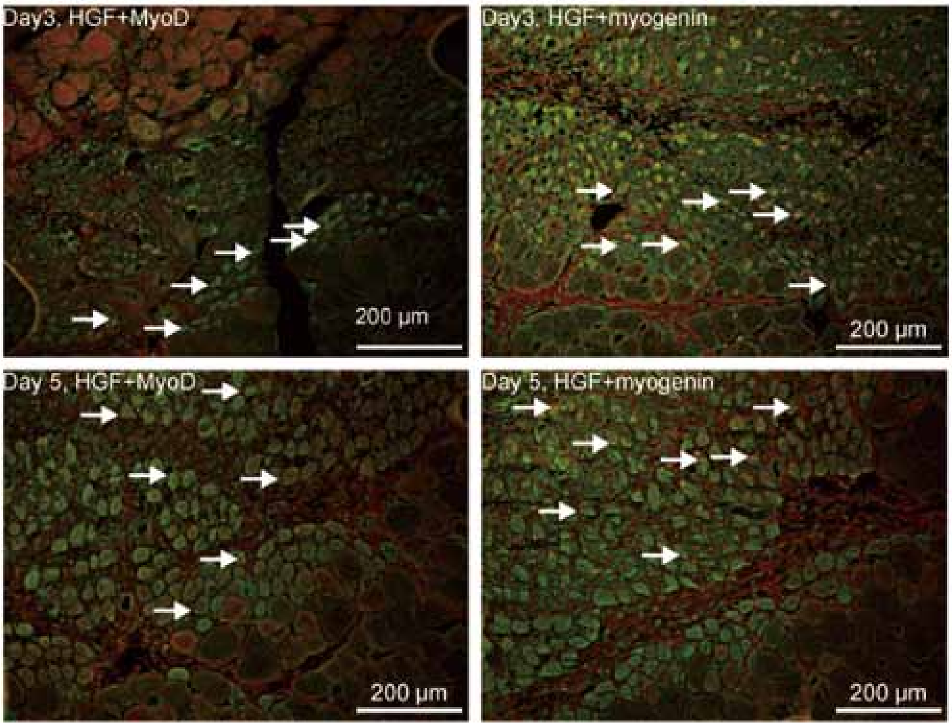

Figure 5 Immunofluorescent analysis of the localization of hepatocyte growth factor and myogenic differentiation 1 in a wide area of regenerating muscle on Days 3 and 5 after injury

Sections of regenerating muscle on Days 3 and 5 after injury were stained for HGF (green) and MyoD (red) or myogenin (red). Merged images for HGF and MyoD or myogenin are also shown. The HGF and MyoD or myogenin positive nuclei of regenerating muscle cells are indicated by arrows.

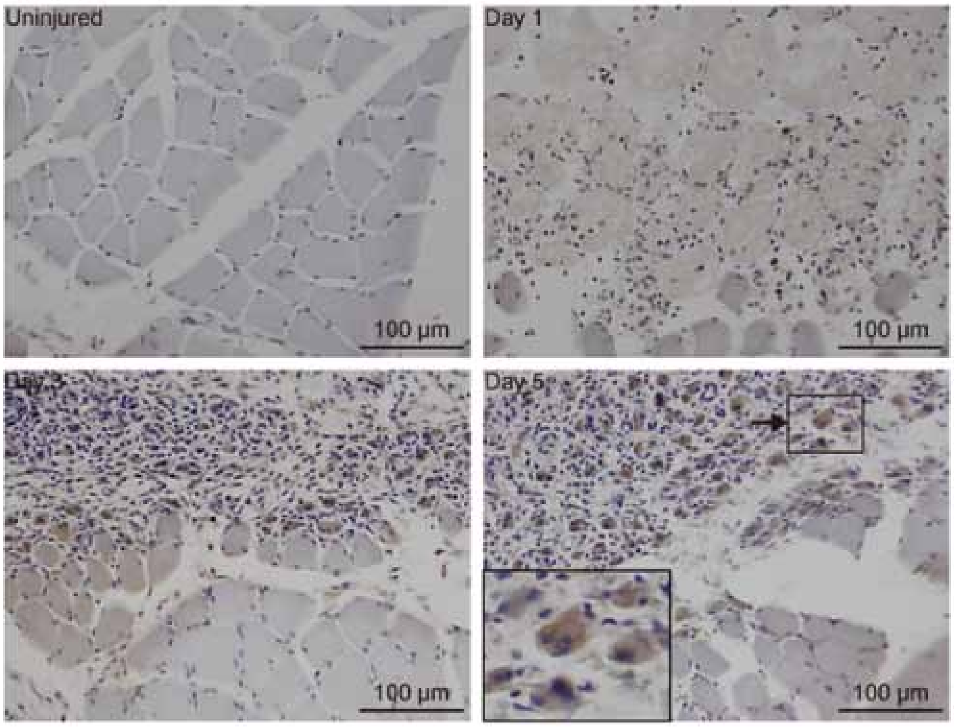

Figure 6 Immunoperoxidase analyses of the localization of phospho-c-Met in regenerating muscle over five days of regeneration

Sections of regenerating muscle on Day 1, 3 and 5 after injury and uninjured muscle were stained for phospho-c-Met (brown). Nuclei were counterstained with hematoxylin (blue). The boxed region indicated by an arrow shown to magnification view.

