THE ANATOMICAL RECORD 241:123–128 (1995)

Regeneration in Denervated Toad (*Bufo viridis*) Gastrocnemius Muscle and the Promotion of the Process by Low Energy Laser Irradiation

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ABSTRACT ***Background:*** **It is known that while denervated skeletal muscles have the ability to regenerate, maturation of regenerated myofibres does not take place under these conditions. Denervation also causes elevation of "invasive" and satellite cells, but the role of these cells in the regeneration process after injury to the denervated muscle is still unknown. Low energy lasers have recently been found to modulate and accelerate physiological processes in cells. The aim of the present study was to compare regeneration in denervated and innervated amphibian muscles and to investigate whether this process in denervated muscles can be stimulated by low energy laser irradiation prior to injury in these muscles.**

Methods: **Denervated gastrocnemius muscles of toads were irradiated with He-Ne laser (6.0 mW, 31.2 J/cm^2) 7 days postdenervation (control muscle received red light irradiation at the same wavelength). Nine days after denervation cold injury was performed on the site of irradiation of both groups of muscles. At 14 days postinjury all muscles were removed and processed for histology and histomorphometric analysis of mononucleated cells, myotubes, and young myofibres in the regenerated zone.**

Results: **The volume fraction (percent of total injured zone) of the various histological structures in the injured zones 14 days after cold injury in the denervated (9 days prior to injury) muscles did not differ from innervated injured muscles at the same time interval postinjury. The mononucleated cells and myotubes in the laser irradiated muscles comprised 49 ± 4% and 6 ± 1% of the injured area, respectively, which was significantly lower than their volume fraction (67 ± 2% and 11 ± 2%, respectively) in the control muscles. The young myofibres populated 34 ± 4% of the total injured area in the denervated and laser irradiated muscles which was significantly higher than their volume fraction (12 ± 2%) in control denervated muscles.**

Conclusions: **It is concluded that initial stages of regeneration can also take place in skeletal denervated and injured muscles of amphibians. The kinetics of the regeneration process are identical in denervated and innervated muscles. The process of regeneration in denervated muscles can be markedly enhanced if the muscle is irradiated by low energy laser prior to injury, probably by activation (stimulation of proliferation and/or differentiation) cells in the muscles that are "recruited" and participate in the process of regeneration.**

Key words: Amphibia, Skeletal muscle, Regeneration, Denervation, Low energy laser, Histomorphometry

The process of skeletal muscle regeneration in mammals has been well documented and reviewed (Allbrook, 1981; Carlson and Faulkner, 1983). It is well established that the dormant satellite cells in the skeletal muscles are stimulated after injury to proliferate and consequently give rise to the newly formed muscle fibers in the injured site during muscle regeneration. Although Bischoff (1986a,b) has reported a mitogen from crushed skeletal muscles that stimulates satellite cells in vitro, and various growth factors have been suggested to play a part in muscle regeneration (White

Received December 14, 1993; accepted July 5, 1994.

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and Esser, 1989; Bischoff, 1990), the mechanisms associated with stimulation of satellite cells to proliferate remain to be elucidated.

It has long been observed that satellite cells can also be stimulated to proliferate by denervation (Reznik, 1976). According to Aloisi et al. (1973), 3 days after denervation the percentage of satellite cells increased from 4.5% to 8.6% and 1.7% to 9.1% in the soleus and extensor digitorum longus muscles, respectively. Ontell (1974) calculated that satellite cells rose from 2% to 12% in the tibialis anterior of the rat 3 weeks after denervation. Murray and Robbins (1982a,b) investigated more thoroughly the phenomenon of cell proliferation in denervated skeletal muscles of mice. They showed that H^3-thymidine incorporation increased twentyfold 4 days following denervation and proposed that the proliferation is related to nerve or muscle mitogen and is not a response to simple disuse of the muscle. Furthermore, it was proposed that the cells involved in the postdenervation events represent a limited regenerative response. The association of the proliferative response of cells postdenervation with the regeneration process of denervated muscle is a phenomenon not yet studied. It is known that regeneration also takes place in denervated muscle, but maturation of newly formed regenerated muscle fibers does not occur in the absence of innervation (Gulati, 1988; Carraro et al., 1989).

