SKELETAL MUSCLE REGENERATION AFTER MYONECROSIS INDUCED BY CRUDE VENOM AND A MYOTOXIN FROM THE SNAKE BOTHROPS ASPER (FER-DE-LANCE)

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J. M. Gutiérrez, C. L. Ownby and G. V. Odell. Skeletal muscle regeneration after myonecrosis induced by crude venom and a myotoxin from the snake Bothrops asper (Fer-de-Lance). Toxicon 22, 719 – 731, 1984. — Skeletal muscle regeneration was studied following injections of Bothrops asper venom and a myotoxin isolated from the crude venom. In toxin-injected muscle regeneration proceeded normally. By 4 days there were myotubes and small regenerating cells. The size of the cells increased by 1 and 2 weeks, and by 4 weeks regenerating cells were fully developed. The regenerated cells retained centrally located nuclei. The regenerative process in venom-injected muscle was not completely normal — by 1 and 2 weeks four main areas, based on the predominant cell type present, were observed in the tissue: (a) necrotic muscle cells; (b) regenerating muscle cells; (c) fibroblasts and collagen; (d) adipocytes. Furthermore, some nerve fibers were demyelinated. Samples obtained 4 weeks after venom injection showed an almost complete regeneration in many areas, whereas in other areas nests of small regenerating cells were surrounded by portions of adipose tissue and collagen. At four weeks regenerating cells in venom-injected muscle were significantly smaller than cells in toxin-injected and saline-injected muscles. There was a significant reduction in capillary/muscle cell ratio in areas of the muscle where hemorrhage and myonecrosis were present 30 min after injection of B. asper venom. Since B. asper venom drastically affects the microvasculature, it is proposed that impairment of regeneration after injection of crude venom is a consequence of diminished blood supply to some areas of the muscle.

INTRODUCTION

REGENERATION of skeletal muscle constitutes a well-described phenomenon observed after several types of injury (Allbrook, 1981). In some cases regeneration proceeds to completion, and there is no functional loss of muscle, e.g. after bupivacaine-induced myotoxicity (Nonaka et al., 1983). In other examples of muscle disease, however, regeneration is impaired and the damaged muscle is substituted by adipose tissue and fibrosis, e.g. in older patients with Duchenne muscular dystrophy (Lipton, 1979). It has been demonstrated that skeletal muscle regeneration proceeds to completion when several requirements are met. These requirements include adequate blood supply, presence of functional innervation, and presence of an intact basal lamina (Allbrook, 1981).

Many snake venoms induce myonecrosis (Homma and Tu, 1971; Ownby, 1982). Experimental studies with the elapine toxins notexin and taipoxin, as well as with the venom of Oxyuranus scutellatus, have shown that they induce a prominent myotoxic effect after which regeneration proceeds rapidly and is completed by one month (Harris et al., 1980; Harris and Maltin, 1982; Maltin et al., 1983). However, venoms from pit
Vipers (family Crotalidae) induce a more complex picture of local effects since they affect the microvasculature in addition to being myotoxic (Homma and Tu, 1971; Ownby, 1982; Mebs et al., 1983). It has been shown clinically that in these cases regeneration is somehow impaired, tissue loss occurs frequently, and amputation is often necessary (Okonogi et al., 1964; Rosenfeld, 1971; Ownby, 1982).

Bothrops asper, a crotaline snake, is responsible for most snakebite cases in Central America (Bolaños, 1982). Its venom induces prominent local effects which are only partially neutralized by polyvalent antivenom (Gutiérrez et al., 1980, 1981). It has been described in the clinical literature that tissue loss is a consequence of B. asper poisonings (Picado, 1931; Jiménez and García, 1969). Recently, a myotoxin was isolated from this venom (Gutiérrez et al., 1984a) and its mode of action on skeletal muscle was studied (Gutiérrez et al., 1984b). In order to gain a better understanding of the phenomenon of tissue loss after B. asper venom injection, we have investigated the characteristics of the regenerative process that takes place in skeletal muscle after injections of B. asper venom or myotoxin in mice.

MATERIALS AND METHODS

Venom and toxin

Bothrops asper venom was a generous gift from Dr. Luis Cerdás, Instituto Clodomiro Picado, Universidad de Costa Rica. The venom is a pool obtained from more than 50 specimens collected in the Atlantic slopes of Costa Rica. Myotoxin was isolated as described by Gutiérrez et al. (1984a). Homogeneity was demonstrated by disc-polyacrylamide gel electrophoresis (pH 4.3) as previously described (Gutiérrez et al., 1984a).

