NERVE GROWTH FACTOR IMPROVES MUSCLE HEALING FOLLOWING MECHANICAL INJURY

By

**Raouf F. Bedir; Dalia M. Saleh,
Mona A. Elshahat and Adel A. El-Hawary**

From

*Department of Anatomy, Faculty of Medicine,
Mansoura University, Mansoura, Egypt*

ABSTRACT

Muscle injuries are very common in sports and trauma. The need for a quick and more complete recovery of the muscle after injury directs the attention towards growth factors, which are hormone-like biologically active polypeptides that control cell growth and differentiation. It was observed that nerve growth factor (NGF) is a potent stimulator of myoblast proliferation and fusion in vitro, thus, it was anticipated that NGF could enhance muscle healing in vivo. Six adult albino rats were used in this study. Both right and left gastrocnemius muscles of the albino rats 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 simple sutures using absorbable catgut sutures. The right gastrocnemius

muscles of all the albino rats were injected directly into the lesion with 100 ng/ml of NGF at one, three, and five days after laceration. The contralateral (left) muscles were injected with the same volume of physiological normal saline and were used as a control. At one week after the injury the animals were sacrificed and the gastrocnemius muscles were removed and processed for paraffin sectioning and stained with Hematoxylin and Eosin, Bcl-2 and P53 immune peroxidase stains. It was observed that NGF enhanced muscle regeneration in vivo compared to the control. It stimulated satellite cells to proliferate either directly or through enhancing the inflammatory cell response e.g. macrophages. Inflammatory cells were found to survive more and express death-inhibiting protein Bcl-2

under the effect of NGF. This lead to enhancement of satellite cell proliferation and differentiation. Proapoptotic protein p53, was found to have no manifest role in muscle regeneration in this in vivo study.

INTRODUCTION

Adult skeletal muscle has a remarkable ability to regenerate following myotrauma. Since adult myofibers are terminally differentiated, the regeneration of the skeletal muscle is largely dependent on a small population of resident cells termed satellite (stem) cells (Hawke and Garry, 2001). In the normal state, these satellite cells remain in a non-proliferative, quiescent state. On the other hand, in response to stimuli such as growth, remodeling, denervation or muscle injury, satellite cells become activated and stimulated to re-enter the cell cycle. They appear as a swelling on the myofiber with cytoplasmic processes extending from one or both poles of the cell. Satellite cells proliferate and express myogenic markers (now are termed myoblasts). Ultimately, during regeneration of damaged skeletal muscle, these cells fuse to existing muscle fibers or fuse together to form new myofibers (Bischoff, 1994; Schultz and McCormick, 1994).

During regeneration, macrophages are essential in the orchestration of the repair process as they secrete a collection of cytokine factors that regulate the satellite cell pool (Nathan, 1987). Importantly, in the presence of an enhanced macrophage response, there is an increase in satellite cell proliferation and differentiation (Lescaudron et al., 1999). Emerging evidence suggests that regulation of macrophage apoptosis may be pivotal in the outcome of an inflammatory response. The cytokines, which are a unique family of growth factors, might either promote or prevent macrophage apoptosis. This process is effectively reversed by the addition of growth stimuli suggesting that apoptosis may represent the mechanism for eliminating activated macrophages that are no longer necessary as inflammation wanes (Tidball and St. Pierre, 1996).

Nerve growth factor is a neurotrophic factor known to play a crucial role in neurite growth and differentiation. It is produced by various cell types including vascular smooth muscle cells and fibroblasts following wounding (Turrini et al., 2002). NGF might play an accessory role in the regulation of immune and inflammatory

ry reactions through stimulation of chemotactic migration of human polymorphonuclear leukocytes both in vitro and in vivo. It also has an additional role in promotion of the proliferation, growth and differentiation of other blood elements (Brodie and Gelfand, 1992). It was also observed that it is a potent stimulator of myoblast proliferation and fusion in vitro. However, its role and effect on muscle healing following trauma needs further investigation. Therefore, the present work was carried out to study the effect of NGF on the early events of muscle regeneration following mechanical injury in vivo.

MATERIALS AND METHODS

Animals used : Six adult male and female albino rats obtained from Animal House, Faculty of Medicine, Mansoura University were used in this study. Age varied from 13-15 weeks, weighting 200-300 g and housed in cages with softwood granules as bedding. They had free access to standard diet and drinking water.