Regeneration in amphibian muscles has been studied previously, and satellite cells were first discovered in these muscles by Mauro (1961). Later on Trupin (1976) found the frequency of satellite cells in anuran skeletal muscles to be approximately 2.7% and probably sufficient to account for regeneration after injury in these muscles. Maruenda and Franzini-Armstrong (1978) in a more detailed study investigated satellite cells and the so-called invasive cells in amphibian muscles. They also found that in denervated muscles for 11–61 days there was no change in the content of the satellite cells, but in the overall population of satellite and invasive cells there was a tendency to increase with time after denervation.

Low energy lasers have recently been found to modulate and accelerate physiological processes like cell proliferation and ATP production in various biological systems (Belkin et al., 1988; Karu, 1989; Galletti, 1992). It has also been demonstrated that low energy laser irradiation can affect regeneration processes, such as acceleration of wound healing (Mester et al., 1973), and cause a significant increase in the amplitude of the compound action potential after crush injury to peripheral nerves (Rochkind et al., 1987). We have recently shown that the process of skeletal muscle regeneration following injury is markedly promoted in mammals and amphibians by He-Ne laser irradiation (Weiss and Oron, 1992; Bibikova and Oron, 1993, in press).

In the present study we have compared the kinetics of the regeneration process in denervated and innervated muscles and investigated the possibility that low energy laser irradiation can activate cells in denervated toad muscles that may participate in the regeneration process and enhance the regeneration process following injury. Comparison of the rate of regeneration in the injured area was performed using quantitative histomorphometric methods. Thus the volume fraction (volume occupied by a certain histological structure out of total volume) of mononucleated cells, myotubes, and young myofibres in the injured zone served as a quantitative criterion of the extent of regeneration during a specific time period.

MATERIALS AND METHODS

Experimental Procedure and Laser Irradiation

Mature (30–35 g body weight) male toads (*Bufo viridis*) kept at 20°C ± 1°C and housed as described previously (Bibikova and Oron, 1993) were used for the experiments. Anaesthesia for surgery and laser irradiation was performed by immersing the toads in a 0.15 w/v solution of Tricain methan sulphonate (Sigma, St. Louis, MO) in tap water for 5 min. All the surgical procedures and laser irradiations (see below) were performed under sterile conditions, and the toads were immersed, following these procedures, in tap water containing 1.4 mg/l Gentamycin (Teva, Israel) for 1 h. Denervation was performed by cutting the skin at the posterior region of the pelvic girdle, exposing the ischiatic nerve in one leg (randomly selected), and creating a gap of about 1 cm in it, approximately 1 cm from its origin from the spinal cord. Seven experimental toads received a single laser irradiation (Bibikova and Oron, in press) at the location in the gastrocnemius muscle to be injured (see below) at the seventh day postdenervation. The location of irradiation in the gastrocnemius muscle was precisely identified and irradiated by four adjacent laser irradiations to give a square region of about 4 mm × 4 mm in the central and lateral part of the gastrocnemius muscle prior to the injury. Laser irradiation was performed using He-Ne laser (Holliston, MA) operating at 632.8 nm (6.0 mW; 1.9 mm beam diameter) for 2.3 min (31.2 J/cm^2). The control group (seven toads) underwent the same experimental procedure as that of the experimental toads but were subjected to red light (660 nm; 0.4 J/cm^2) instead of laser irradiation. In preliminary experiments, we tested the possible adverse effects of the above irradiation on normal and developing gastrocnemius muscle. The laser irradiation did not cause any change in creatine phosphokinase (CPK) enzymatic activity in rat or toad gastrocnemius muscle. Furthermore, there was no effect on CPK enzymatic activity in developing muscle in rats during the period of 1–5 days postnatal. Histological examination of the irradiated muscles did not show any pathological changes (vacuolization, inflammation, etc.) in the muscles. The cold injury to all muscles (control and experimental) was performed 9 days after denervation by placing a copper rod (1.8 mm in diameter), prechilled in liquid nitrogen, for 10 seconds against the area that had been previously irradiated (by laser or red light) in the gastrocnemius muscle. Fourteen days following cold injury, all the toads were sacrificed by an overdose of chloroform, and the injured zones were identified, excised, and immediately immersed in Bouin's fixative. The above experimental procedure (timing of irradiations, injury after denervation, and stage to be analyzed after injury) was determined after a series of preliminary experiments.

