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J. Menetrey, C. Kasemkijwattana, C. S. Day, P. Bosch, M. Vogt, F. H. Fu, M. S. Moreland, J. Huard

From the Children's Hospital and the University of Pittsburgh, USA

Injury to muscles is very common. We have previously observed that basic fibroblast growth factor (b-FGF), insulin growth factor type 1 (IGF-1) and nerve growth factor (NGF) are potent stimulators of the proliferation and fusion of myoblasts in vitro. We therefore injected these growth factors into mice with lacerations of the gastrocnemius muscle. The muscle regeneration was evaluated at one week by histological staining and quantitative histology. Muscle healing was assessed histologically and the contractile properties were measured one month after injury.

Our findings showed that b-FGF, IGF and to a less extent NGF enhanced muscle regeneration in vivo compared with control muscle. At one month, muscles treated with IGF-1 and b-FGF showed improved healing and significantly increased fast-twitch and tetanus strengths. Our results suggest that b-FGF and IGF-1 stimulated muscle healing and may have a considerable effect on the treatment of muscle injuries.

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Injuries to muscle are common; their incidence varies from 10% to 55% of all injuries sustained in sports.[1] They are divided into shearing injuries in which both the myofibres and the framework of the connective tissue are torn, and injuries in situ, in which only the myofibres are damaged.

J. Menetrey, MD
C. Kasemkijwattana, MD
C. S. Day, MD
P. Bosch, MD
F. H. Fu, MD
Department of Orthopaedic Surgery, Musculoskeletal Research Centre, Division of Sports Medicine, Athletic Department.
M. Vogt, PhD
M. S. Moreland, MD
J. Huard, PhD
Department of Orthopaedic Surgery, Musculoskeletal Research Centre Children's Hospital of Pittsburgh and the University of Pittsburgh, Pittsburgh, Pennsylvania 15261, USA.

Correspondence should be sent to Dr J. Huard.

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There are three phases in the healing process of an injured muscle.[2] The destruction phase is characterised by the formation of a haematoma, necrosis of muscle tissue, degeneration, and an inflammatory-cell response. The repair phase includes phagocytosis of the damaged tissue, regeneration of the striated muscle, production of a connective-tissue scar and capillary ingrowth. In the final remodelling phase, the regenerated muscle matures and contracts with reorganisation of the scar tissue. There is often incomplete restoration of the functional capacity of the injured muscle.

The regeneration of the myofibres begins with the activation of myogenic precursor cells, or satellite cells, located between the basal lamina and the plasma membrane of each individual myofibre. They proliferate and differentiate into multinucleated myotubes and eventually into myofibres. Many of these myoblasts are able to fuse with existing necrosed myofibres and may prevent the muscle fibres from completely degenerating.[3] At the same time, fibroblasts invade the gap and begin to produce extracellular matrix to restore the framework of the connective tissue.[4,5] The physiological role of this scaffold is to transmit load across the defect so that the injured limb can be used before the repair process is complete.[2] In extensive muscle injury, the proliferation of fibroblasts can quickly lead to an excessive formation of dense scar tissue, which impedes regeneration of the muscle and results in an incomplete recovery.[6,7] This has already been shown in several injuries including strains, contusions and muscle lacerations.[8-14]

Growth factors are small peptides which bind to membrane receptors to influence the various steps of the growth and development of cells through several signalling pathways.[15,16] It has already been shown that they are capable of stimulating the growth and protein secretion of many musculoskeletal cells.[17] During muscle regeneration, it is presumed that trophic substances released by the injured muscle activate the satellite cells,[18-22] and in growth and development many growth factors have been shown to be capable of eliciting variable responses from the skeletal muscle.[15,16,23] Preliminary data suggest that individual growth factors play a specific role during muscle regeneration[15,16,24-27] and therefore may improve muscle healing.

