Granulocyte-colony stimulating factor enhances muscle proliferation and strength following skeletal muscle injury in rats

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Submitted 15 January 2007; accepted in final form 10 August 2007

Stratos I, Rotter R, Eipel C, Mittlmeier T, Vollmar B. Granulocyte-colony stimulating factor enhances muscle proliferation and strength following skeletal muscle injury in rats. J Appl Physiol 103: 1857–1863, 2007.—Insufficiency of skeletal muscle regeneration often impedes the healing process with functional deficiencies and scar formation. We tested the hematopoietic growth factor granulocyte-colony stimulating factor (G-CSF) with respect to its efficacy to improve functional muscle regeneration following skeletal muscle injury in Wistar rats. After crush injury to the left soleus muscle, animals received daily G-CSF (20 μg/kg ip) or vehicle solution (n = 30 per group each). Sham-operated animals without muscle injury served as controls (n = 15). After in vivo assessment of the fast-twitch and tetanic contraction capacity of the soleus muscles at days 4, 7, and 14 post-injury, sampling of muscle tissue served for analysis of satellite cell proliferation [bromodeoxyuridine (BrdU)/laminin and BrdU/desmin double immunohistochemistry] and cell apoptosis (transferase nick-end labeling analysis). Muscle strength analysis revealed recovery of contraction forces to 26 ± 2, 35 ± 3, and 53 ± 3% (twitch force) and to 20 ± 2, 24 ± 2, and 37 ± 2% (tetanic force) within the 14-day observation period in vehicle-treated animals. In contrast, G-CSF increased contractile forces with markedly higher values at day 7 (twitch force: 42 ± 2%; tetanic force: 34 ± 2%) and day 14 (twitch force: 62 ± 3%; tetanic force: 43 ± 3%). This enhancement of muscle function was preceded by a significant increase of satellite cell proliferation (BrdU-positive cells/mm²: 27 ± 6 vs. vehicle: 12 ± 3) and a moderate decrease of cell apoptosis (transferase nick-end labeling-positive cells/mm²: 11 ± 2 vs. vehicle: 16 ± 3) at day 4. In conclusion, G-CSF histologically promoted viability and proliferation of muscle cells and functionally enhanced recovery of muscle strength. Thus G-CSF might represent a therapeutic option to optimize the posttraumatic course of muscle tissue healing.

apoptosis; crush injury; muscular biomechanics; bromodeoxyuridine; transferase nick-end labeling

MUSCLE INJURY STATES A disabling condition in sports medicine and a challenging problem in current traumatology. Patients having experienced a heavy soft tissue trauma are frequently forced to deal with life-long pain or physical disability. Contusion injuries occur, when a muscle is subject to a sudden, heavy compressive force, such as a direct blow to the muscle (21). Thereby the healing process of the muscle is very slow or inefficient and often hindered by scar tissue formation. However, even if muscular regeneration is imperfect, the muscles can be used like before the injury, whereas their full functional pretraumatic contractibility cannot be achieved.

According to Huard et al. (18), the healing process of an injured muscle can be divided into three different phases. The first phase, or the necrosis-degeneration and inflammation phase, occurs within the first minutes and continues for up to 1–2 wk post-injury. The second phase, or the regeneration-repair phase, begins in the first week post-injury and peaks at ~14 days. Finally, scar-tissue formation (third phase) takes place during week 2 and increases over time for up to 4 wk post-injury. All procedures are interrelated and time dependent. Furthermore, the time between injury and initiation of the proliferation is affected by several factors, including species, type of injury, and metabolic state of the muscle.

Satellite cells are located beneath the basal lamina that surrounds each myofiber (27) and act as myogenic precursors for repair with stem cell-like characteristics after muscle injury (6). Many growth factors, like insulin-like growth factor-1, hepatocyte growth factor, epidermal growth factor, transforming growth factors, and platelet-derived growth factors as well as cytokines have been identified to cause proliferation of satellite cells with subsequent transformation into myotubes and muscle fibers (5), to regulate myoblast proliferation and differentiation, and to promote muscle regeneration or repair (18). Accordingly, granulocyte-colony stimulating factor (G-CSF) also seems to be attractive with respect to muscle tissue regeneration, since myocardial infarction studies have shown beneficial effects of G-CSF treatment. In detail, G-CSF was found to be responsible for homing of mobilized bone marrow-derived cells to the damaged myocardium and for enhancing the vascularization of the damaged heart (30).

