Hepatocyte growth factor affects satellite cell activation and differentiation in regenerating skeletal muscle

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Hepatocyte growth factor (HGF) is the only known growth factor that activates quiescent satellite cells in skeletal muscle. We hypothesized that local delivery of HGF may enhance regeneration after trauma by increasing the number of myoblasts available for restoring normal tissue architecture. Injection of HGF into muscle at the time of injury increases myoblast number but does not enhance tissue repair as determined using quantitative histological analyses. Rather, depending on the dose and the timing of HGF administration relative to the injury, regeneration can be inhibited. The greatest inhibitory effect is observed when HGF is administered on the day of injury and continued for 3 days, corresponding to the time when satellite cell activation, proliferation, and early differentiation normally occur. To establish a mechanism for this inhibition, we show that HGF can act directly on primary muscle cells to block differentiation. These results demonstrate that 1) exogenous HGF synergizes with factors in damaged muscle to increase myoblast number, 2) regeneration is not regulated solely by myoblast number, and 3) HGF inhibits muscle differentiation in vitro and in vivo.

Various growth factors are thought to play a role in different stages of muscle regeneration (3, 19). Of these, the most extensively studied in vitro are insulin-like growth factor I (IGF-I) and IGF-II, basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), interleukin-6 (IL-6), and leukemia inhibitory factor (LIF). Such factors differ in their effects on myogenesis: IL-6 and LIF only stimulate myoblast proliferation (7), whereas bFGF stimulates myoblast proliferation but also inhibits muscle differentiation (21). IGF-I stimulates both proliferation and differentiation (18), whereas TGF-β inhibits proliferation (2). On the basis of these observations, the factors that regulate muscle regeneration in vivo must act to maintain a balance between growth and differentiation in order for restoration of normal tissue architecture to occur.

Bischoff (11) first demonstrated the presence of satellite cell mitogens in extracts of isolated muscles lightly crushed in vitro. Mitogens such as bFGF, platelet-derived growth factor-BB, and transferin were later identified in such extracts (15). The factor in these extracts responsible for the activation of quiescent satellite cells was recently identified as hepatocyte growth factor (HGF) on the basis of immunoneutralization experiments (34). HGF, a ligand for the c-met receptor, is a factor that elicits mitogenic, motogenic, and morphogenic activities during development and tissue regeneration (36). HGF has emerged as an important candidate molecule in muscle regeneration for several reasons. 1) The c-met receptor is present on quiescent satellite cells in normal muscle tissue (17, 34) and precedes expression of other myogenic genes (17). Addition of HGF to either cultured satellite cells or normal muscle promotes entry of quiescent satellite cells into the cell cycle (4, 34). 2) HGF is expressed not only in regenerating muscle but also in normal muscle tissue, and it can be released upon injury (34). 3) HGF increases migration of muscle cells in vitro (9). Together, these studies imply that HGF plays a prominent role in regulating the early phases of muscle regeneration.

Purified growth factors such as bFGF and LIF have been delivered locally to injured muscle to increase muscle repair, with limited success (8, 28). In light of the association of HGF with activation and migration of myogenic precursor cells, we hypothesized that local delivery of HGF would augment satellite cell activation in regenerating muscle and that this increased number...
of myogenic precursor cells would lead to an enhancement of muscle repair. We used a local muscle freeze injury model in mice to test the effects of HGF on muscle regeneration. Our results demonstrate that exogenous HGF can increase the number of myoblasts present in regenerating muscle. This increased number of myoblasts does not lead to an enhancement of muscle regeneration. Instead, HGF administration for 4 days during the early stages of regeneration when primarily satellite cell activation, proliferation, and early fusion occur leads to an inhibition of muscle regeneration. Administration of HGF at later stages of regeneration when primarily myofiber growth occurs leads to little or no inhibition of regeneration. The inhibition of differentiation observed at early phases is reversible upon cessation of HGF treatment. These data provide in vivo evidence that HGF plays dual roles in regulating satellite cell activation and differentiation in regenerating muscle.

