Skeletal Muscle Regeneration in Chronic Critical Limb Ischemia

Zygmunt Mackiewicz[1, 2], Arvydas Rimkevičius[2], Elżbieta Dudek[1], Yrjö T. Konttinen[3, 4]

[1] *Department of Cell Biology, University of Opole, Poland*
[2] *Vilnius University Institute of Experimental and Clinical Medicine, Lithuania*
[3] *Department of Medicine/ invärtes medicin, Helsinki University Hospital,*
[4] *ORTON Research Institute and the Ortopaedic Hospital of the Invalid Foundation, Helsinki, Finland*

Skeletal muscle specimens from ten amputated atherosclerosis- and/or diabetes mellitus- damaged limbs and five similar biopsies from acute trauma patients (controls) were subjected to histological and immunohistochemical analysis.

A widespread skeletal muscle fiber atrophy, dystrophy and fibrolysis in parallel to satellite cell proliferation and myogenic tube formation were found in the same area or in adjacent ischemia-damaged areas. Different phases of muscle degradation and regeneration in the same area were observed. As a rule, the regeneration process was not finalised and resulted in smaller or larger fields of fibrosis. No histological signs of inflammation in the zones of intensive formation of myotubes were registered.

Since in amputated ischemic human limbs signs of the continuous muscle regeneration process seen always have been, we conclude that the biological indications for surgical limb revascularization still existed. Limb amputation probably should be practiced more rarely than it is done nowadays.

Key words: skeletal muscle, ischemia, regeneration

INTRODUCTION

Peripheral arterial ischemia is an increasingly important problem in rapidly aging population. Ischemia-induced ulcer or gangrene in the lower extremity is not uncommon in the aging people. Claudication and muscle pain at rest are common symptoms of limb arterial occlusions. Major amputation is no longer an easily acceptable treatment without prior assessment for a limb salvage procedure, often surgical revascularization. The final results of revascularization depend also on the functional and structural state of chronically ischemia-damaged muscles. The choice of treatment is a challenging problem in traumatology and orthopaedic surgery. No real guidelines for treatment of muscle ischemia exist.

Skeletal muscles of adult mammals after injury and ischemia (1), are capable of regeneration although very slow and not always with complete functional recovery. If a muscle does not receive blood supply, necrosis occurs. Nevertheless, even in some necrotic zones the regeneration capacity of a muscle can persist. Usually the damage–regeneration process consists of three phases: necrosis, revascularization, and remodeling (2). The injured muscle undergoes a rapid process of skeletal muscle regeneration, which is hindered by the development of scar tissue. Injury- and ischemia-induced regeneration in a skeletal muscle is due to satellite cell functions *in vivo* (3). Quiescent myogenic stem cells (satellite cells) are ischemia/hypoxia activated to reenter the cell cycle and to differentiate for repair of damaged myofibers, thereby recapitulating the features of myogenesis during embryonic development (4). The capacity for tissue repair is crucially conferred by satellite cells located between the basal lamina and sarcolemma of mature myofibers (5, 6).

Limb preservation should be the main goal in most patients with chronic critical limb ischemia. The feasibility of revascularization is determined by arteriographic findings as well as the availability of a bypass conduit. The state of skeletal muscle viability is very important.

Correspondence to: Zygmunt Mackiewicz, Department of Cell Biology, University of Opole, 4 Kominka Street, 45-035 Opole, Poland. E-mail: zmackiewicz@uni.opole.pl

The objective of the study was to analyze the state of skeletal muscle regeneration in conditions of extreme limb ischemia/hypoxia in patients to whom an ischemic limb was amputated.

PATIENTS AND METHODS

Muscle specimens were taken from amputated atherosclerosis- and/or diabetes mellitus-damaged limbs of 10 patients in Vilnius University Hospital. Biopsies from five fresh acute trauma patients served as control. All specimens were fixed in 10% neutral formalin, embedded in paraffin and processed for staining with hematoxilyn-eosin (H&E), safranin, and for immunohistochemical analysis.

Immunohistochemistry. The primary antibodies used were serum protein absorbed rabbit anti-human vWF IgG (1:400, Dakopats A/S, Glostrup, Denmark); anti-human CD68 monoclonal mouse antibody (1:100, DAKO A/S, Glostrup, Denmark); anti-human T cells, CD8 monoclonal mouse antibody (1:100, DAKO A/S, Glostrup, Denmark).