Up to now, however, the influence of G-CSF on peripheral skeletal muscle regeneration has not been investigated. Therefore, the present study evaluates the hypothesis that daily injection of G-CSF improves both the regeneration and functionality of the peripheral skeletal muscle tissue after a blunt injury.

MATERIALS AND METHODS

Animal model. Male Wistar rats [275–325 g body weight (bw); Charles River Laboratories, Sulzfeld, Germany] were used for the experiments and were kept on water and standard laboratory chow ad libitum. The experiments were conducted in accordance with the German legislation on protection of animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council).

Under pentobarbital sodium anesthesia (55 mg/kg bw ip; Narcoren, Merial, Hallbergmoos, Germany), the left lower limb was shaved and disinfected with povidone-iodine. Through a 2-cm posterolateral lon-
gitudinal incision of the skin and the underlying fascia from the lateral gastrocnemius head to the Achilles tendon, the soleus muscle was mobilized, as described previously by Matziolis et al. (26). The blunt injury was induced via an instrumented clamp, which allowed the standardized force application of 25 N during each muscle contusion for 10 s (DMC PLUS, HBM Hottinger Baldwin Messtechnik). The muscle was manually clamped over its complete length, with the exception of the entry point of the supplying neurovascular structures, which arise from the mid part of the medial gastrocnemius (26). The superficial muscle and the skin were closed using 4-0 Vicryl sutures (Ethicon, Norderstedt, Germany). During the procedure, body temperature was maintained at 36–37°C by means of a heating plate. Animals were allowed to awake from anesthesia and remained in single cages with free access to water and laboratory chow until the final experiment. For immunohistochemical analysis of muscle cell proliferation, animals received a single intraperitoneal injection of bromodeoxyuridine (BrdU; 50 mg/kg 48 h before the final experiments at either day 4, 7, or 14 post-injury) similarly as described by others (13).

Muscle strength measurements. For in vivo assessment of muscle strength as described by Matziolis et al. (26), animals were reanesthetized at days 4, 7, and 14 post-injury with pentobarbital sodium (55 mg/kg bw ip) and placed on the heating pad. After bilateral exposure of the sciatic nerve and the soleus muscle, the Achilles tendon was cut and the lower extremity was fixed into the muscle force measuring device (Experimetria, Budapest, Hungary). The distal part of the soleus muscle was connected to the force transducer through a suture (4-0 Vicryl). All muscles that were inserted through the Achilles tendon, except to the soleus muscle, were cut through closest to the Achilles tendon.

The sciatic nerve was subsequently stimulated with 9 mA/75 Hz bipolar five times, 0.1 s (8 periods) with 5-s interval between the pulses (26). After this fast-twitch stimulation protocol, maximal strength was measured by application of 9 mA/75 Hz pulses for five times, 3 s each with 5-s intervals, reaching tetany in all cases (26).

Contraction forces under fast-twitch and tetanic stimulation were analyzed by calculating the mean of the maximal values from the first five consecutive contractions and given as percentage of the corresponding values of the contralateral non-injured muscle. After completion of muscle strength measurements, experiments were terminated by direct puncture of the heart for blood sampling and by excision of the left soleus muscle for subsequent immunohistochemistry.

Histology and immunohistochemistry. At the end of the in vivo experiment, muscle tissue was fixed in 4% phosphate-buffered formalin for 2–3 days, straight embedded in paraffin, and strictly cut in a longitudinal fashion from the proximal to the distal insertion. This procedure guarantees the assessment of non-injured muscle tissue (survival zone), injured muscle tissue (central zone), and muscle tissue next to the injury (“penumbra,” regenerating zone) (Fig. 1). For immunohistochemical demonstration of BrdU, tissue specimens collected on poly-l-lysine-coated glass slides were treated by microwave for antigen unmasking. Monoclonal mouse anti-BrdU (1:50; Dako Cytomation) was used as primary antibody and incubated for 18 h at 4°C. After equilibrating to room temperature, sections were incubated with a horseradish peroxidase-conjugated secondary antibody for 30 min according to the manufacturer’s instructions (Dako Cytomation). 3,3’-Diaminobenzidine was used as chromogen. Sections were counterstained with hemalaun and examined by light microscopy (Axioskop 40, Zeiss, Göttingen, Germany).