Histological and ultrastructural studies

Groups of 4 mice (Charles River, CD-1) weighing 20 ± 2 g were injected i.m. dorsolaterally in the thigh with venom (2.5 μg/g), toxin (2.5 μg/g) or physiologic saline solution. At 5 time intervals (2 days, 4 days, 1 week, 2 weeks, and 4 weeks) mice were killed by cervical dislocation and a sample of muscle obtained from the ventromedial aspect of the thigh. The tissue was processed as previously described by Ownby et al. (1976). Thick sections were stained with Mallory’s trichrome, and thin sections (silver to light gold) were stained with methanolic uranyl acetate and lead citrate, and examined in a Philips EM 200 electron microscope. A total of 52 mice were used.

Measurement of cell diameters

The diameter of regenerating muscle cells was measured using a Zeiss light microscope equipped with a Zeiss MOP 3 Image Analyzer in samples obtained 1 month after injection. The tissue was oriented to provide cross-sections of muscle cells, and the smallest diameter of muscle cells was determined. A regenerating cell was defined as a muscle cell with a centrally located nucleus. A total of 1000 cells were measured in each experimental group, and the mean and SE of each group were determined. To determine the significance of the differences observed a t test was used comparing the calculated value to the Z table because of the large sample size.

Estimation of capillary/muscle cell ratio

In order to study the alterations induced by crude B. asper venom in the microvasculature, the capillary/muscle cell ratio was estimated in samples obtained 30 min after injection, as compared with control mice injected with physiologic saline solution. The ratio was estimated in envenomated muscle only in areas that had been affected by the venom (i.e. areas that showed either myonecrosis or hemorrhage). Also, to study the revascularization of damaged muscle, the ratio was estimated in samples obtained 1 month after injection. A blood vessel was counted as a capillary when it had a well-defined lumen and a diameter between 5 and 10 μm (Simionescu and Simionescu, 1983). Four sections of tissue were studied from each mouse; in each section the total number of capillaries and skeletal muscle cells were counted using a Zeiss light microscope equipped with a Zeiss MOP 3 Image Analyzer. For each experimental group, the mean and S.E. were determined, and the Student’s t test was used to determine the significance of the differences observed.

RESULTS

Regeneration after toxin-induced myonecrosis

Forty-eight hr after toxin injection there was a widespread necrosis of skeletal muscle cells. An active process of phagocytosis was indicated by the presence of many phagocytes in the interstitial connective tissue, as well as inside necrotic muscle cells. The basal lamina
Fig. 1. Light micrographs of thick sections of muscle taken after injection of myotoxin (2.5 μg/g).

(A) Four days. Small regenerating muscle cells (R) are observed within the basal lamina of necrotic muscle cells (N). (B) Two weeks. Regenerating muscle cells (R) with centrally-located nuclei are observed.
Fig. 2. Electron micrographs of portions of regenerating muscle cells one week (A) and two weeks (B) after injection of myotoxin (2.5 μg/g).

(A) Myofibrils (My) appear in an early stage of organization. Mitochondria and ribosomes are abundant. Note the presence of sarcoplasmic reticulum (SR) and membrane whorl (arrow).

(B) Myofibrils are organized into sarcomeres (S), and the nucleus (Nu) is centrally located. The basal lamina and plasma membrane appear intact. Note the presence of a Golgi complex (G) as well as mitochondria (M).
FIG. 3. LIGHT MICROGRAPHS OF THICK SECTIONS OF MUSCLE TAKEN ONE WEEK AFTER INJECTION OF B. asper VENOM (2.5 μg/g).

(A) Area of necrotic muscle cells (N). All the cells are necrotic, with disorganization of myofibrils. (B) Area of regenerating muscle cells. Note that regenerating cells (R) have centrally-located nuclei. (C) Area of fibroblasts and collagen. Note the numerous fibroblasts (F) and collagen fibers in contrast to the small number of regenerating muscle cells (R). Also note the presence of three nerves (one indicated by arrows) in which axons are demyelinated. (D) Area of adipocytes. Some adipocytes (Ad) contain large lipid inclusions. There are also many adipocyte precursors (arrows) which contain several small lipid inclusions in their cytoplasm.
Fig. 4. Electron micrographs obtained after injection of B. asper venom (2.5 μg/g).

(A) Area of fibroblasts and collagen, 2 weeks after injection. Note the presence of abundant collagen fibers (arrows) and a portion of a fibroblast (F). (B) Muscle one week after injection. This adipocyte precursor contains many small lipid inclusions (L) of variable size. The nucleus (Nu) with peripheral chromatin is observed.
FIG. 5. LIGHT MICROGRAPHS OF THICK SECTIONS OF MUSCLE TAKEN 4 WEEKS AFTER INJECTION OF B. asper VENOM (2.5 μg/g).