**MATERIALS AND METHODS**

Animals and reagents. Sixty adult C57BL/6 male mice (4–6 wk old) were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed in virus- and pathogen-free conditions. All animals were handled in accordance with the guidelines of the Administrative Panel on Laboratory Animal Care of Emory University.

Human recombinant HGF was purchased from R&D Systems (Minneapolis, MN). Mouse ascites, normal goat serum, and calf skin collagen were purchased from Sigma (St. Louis, MO). The antibody against MyoD was from Vector Laboratories (Burlingame, CA), and the antibody against embryonic myosin heavy chain (EMHC) was from the Developmental Studies Hybridoma Bank (Iowa City, IA) and was contributed by Dr. Helen Blau. Secondary antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA). bFGF was purchased from Promega (Madison, WI). The enhanced chemiluminescence kit was purchased from Amersham (Arlington Heights, IL). All tissue culture reagents were from Gibco BRL (Gaithersburg, MD), unless noted. Protease inhibitors were obtained from Boehringer Mannheim (Indianapolis, IN). Immobilon membrane was purchased from Millipore (Bedford, MA).

Induced regeneration of skeletal muscle. Tibialis anterior (TA) muscles were subject to a standardized local freeze damage as described (29). Mice were anesthetized with an intraperitoneal injection of 87 mg/kg ketamine and 13 mg/kg xylazine, and muscle damage was induced by direct application to the muscle for 5 s of a 4-mm metal probe precooled in dry ice. The damaged muscles were injected daily along the longitudinal axis of the muscle with either HGF or vehicle (PBS containing 0.1% BSA) in a total volume of 15 µl using a Hamilton syringe and a 27-gauge needle. Animals were killed using CO₂ inhalation at different times after damage, and the muscles were removed and used in subsequent assays as described below.

Analyses of myoblast populations after damage. The number of myoblasts in regenerating muscles in the presence or absence of HGF was compared using MyoD as a marker as described (29), with slight modifications. Both TA muscles were injected and then injected intramuscularly with either vehicle or HGF at two different doses (6.25 or 50 ng), starting on the day of injury and continuing daily. These doses of HGF were determined empirically. The tissues were collected 1, 2, and 3 days later. The muscles were enzymatically dissociated, and the liberated cells were allowed to attach to culture dishes. The entire cell suspension generated from each muscle was plated on two collagen-coated 35-mm dishes in growth medium [GM; Ham's F-10, 20% FBS, and 5 ng/ml bFGF] in a humidified 5% CO₂ atmosphere at 37°C (30). After 2 days in culture, before the initiation of proliferation, the cells were fixed and processed for immunohistochemistry.

To identify MyoD-positive cells, cultures were fixed in formaldehyde and incubated with blocking buffer containing 5% goat serum, 0.5% BSA, and 0.25% Triton-X 100 in PBS, followed by a mouse monoclonal antibody against MyoD used at a dilution of 1:20. MyoD expression was detected with a 1:200 dilution of Texas red-conjugated goat anti-mouse IgG. Cells in 9–15 random fields were analyzed using an Axiovert Zeiss microscope (Carl Zeiss, Thornwood, NY). Controls included Texas red-conjugated secondary antibody alone and mouse ascites. No staining was observed in any of these control situations. One-way ANOVA with posttesting by the Bonferroni method was used to evaluate the significance of differences in the mean numbers of cells between control and HGF-treated muscles.

These analyses provide an estimate of the number of myoblasts present in the regenerating muscles of control and HGF-treated animals. This type of analysis was not intended to provide a quantitative measure of myoblast number after injury, but rather a relative comparison between untreated and treated muscles. Previous studies indicate correlation between this assay and direct analyses of MyoD-positive cells in tissue sections (29).