Using a ×10 objective (numerical aperture 0.65), BrdU-positive cells were counted within the muscle tissue from the proximal to the distal insertion (~25 consecutive observation fields), comprising both injured and noninjured areas as well as penumbra areas (Fig. 1). BrdU-positive cells were given as cells per mm² muscle tissue, disregarding the heterogeneous morphology of muscle tissue as well as per mm² muscle tissue in the survival zone.

To better discriminate between proliferating satellite cells and other cell types within the regenerating muscle tissue, double immunohistochemistry for either desmin or laminin and BrdU was performed, according to the procedure described above. Herein, a goat polyclonal desmin antibody (1:100; 1-h incubation at 37°C; Santa Cruz Biotechnology, Santa Cruz, CA) and a rabbit polyclonal laminin antibody (1:50; 1-h incubation at 37°C; Dako Cytomation) were used with 3,3’-diaminobenzidine and fuchsin as chromogens. By labeling of laminin, which is present in the basement membrane, confirmation of proliferating satellite cells was able through their localization beneath the laminin-positive basal lamina in the survival zone, as described by Smith et al. in the soleus muscle of rats (39). Desmin, a cytoskeletal class III intermediate filament, is expressed during skeletal muscle development, i.e., myotube formation, and is widely used as marker for distinguishing individual cell types within a tissue (37), such as myoblasts from fibroblasts in the regenerating and central zone of muscle injury. By using this double immunohistochemical approach, satellite cells were given in percentage of a total of 100 BrdU-positive cells both within muscle tissue, disregarding the heterogeneous morphology, as well as within the survival zone of the injured skeletal muscle (~100 oil objective, numerical aperture 1.25).

To determine apoptotic cell death, terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) analysis was performed using a commercially available in situ apoptosis detection kit (ApopTag, S7100, Chemicon, Temecula, CA). Using a ×40 objective, TUNEL-positive cells were counted within the muscle tissue from the proximal to the distal insertion (~25 consecutive observation fields), comprising both injured and noninjured areas, as well as penumbra areas, and are given as cells/mm².

To look in more detail on the effect of G-CSF on inflammation, we identified tissue-infiltrating inflammatory cells (mainly leukocytes but also monocytes/macrophages) by chloroacetate esterase (CAE) staining and subsequent quantitative analysis of the number of CAE-positive cells per mm² observation area using a ×40 objective (14).

In addition, fibrotic tissue transformation on muscle trauma was identified by collagen staining of muscle tissue using Sirius Red. For this purpose, tissue specimens were stained using a 0.1% solution of Sirius Red (Direct Red 80, Aldrich, Toronto, Canada) in Bouin’s solution. Quantitation of Sirius Red staining was performed on tissue sections of all animals within each group. High-density digital images acquired by means of a digital camera (Zeiss Axiocam MRC 5; Göttingen, Germany; ×20 objective, numerical aperture 0.50) were processed using the thresholding function of Photoshop (version 7, Adobe Systems Europe, Uxbridge, UK), as described by others in cardiac muscle tissue (23). Collagen deposition was expressed as a percentage of the total tissue area occupied by Sirius Red-stained muscle tissue.

Fig. 1. Light microscopic images [A: ×10 magnification; B–D: ×40 magnification: hemalaun staining/bromodeoxyuridine (BrdU) negative control] of the soleus muscle at day 7 after crush injury. Please note the differing areas of muscle tissue, comprising both injured (D) and noninjured areas (B) as well as penumbra areas (C).
To elucidate the impact of granulation tissue vs. regenerated muscle tissue on muscle strength, we assessed the area covered by muscle tissue in percent to the entire observation field by planimetric analysis (Photoshop) of hemalan-stained tissue specimen, assuming that muscle tissue serves as “reciprocal value” to granulation tissue. Using a ×4 objective (numerical aperture 0.10), four to five consecutive observation fields were acquired to fully assess the muscle tissue from the proximal to the distal insertion.

**Laboratory analysis.** Systemic blood cell counts were performed using a SYMSMEX analyzer (KX-21; Norderstedt, Germany). In addition, creatine kinase was assessed by means of a commercially available photometric kit (UnCel DxS 800 Synchon R Clinical System, Beckman Coulter, Heidelberg, Germany).