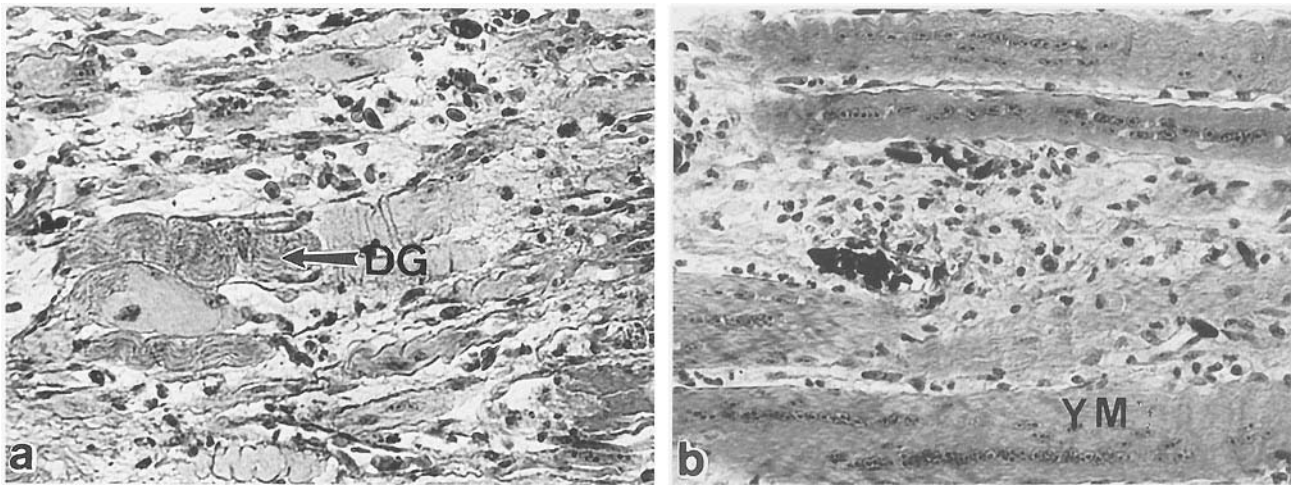

Fig. 1. Light micrographs of regenerated zones in the toad gastrocnemius muscle at 9 (**a**) and 14 (**b**) days postinjury. Note degenerative muscle fibres (DG) with dispersed cytoplasm in a and young myofibres (YM) with central nuclei and organized contractile proteins in b. Masson's trichrome. ×220.

Morphometric Analysis and Statistics

The injured area and vicinity were fixed in Bouin's fixative for 24 h, dehydrated, and embedded in paraffin. Eight micrometer serial longitudinal sections were prepared and stained with hematoxylin-eosin or Masson's trichrome stain.

The number of nuclei (within the basal lamina) per unit area in the muscle fibers of sections of intact muscles and denervated muscles (14 days postdenervation) was calculated in six randomly selected sections (0.15 mm^2 each) from each muscle (six intact and six denervated) at a final magnification of 400.

The characteristics of the mononucleated cells were verified by visualization of 80–120 cells in each of the two sections (randomly selected) in each rat (five control or laser irradiated) under ×400 or ×1,000 magnification. A total of 1,900 and 1,450 cells were counted in the control and laser irradiated rats, respectively. Cells were identified as fibroblast/myoblast according to their shape; endothelial cells were traced according to shape and formation of new blood vessels; macrophages, leukocytes, and lymphocytes were traced according to their shape and nuclei; pigment cells were identified by the melanin contamination.