Paraffin sections 5 μm thick were mounted on DAKO Capillary slides (TechMate™, DAKO, Glostrup, Denmark), deparaffinized in xylene and rehydrated in graded ethanol series and 10 Mm phosphate-buffered, 0.9 saline, pH 7.4 (PBS). For antigen retrieval, the slides were placed into the Buffer for Antigen Retrieval for the use with TechMate™ Instruments (Dako A/S, Denmark) and microwaved for 10 min at 600 W, then kept at room temperature for 30 min, washed in PBS and stained automatically according to the following protocol: 1) the primary antibody, diluted with DAKO ChemMate™ antibody diluent, for 1 h; 2) secondary antibody containing both biotinylated goat anti-rabbit IgG and biotinylated goat anti-mouse IgG antibodies for 30 min; 3) peroxidase block for 30 min; 4) peroxidase-conjugated streptavidin 3 times for 3 minutes; 5) HRP Substrate Buffer and finally 6) substrate working solution containing 3,3'-diaminobenzidine tetrachloride (ChemMate™ Detection Kit) for 5 min. Between each step, the sections were washed with DAKO ChemMate™ washing buffers three times and dried in absorbent pads. Replacement of the primary antibodies with normal rabbit IgG or monoclonal mouse IgG of the same isotype and concentration as the specific primary antibodies but with irrelevant specificities, diluted with DAKO ChemMate™ antibody diluent, were used as negative controls. All incubations were performed at +22 °C. After immunostaining the sections were removed from machine, counterstained with hematoxylin or left without counterstaining, washed, dehydrated in ethanol series, cleared in xylene and mounted in synthetic mounting medium (Diatex, Beckers Industrifäg AB, Märsta, Sweden).

RESULTS

Individual myofiber cut in a cross-section in the control group were polygonal (often hexagonal), had peripheral nuclei and relatively uniform diameters of 30 to 80 microns in different areas. On routine histologic sections stained with H&E or safranin, myofibers appeared to lie immediately adjacent to each other. A thin network of collagen fibers that surrounds the myofibers, the endomysium, was not readily seen. Small arteries usually were damaged (Fig. 1), their lumina narrowed.

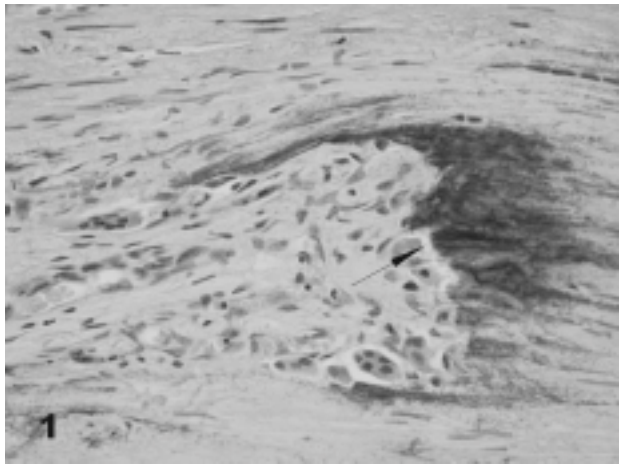

Fig. 1. Calcium deposits (arrow) and multinucleated giant cells in the tunica media of occluded small artery. H & E. ×400

Rapid degeneration of ischemia-damaged myofibers spared the satellite cells or stimulates their proliferation in the same area (Fig. 2). The key for rapid and successful regeneration seemed to be the preservation of reasonable intact basal laminae surrounding the infused muscle fibers. Fragmentation

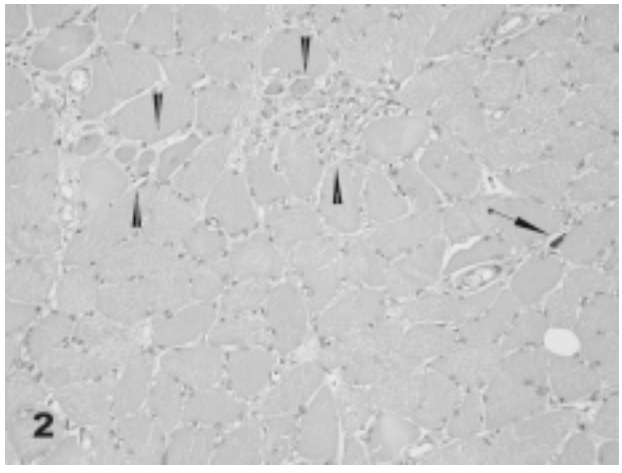

Fig. 2. Proliferating satellite cells (arrow) in the area of atrophic muscle fibers (arrowheads). Safranin staining. ×400