**Experimental groups.** Three experimental groups of animals were included in the present study: group trauma/G-CSF, i.e., animals that underwent crush injury of the left soleus muscle and received a daily injection of G-CSF (20 μg/kg bw ip; Filgrastim, Amgen, Munich, Germany; n = 30); group trauma, i.e., animals that underwent crush injury of the left soleus muscle and received a daily injection of 0.9% saline (0.6 ml/kg bw ip; n = 30). Sham-operated animals without muscle crush injury served as controls and received a daily injection of 0.9% saline (0.6 ml/kg bw ip; n = 15). First application of G-CSF and saline was performed at the time point of muscle crush injury and then repeated in 24-h intervals.

**Statistical analysis.** All data are expressed as means ± SE. Differences between groups and time points were assessed using a two-way ANOVA, followed by the Holm-Sidak test for pairwise comparison. Regression analysis was performed using Pearson’s product-moment correlation. Statistical significance was set at P < 0.05. Statistics were performed using the software package SigmaStat (version 3.0, Jandel, San Rafael, CA).

**RESULTS**

**General observations.** All animals that underwent open crush injury to the left soleus muscle or sham operation awoke from anesthesia without complications. Usage of the left hind limb was found to be only slightly reduced during the first 1–3 days. There were no other signs of discomfort or illness.

**Muscle strength.** Noninjured muscles in sham-operated animals revealed a mean twitch force of 0.67 ± 0.02 N and a mean tetanic force of 1.09 ± 0.04 N. Calculation in percentage of the corresponding values of the contralateral muscle averaged 95–100% at the distinct time points after intervention, indicating constancy of muscle strength (Fig. 2, A and B). In contrast, open crush injury caused a marked loss of contraction forces. At day 4, muscle tissue demonstrated only a recovery of contraction force to ~26% (twitch) and ~20% (tetanic) of the force of the contralateral noninjured muscle (Fig. 2, A and B). At the subsequent observation time points, muscle tissue showed a continuous improvement of strength, however, far from reaching values of non-injured controls. Twitch force amounting to 53%, but in particular tetanic force amounting to 37%, implied the still massive impairment of muscle force at day 14 after open crush injury (Fig. 2, A and B).

Daily application of G-CSF caused a marked improvement of muscle force at day 7 and day 14, as given by an increase of twitch and tetanic force of ~6–10% compared with the vehicle-treated animals (Fig. 2, A and B). At the early observation time point of day 4, both groups with muscle crush injury did not notably differ with respect to muscle strength (Fig. 2, A and B).

Calculation of the twitch-to-tetanic force ratio of the left soleus muscle revealed significantly higher mean values at all time points of observation in the group with crush injury compared with sham-treated controls. G-CSF application in animals with muscular crush injury only modestly reduced the values for twitch-to-tetanic force ratio at days 4 and 7 toward that of control animals (Fig. 3A). Regression analysis showed significant (P < 0.001) linear correlations between fast-twitch and tetanic contraction forces of the left soleus muscle in all groups studied (Fig. 3B). Of interest, the slopes of the correlation straight lines were found to be comparable in the sham-treated controls and the G-CSF-treated animals with muscular crush injury, whereas untreated animals with crush injury revealed a slightly steeper line of best fit, probably indicative for the morphological switch of muscle to a fast-twitching muscle (Fig. 3B).

**Muscle cell proliferation and apoptosis.** Quantitative histochmical analysis of muscle tissue upon open crush injury demonstrates a distinct kinetics of both cell proliferation and cell apoptosis (Figs. 4 and 5). Incorporation of BrdU, serving as an indicator of muscle cell proliferation, was found increased in both groups of animals compared with sham-treated controls (Fig. 4). Notably, however, G-CSF-treated animals revealed significantly higher numbers of BrdU-positive cells within the injured muscle compared with the corresponding
values in vehicle-treated traumatized animals (Fig. 4). Double immunohistochemistry for laminin and BrdU allowed for reliable assessment of satellite cells/myoblasts in the survival zone of the injured skeletal muscle and revealed that, in all three groups studied, ~70% of BrdU-positive cells were beneath the laminin-positive basal lamina. Double staining for BrdU and desmin further demonstrated that almost 90% of the BrdU-positive cells expressed desmin, again without notable differences between the experimental groups. By multiplying the absolute numbers of BrdU-positive cells per mm² with the percentage of satellite cells, as confirmed by double immunohistochemistry, G-CSF-treated animals revealed significantly higher numbers of proliferating satellite cells at day 4 compared with the vehicle-treated animals (Table 1). At later time points, the differences are still evident, however, without reaching statistical significance (Table 1).