(A) Small regenerating cells (R) are surrounded by a dense fibrotic area in which many fibroblasts (F) are present. (B) A nest of small regenerating muscle cells (outlined by arrows) is surrounded by normal unilocular adipocytes (NA).
remained apparently intact in necrotic muscle cells. The presence of many small, regenerating cells located within the basal lamina of necrotic cells at 4 days indicated that regeneration was underway (Fig. 1a). At this time myotubes were evident in longitudinal sections. By 1 and 2 weeks there were many regenerating cells with central nuclei (Fig. 1b). These cells contained normal Golgi complexes, mitochondria, sarcoplasmic reticulum, and T tubules (Fig. 2). The plasma membrane and basal lamina were intact. In some cells there were membrane whorls near the plasma membrane. Myofibrils were observed in different stages of development. Some cells had short, randomly dispersed myofibrils that may have been undergoing a process of assembly (Fig. 2a). Other cells were in a more advanced stage of myofibrillar organization, with longitudinally oriented bundles of myofilaments and with a normal alignment of sarcomeres (Fig. 2b). Regenerating cells contained many ribosomes, either free or as polyribosomes. At the fourth week muscle cells had all the characteristics of mature cells, with the exception of centrally located nuclei. Nerves and blood vessels remained intact throughout the process of regeneration, and the amount of interstitial collagen did not increase significantly.

Regeneration after venom-induced myonecrosis

By 48 hr abundant phagocytes were located inside necrotic muscle cells. Four days after injection there were many fibroblasts in the interstitial connective tissue. Small myotubes were located within the basal lamina of necrotic cells, indicating that regeneration was taking place. However, the process of regeneration after venom-induced myonecrosis did not show the same characteristics observed after toxin inoculation. Observations made at 1 and 2 weeks indicated that there were four main areas based on the predominant cell type present: (a) necrotic muscle cells; (b) regenerating muscle cells; (c) fibroblasts and collagen; (d) adipocytes (Fig. 3). There were groups of necrotic muscle cells which lacked organized myofibrils (Fig. 3a). At the light microscopic level regenerating muscle cells had centrally-located nuclei (Fig. 3b). At the electron microscopic level there were many free ribosomes and polyribosomes in the cytoplasm, and the myofibrils showed various degrees of development. In some cells there were bundles of myofibrils which were apparently undergoing synthesis and longitudinal assembly, whereas in other cells the myofibrils had the typical fully-developed striated pattern. Thus, these cells had the same morphologic characteristics described for regenerating cells after toxin-induced myonecrosis (see Fig. 2).

There were areas in which fibroblasts were the predominant cell type (Fig. 3c). In these areas there were isolated regenerating muscle cells which were usually of small diameter and surrounded by fibroblasts and dense bundles of collagen. These areas contained nerves in which nerve fibers were demyelinated (Fig. 3c). At the electron microscopic level there was a well-developed granular endoplasmic reticulum was observed in the fibroblasts, as well as abundant collagen fibers (Fig. 4a). Many fibroblasts had a ruffled surface with cellular projections in many directions.

At the light microscopic level areas of adipocytes contained cells with their cytoplasm filled with large lipid inclusions, as well as cells which resembled adipocyte precursors (Fig. 3d). Other cells had a larger lipid droplet with a rim of cytoplasm surrounding it. These cells had an ovoid shape and a peripherally located nucleus (Fig. 3d). Thus, they presented the characteristic features of mature adipocytes (GREENWOOD and JOHNSON, 1983). Ultrastructurally, these cells contained few mitochondria and scanty endoplasmic reticulum, and the lipid inclusions present in the precursor cells were of different sizes (Fig. 4b).
Samples obtained 4 weeks after venom injection showed an almost complete regeneration in most of the areas examined. However, in some zones regenerated cells were small. Moreover, in other areas there was an increase in collagen in the interstitial space (Fig. 5a) and nests of small regenerating cells were surrounded by adipose tissue and collagen (Fig. 5b).

**Measurements of cell diameters**

The diameter of regenerating cells in samples obtained 4 weeks after injection of crude venom (14.9 μm ± 0.3; n = 1000) was significantly smaller (P < 0.001) than the diameter of regenerating cells in mice injected with toxin (48.1 μm ± 0.2; n = 1000) and the diameter of normal muscle cells (46.6 μm ± 0.2; n = 1000) (Fig. 6). On the other hand, no significant difference (P > 0.01) was observed between the diameter of regenerating muscle cells in mice injected with toxin and the diameter of control muscle cells (Fig. 6).

