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Growth Hormone Stimulates Mechano Growth Factor Expression and Activates Myoblast Transformation in C2C12 cells

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Mechano growth factor (MGF) is an alternatively spliced variant of insulin-like growth factor-I (IGF-I). Previous reports have revealed that the MGF in skeletal muscles is induced by mechanical overload or muscle injury. In the present study, we examined the effect of growth hormone (GH) on MGF expression in C2C12 mouse muscle cell lines since GH is the principal regulator of IGF-I. The MGF mRNA increased 1 h following GH stimulation whereas IGF-IEa mRNA, which encodes a systemic type of IGF-I, increased 4 h following GH stimulation. The diverse expression of MGF and IGF-IEa was also observed in the case of muscle injury by using bupivacaine in the same cell line. Furthermore, GH induced the increase of MyoD as well as M-cadherin expression, the peak of which was parallel to that of MGF. These results indicate that GH directly and preferentially increased MGF prior to the IGF-IEa expression in C2C12 cells, which may lead to the activation of muscle satellite cells.

Insulin-like growth factor-I (IGF-I) plays a role in the skeletal muscle in the development, growth, repair, and maintenance of the tissues in an autocrine/paracrine as well as endocrine fashion (7, 28). Alternative splicing of the *IGF-I* gene is known to generate several different variants (28). Yang et al. (31) cloned the cDNAs of *IGF-I* splice variants that are expressed in the muscle. These include IGF-IEa and the mechano growth factor (MGF). The former is the same as the circulating IGF-I produced by the liver, and the latter is reported to be markedly upregulated after mechanical stimulation or damage of muscle tissue (18, 31). *MGF* mRNA is derived by alternative precursor mRNA splicing from the *IGF-I* gene, which has a 52-base-pair insert in rodents or a 49-base-pair insert in humans within the E domain. This insertion causes a translational frameshift that results in a different carboxy terminal sequence from that of IGF-IEa. Previous reports suggest that MGF possesses a different function as compared to IGF-IEa in the muscle cells (27, 29, 31). The IGF-IEa peptide transfected into C2C12 myoblast cells caused an increase in cell density and myoblasts fused

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to form myotubes. On the other hand, transfection of the MGF peptide into C2C12 myoblasts led to an increase in the number of mononucleated myoblasts but prevented their differentiation (32). Although IGF-I is reported to be involved in the maintenance of muscle tissue and plays a critical role in activation of muscle satellite (stem) cells that are required for local muscle repair (1, 22), the type of IGF-I isoform that may be involved in the process of muscle regeneration remains unknown.

Growth hormone (GH) is the principal regulator of *IGF-I* expression in tissues (5, 7). *In vivo*, hypophysectomized rats demonstrated a reduced *IGF-I* expression in muscle that was recovered by GH administration (15, 30). There are also *in vitro* evidences for *IGF-I* regulation by GH in a mouse C2C12 cell line (9, 24). However, there are no reports to date regarding the regulation of *MGF* by GH *in vitro*. We previously reported that *IGF-IEa* and *MGF* in skeletal muscle were differentially regulated by GH administration in GH-deficient mice (13). Therefore, we speculate that if GH directly regulates the expression of both *IGF-IEa* and *MGF* in the muscle cells, GH administration might be able to repair and regenerate muscle cells more efficiently via the production of both IGF-IEa and MGF than the administration of IGF-I alone.

The aim of this study was to evaluate the direct effect of GH treatment on the expression of *IGF-I* isoforms in the muscle cells and to clarify whether GH treatment could activate satellite cells via MGF production.

MATERIALS AND METHODS

1. Cell culture

Mouse C2C12 myoblast cells can differentiate into myotubes under certain experimental conditions. The C2C12 cells were incubated at 37°C with 5% CO_2 in growth medium containing Dulbecco's modified Eagle medium (DMEM) with 10% horse serum (HS). To enable differentiation into myotubes, the medium was exchanged with DMEM containing 2% HS for 5 days. The cells were then differentiated to myotubes exhibiting morphological changes (Fig. 1a).