Morphometric analysis was performed on the entire injured zone. Mononucleated cells were defined as all cells with a single nucleus in the regenerated area. Degenerated myofibres were myofibres with a diameter similar to mature myofibre (larger than young myofibres); nuclei were usually dispersed or absent, and the cytoplasm did not show the organization of striation (Fig. 1a). Myotubes were identified by their low diameter relative to young myofibres and their central nuclei and basophylic cytoplasm. Young myofibres were characterized by larger diameter (about fivefold the diameter of control nuclei) than myotubes, central nuclei, and more eosinophylic cytoplasm (Fig. 1b). Three level nested analysis of variance (Sokal and Rohlf, 1981) was carried out on the morphometric results of a preliminary experiment in order to determine the number of slides and number of sections in each slide to be taken from each muscle according to the magnitude of variability of the structures within the sections on each slide, all slides, the animals, and among the control/treated group. In this preliminary experiment, six injured zones (three slides and two sections per slide) were randomly chosen and analyzed from each muscle (three control muscles and three laser irradiated muscles). The results of the three level nested analysis of variance revealed variance components (in %) as follows: 10% between sections within the same microscopic slide; 13% among slides taken from the same muscle; 7% among the muscles (toads) of the same group (control or experimental); and 70% between control and experimental muscles. According to this test, six injured zones (from three microscopic slides and two sections per slide) were randomly chosen for morphometric analysis. The sections were viewed through a microscope equipped with a videoscan, and the volume fraction (percent of the total volume of the injured zone) of each histological structure (myotubes, young myofibres, mononucleated cells) was calculated using the point-counting method (Weibel, 1980). The above sampling procedure from the total sections and toads was used following three level nested ANOVA as described by us previously (Bibikova and Oron, 1993). The results obtained from the morphometric analysis were finally statistically analyzed using ANOVA test with statistical significance level of $P < 0.05$ (Sokal and Rohlf, 1981).

RESULTS

The number of nuclei per unit area in the uninjured region of the denervated (14 days postdenervation) muscle was 65 ± 2, which was significantly ($P < 0.05$) higher than its value (47 ± 2) in normal uninjured gastrocnemius muscle (Fig. 2). The relative frequencies of the various cell types that were considered as mononucleated cells in the above injured zones are presented in Table 1. Fibroblasts and myoblasts comprised 29 ±

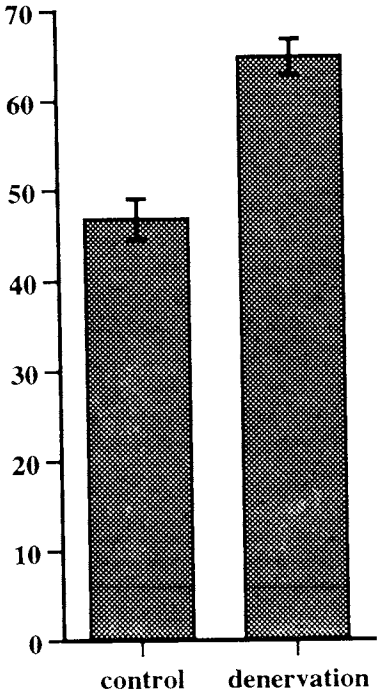

Fig. 2. Number of nuclei (in 0.15 mm^2 of area section) within myofibres of a control and denervated (14 days postdenervation) gastrocnemius muscle. Results are presented as mean ± S.E.M. of six areas that were analyzed in each of the six muscles (control or denervated) used for the experiment. *$P < 0.05$.

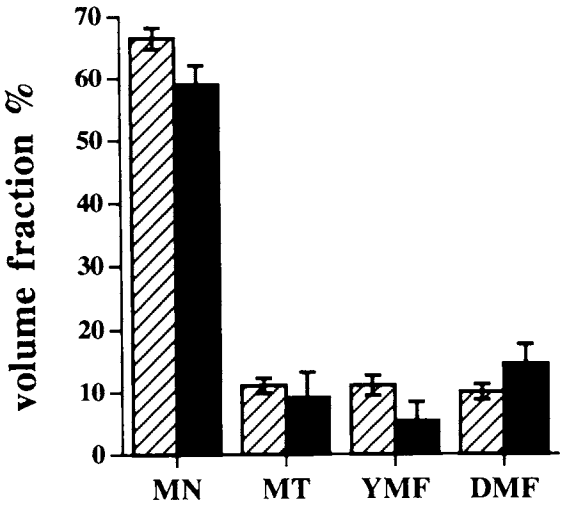

Fig. 3. Histograms of volume fractions of mononucleated cells (MN), myotubes (MT), young myofibres (YMF), and degenerated muscle fibers (DMF) in the injured zones of denervated (dashed columns) and innervated control (solid columns) gastrocnemius muscle 14 days postinjury. The characteristics of the mononucleated cells are detailed in Table 1. The results are mean ± S.E. from ten control sham operated toads and seven toads that were denervated 9 days prior to injury.