Anesthesia : Rats were anesthetized with ether inhalation through soaked cotton. This method of anesthesia was suitable as the surgical

procedure required only few minutes and the animals were fully recovered in about 5 minutes.

Injury of skeletal muscle : Gastrocnemius muscles were subject to a cut injury as described by Menetrey et al. (2000). Under sterile precautions both right and left gastrocnemius muscles of the albino rats 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 simple sutures using absorbable catgut sutures (6-0) with rounded non traumatic needles. The severity of the lesion sustained by the muscle had been previously determined as a grade-III injury according to the classification of Buckwalter et al., (1996). Non-absorbable suture such as Prolene® was used for skin sutures and served as a landmark for injection.

NGF injection : The right gastrocnemius muscles of all the albino rats were injected directly into the lesion with 100 ng/ml of NGF (Cerebrolysin®, EBEWE Arzneimittel Ges.m.b.H., pharmaceutical Laboratories, A-4866 Unterbach, Austria, Europe) at one, three, and five days after laceration using a micro-syringe.

The contralateral (left) muscles were injected with the same volume of physiological normal saline and were used as a control. Animals were sacrificed using over dose of ether inhalation followed by cervical dislocation at seventh day after the injury, and the gastrocnemius muscles were removed and processed for histological examination.

Histological and immunohistochemical studies : The isolated gastrocnemius muscles of both sides were fixed in 10% neutral formalin and processed for paraffin sectioning. For histological analyses, 4-6 μm cross and longitudinal sections were collected along the entire length of the muscle and at the site of the lesion to evaluate muscle regeneration. The sections were stained with Hematoxylin and Eosin and with Bcl-2 and P53 immune peroxidase stains. The immunohistochemical study was performed using streptavidin-biotin peroxidase complex technique (Bhatavdekar et al., 1998) using monoclonal antibodies for Bcl2 (Bcl2 Ab-3 1:50; labvision, Westinghouse, Fremont CA, USA) and P53 (Do-7 prediluted IgG, Kappa; Dako AS, Copenhagen, Denmark) and counterstained with hematoxylin. All selected sections

were examined and photographed using an Olympus microscope.

Quantitative study : Sections containing the largest area of damage were selected for analysis. An image of the core of the damaged area, defined as the region that was least regenerated, was photographed. For analysis of control muscles, sections were selected from the same anatomical region as those from injured muscles. The diameter of the regenerating myofibers in both the injected and the contralateral control muscles was measured from photomicrographs. The photographs were scanned using Acer scanner, saved as "jpeg" files and used for the statistical studies using image processing computer software "ImageJ". Centronucleated cells were considered as regenerating myofibers. Nuclei with no discernible surrounding cytoplasm were discarded. All the centrally nucleated myofibers within this field were counted and their diameters were measured and were compared with those of the control contralateral non-injected muscle. Since satellite cells lie between the sarcolemma and the basal lamina at the periphery of myofibers and constitute about 5% of the number of the peripheral nuclei found in

the myofibers, it was difficult to count them alone without using special stains, which were not used in this study. Nuclei at the periphery of the myofibers (Satellite cells nuclei and myonuclei) were counted and the mean number and ratio of their diameter compared to myofibers diameter (nucleo-cytoplasmic ratio) were calculated and measured in 200 myofibers in 10 different randomized areas close to the site of the lesion both in injected and contralateral control muscles. The data obtained from the control and the experimental muscles were compared using the appropriate statistical test.

Statistical analysis : Depending on the number of the sections examined, either independent sample t-test for sections number more than 30 with normal probability distribution or Mann-Whitney test for sections number less than 30. The diameters of the regenerating muscle fibers and the nucleo-cytoplasmic diameter ratio were compared in the experimental and contralateral muscles using a paired student t-test. The satellite/myonuclear number was compared in the experimental and contralateral muscles using Mann-Whitney test. Statistical analysis was not performed

for the number of regenerating myofibers because the number of the animals was small for formal statistical comparison. All values are expressed as means $\pm$ SD. Significant differences were attributed with $P < 0.05$.

RESULTS

Evaluation of muscle regeneration in vivo : One week following the injury, the control and the injected muscles showed signs of degeneration and acute inflammation. There was invasion of the site of laceration by inflammatory cells seen perimysial and endomysial. Some degenerated muscle fibers appeared pale others with interstitial edema (Figs. 1, 2). In longitudinal sections, degenerated muscle fibers appeared pale and contracted and were invaded by phagocytic inflammatory cells (Figs. 3, 4). In the control non-injected muscles, new regenerating myofibers, identified by their centrally located nuclei and basophilic sarcoplasm, were found deep to the site of laceration while the superficial part was infiltrated by inflammatory cells (Fig. 5). The muscles injected with NGF showed many regenerating myofibers uniformly distributed both in the superficial and the deep parts of the laceration (Fig. 6).