2. Stimulation

We added 100 ng/ml of recombinant human GH (kindly provided by Eli Lilly and Company, Tokyo, Japan) or phosphate-buffered saline (PBS) as the control into the cultured medium, and cells were harvested after 1, 2, 4, 6, and 8 h for RNA preparation. In the other experiment, we used bupivacaine to induce the expression of the IGF-I isoforms resulting from myotoxicity in C2C12 cells as described previously (12, 20). We added 0.3 mM of bupivacaine or PBS as a control in the culture medium and harvested the cells after 1, 2, 4, and 7 days.

3. Total RNA preparations

The RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity of extracted total RNA was determined using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

4. Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Structures of mouse IGF-IEa and MGF cDNAs and the position of primers are illustrated in Fig. 1b: namely, the reverse primer specific for *IGF-IEa* was produced complementary to the sequences including the boundaries of exon 4 and 6 of the mouse *IGF-I* cDNA. On the other hand, the forward primer specific for *MGF* was produced complementary to the sequences including the boundaries of exon 4 and 5 of the mouse *IGF-I* cDNA. Primer sequences for *IGF-IEa* and *MGF* are listed in Table. 1. Primers for 18S rRNA, MyoD, and

M-cadherin were designed as described previously (4, 11). We reverse transcribed 500 ng of total RNA from C2C12 cells in a total volume of 10 µl using the iScript cDNA Synthesis Kit (Bio Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instruction. Real-time PCR detection system (model 7500, Applied Biosystems, Foster, CA, USA) with SYBR Premix Ex Taq (Takara, Tokyo, Japan) was used for sample cDNA quantification. The nucleotides of PCR products were confirmed by sequencing using a DNA sequencer (model 310, Applied Biosystems, Foster, CA, USA).

5. Data analysis

Results of the expression of the *IGF-I* isoforms, *MyoD*, and *M-cadherin* were adjusted to those of the amplified 18S rRNA and expressed as mean ± SEM. Differences were determined by the unpaired *t* test or ANOVA, as appropriate. A value of $p < 0.05$ was considered significant.

Table 1. Primer pairs of *IGF-IEa* and *MGF* used for real-time quantitative PCR.

IGF-I Ea forward	5′-TGACATGCCCAAGACTCA-3′
IGF-I Ea reverse	5′-TGTGGCATTTTCTGCTCCGTGG-3′
MGF forward	5′-AGCTGCAAAGGAGAAGGAAAGGAAG-3′
MGF reverse	5′-GGTGATGTGGCATTTCCTGCT-3′