7% of the cells in the denervated and injured muscles, which tended to be higher than the frequency of these cells in the denervated, laser irradiated, and then injured muscles. Most of the cells in the regenerating zones of the denervated muscles were leukocytes and macrophages, with a similar relative frequency of these cells in the laser irradiated and nonirradiated (control) muscles. The relative frequency of the endothelial cells was significantly higher ($P < 0.05$) in the regenerating zone of the denervated and then laser irradiated muscles as compared to the nonirradiated denervated muscles. Figure 3 represents the comparison between the volume fraction of the various cytological structures of control innervated muscle regenerate and a denervated (9 days prior to injury) muscle regenerate 14 days postinjury. There was no statistical difference between the volume fraction of the various cytological structures of the control and denervated muscle regenerates.

There were differences in the volume fraction (percent of total injured area) of the various histological structures in the regenerated area between the control muscles that were denervated and injured and those that were denervated, irradiated with laser, and then injured (Fig. 4). The mononucleated cells occupied most of the injured area at 14 days post–cold injury. In the control muscles, their volume fraction comprised 67 ± 2%, but in the laser irradiated muscles their volume fraction was significantly ($P < 0.01$) lower than control, comprising 49 ± 4% of the injured area. The young myofibres populated 34 ± 4% of the area in those muscles that were irradiated with laser prior to the injury. This value was 2.8-fold significantly ($P < 0.01$) higher than control injured muscles. The rest of the injured area was occupied by degenerating myofibres which did not show a significant difference between control and experimental muscles and myotubes. The volume fraction of the myotubes in the laser irradiated muscles was significantly ($P < 0.01$) lower than in control.

DISCUSSION

The results of the present study clearly indicate that a single irradiation of denervated amphibian skeletal muscles with low energy laser causes a marked enhancement of the process of muscle regeneration in injured muscles. In our experimental model, an approximate threefold significant increase in the volume fraction of the young myofibres (providing a good indication for the rate of maturation of myogenic structures in the injured area) was observed in the muscles that were laser irradiated prior to injury as compared to control, nonirradiated muscles. The fact that following denervation there is an increase in the cells (satellite cells) in the denervated muscle, as shown in this study and also by others (Ontell, 1979; Murray and Robbins, 1982a,b), supports the hypothesis that more cells may be stimulated by the single laser irradiation and "recruited" for regeneration when the muscle is injured. Indeed, we have recently shown (Bibikova and Oron, in press) that in amphibian muscles a single irradiation was sufficient to enhance the process of muscle regeneration. We have also shown previously that regeneration in toad and rat gastrocnemius muscles is enhanced by the same laser (He-Ne) irradiation used in this study (Weiss and Oron, 1992; Bibikova and Oron, 1993). Thus it may be hypothesized that the laser irradiation increases the rate of proliferation or the rate

TABLE 1. Relative frequency (% of total cells counted) of cell types in the regenerating zones of denervated and denervated, laser irradiated muscles 14 days following injury[1]

Cell type	Control muscles (M ± SEM)	Laser irradiated muscles (M ± SEM)
Fibroblasts/myoblasts	29 ± 7	20 ± 3
Endothelial cells	19 ± 2	32* ± 4
Macrophages	13 ± 3	10 ± 1
Leukocytes (other than lymphocytes)	17 ± 2	16 ± 2
Lymphocytes	15 ± 6	19 ± 3
Pigment cells	7 ± 6	3 ± 1

[1]Results are expressed as percentage (mean ± SEM) of each cell type out of the total cells counted, as detailed in Materials and Methods. The results were statistically analyzed using ANOVA.
*Statistically different ($P < 0.05$) from control.