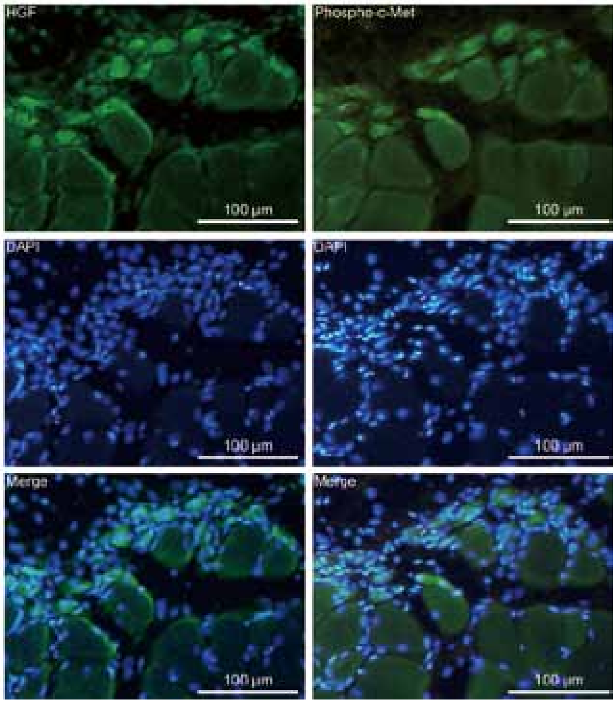

Figure 7 Immunofluorescent analysis of the localization of hepatocyte growth factor and phospho-c-Met in regenerating muscle on Day 3
Serial sections of regenerating muscle on Day 3 were fixed with 4% paraformaldehyde (PFA) and were stained for HGF (green, left side) and phospho-c-Met (green, right side). Nuclei were counterstained with DAPI (blue). Merged images for HGF or phospho-c-Met and DAPI were also shown.

which cells phosphorylated c-Met was present. Here, we demonstrated that c-Met that is on the cell surface of regenerating muscle cells is phosphorylated (Figure 6) and that HGF and phospho-c-Met are localized in the same cells (Figure 7). In this study, the area occupied by phospho-c-Met-positive cells, similar to the area occupied by HGF-, MyoD- and myogenin-positive cells, extended with expansion of the regenerating area from the superficial to the deeper parts of the injured muscle. These cells have distended cytoplasm and are multinucleate, indicating that they are in the process of differentiating into mature muscle cells. Our findings regarding the quantification of c-Met expression and its localization over time suggest that c-Met expression is initiated immediately after injury in regenerating muscle cells and that c-Met kinase activity is activated by increasing HGF levels.

In summary, in this paper we have shown the temporal expression and subcellular localization of HGF, and the temporal expression, subcellular localization and activation status of c-Met, in regenerating muscle. These data will be useful for the understanding of muscle injury at a molecular level and the future therapy of muscle injuries.

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肝細胞増殖因子の発現は骨格筋再生領域の拡大と同期する

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要　　旨

肝細胞増殖因子（HGF）受容体である c-Met は、HGF の結合によるリン酸化を契機に下流のシグナリング伝達を開始するが、骨格筋再生中における HGF 発現と骨格筋衛星細胞の活性化の関係性については十分に解明されていない。そこで、骨格筋損傷後の再生過程において限定的に活動する活性化筋衛星細胞の再生中の発現経過と広がりと、HGF およびリン酸化 c-Met の発現について調査した。HGF、c-Met、活性化筋衛星細胞の指標として使用した myogenic differentiation 1（MyoD）および myogenin mRNA の発現は、損傷後の再生中に著しく増加した。リン酸化 c-Met は再生筋細胞表面に局在していて、HGF はリン酸化 c-Met、MyoD あるいは myogenin と同様の細胞に局在していた。HGF、リン酸化 c-Met、MyoD あるいは myogenin が発現している領域は筋再生の拡大に同期して広がっていた。これらの結果は、再生筋細胞に発現した c-Met および HGF が、in vivo における筋形成に寄与していることを示していた。