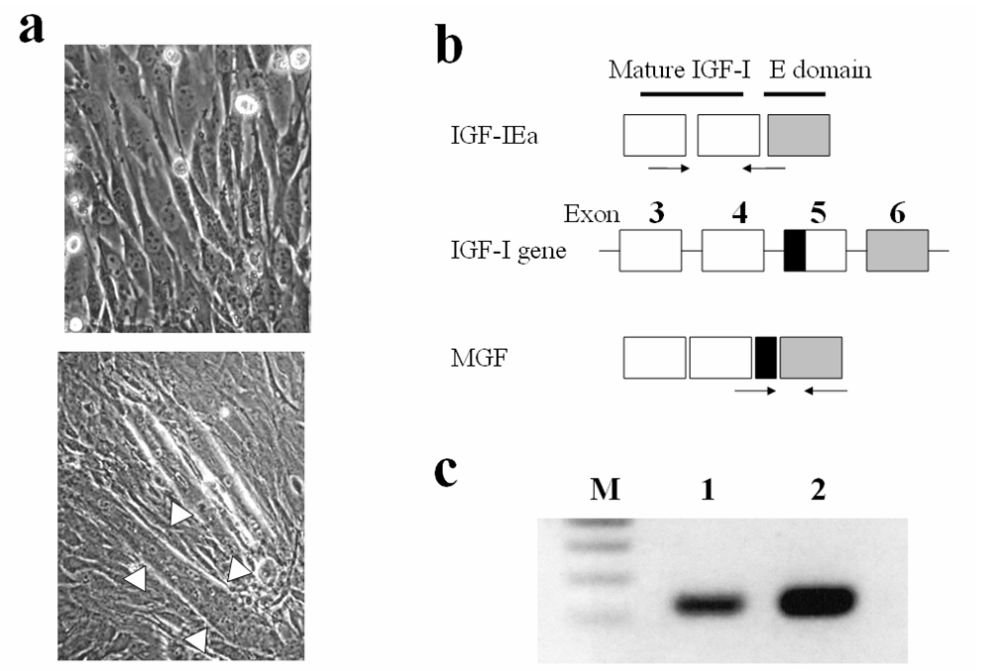

Figure 1. (a) Morphological changes in the C2C12 cells before and after differentiation from mononucleated myoblasts (upper panel) into mature myotubes (lower panel, arrowhead). (b) Schema of the mouse IGF-IEa and MGF cDNAs. The arrows indicate the position of specific primers for PCR amplification. (c) Detection of a specific band of IGF-IEa (lane 1) or MGF (lane 2) cDNA in the C2C12 cells. Products amplified by RT-PCR were separated on 2% agarose gel and visualized with ethidium bromide staining. M denotes the molecular weight marker.

RESULTS

1. Expression of *IGF-IEa* and *MGF* in C2C12 cells

In a preliminary study, we used RT-PCR to examine whether the *IGF-IEa* and *MGF* mRNAs are expressed in C2C12 cells. Using the primer pairs as described, specific bands were detected (Fig. 1c) and their nucleic sequences were confirmed as mouse *IGF-IEa* and *MGF* (*IGF-IEb*), respectively, by sequencing analysis (data not shown).

2. Effects of GH on IGF-IEa and MGF expression in C2C12 cells

The mRNA levels of both *IGF-IEa* and *MGF* were upregulated by 100 ng/ml GH. As illustrated in Fig. 2, the *MGF* mRNA increased 1 h after stimulation by GH. In contrast, *IGF-IEa* mRNA slowly increased at 4 h and reached the peak level at 6 h after stimulation.

3. Effects of chemical damage by bupivacaine on the expression of *IGF-IEa* and *MGF* in C2C12 cells

To evaluate whether or not the diverse expression of *MGF* from *IGF-IEa in vitro* is specific for the stimulation by GH, we next investigated the expression of *IGF-I* isoforms after the stimulation by bupivacaine, which caused cell damage in muscle tissue and induced *MGF* expression (11, 12, 20). As illustrated in Fig. 3, the *MGF* mRNA increased at 1 day whereas *IGF-IEa* significantly increased 7 days after the addition of bupivacaine.

4. Expression of markers of activated satellite cells after the stimulation by GH in C2C12 cells

Proliferating myoblasts derived from satellite cells express *MyoD*, a member of muscle regulatory transcription factors (25), or adhesion molecules such as M-cadherin (14). Therefore, we examined the *MyoD* and *M-cadherin* gene expressions after the stimulation by GH in C2C12 cells as a marker of activated satellite cells. As illustrated in Fig. 4, bimodal increase of *MyoD* was observed 1 h and 6 h after stimulation by GH. The significant increase in *M-cadherin* was observed at 1 h but not at 6 h after the stimulation.