of muscle fibers indicated an early process of injury, usually no older than 6 h. The areas with dominating phagocytosis showed the deterioration process that had lasted at least 10 days. After 13 days of the ischemic injury the areas with regeneration of myotubes were present (Fig. 3), showing the process to be no less than two weeks old. Histological examination of serial sections revealed large numbers of differentiating muscle fibers. Regenerating myofibers were identified by their basophilic cytoplasm and nuclei containing prominent nucleoli. In proximity to them cross-striated muscle fibers appeared; this phenomenon appears after 18 days of the onset of muscle regeneration. The data showed that activation of the myogenic response did not depend directly on the surrounding cell death and degeneration process. Occasionally it was at some distance from atrophic or dystrophic muscle fibers. The regenerative process frequently was not finalized, resulting in development of abnormal muscle fibers that branched or formed small autonomous-looking clusters. In some areas larger or smaller amounts of scar tissue was formed.

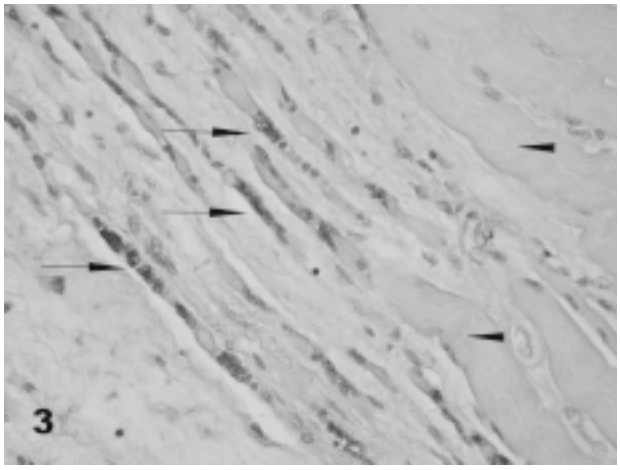

Fig. 3. Clusters of regenerating myotubes (arrows) among degenerating muscle fibers (arrowheads). Safranin staining. ×400

Usually the picture showed simultaneous muscle degradation and regeneration that followed chronic ischemic injury, nevertheless almost always with incomplete structural recovery. Degenerating myofibers were engulfed by CD68+ macrophages in the process of myofiber phagocytosis (Fig. 4). Inflammatory cells in the phase of necrosis usually were lymphocytes, CD8+ cells, histiocytes, and plasma cells, surrounding blood vessels and infiltrating the widened interstitium between the myofibers. Polymorphonuclear phagocytes were seen only occasionally. No mitosis figures in fibers or myotubes were found.

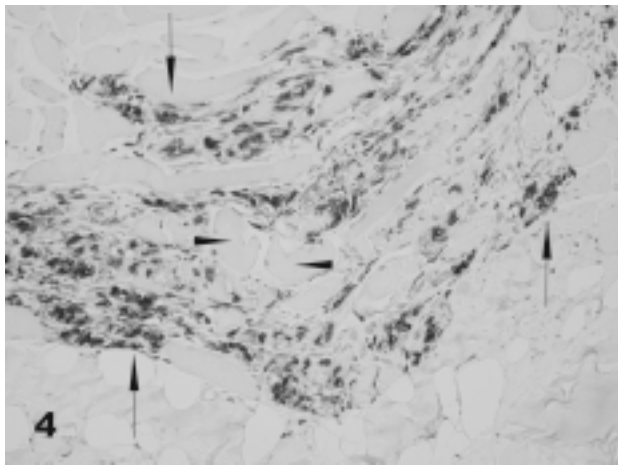

Fig. 4. Phagocytosis of degenerating muscle fibers (arrowheads). Immunohistochemical staining for CD68 (arrows). Conterstained with H & E. ×200

No dividing cells were in close proximity with a regenerating muscle fibers. No inflammatory cells in the proximity to myotubes were seen. There were no inflammatory giant cells among necrotising muscle fibers. No inflammation signs were present in the spots of regenerating myotubes. Proliferative satellite cells were often detected on a large cleft in degenerating myofiber beneath a single basal lamina. There was no evidence for migration of satellite cells through the basal lamina. Myotubes with central nuclei and filaments of nascent sarcomeres were seen as well. Adjacent to the myotubes there were many small mononuclear cells with condensed heterochromatin and little cytoplasm. These cells were universally in contact with both the basal lamina and the cell membrane of the growing myofiber and were likely to represent the myogenic precursor cells that have reverted to a quiescent state.