In a previous study, we have found that basic fibroblast growth factor (b-FGF), insulin growth factor type 1 (IGF-1)

Table I. Effect of growth factors on the proliferation and fusion of myoblasts in vitro

Growth factor*	Proliferation	Fusion
b-FGF	Stimulates†	Stimulates†
IGF-1	Stimulates†	Stimulates†
NGF	Stimulates†	Stimulates†
a-FGF	Inhibits	Stimulates†
PDGF-AA	Inhibits	Inhibits
EGF	Inhibits	Inhibits
TGF-α	Inhibits	Inhibits
TGF-β	Inhibits	Inhibits

* b-FGF, basic fibroblast growth factor; IGF-1, insulin-like growth factor type 1; NGF, nerve growth factor; a-FGF, acidic fibroblast growth factor; PDGF-AA, platelet-derived growth factor, AA form; EGF, epidermal growth factor; TGF-α, transforming growth factor α; TGF-β, transforming growth factor β
† $p < 0.05$

and nerve growth factor (NGF) are potent stimulators of the proliferation and fusion of myoblasts in vitro. These results are summarised in Table I.

Our aim in this study was to assess the effect in vivo of these growth factors on muscle regeneration after injury and to evaluate their influence on muscle healing.

Materials and Methods

Evaluation of muscle regeneration in vivo. We used mice from the Rangos Research Centre Animal Facility of the Children's Hospital of Pittsburgh, the policies and procedures of which are in accordance with those detailed by the US Department of Health and Human Services. The research protocol had been reviewed and approved by the Animal Research and Care Committee (ARCC) at the University of Pittsburgh.

Both gastrocnemius muscles of six mice were cut at 60% of their length from their distal insertion, through 75% of their width and 50% of their thickness, and then sutured with a modified Kessler stitch and simple sutures using a PDS 7.0 wire (Ethicon, Somerville, New Jersey). The advantages of this model are its reproducibility and the ability to apply consistently precise injections into the laceration site. The severity of the lesion sustained by the muscle had been previously determined as a grade-III injury,[13] according to the classification of Buckwalter et al.[28]

Two mice received an injection of b-FGF (100 ng per injection; micro-syringe (Hamilton Co, Reno, Nevada) through a #30 gauge needle (Becton Dickinson Co, Franklin Lakes, New Jersey) into the right leg on days 1, 3 and 5 after laceration. The suture wire served as a landmark for the injection of the growth factor directly in the lesion. The delivery of the growth factor as close to the injured site as possible was important, because the short biological half-life and quick systemic lavage lead to a rapid disappearance of these substances.[29,30] The left leg was injected with the same volume of physiological solution as the control. Another two mice received IGF-1 and the last two NGF, following the same protocol. The doses of growth factors injected in vivo were determined by reference to previous studies in which growth factors had been injected into rat ligaments.[31,32] The efficient dose in vivo was a hundred times higher than the optimal dosage in vitro. Because of the smaller size of the mouse, we decided on a dosage of 100 ng/ml since the optimal concentration in vitro was 1 ng/ml.

During the process of muscle regeneration following any injury, b-FGF is present in the extracellular space at eight hours after the injury, reaching a peak at 24 hours, with the levels slowly decreasing over a period of one week.[33] IGF-1 is present after two days, reaches its peak at three days and decreases over a period of one week.[25,26] Thus, to cover the period of expression of growth factors the injections were performed at 1, 3 and 5 days after the injury.

The animals were killed by CO_2 inhalation followed by cervical dislocation seven days after injury. The gastrocnemius muscles were isolated and frozen in 2-methylbuthane precooled in liquid nitrogen. The level of muscle regeneration at different intervals after injury was evaluated by histological and immunohistochemical techniques characterising the expression of desmin.

Quantitative histological examination. One week after injection the number of regenerating myofibres in both the injected and contralateral muscles, which had not been injected, was counted on photomicrographs of sections stained with haematoxylin and eosin. These were coded and this was blinded to the observer performing the counting. Centronucleated cells were considered as regenerating myofibres. Nuclei with no discernible surrounding cytoplasm were discarded. The diameter of 200 regenerating myofibres was measured in 10 different randomised areas on the slide using a micrometer ruler. The number and the mean diameter of the regenerating myofibres were calculated and compared with those of the control non-injected muscle.