Opposite to cell proliferation, apoptotic cell death was found to be lower in injured muscle tissue of G-CSF-treated animals (Fig. 4). Thus specific kinetics of cellular turnover with the final net result of an increase of muscle cells seems to precede muscle strength recovery.

To assess the issue as to whether supplementation of G-CSF accelerates muscle regeneration via induction of inflammatory cell infiltration and subsequent granulation tissue production and scar formation, collagen deposition and inflammatory cell infiltration was determined (Table 2). Although collagen deposition within the muscle tissue studied did not markedly differ among groups, traumatized muscle tissue per se revealed higher numbers of CAE-positive cells compared with sham control tissue. Of interest, G-CSF did not notably increase inflammatory cell infiltration (Table 2). Along with this finding, G-CSF supplementation did not cause an increase in the granulation tissue production but was rather characterized by a higher area covered with muscle tissue (Table 2).

Systemic creatine kinase activity and systemic leukocyte counts. Photometric analysis of creatine kinase revealed lower serum levels between the G-CSF-treated vs. vehicle-treated and sham-operated animals at day 4 (Table 3). At day 4, saline-treated animals with muscle tissue trauma revealed significantly lower numbers of systemic leukocyte counts in contrast to G-CSF-treated animals with trauma and nontraumatized animals (Table 3).

**DISCUSSION**

Our results demonstrate that, after muscle crush injury, daily injection of G-CSF improves muscular regeneration, as indicated by enhanced satellite cell proliferation and slightly reduced cell apoptosis at 4 days after injury. Muscle tissue of...
G-CSF and muscle regeneration

Table 1. Quantitative analysis of proliferating satellite cells in the survival zone of the injured skeletal muscle and in overall muscle tissue, disregarding the heterogeneous morphology

<table>
<thead>
<tr>
<th>Day</th>
<th>Survival Zone</th>
<th>Overall Muscle Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Trauma</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>Trauma</td>
</tr>
<tr>
<td>4</td>
<td>14.1±2.5</td>
<td>37.5±9.9</td>
</tr>
<tr>
<td>7</td>
<td>2.0±0.7</td>
<td>17.6±6.2</td>
</tr>
<tr>
<td>14</td>
<td>2.8±0.2</td>
<td>16.4±3.6</td>
</tr>
<tr>
<td>14</td>
<td>6.1±2.0</td>
<td>10.6±2.4</td>
</tr>
<tr>
<td>14</td>
<td>0.9±0.4</td>
<td>7.8±2.5</td>
</tr>
<tr>
<td>14</td>
<td>1.1±0.5</td>
<td>7.4±0.8</td>
</tr>
</tbody>
</table>

Data are means ± SE. Cells were characterized by either bromodeoxyuridine (BrdU) positivity and their localization beneath the laminin-positive basal lamina (survival zone) or by double-positive staining for BrdU and desmin (overall muscle tissue). Animals underwent a standardized open crush injury to the left soleus muscle and chronic treatment with granulocyte-colony stimulating factor (G-CSF) or vehicle solution for a total of 14 days after injury (n = 10 animals per time point and group). Sham-operated animals without muscle injury served as controls (sham; n = 5 animals per time point). Significant difference via 2-way ANOVA and Holm-Sidak test: *P < 0.05 vs. sham; †P < 0.05 vs. trauma. For satellite cells in the survival zone, 2-way ANOVA revealed an overall significant difference among all three groups studied.

G-CSF-treated animals showed neither increased numbers of inflammatory cells nor higher extent of granulation tissue, thus disproving the idea that acceleration of muscle healing by G-CSF supplementation is simply due to increased inflammation-induced granulation tissue production at the site of injury. In contrast, double immunohistochemistry using specific markers allowing the differentiation of satellite cells/myoblasts from fibroblasts and other cell types within the injured muscle tissue revealed a two-times higher number of proliferating satellite cells. Most supposedly, these cellular events are responsible and preceded the improvement of muscle function with significantly enhanced contraction forces at days 7 and 14 in G-CSF-treated animals.