Numerous, closely packed and centrally positioned nuclei in newly-formed muscle fibers were noticed in regeneration spots. When the nuclei migration to the periphery of fiber as the sign of further differentiation progresses were seen, an augmentation of the diameter of fiber was simultaneously registered. The more distinctly fibrils forming contractile apparatus exhibiting the cross-striation appeared, the more mature newly regenerated fibers were. Some hypercontracted (opaque) fibers were scattered among other newly-formed skeletal muscle fibers. A heterogeneity in the levels and amount of atrophic, degenerative and regenerative muscle fibers was usually found.

DISCUSSION

Patients with critical limb ischemia require a multi-disciplinary approach to effective management (7–9).

Chronic critical limb ischemia is manifested by pain at rest, non-healing wounds and gangrene. Chronic critical limb ischemia is the end result of arterial occlusive disease, most commonly atherosclerosis. Narrowed vessels cannot supply a sufficient blood flow to exercising leg muscles. Critical limb ischemia can develop when the blood flow does not meet the metabolic demands of tissues at rest. Patients with diabetes are more likely than other patients to have distal disease (10). Compared with amputation, revascularization is more cost-effective and is associated with better perioperative morbidity and mortality.

Skeletal muscle are damaged and repaired repeatedly throughout life. Skeletal muscle is one of the regenerative tissues in mammals. This process is dependent on highly coordinated gene regulation (11). The primary events are essentially the same as those in the differentiating muscle in the embryo. Both cell proliferation and differentiation programs are essential for myogenesis. Upon injury satellite cells reenter the cell cycle, proliferate and then exit the cell cycle either to renew the quiescent satellite cell pool or to differentiate into mature myofibers. Regeneration starts when satellite cells located between the sarcolemma and basement lamina of the muscle fibers are activated by growth factors released by infiltrating mononuclear cells and platelets associated with early hematoma. Neutrophils appear within hours becoming a source of pro-inflammatory cytokines. Macrophages remain for several weeks. Lymphocytes debride the necrotic tissue and secrete a variety of growth factors that promote regeneration. Greater understanding of the molecular mechanisms by which satellite cell activity is regulated could promote the development of novel countermeasures to enhance muscle performance compromised by disease or aging. Mammalian cell proliferation involves the retinoblastoma protein (Rb)/E2F signaling pathway (12, 13), activation of which leads to increased activities of E2F transcription factors (14, 15) and upregulation of E2F-responsive genes encoding proteins directly involved in DNA replication and cell cycle progression (16, 17). Myogenic differentiation is controlled by interactions of transcription factors (18). Paired-box proteins (Pax 3 and Pax 7) are involved in myogenic cell lineage determination and specification (19). The protein dystrophin is essential for the functioning muscles.

However, the functional roles of many regulatory proteins and the molecular mechanisms that coordinate the proliferation and differentiation programs in adult skeletal muscle are not well defined.

Some cells move into a wasted muscle – and prompt it to regrew. Coming cells start to become muscle cells and start to produce all muscle-specific proteins. How is this hormonally regulated and which genes switch this on? Transient, intermittent ischemia stimulates muscle reserves of regeneration. Prolonged ischemia results in cell death. These events suggest that genes involved in cell survival have an enhanced expression.

Typhoid can cause a particular, Zenker's waxy degeneration. Pathologist and anatomist Heinrich von Waldeyer found myotubes in microscopic sections of muscle that exhibited waxy degeneration. Cardinal signs of the regenerative process are basophilic multinucleated myotubes described by Waldeyer in 1865 (20).

Regenerative myotubes closely resemble those of newly differentiating embryonic muscles (20). The first wave of myotube formation is highly synchronized and takes only hours and days after injury (20). One also sees myotubes in muscular dystrophy.

Enhanced regeneration in dystrophic as well as injured muscle was described (21, 22). The molecular signals resulting in myoblast recognition and the fusion appear to be calcium-regulated (23).