Histological analyses. The isolated TA muscles were embedded in OCT mounting medium and frozen in isopentane cooled in liquid nitrogen. For histological analyses, 10–12 14-µm cross sections were collected along the entire length of the muscles at 400- to 500-µm intervals and stained with hematoxylin and eosin. For quantitation, the section that contained the largest lesion area was selected. All analyses and photography were performed on an Axiovert microscope equipped with a video camera and Scion Image software. Sections were analyzed by two independent observers. The core of the lesion, as defined by the region that was least regenerated, was visualized using a ×10 objective, and the image was captured to a computer screen. All the centrally nucleated fibers within this 307,000-µm² field were counted. Fibers smaller than 100 µm² were not included in the analysis, so as to unambiguously identify the regenerated fibers. The area of individual regenerated fibers was also measured. Depending on the particular experiment, either unpaired t-tests or one-way ANOVA with posttesting by the method of Dunnett was used to evaluate the significance of differences in the mean number of fibers among various treatment protocols. Unpaired t-tests were used to evaluate the significance of differences in the mean fiber area between treatment protocols.

Cell culture and in vitro differentiation assays. Primary mouse myoblasts were isolated 2 days after induced muscle damage in adult mice and expanded to >99% purity by culture. To induce differentiation, confluent cell cultures were switched to fusion medium (FM; DMEM with 2 or 5% horse serum) containing various doses of HGF. The FM was replaced daily. Cells were lysed after 36–48 h in ice-cold RIPA-2 (50 mM Tris, pH 8.0, 150 mM NaCl, 1% N-P-40, 0.5% deoxycholate, and 0.1% SDS) containing protease inhibitors (Complete Mini, Boehringer Mannheim), and the lysates were processed for immunoblotting. Equal amounts of protein (5 µg) (13) were separated on 7.5% SDS-polyacrylamide gels and transferred to an Immobilon membrane. After blocking of
nonspecific protein binding with 5% nonfat dry milk, the membranes were incubated overnight at 4°C with a mouse monoclonal antibody against EMHC (16). The membranes were washed and further incubated with a peroxidase-conjugated anti-mouse IgG. Immunoreactivity was detected using enhanced luminescence. Immunoblots were subsequently stained with Coomassie blue to ascertain equal loading of proteins among various samples.

RESULTS

Dose-dependent effect of HGF on the number of myoblasts in regenerating muscles. To determine whether exogenous HGF can increase the number of myoblasts in regenerating muscles, we performed single-cell analyses of control and HGF-treated muscles to compare the numbers of myoblasts. Two different doses of HGF, 6.25 and 50 ng, were used in these studies. Treatment with 6.25 ng HGF does not significantly increase the number of myoblasts compared with control at any time tested (Fig. 1A). In contrast, muscles treated with 50 ng HGF on the day of injury and analyzed 1 day later (Fig. 1B) yield about threefold more MyoD-positive cells than controls. In muscles further treated with HGF on subsequent days and analyzed either 2 or 3 days after injury, no significant increase occurs in the number of myoblasts. Thus exogenous HGF can synergize in a dose-dependent manner with factors in damaged muscle to increase the number of myoblasts.

An increase in the number of myoblasts does not lead to enhanced muscle regeneration. To study the effects of HGF on muscle regeneration, it was first necessary to ascertain that any differences in regeneration would be due to HGF treatment and not to variability in the injury protocol. To determine the reproducibility of the local injury model in quantitative histological analyses, we injured both TA muscles of individual animals and collected the muscles 7 days later. The number and the area of centrally nucleated fibers within the core of the injury were quantitated as described in MATERIALS AND METHODS and compared between the left and right TA muscles. No significant difference exists in either the number (Fig. 2A) or the area (Fig. 2B) of fibers. Data are means ± SD for fiber number and means ± SE for fiber area from 5 animals.

To determine whether the increased number of myoblasts as a result of HGF treatment leads to enhanced repair of injured muscle, 50 ng HGF or vehicle was injected into the TA muscles on the day of injury, and the muscles were collected 7 days later. The number (Fig. 3A) and the area (Fig. 3B) of the regenerated fibers within the core of the lesion were determined for each treatment group. No significant difference exists between control and HGF treatment in either of these indexes of regeneration. These results demonstrate that although one injection of HGF can increase the number of myoblasts in regenerating muscles, no enhancement of the repair process occurs.