Evaluation of growth factors on muscle healing at one month. Both gastrocnemius muscles of 36 mice were lacerated and repaired as described above. IGF-1, b-FGF and NGF (100 ng per injection) were injected into the injured site of 12 mice each at days 1, 3 and 5 after injury and the same volume of physiological solution was introduced into the contralateral leg. At one month after the injury, three animals were killed for each growth factor and their muscles were prepared for histological examination as described above. Muscle healing was assessed on sections stained with haematoxylin and eosin.

Contractile properties. The physiological evaluation of contractile properties was performed on the remaining nine animals for each growth factor. Both gastrocnemius muscles were removed under methoflurane anaesthesia and mounted in a double-jacketed organ bath of 5 ml at 36°C in Krebs solution (mmol/l: NaCl 113, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11.5) and constantly bubbled with a mixture of 95% O_2 and 5% CO_2.

The initial tension was set at 20 mN; isometric contractions were measured by strain-gauge transducers coupled to a TBM4 strain-gauge amplifier (World Precision Instruments, Sarasota, Florida) and recorded on a computer using a data acquisition program (Windaq; DATAQ Instruments Inc, Akron, Ohio). The sampling rate per channel was set at 500 Hz. The amplitude of the stimulation-evoked contractions was computed by a calculation program (WindaqEx; DATAQ). After 20 minutes of equilibration, electrical field stimuli were applied through two platinum wire electrodes positioned on the top and bottom of the organ bath separated by 4 cm. The muscles were stimulated with square-wave pulses of duration 0.25 ms with a maximal voltage of 50V. First, stimulation of 1Hz was applied and the muscle twitches recorded, then six tetanic stimulations were applied. The stimulation lasts 0.5 s, train shape, and this stimulation is performed every 10 s. Finally, the muscle was weighted using a microbalance (Mettler Toledo Inc, Hightstown, New Jersey). The strength measurements were reported by weight unit and expressed in mN/g.

Statistical analyses. For the regeneration study, the diameters were compared in the experimental and contralateral muscles using a paired Student *t*-test. Statistical analysis was not performed for the number of regenerating myofibres, because only two animals per growth factor were used making the numbers extremely small for formal statistical comparison. Regarding the physiological evaluation of contractile properties and based on information obtained in preliminary experiments, data from six animals gave 80% power (type-1 error rate of 0.05) for detection of one to two standard deviation differences between controls and experiments. Statistical significance was set at $p < 0.05$. Absolute fast twitch and tetanic strength were compared in the experimental and contralateral muscles using a paired Student *t*-test. The results were expressed as a percentage of the control side in each animal and the mean percentage for the nine animals tested was reported for each growth factor.