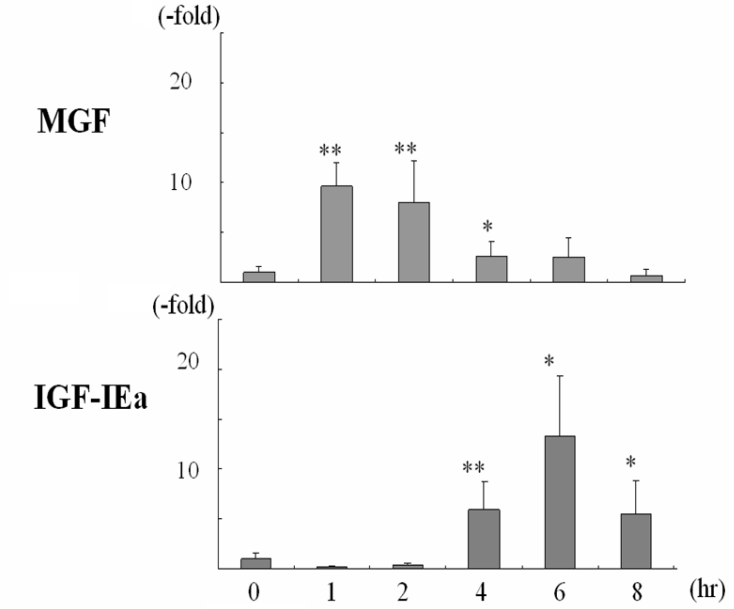

Figure 2. GH increased the expression of *MGF* and *IGF-IEa* in C2C12 cells. The mRNA levels of *MGF* (upper panel) and *IGF-IEa* (lower panel) was increased and the peak levels were reached at 1 h and 6 h, respectively, following stimulation by 100 ng/ml of GH. *$p < 0.05$, **$p < 0.01$ vs. time point 0 ($n = 5$ in each group).

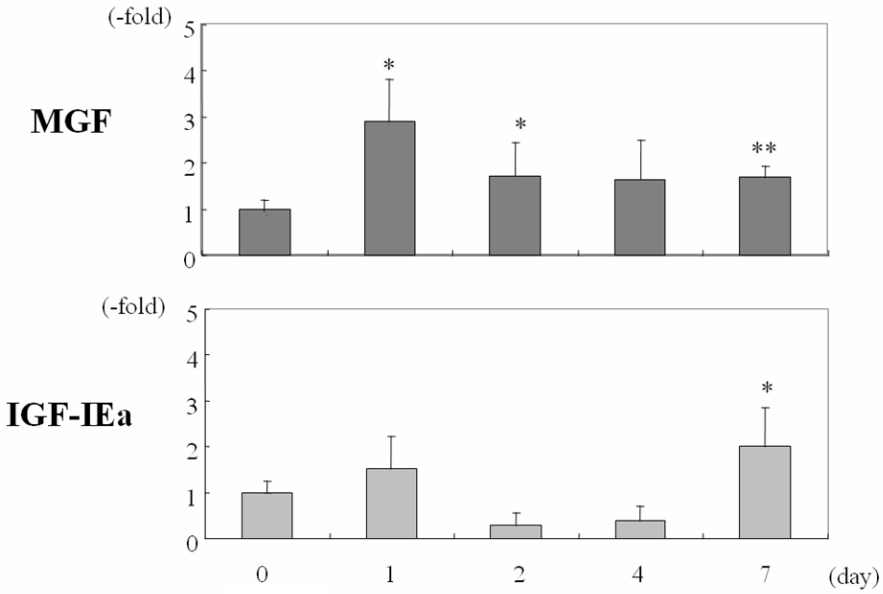

Figure 3. Chemical damage on the C2C12 cells induced the expressions of *MGF* (upper panel) and *IGF-IEa* (lower panel) on days 1 and 7, respectively, after treatment with bupivacaine. *p < 0.05, **p < 0.01 vs. time point 0 (n = 5 in each group).