Multiple injections of HGF inhibit regeneration. We hypothesized that multiple injections of HGF during the early phases of regeneration may be required to observe an enhancement in the restoration of normal tissue architecture after injury. Therefore, either 50 ng HGF or vehicle was injected into the TA muscles on the day of injury and again once daily through day 3. The muscles were collected 7 days later. The HGF-treated muscles are notable for the large areas of the lesions...
that are devoid of regenerated myofibers (Fig. 4, inset). Both the number (Fig. 4A) and the area (Fig. 4C) of the regenerated fibers in the core of the injury are significantly decreased with HGF treatment. Approximately twofold fewer fibers and twofold smaller fibers are present in muscles treated with HGF compared with vehicle. A 2.1-fold difference in the proportion of the lesion that contains nonregenerated areas is observed in the HGF treatment group compared with controls (Fig. 4E).

To determine whether the inhibition of regeneration is dose dependent, 6.25 ng HGF or vehicle was injected into TA muscles using the same protocol as for the 50-ng dose in the previous set of experiments. A different pattern of inhibition is observed with the lower dose of HGF. Virtually the entire area of the lesion is filled with regenerated myofibers (Fig. 4, inset; compare middle and bottom); hence, there is no difference in the number of fibers within the core of the lesion (Fig. 4B). The 6.25-ng dose of HGF is not without an effect on regeneration, however. The area of the regenerated fibers in these muscles is significantly smaller than in vehicle-treated ones (Fig. 4D). Differences between the two doses are noted in the degree to which fiber area is inhibited, with the higher dose leading to a greater inhibition (compare Fig. 4, C and D). Thus multiple injections of HGF at early times after injury do not enhance regeneration but rather lead to inhibition of regeneration. This inhibition is dose dependent.

Inhibition of regeneration is dependent on the timing of HGF treatment. We hypothesized that the timing of HGF administration relative to the injury may be a key factor in the inhibitory effect of HGF. Perhaps the addition of exogenous HGF during the early stages of regeneration (characterized by satellite cell activation and proliferation), middle stages (characterized by satellite cell differentiation and fusion), or late stages (characterized by growth of myofibers) results in different effects on tissue architecture. The muscles were injured on day 0, injected either with vehicle or 50 ng HGF as indicated (Fig. 5), and then collected for analysis on day 7. The number of regenerated myofi-

![Fig. 4](http://ajpcell.physiology.org/)

**Fig. 4.** Muscle regeneration is inhibited with local HGF treatment in a dose-dependent manner. TA muscles were injected daily with either vehicle or HGF (50 or 6.25 ng), starting on day of injury and continuing once daily through day 3. Seven days after injury, muscles were collected and analyzed for number and area of regenerated myofibers in central region of lesion. The 50-ng dose of HGF leads to a significant decrease in number (A) and area (C) of regenerated myofibers. A larger proportion of lesion is occupied by nonregenerated tissue in 50 ng HGF-treated animals than in controls (E). A 6.25-ng dose of HGF does not affect number (B) of regenerated myofibers but does significantly decrease area (D) of these myofibers. For myofiber density, data are means ± SD; n = 4 or 5 for vehicle, n = 5 for 6.25 ng HGF, and n = 4 for 50 ng HGF. For fiber area, data are means ± SE; n = 5. *P < 0.05 vs. control.

Inset: representative hematoxylin and eosin-stained muscle sections from muscles treated with vehicle (top), 50 ng HGF (middle), or 6.25 ng HGF (bottom). Large areas of lesion are devoid of regenerated fibers in 50-ng treatment group. Bar, 40 µm.
HGF inhibits muscle regeneration

Effect of HGF on muscle regeneration in vivo. HGF could be inhibiting muscle regeneration either by acting directly on muscle cells (20) or by affecting other cell types necessary for optimal regeneration to occur. To determine whether HGF exerts a direct effect on differentiation of primary mouse muscle cells, differentiation assays were performed in vitro. High-density myoblasts were treated with different concentrations of HGF in fusion medium, and the cells subsequently were collected for immunoblotting. Immunoblots of HGF-treated cells were analyzed for EMHC expression, a marker of myotubes in vitro (33). HGF exerts a dose-dependent decrease in EMHC levels in these cultures (Fig. 7). Thus inhibition of muscle regeneration in vivo is likely to result, in part, from a direct action of HGF on muscle cells.