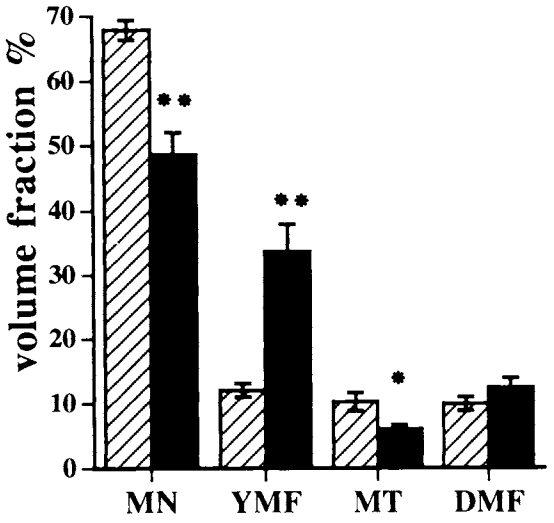

Fig. 4. Histograms of volume fractions of mononucleated cells (MN), myotubes (MT), young myofibres (YMF), and degenerated muscle fibers (DMF) in the injured zones (14 days postinjury) of denervated (dashed columns) and denervated muscles that were laser irradiated 2 days prior to injury (solid columns). Results are mean ± S.E. of 7–10 toads. Levels of statistical difference between groups are $*P < 0.05$ or $**P < 0.01$.

of differentiation (Belkin et al., 1989; Karu, 1989), or both, in certain cells within the denervated muscles. Thereafter, when the muscle is injured, these cells, the origin of which has not been defined in this study, most probably take part in the regeneration process in conjunction with other cells that participate in the formation of new muscle fibres in the injured amphibian muscle. Indeed, it was suggested by Grounds (1990) that cells, even of nonmuscle origin (particularly mesodermal), might well develop into muscle precursor cells in vivo in situations in which normal muscle tissue architecture and communication are severely disrupted. Such conditions in regenerating muscle might induce the expression of muscle differentiation gene in nonmuscle mononuclear cells and in this way recruit additional muscle precursors.

The "beneficial" effect of denervation on muscle transplantation has also been shown in previous studies. It was demonstrated that by denervating muscles prior to transplantation, it is possible to enhance muscle regeneration in the transplanted muscle (Carlson and Gutman, 1975, 1976; Carlson and Faulkner, 1983). Coan and Tomanek (1981) showed that enhancement of growth of muscle transplants can be achieved by ablation of the synergist muscle, suggesting that overload may be another factor that promotes the regenerative response of the muscle.

In the present study we were also able to show that there is a significant increase in the number of nuclei per unit area in the amphibian muscle fibres even as late as 14 days after denervation, as compared to control innervated muscle. This fact suggests that in these muscles, as in mammalian muscles (Reznik, 1976; Robbins, 1982a,b), there may be a relatively long phase of proliferative response of cells following denervation. The differential analysis of the mononucleated cells of denervated and injured muscles revealed that there was still a significant amount of leukocytes and macrophages in the regenerating zone, indicating that the inflammatory response is evident even at this stage in the denervated muscles as well as in the denervated muscles that were laser irradiated prior to injury. The relative frequency of the endothelial cells in the regenerating zone of the denervated and laser irradiated muscles was 1.6-fold, significantly higher than the denervated and nonirradiated muscles, indicating a higher degree of neoformation of blood vessels in these muscles. This phenomenon could account, among other factors, for the enhancement in maturation of newly formed muscle fibers in the regenerating zone of these muscles due to a better supply of oxygen and nutrients compared to the nonirradiated ones.

Another outcome of the present study is the comparison of rate of regeneration in innervated and denervated gastrocnemius muscles in amphibians. The histomorphometric data indicate that the process of regeneration, at least up to 14 days postinjury, can proceed at the same rate in innervated muscles and in muscles that were denervated even 9 days prior to the injury. Since at 14 days postinjury to the toad gastrocnemius muscle most of the regenerated area is populated with mononucleated cells, some myotubes, and a very few young myofibres, it can be concluded that early phase of regeneration is not dependent on innervation. Thus, the phenomenon that myotubes are formed in muscle regenerated even after long-term denervation, as found previously for mammalian muscles (Carlson and Gutmann, 1975; Carraro et al., 1983; Gulati, 1988), has been confirmed in the present study by quantitative measurements and is valid also for amphibian muscles. However, it may be assumed that maturation of newly formed myofibres may be dependent on innervation, as was previously found for mammalian muscles (Gulati, 1988).

ACKNOWLEDGMENTS

The authors thank Mrs. C. Shapiro for typing the manuscript. This study was partially supported in part by the George S. Wise post-doctoral fellowship for Dr. Bibikova.

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