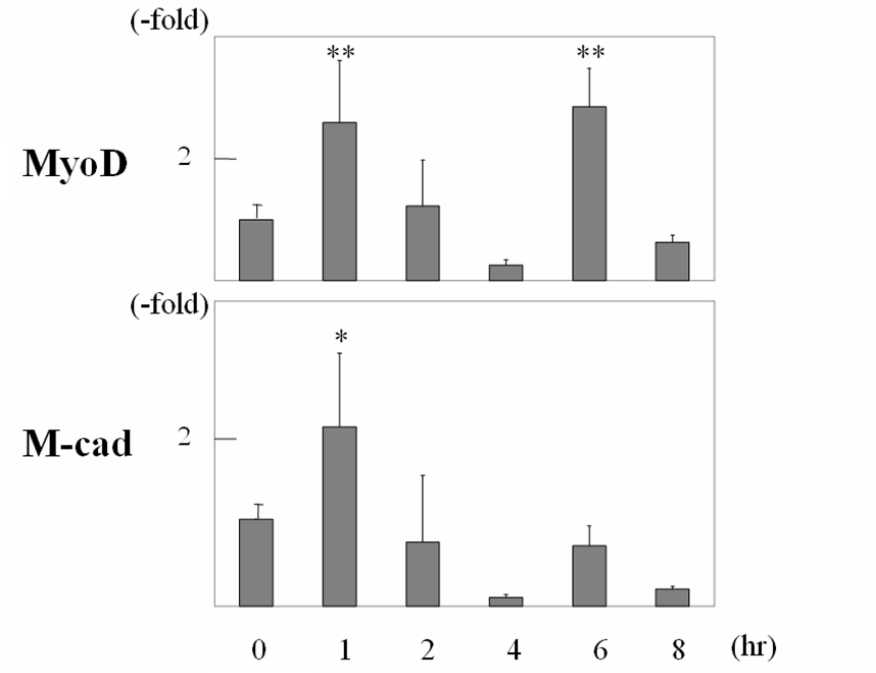

Figure 4. GH induced the bimodal expression of *MyoD* (upper panel) 1 h and 6 h after the stimulation by GH. On the other hand, GH increased *M-cadherin* expression 1 h but not 6 h after the stimulation by GH (lower panel). *p < 0.05, **p < 0.01 vs. time point 0 (n = 5 in each group).

DISCUSSION

The main finding of the present study is that in C2C12 cells, the expression of both *MGF* and *IGF-IEa* was directly but differentially regulated by GH. Furthermore, GH probably activated the muscle satellite cells via the production of MGF and IGF-IEa. We believe that this is the first report in terms of the direct effect of GH on the expression of *IGF-I* isoforms and on the elevation of markers for the activation of satellite cells. Since GH preferentially increased MGF prior to IGF-IEa production, our results suggest that GH may have a potential to regenerate muscle cells more effectively than IGF-I alone.

Several lines of evidence indicate that *MGF* is regulated by mechanical overload or chemical damage to the muscle tissue. *In vivo*, *MGF* and *IGF-IEa* were maximally expressed as early as 1 day and 7 days following stretch stimulation in the rat anterior tibialis muscle, respectively (12). On the other hand, *MGF* and *IGF-IEa* were maximally expressed 4 days and 11 days following the chemical damage by the bupivacaine injection, respectively, in the same animal models (12). In the C2C12 cells *in vitro*, *MGF* was upregulated by a single ramp stretch and by cycling loading whereas *IGF-IEa* was increased by a single ramp stretch but reduced by repeated cyclic stretch (3). Thus, previous reports regarding the regulation of *MGF* mRNA expression in skeletal muscles have emphasized the role of mechanical signals or muscle injury. On the other hand, we previously reported that GH preferentially induced expression of *MGF* prior to that of *IGF-IEa in vivo* in GH-deficient mice (13). However, since the acute injection of GH *in vivo* had an effect on several parameters such as serum glucose or insulin levels and freely moving animal models were used, it remains unclear whether the increased *MGF* in GH-deficient mice was achieved due to the direct effect of GH or via other factors including exercise *per se*. In this study, to investigate whether or not GH directly regulates *MGF* expression and production, we used an *in vitro* approach using C2C12 cells subjected to addition of GH in the culture medium. As a result, we demonstrated that GH directly regulated both *MGF* and *IGF-IEa* mRNA expressions. More importantly, *MGF* expression increased more rapidly than that of *IGF-IEa* by GH stimulation; that was consistent with the *in vivo* result we previously described in GH-deficient mice (13). This result suggests that GH not only upregulated the primary transcript of the *IGF-I* gene but also affected the splicing toward the preferential induction of *MGF*. We next investigated the effect of myotoxicity by bupivacaine in the same cell line to address whether or not the preferential expression of *MGF* in C2C12 cells was specifically due to the stimulation of GH. In this experiment, *MGF* mRNA was preferentially expressed followed by the increased expression of *IGF-IEa* although both the expressions stimulated by bupivacaine were slower than those stimulated by GH. Our results are consistent with the *in vivo* result described by Hill et al. (12). Taken together, it appears that the expression pattern for each *IGF-I* splice variant is considerably similar in both myotoxin-induced chemical damage and GH-stimulated cell models, with MGF peaking before IGF-IEa.