Methodological considerations. Although phases of the muscle regeneration process are described to be quite comparable among different muscle types and varying causes of injuries, the kinetics and amplitude of each phase might strongly vary in dependency to the experimental model used. Other than usage of myotoxins, which have the advantage of a highly reproducible way of inducing muscle regeneration but lack pathophysiological relevance (5, 24), induction of tissue trauma has been described by clinically more relevant methods, including the employment of a spring hammer (20) as well as the use of a drop-mass technique (7, 25, 38) and use of a controlled impact technique (32, 33). In addition, muscle lacerations (28), compression-induced ischemia followed by reperfusion (1), and direct blunt injuries by usage of clamps (26) are commonly used models to study muscle tissue healing. The present technique comprised of an instrumented clamp further bears the advantage of controlling the injury by quantitative assessment of the applied force. By controlling clamping of the muscle 10 times with 25 N for 10 s, each extent of muscle injury allows only a 50% recovery of muscle force within the 14-day observation period, as was similarly described by others who used a clamp for muscle injury (26). Thus injury severity seems to be more pronounced compared with the muscle laceration model, which is described to show a recovery in fast-twitch and tetanus strength ratio of up to 72% and 80% at postoperative day 14 (28).

The methodology used in the present study focuses on both histological and functional recovery of injured muscle tissue, thereby assessing cellular mechanisms, such as proliferation and apoptosis, by BrdU staining and TUNEL analysis, as well as muscle tissue force by direct strength measurements. BrdU is reported to be a reliable marker to label proliferating satellite cells (35) and other nonmuscle mitotic cells, such as endothelial cells and fibroblasts (13). We further stressed the specification of proliferating satellite cells and confirmed this cell type by either its localization beneath the laminin-positive basal lamina in the survival zone of the muscle or by its expression of desmin in the regenerating and central zones of the injury. Given that muscle response on injury closely relates not only to mitogenic stimuli but also to pro- and anti-apoptotic events, TUNEL analysis was performed to detect apoptotic cell loss (15, 22, 42).

Although important data, such as cell type, integrity, and localization, can be deduced from histomorphology, muscle strength analysis will finally allow appreciating the recovery of in vivo function. In line with others (26, 28), contraction...
capacity most reliably reflects functional regeneration and outcome after skeletal muscle crush injury. The slope of the twitch-to-tetanus correlation allows one to interpret the muscle regeneration with respect to fast motor units (type II fibers) and slow motor units (type I fibers) and shows that the slow soleus muscle slightly shifts on injury-induced regeneration toward the phenotype of a fast muscle (4).

Muscle cell response on injury and effects of G-CSF. The most important cell source for regeneration of skeletal muscle tissue is the classic satellite cell (2, 10), although muscle-derived stem cells located extralaminally within the connective tissue are also reported to give rise to determine myoblasts and to differentiate to myotubes (5, 9, 19). Proliferation of endothelial and interstitial nonendothelial cells, such as fibroblasts, further contributes to the repair process of the injured muscle tissue, comparably as shown for cell proliferation in atrophic muscle tissue due to weightlessness (13). In the case of severe injury, as induced in the present study, the pool of stem and stem-like cells might be utilized due to their employment in tissue restoration. Thus muscle regeneration might benefit from adjunct pharmacological strategies, like application of G-CSF, presumably through mobilization of cells that will subsequently help to regenerate more intensely and rapidly (12, 31) or through extracellular signals (5). In line with this view, G-CSF caused an overall increase of the number of BrdU-positive satellite cells with finally improved muscle contraction capacity compared with vehicle-treated animals with muscle injury. Although we analyzed the response of myocytes and other nonmuscle mitotic cells to the insult, it is not possible to deduce from the present results by which mechanisms G-CSF finally exerts its beneficial effects. Since leukocytes are supposed to play an essential role in the proteolytic modification of damaged tissue and subsequent phagocytosis of debris (11) and the classic action of G-CSF is the modulation of neutrophil function by increasing phagocytosis (8), it is self-evident to assume that the better removal of tissue debris in the injured muscle by G-CSF primed neutrophils may accelerate the regenerative process. In support of this view, mice depleted of neutrophils showed a more deficient regenerative response than control mice in a model of myotoxin-I-induced muscle injury (41). On the contrary, one could speculate that G-CSF induced inflammatory cell infiltration could have caused increase in the granulation tissue production at the site of injury. However, the comprehensive data from quantitative analysis of inflammatory cell infiltration, collagen deposition, and muscle tissue area is more likely indicative of the conclusion that the supplementation of G-CSF stimulates skeletal muscle regeneration.