Bcl-2 protein expression : In the control sections stained with immunoperoxidase stain with anti Bcl-2 monoclonal antibody, there was an invasion of the injury site by inflammatory cells mainly granulocytes and were negatively expressing Bcl-2 (Fig. 7). Fibroblasts, fibrocytes and lymphocytes were seen among the regenerating myofibers and were positively stained with Bcl-2 (Fig. 8). In NGF injected muscles, Bcl-2 protein was strongly positive (dark brown) in the cytoplasm of the inflammatory cells. The positive cells were mainly lymphocytes and macrophages; some macrophages showed double nuclei and others showed typical cleaved and kidney shaped nuclei (Figs. 9, 10).

p53 protein expression: Sections stained with immunoperoxidase stain with anti p53 monoclonal antibody, showed that p53 protein was not expressed in the nuclei of the satellite cells (peripheral nuclei) either in the quiescent (Fig. 11), active or differentiated stages (Fig. 12).

Quantitative histological examination : Table (1) and figures (13-14) summarize the mean diameter $\pm$ SD of the regenerating myofibers, the mean number $\pm$ SD of the satellite-myonuclear cells and the mean ratio of the myonuclear/myofibers diameter $\pm$ SD both in the control and the NGF injected muscles. The mean diameter of the regenerating myofibers was 28.2 ± 7.7 for the control and 41.4 ± 12.4 for the NGF injected muscles. The mean ratio of the myonuclear/myofiber diameter was 0.08 ± 0.03 for the control and 0.001 ± 0.04 for the NGF injected muscles. The mean number of satellite-myonuclei cells was 35.8 ± 8.5 for the control and 45.2 ± 15.7 for the NGF injected muscles. The mean diameter of the regenerating myofibers and the mean ratio of the myonuclear/myofiber diameter were significantly higher in the NGF injected muscles compared to the control while the mean number of satellite-myonuclei cells in the NGF injected muscles was not significantly different from that of the control.

Grouping variable	Mean $\pm$ SD		P value
	Control	NGF	
Satellite cell/myonuceli number	35.8 $\pm$ 8.5	45.2 $\pm$ 15.7	0.3
Myonucleo/cytoplasmic diameter ratio	0.08 $\pm$ 0.03	0.001 $\pm$ 0.04	0.000*
Diameter of regenerating myofibers (μ)	28.2 $\pm$ 7.7	41.4 $\pm$ 12.4	0.000*

Values are mean $\pm$ SD;

* highly significant

Figure 1. Photomicrograph of a transverse section of the control muscle one week after the injury at the site of the lesion showing, degenerated muscle fibers (arrows) and infiltration of the area by inflammatory cells (arrowheads). Hx & Eosin, X100.

Figure 2. Photomicrograph of a transverse section of NGF injected muscle one week after the injury at the site of the lesion showing, degenerated muscle fibers with interstitial edema infiltration of the area by inflammatory cells both seen perimysial (curved arrows) and endomyssial (arrowheads). Hx & Eosin, X100.

Figure 3. Photomicrograph of a longitudinal section of the control muscle one week after the injury at the site of the lesion showing, contracted muscle fibers invaded by inflammatory cells (arrows). Hx & Eosin, X400.

Figure 4. Photomicrograph of a longitudinal section of NGF injected muscle one week after the injury at the site of the lesion showing, pale necrotic (M) and lacerated (L) muscle fibers invaded by inflammatory cells (arrows). Hx & Eosin, X400.

Figure 5. Photomicrograph of a transverse section of the control muscle one week after the injury at the site of the lesion showing, few regenerated myofibers (arrowheads) in the deep part of the section (d) and infiltration of the inflammatory cells in the superficial part of the section (s). Hx & Eosin, X100.

Figure 6. Photomicrograph of a transverse section of NGF injected muscle one week after the injury at the site of the lesion showing, regenerated myofibers (arrowheads) in the deep (d) and the superficial (s) parts of the section. Hx & Eosin, X100.