Reversible inhibition. To determine whether the inhibitory effect of HGF on regeneration is reversible, 50 ng HGF or vehicle was injected into TA muscles, starting on the day of injury and continuing once daily through day 3. The muscles were collected 10 days after injury. At this time point, the core of the lesion in HGF-treated muscles is no longer characterized by large areas devoid of regenerated fibers (Fig. 6B). The area of the regenerated fibers in both control and HGF-treated muscles is greater than that observed for each at day 7 (Fig. 4C), but the area of the fibers at day 10 in the HGF-treated muscles is still significantly smaller than that of the controls (Fig. 6C). Thus, once HGF administration is stopped for long enough, the muscle does recover and is able to continue the regenerative process. Because regeneration was greatly inhibited, the HGF-treated muscles lag behind in terms of fiber area at a time later than those analyzed in these experiments.

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Among growth factors studied to date, HGF has the unique ability to activate quiescent satellite cells, which led us to propose that local delivery of HGF to regenerating muscles would augment satellite cell activation and subsequently lead to a stimulation of muscle repair after trauma. In this study, we demonstrate that local delivery of HGF to muscle on the day of injury increases the number of myoblasts 3.2-fold in these tissues when assayed 1 day later. Despite this increase in the number of myoblasts, restoration of normal tissue architecture is not enhanced. Additional HGF treatments do not further increase the number of myoblasts but rather, depending on the time of administration, can inhibit regeneration. If HGF is given on the day of injury and continued for 3 additional days during which satellite cell activation, proliferation, and fusion ordinarily occur, a marked inhibition in the number and size of regenerated myofibers is observed. The inhibition of these indexes of regeneration is reversed upon cessation of HGF administration. On day 10 after injury, HGF-treated muscles no longer contain large areas devoid of muscle fibers. The fibers are larger than those on day 7 but are still smaller than controls.

Several possibilities can account for the observation that administration of HGF on days 1 and 2 after injury does not further increase the number of myoblasts. First, c-met levels may be downregulated later in regeneration so that the myoblasts are less responsive to the exogenous HGF. Indeed, the relative levels of the c-met epitope do decrease as regeneration progresses, as assessed using immunohistochemistry (34). The second possibility is that HGF levels in muscle tissue reach saturating levels after several days of regeneration. Both normal and regenerating muscles are known to express HGF (34). The last possibility is that HGF acts only as an activation factor for quiescent satellite cells in vivo and not as a growth factor after the cells have entered the cell cycle. Activation is defined as quiescent satellite cells entering the G1 phase of the cell cycle. Activation and proliferation of satellite cells are apparently separate but sequential events. Freshly isolated quiescent satellite cells differ from proliferating satellite cells in protein expression and in the lack of responsiveness to numerous growth factors (5). HGF activates quiescent satellite cells both in vitro (4, 20) and in normal muscle in vivo (34). Perhaps factors found in culture media allow a growth-promoting activity for HGF in vitro (4, 20) but not in vivo.

Although the number of myoblasts is increased with HGF administration at the time of injury, restoration of normal tissue architecture as assayed 7 days later is not enhanced. Manipulation of myoblast number in regenerating tissues by implantation of exogenous myo-

**Fig. 6.** Inhibition of muscle regeneration due to HGF administration at early time points after injury can be overcome. TA muscles were injected with either vehicle or 50 ng HGF, starting on day of injury (day 0) and continuing daily through day 3. Muscles were collected 10 days after injury. Representative hematoxylin and eosin-stained muscle sections are shown from core of lesion in muscles treated with vehicle (A) and 50 ng HGF (B). Unregenerated areas are not present in HGF-treated muscles at this time point. C: fibers of HGF-treated muscles are still significantly smaller in area than those of controls. Data are means ± SE for n = 5. *P < 0.05 vs. control.