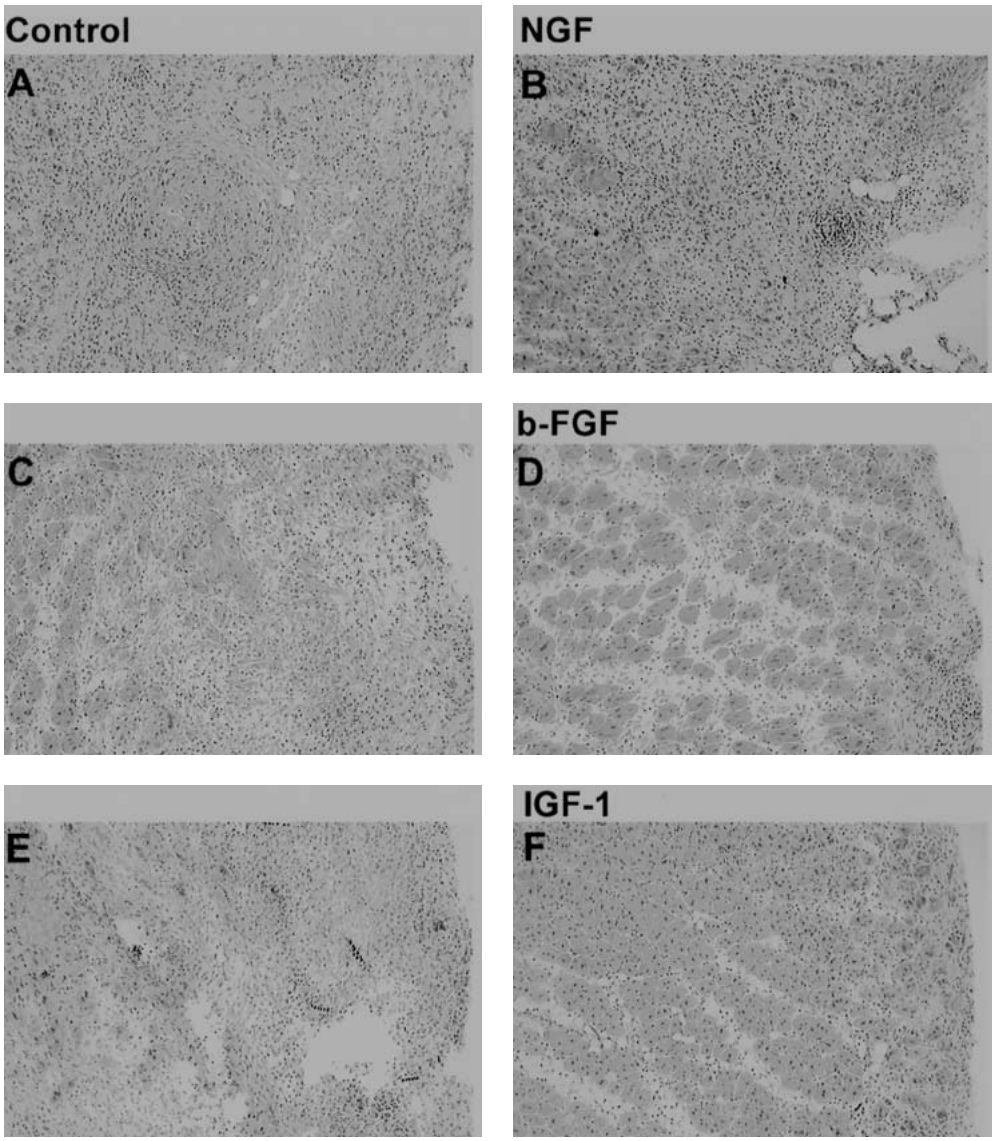

Fig. 1

Photomicrographs of the site of laceration seven days after injury. Figures 1A, 1C and 1E – Control muscle with regenerating myofibres in the deep part of the muscle and infiltration of mononucleated cells in the superficial part. Figures 1B, 1D and 1F – Laceration injected with NGF (B), b-FGF (D) and IGF-1 (F). Regenerating myofibres are located throughout the injured site in both the deep and superficial areas (haematoxylin and eosin × 10).