**Estimation of capillary/muscle cell ratio**

The capillary/muscle cell ratio in muscle injected with physiologic saline solution was 1.45 ± 0.04 (n = 4). This is very similar to the value of 1.49 ± 0.07 obtained for the same ratio in a variety of muscles from 5 mammalian species (PLYLEY and GROOM, 1975). In contrast, the capillary/muscle cell ratio in areas of hemorrhage and/or myonecrosis present in samples taken 30 min after injection was significantly lower (0.30 ± 0.06; P < 0.01). This corroborates an earlier finding which indicates that *B. asper* venom induces a hemorrhagic effect as early as 30 min after injection (GUTIÉRREZ et al., 1984b). When the ratio was estimated in samples obtained 4 weeks after venom injection a value of 0.85 ± 0.09 (n = 4) was obtained. This indicates that there was revascularization; however, the ratio at 4 weeks was still significantly lower (P < 0.01) than that from control muscle.
DISCUSSION

In our experimental conditions, i.e. using a dose of 2.5 μg/g, regeneration after toxin-induced myonecrosis corresponds closely with normal regeneration (ALLBROOK, 1981), whereas in mice injected with crude venom regeneration was not normal. In muscle taken from mice injected with crude venom there were areas of fibrosis and adipocytes as well as areas of regeneration in which the diameter of regenerating muscle cells was abnormally small.

In order for muscle regeneration to proceed normally, several requirements must be met: (a) adequate blood flow to the tissue (ALLBROOK, 1981); (b) the presence of an intact basal lamina which plays the role of a scaffold for regeneration (VRACKO and BENDITT, 1972); (c) adequate innervation to muscle (HALL-CRAGGS and SEYAN, 1975). It has been shown that B. asper myotoxin does not affect the integrity of blood vessels, nerves, or basal laminae (GUTIÉRREZ et al., 1984b); this may explain why regeneration proceeded in the normal way after myotoxin injection. This observation is consistent with other studies which showed that regeneration after myonecrosis induced by the elapine toxins notexin (HARRIS et al., 1980) and taipoxin (HARRIS and MALTIN, 1982; MALTIN et al., 1983) proceeded rapidly and was completed by one month. Similar findings were described in the case of myotoxicity induced by the local anesthetic bupivacaine (NONAKA et al., 1983). It is significant that in all these cases there was no damage to the microvasculature, and that the muscle fiber basal laminae were also preserved.

After the injection of crude B. asper venom there was prominent hemorrhage (GUTIÉRREZ et al., 1984b). Our results show that there was a significant decrease in the capillary/muscle cell ratio in samples taken as early as 30 min after injection. This would obviously affect the blood supply to the regenerating muscle. However, in tissue examined 4 weeks after venom injection there was a partial revascularization of bundles of muscle cells. It has been shown that after transplantation of skeletal muscle most blood vessels degenerate initially, but revascularization takes place due to vessel growth either from the host tissue (HANSEN-SMITH et al., 1980) or from surviving vessels in the graft (FAULKNER et al., 1983). Nevertheless, it is possible that in our experimental conditions revascularization after venom injection is not complete by the time critical steps in muscle regeneration are taking place. Thus, in some areas there may be a functional ischemia during regeneration. Furthermore, the increased collagen deposition and fibrosis observed in some areas may affect the diffusion of oxygen to regenerating cells. In the case of Duchenne muscular dystrophy, it has been proposed that the decrease in muscle regeneration observed in older patients may be due to a progressive accumulation of interstitial collagen which would affect oxygen diffusion and create local ischemia (LIPTON, 1979). In muscle samples obtained after venom injection there were alterations in peripheral nerves; although the basis for this damage was not studied, this may be an additional factor that interferes with normal regeneration. HARRIS et al. (1975) observed that the diameter of regenerating skeletal muscle cells was small in denervated muscle as compared with normally innervated muscle.

In conclusion, this study shows that muscle regeneration does take place after myonecrosis induced by injections of B. asper venom and myotoxin, despite that fact that it is partially impaired when using the crude venom. These observations bring into question the effectiveness of therapeutic devices based on the debridement of necrotic tissue after snake-bite accidents, since such procedures probably eliminate the myogenic cells along with the necrotic tissue. Our findings also suggest that regeneration is partially impaired in muscle injected with venom perhaps as a consequence of diminished blood
supply to some areas due to the action of venom components on the microvasculature.

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REFERENCES