Another relevant finding in the present study is that GH induced the bimodal increase of the *MyoD* and an early induction of *M-cadherin* expression. We selected MyoD and M-cadherin in the present study as markers for proliferating myoblasts since previous work demonstrated that proliferating myoblasts derived from satellite cells express myoblast markers such as MyoD and adhesion molecules such as M-cadherin, but not terminal differentiation markers such as myogenin or myosin heavy chains (17, 21). Our results demonstrate the early induction of *M-cadherin* as well as *MyoD* in parallel with *MGF* expression after the addition of GH, consistent with a notion that GH plays a role in the activation of the satellite cells via MGF production. The further induction of *MyoD* was probably caused via IGF-IEa production. It remains unclear why no further induction of

M-cadherin by GH was observed; however, this suggests that IGF-IEa possesses a relatively weak potential in terms of satellite cell proliferation as compared with MGF (31). Liu et al. (16) reported that the expression of *myostatin*, negative regulator of muscle regeneration, in C2C12 cells was reduced by GH administration and increased by the administration of a GH receptor antagonist. This finding supports the role of GH on muscle cell regeneration. There are several lines of evidence indicating that IGF-I can potentially maintain the skeletal muscle volume and prevent cell death in skeletal muscles (1, 22). Coleman et al. (6) reported that myogenic vector expression of *IGF-I* stimulated muscle cell differentiation and myofiber hypertrophy. Musaro et al. (19) demonstrated that localized *IGF-I* transgene expression in muscle induced local muscle hypertrophy and prevented the age-related decline in muscle mass. However, these experiments did not distinguish the role of each IGF-I isoform. On the other hand, transgenic mice with skeletal muscle-specific overexpression of *IGF-IEa* did not increase myoblast proliferation (26). Mice with muscle-specific inactivation of the *IGF-I receptor* exhibited marked muscle hypoplasia from birth to 3 weeks of age, but the mice exhibited hyperplasia in skeletal muscle from 3 weeks to adulthood (8). These results suggest that other factors independent of IGF-I receptor signaling such as MGF may play a relevant role in the early events of tissue repair in skeletal muscle. Repair and regeneration of muscle tissue require 2 distinct processes; one is the replication of the mononucleated myoblasts and the other is the fusion of myoblasts with muscle fibers. MGF has a potential of inducing myoblast proliferation but prevents their differentiation (32), which would be favorable as it facilitates conservation of the satellite cell pools. Thus, IGF-IEa in combination with MGF could repair the muscle tissue more efficiently than IGF-IEa alone. Aging in humans is characterized by a loss of muscle strength and mass, and also by a gradual decline in circulating GH (23). As discussed by Hameed et al. (10), GH may be crucial to facilitate the increase of MGF in combination with exercise. It is possible that sarcopenia in elderly may be linked to MGF impairment as well as reduced serum GH levels (2).

In conclusion, we demonstrated that GH directly and preferentially induces *MGF* followed by *IGF-IEa* expression, which may lead to the activation of muscle satellite cells.

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