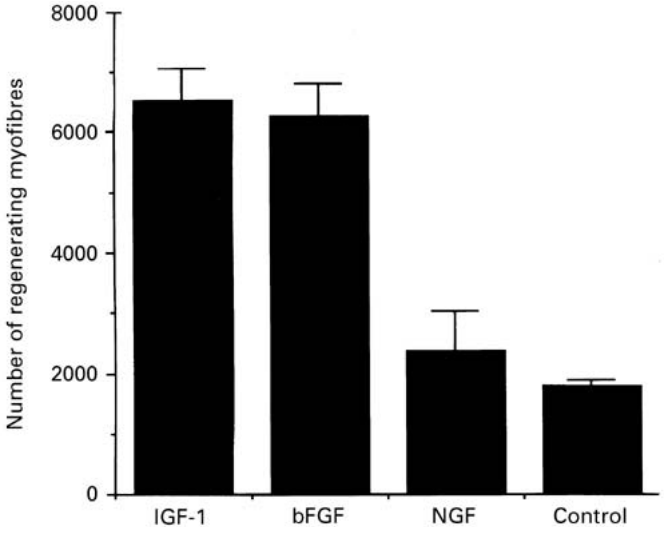

Fig. 2a

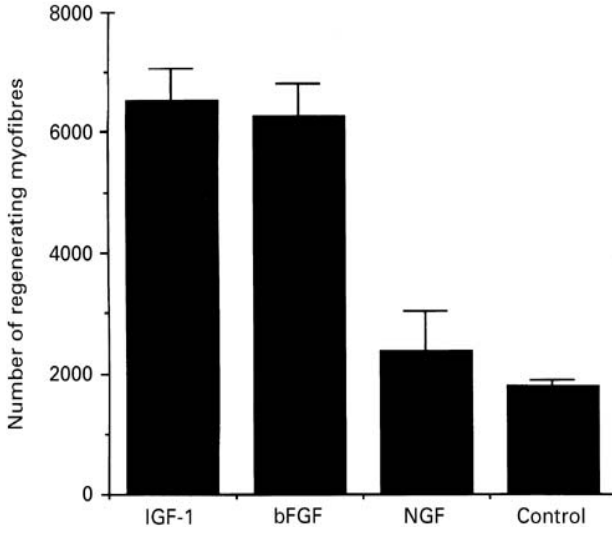

Fig. 2b

Histograms showing a) the mean number of regenerating myofibres counted in the muscle and b) their mean diameter.

Results

Evaluation of muscle regeneration in vivo. The muscles into which growth factors had been injected showed numerous regenerating myofibres in the site of laceration. These were uniformly present throughout the injured region in the superficial as well as in the deep part of the muscle (Figs 1B, 1D and 1F). The control muscles also contained regenerating myofibres but they were predominantly located in the deep part of the laceration. In the superficial region of the control muscles there was infiltration of mononucleated cells with only a few regenerating myofibres (Figs 1A, 1C and 1E).

Quantitative histological examination. Figure 2 shows the mean (SD), number and diameter of the regenerating myofibres. All the centronucleated myofibres present in the injured muscle were counted and the diameter was measured. There was an increase in the number of regenerating myofibres in the muscles receiving growth factors, 3.5 times for b-FGF and IGF-1 and 1.5 times for NGF. The mean diameter of the regenerating myofibres was 31.3 (10.2) μm for the control, 34.2 (10.8) μm for NGF ($p = 0.0061$), 37.4 (10) μm for b-FGF ($p < 0.0001$) and 37.9 (8.1) μm for IGF-1 ($p < 0.0001$). The medians for each group were 30 (10 to 60), 30 (20 to 80), 40 (20 to 60) and 40 (20 to 80), respectively. The NGF group differed from the b-FGF and IGF-1 groups ($p = 0.0022$ and $p < 0.001$, respectively). Only the distribution for IGF-1 and b-FGF showed no statistically significant differences.

Evaluation of the effect of growth factor on muscle healing at one month. At one month, the non-treated muscle showed numerous centronucleated regenerating myofibres in the deepest part of the laceration. Superficially, the laceration was covered by a fibroblastic tissue in which there were many regenerating myofibres (Figs 3A, 3C and 3E). In the muscle treated with IGF-1 and b-FGF, the regenerating myofibres were uniformly located in the deep and superficial parts of the muscle. Their diameter was similar to the surrounding normal myofibres, and many of their nuclei were already peripherally located. The development of fibroblastic tissue was also reduced in the treated muscle (Figs 3D and 3F). These findings suggested that muscle healing was accelerated in these muscles when compared with control muscle. The muscle treated with NGF contained numerous mononucleated regenerating myofibres in its deep part. In the superficial part, regenerating myofibres of small diameter were observed as well as areas of fibroblastic tissue (Fig. 3B). These muscles had the same histological appearance as in the control muscle.