Figure 7. Photomicrograph of a longitudinal section of the control muscle one week after the injury at the site of the lesion showing, degenerated muscle fibers (arrows) and infiltration of the area by negatively stained inflammatory cells mainly granulocytes (arrowheads). Bcl-2 immunoperoxidase stain, X100.

Figure 8. Photomicrograph of a transverse section of the control muscle one week after the injury at the site of the lesion showing, positively stained fibrocytes (arrows) and inflammatory cells both lymphocytes (arrowhead) and macrophage (short arrows). Bcl-2 immunoperoxidase stain, X400.

Figure 15 : The mean diameter of the regenerating myofibers of the control and NGF injected rats.

DISCUSSION

Skeletal muscle has an impressive ability to regenerate itself on a daily basis as well as in response to injury. The development of the use of growth factors, which will give quicker and more complete recovery, may significantly affect the downtime after injury. Muscle regeneration following injury depends primarily upon satellite cells and upon other cellular processes involving non-muscle cells. One of the earliest of these is the inflammatory response, which facilitates myogenesis via phagocytosis of cellular debris and the release of chemoattractants and growth factors (Chazaud et al., 2003). In this study it was found that when muscle fibers are damaged by

cut injury, several overlapping cellular processes, including inflammation and myogenesis took place. These initial data confirm those of Grounds (1991). Skeletal muscle regeneration, initiated by muscle injury, involves the degeneration of damaged myofibers, formation of a hematoma, necrosis of muscle tissue and an inflammatory cell response. Haslett et al. (1994) reported that neutrophils and macrophages accumulate at the injury zone as early as 30 minutes post injury. Neutrophils prepare the site for further events in muscle regeneration by releasing compounds, which break down both foreign and cellular materials, thus decreasing the likelihood of the wound becoming a route for sys-

temic infection. They then undergo apoptosis (programmed cell death).

Macrophages migrate to the damaged area through bloodstream and from the muscle and surrounding tissue by direct chemotaxis under the effect of soluble signals from tissue destruction (Carlson and Faulkner, 1983; Armstrong, 1990; Robertson et al. 1993). Macrophages are thought to be responsible for phagocytosis of the bulk of the macromolecules resulting from damaged myofibers, extracellular matrix and apoptotic neutrophils. They can also orchestrate localized immune responses in the injury zone by producing a large variety of soluble factors (Nathan, 1987). They release factors such as platelet derived growth factor (PDGF) and an undefined satellite cell-specific factor, which stimulate satellite cell proliferation in the injury zone (Cantini et al. 1994; Cantini and Carraro, 1995).

Skeletal muscle repair includes activation of satellite cells (Bischoff, 1990). When a muscle is damaged, these cells are activated within 18 hours after the injury (Wanek, 1997). Quiescent satellite cells are activated by various growth factors or myogenic regulatory factors "wound hormones",

which are released from the damaged myofibers and the extracellular matrix surrounding them (Dodson et al. 1996). This is done through interaction with growth factors receptors on the surface of these cells. Once these satellite cells have undergone differentiation they can then fuse to existing muscle cells. This fusion allows the satellite cell to donate needed myonuclei to the wounded or developing muscle cell. It also appears that these cells are able to migrate, and may move from healthy areas of the muscle to the injured area in response to chemical stimuli. They then fuse together to form myoblasts, which in turn fuse to form myotubes. One of the hallmarks of regenerating myofibers is the centrally located position of the myonuclei. Finally, the regenerated muscle matures and contracts with reorganization of the scar tissue (Best, 1995). Upon maturing, muscle fiber nuclei are located along the cell periphery (Hawke and Carry, 2001).

NGF may have some significance in muscle regeneration, notably at the reinnervation phase. It is anticipated that NGF has a potent stimulator of muscle regeneration in vivo as those seen in vitro (Toti et al. 2003). In this

study there was a significant increase in the mean myonuclei/myofiber diameter ratio and the size of the regenerating myofibres as an index of muscle regeneration in the NGF injected group compared to the control. It has also been found that regenerating myofibres were located both in the deep and the superficial areas of the injured site of muscles only when treated with NGF. Thus demonstrating greater initial healing when the injured muscle is treated with NGF. This could be a direct effect of NGF upon satellite cells or indirectly through up-regulation of macrophages, which clean the site of injury by initiating phagocytosis of tissue debris in preparation for the healing process and produce factors influencing satellite cell proliferation and differentiation through stimulation of its migration and delay its apoptosis by activation of death inhibiting proteins (Lescaudron et al., 1999).