**Fig. 7.** HGF can inhibit muscle differentiation by a direct action on muscle cells. High-density primary mouse myoblasts were treated with either vehicle or different doses of HGF for 36–48 h in fusion media. Top: representative immunoblot of electrophoretically separated proteins. HGF treatment results in a dose-dependent inhibition of embryonic myosin heavy chain protein. Bottom: Coomassie blue-stained 45-kDa proteins showing equal transfer of proteins on blot.
blasts can have a salutary effect on the repair process (1, 6, 25). In a model in which regeneration of minced muscle fragments was assessed by determination of the muscle-specific form of creatine kinase, addition of exogenous myogenic cells resulted in a threefold enhancement of creatine kinase activity (12). Our studies indicate that simply increasing the number of the animal’s own myoblasts does not lead to an increase in the number or size of regenerating fibers. Perhaps further stimulation of myoblast numbers beyond those reported in our study must be obtained in injured muscles in order to have a measurable effect on the restoration of normal tissue architecture.

The fact that HGF augments satellite cell activation after injury contrasts with its ability to inhibit the repair process. Such inhibition was not observed by others using local delivery of other growth factors, such as bFGF and LIF, in vivo. Using a crush injury model, Mitchell et al. (28) demonstrated no measurable effect of bFGF on muscle regeneration: the proportions of myotubes, connective tissue, and necrotic muscle were not statistically different in treated muscles. In contrast, Barnard et al. (8) showed that local delivery of LIF to crush-injured muscle led to an increase in fiber size but not an increase in total fiber number in the area of injury. Thus the ability to inhibit muscle regeneration is specific to HGF and not due to the administration of growth factors into injured muscle per se.

The timing of HGF treatment relative to the injury can modify the degree and the manifestation of its inhibitory action (fiber number or area) on myogenesis. Several models exist to explain the dependence of this inhibition on the timing of HGF administration relative to the injury. 1) As discussed earlier, c-met levels in muscle cells may be downregulated with time after injury. The lack of effect with treatment during days 4–6 after injury could stem from such a decrease in receptor levels. However, the marked difference between treatments occurring during days 0–2 and 0–3 cannot be explained by this model. 2) HGF may block muscle differentiation directly. We show using cultured primary mouse muscle cells that HGF can act directly on myoblasts to inhibit their differentiation, confirming the previous in vitro work of Gal-Levi et al. (20) with chicken satellite cells. This model could explain the difference in the effect of HGF treatment during days 0–2 compared with days 0–3. Because fiber number and size are not affected in the day 0–2 treatment but are significantly decreased in the day 0–3 treatment, days 2–3 must be a critical period in regeneration. Indeed, the period between day 2 and day 3 in vivo is normally a time when a great deal of myoblast fusion occurs, as evidenced by the large decrease in the number of myoblasts obtained from injured muscle on day 3 relative to day 2 (29). However, this model is hard to reconcile with the fact that HGF treatment during days 2–4 does not affect fiber number but only fiber area. Perhaps some differentiation occurs before day 2 that establishes the number of fibers to be formed, but HGF treatment inhibits subsequent fiber growth. 3) HGF may affect nonmuscle cell types found within regenerating muscle tissue. Infiltration of immune cells into damaged muscle is required for regeneration (24), and perhaps HGF alters this process. Because no one model is sufficient to explain the observed effects of HGF on regeneration, most likely a combination of mechanisms is involved.

The inhibition of regeneration in vivo is reversed when HGF administration is stopped, which demonstrates the pleiotropic role that HGF probably plays in the early stages of muscle regeneration. HGF is expressed by satellite cells in regenerating muscles (26, 34). At initial stages after injury, HGF serves to activate quiescent satellite cells. By blocking the differentiation of these activated satellite cells until there are sufficient cell numbers, HGF could serve to increase the pool of proliferating myoblasts to some optimal cell density, whereupon fusion could commence. Such a model would predict that the levels of HGF should decrease within regenerating muscles. By immunohistochemistry, the intensity of HGF staining does decrease with time after injury (34), but quantitative biochemical measurements have not been made to date.

In summary, this study demonstrates the effects of exogenous HGF administration on satellite cell activation and differentiation in regenerating mouse muscles after trauma. The timing of HGF administration relative to the ongoing cellular events in myogenesis during the repair process is critical for its effects on regeneration. We conclude that HGF levels in regenerating muscle are regulated so as to maintain a balance between activation of satellite cells and their subsequent fusion and differentiation in order to restore normal tissue architecture.