Contractile properties. One month after the injury, the twitch and tetanus strengths were increased in muscles treated with IGF-1 and b-FGF (Fig. 4). To minimise inter-animal variation, the data were normalised with respect to untreated controls, i.e., the strength in the experimental muscle was divided by that in the control muscle in the contralateral side and multiplied by 100 to determine the percentage of change. The fast twitch strength was increased by 76 (14%) ($p = 0.001$) for b-FGF and 164 (36%) ($p = 0.005$) for IGF-1 when compared with the control (Fig. 4a). The tetanus strength was increased by 74 (20%) ($p = 0.002$) for b-FGF and 106 (34%) ($p = 0.003$) for IGF-1. The muscles treated with NGF regularly produced a mean twitch and tetanus strength which was less than those of control muscles, but the difference was not statistically significant (Fig. 4b).

Discussion

The development of the use of growth factors which will give quicker and more complete recovery may significantly affect the 'down-time' after injury of a muscle. Our study has shown that the direct injection of specific growth

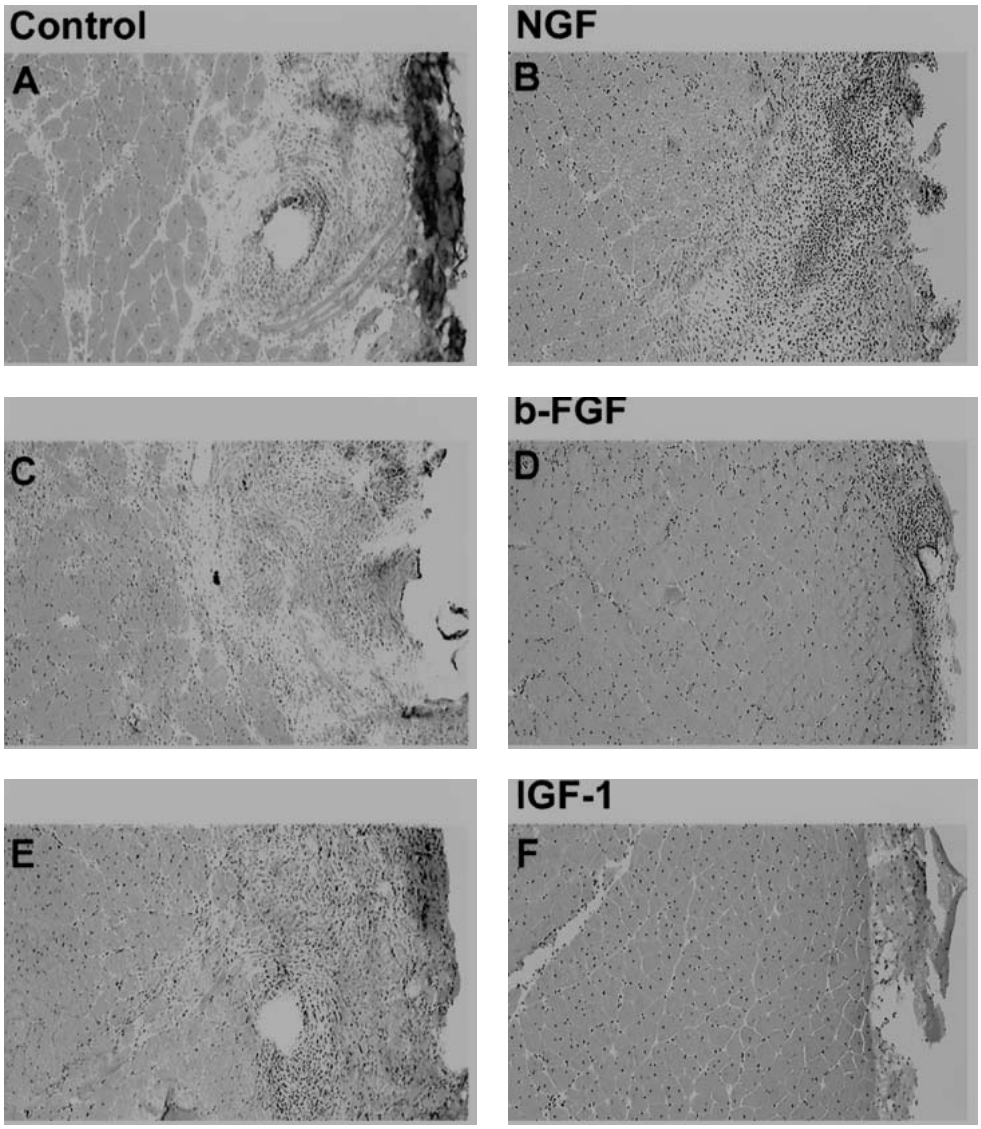

Fig. 3

Photomicrographs of the site of laceration one month after the injury. Figures 3A, 3C and 3E – Control muscle with a superficial layer of fibrotic tissue and small regenerating myofibres with a deep layer containing numerous regenerating myofibres. Figures 3B, 3D and 3F – Laceration injected with NGF (B), b-FGF (D) and IGF-1 (F). The appearance of the muscle injected with NGF is similar to that of control muscle. The muscles treated with b-FGF and IGF-1 show large regenerating myofibres filling the entire site (haematoxylin and eosin × 10).

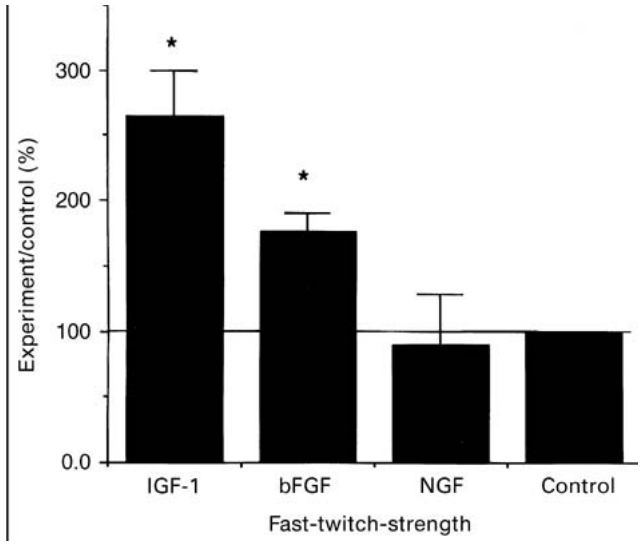

Fig. 4a

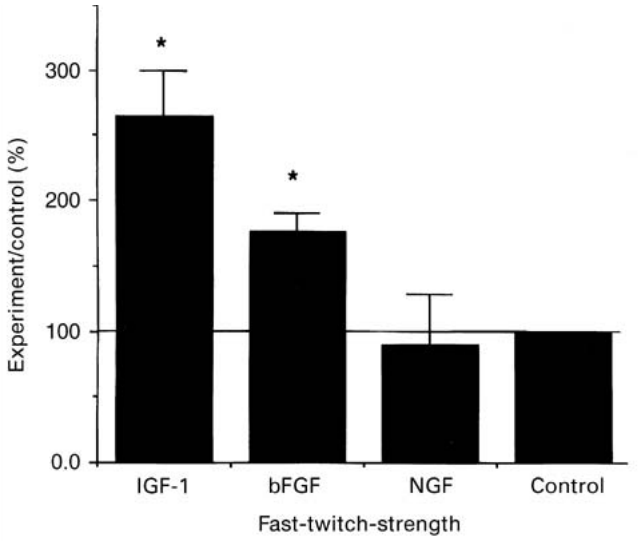

Fig. 4b

Histograms of the contractile properties of the muscles at one month after injury showing a) fast twitch strength and b) tetanus strength (*the ratio of experimental to control mean values (%) differs significantly ($p < 0.05$) from the control).

factors into a muscle can significantly improve healing.