Inflammation, on the other hand, results in an early and maintained elevation in the levels of NGF in injured tissue (McMahon, 1996; Woolf, 1996). Neutralizing 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 (Woolf, 1996). The systematic and local application of exogenous NGF has been shown to produce a rapid and prolonged behavioral hyperalgesia in both animals and man (McMahon, 1993). Such behavior was observed in rats injected with NGF in this study as they frequently bit their sutures after the injection.

NGF, on the other hand, has been shown to regulate the life span of both granulocytes and macrophages *in vitro* (Kannan et al., 1992). Orlofsky et al. (1999) documented the occurrence of a protective Bcl-2 family member in inflammatory neutrophils and macrophages. In this study it was found that NGF retarded apoptosis in neutrophils, macrophages and other inflammatory cells by activating the anti-apoptotic Bcl-2 protein leading to accumulation of these inflammatory cells.

Although many studies strongly supported the role of p53 in myogenesis *in vitro*, as expression of p53 increases during differentiation and fusion of skeletal muscle cells (Halevy et al., 1995; Soddu et al., 1996; Tamir and Bengal, 1998). Also, myotube for-

mation in vitro is impaired in myogenic cells expressing a dominant negative p53 protein and in primary myoblasts derived from p53 $-/-$ muscle (Porrello et al., 2000). This role was not manifested in this in vivo study. This finding is supported by (White et al., 2002) who indicated that p53 is not required for the regulation of myoblast proliferation, differentiation and myotube formation in vivo during myogenesis of adult skeletal muscle. This could be explained by the possibility that genes responsible for myoblasts differentiation could be upregulated in a way independent of p53 in vivo and the critical role of cell contact may possibly account for the differences between in vitro and in vivo results.

CONCLUSION

In conclusion, under these experimental conditions, this study has shown that serial injections of NGF into an injured muscle improved muscle healing in vivo. Further studies will be needed to investigate an eventual dose-dependent response. The current study also documents the occurrence of a protective Bcl-2 family member in inflammatory neutrophils and macrophages, which was expressed in NGF treated

muscles leading to enhancement of the effect of these inflammatory cells. It is also concluded that inflammatory cells play a role in satellite cell activation and proliferation. We have also found that the tumor suppressor gene p53, which is recognized as a central regulator of the cell cycle and apoptosis, had no manifest role in muscle regeneration in this in vivo study.

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عامل النمو العصبى يحسن التئام العضلات بعد الجرح الميكانيكى

الهدف من الدراسة :

إن إصابات العضلات شئ شائع الحدوث نتيجة الرياضة ومن الرضوض، فالحاجة الملحة إلى شفاء كامل وسريع للعضلات بعد الاصابة وجهت الاهتمام ناحية عوامل النمو والتي تشبة الهرمونات، فهى من الناحية البيولوجية مواد متعددة البيتيد نشطة تسيطر على نمو وتمايز الخلايا فقد لوحظ أن عامل النمو العصبى محفز قوى لتكاثر الخلايا العضلية واتحادها خارج الجسم وعليه فإن من المتوقع أن عامل النمو العصبى قد يعزز من التئام العضلة داخل الجسم ولهذا السبب فقد تم إختيار عامل النمو العصبى للتحقق فى هذا البحث.

خطوات البحث :

تم عمل جرح للعضلة ذات البطن فى الفئران البيضاء وقيم تجدد العضلة بعد أسبوع من العملية الجراحية بواسطة الصبغات الهستولوجية والإحصاءات الهستولوجية.

نتائج الدراسة :

وقد لوحظ فى هذا البحث أن عامل النمو العصبى قد حفز تجدد العضلة المصابة داخل الجسم مقارنة بالمجموعة الضابطة وذلك عن طريق تحفيز الخلايا البديلة للنمو إما مباشرة أو عن طريق تعزيز استجابة الخلايا الملتهبة مثل الخلايا الآكلة، فقد وجد أن الخلايا الملتهبة ويظهر فيها بروتين الـ ب.س.: الـ ٢ المسئولة عن وقف موت الخلية المقدر والذي يؤدي إلى زيادة تحفيز الخلايا البديلة للنمو والتجدد. وقد وجد أيضاً فى هذا البحث أن عاملاً إخماد الأورام ب ٥٣ ليس له دور مؤثر فى التئام العضلات داخل جسم الإنسان.

خلاصة البحث

حقن عامل النمو العصبى يحسن التئام العضلات المصابة فى جسم الإنسان بطريقة ملحوظة.