Beside that, it can be stated that apoptosis plays a major role in regulating myoblast proliferation during muscle regeneration (3). Thus G-CSF might also have exerted protection in the present study via its anti-apoptotic properties, as described for cardiomyocytes and endothelial cells on acute myocardial infarction (16) or adriamycin-induced cardiomyopathy (17) as well as for neurons after stroke (34). Finally, the mobilization of bone marrow-derived cells by G-CSF may contribute to the improvement of muscle healing, since bone marrow-derived cells have been shown to enhance muscle strength following blunt muscle injury (26). However, there is accumulating evidence for the fact that satellite cells rather than circulating cells are the major contributor to muscle regeneration (10, 36), which is further supported by the present results of double immunohistochemistry, confirming the major fraction of BrdU-positive cells as satellite cells. In line with this, it was originally believed for the heart that therapeutic effects following bone marrow transplantation by G-CSF were based on the homing and transdifferentiation of bone marrow cells into cardiomyocytes (29, 40), whereas new data support the notion that the most important mechanism might be via paracrine and/or direct effects of G-CSF (29).

In summary, daily G-CSF application provoked an acceleration and better restoration of skeletal muscle function after severe muscle injury, most probably through an enhancement of satellite cell proliferation and slightly reduced apoptosis in

### Table 2. Collagen deposition, CAE-positive cells, and muscle tissue area in tissue sections of animals

<table>
<thead>
<tr>
<th>Day</th>
<th>Collagen Deposition, %</th>
<th>CAE-Positive Tissue Infiltrating Cells, n/mm²</th>
<th>Muscle Tissue, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Trauma/G-CSF</td>
<td>Sham</td>
</tr>
<tr>
<td>4</td>
<td>4.2±2.3</td>
<td>2.4±0.6</td>
<td>2.2±0.5</td>
</tr>
<tr>
<td>7</td>
<td>3.5±1.2</td>
<td>1.7±0.6</td>
<td>2.6±0.6</td>
</tr>
<tr>
<td>14</td>
<td>4.4±1.3</td>
<td>5.9±1.4</td>
<td>3.5±1.4</td>
</tr>
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</table>

Data are means ± SE. Collagen deposition, chloroacetate esterase (CAE)-positive cells, and muscle tissue area in tissue sections of animals that underwent a standardized open crush injury to the left soleus muscle and chronic treatment with G-CSF or vehicle solution for a total of 14 days after injury (n = 10 animals per time point and group). Sham-operated animals without muscle injury served as controls (sham; n = 5 animals per time point). *Significant difference vs. sham (2-way ANOVA and Holm-Sidak test): P < 0.05.

### Table 3. Systemic creatine kinase activities and white blood cell count in animals

<table>
<thead>
<tr>
<th>Day</th>
<th>Creatine Kinase, U/liter</th>
<th>White Blood Cell Count, ×10⁹/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Trauma/G-CSF</td>
</tr>
<tr>
<td>4</td>
<td>1,467±203</td>
<td>1,209±92</td>
</tr>
<tr>
<td>7</td>
<td>1,823±543</td>
<td>1,864±194</td>
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<tr>
<td>14</td>
<td>1,371±315</td>
<td>1,602±187</td>
</tr>
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</table>

Data are means ± SE. Systemic creatine kinase activities and white blood cell count in animals that underwent a standardized open crush injury to the left soleus muscle and chronic treatment with G-CSF or vehicle solution for a total of 14 days after injury (n = 10 animals per time point and group). Sham-operated animals without muscle injury served as controls (sham; n = 5 animals per time point).

*J Appl Physiol • VOL 103 • NOVEMBER 2007 • www.jap.org*
the traumatized tissue. Therefore, G-CSF might represent an attractive adjuvant therapy option for patients suffering from muscle injury.

ACKNOWLEDGMENTS

The authors kindly thank Berit Blendow, Doris Butzlaff, Dorothea Frenz, and Maren Nerowski (Institute for Experimental Surgery, University of Rostock) for excellent technical assistance.

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