We have found that IGF-1, b-FGF and NGF are potent stimulators of the proliferation and fusion of myoblasts in vitro (Table I). These initial data confirm previous studies which showed that b-FGF stimulates cell proliferation in bovine and chick myoblasts in culture.[34-37] The mechanism for b-FGF-stimulated proliferation appears to be the advancing of cells from G0 to G1 in the cell cycle.[18,25] Previous studies have also shown that IGF-1 is capable of powerful stimulation of the proliferation and differentiation of myoblasts in vitro.[23,38-40]

NGF was the first growth factor to be identified and is known to regulate the development and maintenance of sympathetic and some sensory neurones.[41] Based on our data, NGF is a potent stimulator of the proliferation and fusion of myoblasts in vitro. To our knowledge, the effect of NGF on myoblasts has never before been reported. Low-affinity NGF receptors, however, have been identified at the surface of human regenerating myofibres[42] and developing muscle from rat and chicken embryos.[43,44] These findings may have some significance for muscle regeneration, notably at the reinnervation phase.

It is important to recognise that regeneration in vivo is more complex than that in vitro because of the involvement of circulatory and intercellular communication.[15,16] Some preliminary characterisation of the role of certain growth factors during muscle regeneration suggests that the individual growth factors have similar effects to those seen in vitro.[27,45] Thus, we anticipated that b-FGF, NGF and IGF-1 would enhance muscle growth and regeneration in vivo and our findings have confirmed this; b-FGF, IGF-1 and to a less extent NGF improved muscle regeneration in mice. We have shown an increase in the number and size of the regenerating myofibres as an index of muscle regeneration. We have also shown in our model that regenerating myofibres were located in the superficial area of the injured site of muscles only when treated with growth factors, thus demonstrating greater initial healing when the injured muscle is treated with specific growth factors.

The discrepancy between the in vitro and in vivo results for NGF is interesting. In vivo, inflammation results in an early and maintained elevation in the levels of NGF in injured tissue.[46,47] Neutralising the action of the increased NGF with specific anti-NGF antibodies decreases inflammatory hypersensitivity, indicating that this neurotrophin is very important in the production of inflammatory pain.[46] The systematic and local application of exogenous NGF has been shown to produce a rapid and prolonged behavioural hyperalgesia in both animals and man.[47] We have observed such behaviour in mice injected with NGF. These mice frequently bit their sutures after the injection, and their wounds had to be resutured. Because of its effect on inflammatory cells, NGF certainly has an important biological role in the repair process,[48] although the only effect seen in our study was a small stimulation of the initial muscle regeneration. The absence of effect on muscle healing may be explained by the hyperalgesic state induced by the injection of NGF. Consequently, the animal may protect the injected leg and disuse will result. This disuse, and the reopening of the wound, may have a negative effect on muscle healing. Under these experimental conditions, NGF has shown no significant effect on muscle healing in vivo.

Specific growth factors were not only able to improve muscle regeneration but also produced more complete muscle healing. At one month, the muscles treated with IGF-1 and b-FGF had accelerated healing and a higher functional recovery compared with control muscles, and contained only a few areas of fibrosis. The injection of growth factor seemed to reduce the formation of scar tissue in the muscle, but this requires further study. The functional improvement in these muscles at one month after the injury was approximately 160% for b-FGF and 200% for IGF-1. A treated muscle can therefore generate greater strength earlier and perform at a higher level sooner.

Our results are important in terms of the treatment of all types of muscle injury and may give a better quality of healing of muscle tissue. Despite a high capacity for regeneration, the response of muscle tissue to serious injury typically involves the formation of dense fibrotic scar tissue between normal muscle. By enhancing muscle growth and regeneration, it may be possible to prevent this and thus reduce the risk of reinjury at the junction between the scar tissue and regenerated muscle as well as the incidence of muscle pain after scarring.

In conclusion, our study has shown that serial injections of IGF-1 and b-FGF into an injured muscle improve healing in vivo. Further studies will be required to investigate an eventual dose-dependent response, the potential synergetic effect of the association of two growth factors, the limitation of the development of fibrosis, and the application of this treatment to different muscle conditions such as fibrosis after limb-lengthening, the compartment syndrome and muscular dystrophy.

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