The capacity of muscle to regenerate is limited. The injured muscle initiates the regeneration of myofibers, but the process is almost always accompanied by an overgrowth of fibroblast cells located within the connective tissue network.

When considering any muscular disease, it is important to remember that intact central and peripheral nervous system is critical for normal muscle function (24).

Intermittent exposure to hyperbaric hyperoxia serves to interrupt the injury cycle of edema, ischemia and tissue necrosis. Edema is reduced secondary to hyperoxia-induced arteriolar vasoconstriction. Muscle heals by combination of regeneration and scar formation. Although fibrous scar tissue provides tensile strength and plays a role in normal muscle healing, excessive scar tissue impedes muscle fiber regeneration and interferes with muscle contraction.

The results of our study show that even in the cases considered as strong indication for limb amputation there still existed foci of muscle regeneration. This suggests that indications for surgical revascularization instead of limb amputation could be wider than actual practice suggests.

Received 14 May 2003
Accepted 29 May 2003

References

1. Carlson BM. Muscle regeneration in amphibians and mammals: Passing the torch. Dev Dyn 2003; 226: 167–81.

2. Borisov AB, Dedkov EI, Carlson BM. Interrelations of myogenic response, progressive atrophy of muscle fibers, and cell death in denervated skeletal muscle.
3. Garry DJ, Meeson A, Elterman J, Zhao Y, Yang P, Bassel-Duby R, Williams RS. Myogenic stem cell function is impaired in mice lacking the forhead/winged helix protein MNF. Proc Natl Acad Sci USA 2000; 97: 5416–21.
4. Buckingham M, Bajard L, Chang T, Daubas P, Hadchouel J, Meihac S, Montarras D, Rocancourt D, Relaix F. The formation of skeletal muscle: from somit to limb. J Anat 2003; 202: 59–68.
5. Bischoff R. Myogenesis. Ed. Franzini-Armstrong. McGraw-Hill, New York 1994: 97–118.
6. Asakura A. Stem cells in adult skeletal muscle. Trends Cardiovasc Med 2003; 13: 123–8.
7. Rajagopalan S, Grossman PM. Management of chronic critical limb ischemia. Cardiol Clin 2002; 20: 535–45.
8. Vale PR, Isner JM, Rosenfield K. Therapeutic angiogenesis in critical limb and myocardial ischemia. J Interv Cardiol 2001; 14: 511–28.
9. Dawson DL, Hagino RT. Critical limb ischemia. Curr Treat Options Cardiovasc Med 2001; 3: 237–49.
10. Beckman JA, Creager MA, Libby P. Diabetes and atherosclerosis: Epidemiology, pathophysiology, and management. JAMA 2002; 287: 2570–81.
11. Yan Z, Choi S, Liu X, Zhang M, Schageman JJ, Lee SY, Hart R, Liu L, Thurmond FA, Williams RS. Highly coordinated gene reulation in mouse skeletal muscle regeneration. J Biol Chem 2003; 278: 8826–36.
12. Nevins JR. Towards an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ 1998; 9: 585–93.
13. Dyson N. The regulation of E2F by pRB-family proteins. Genes Dev 1998; 12: 2245–62.
14. Sears R, Ohtani K, Nevins JR. Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals. Moll Cell Biol 1997; 17: 5227–35.
15. Adams MR, Sears R, Nuckolls F, Leone G, Nivens JR. Complex transcriptional regulatory mechanisms control expression of the E2F3 locus. Moll Cell Biol 2000; 20: 3633–9.
16. DeGregori J, Kowalik T, Nevins JR. Cellular targets for activation by E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. Moll Cell Biol 1995; 15: 4215–24.
17. Leone G, DeGregori J, Yan Z, Jakoi K, Ishida S, Williams RS, Nevins JR. E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. Genes Dev 1998; 12: 2120–30.
18. Olson E. Activation of muscle-specific transcription by myogenic helix-loop-helix proteins. Symp Soc Exp Biol 1992; 46: 331–41.
19. Seale P, Asakura A, Rudnicki MA. The potential of muscle stem cells. Dev Cell 2001; 1: 333–42.
20. Pietsch P. The effects of colchicine on regeneration of mouse skeletal muscle. Anat Rec 1961; 139: 167–72.
21. Pietsch P, Harrold J. Enhanced muscle regeneration and increased strength in myopathic animals treated with ethylenedinitramine. Nature 1967; 213: 601–3.
22. Pietsch P, Chenoweth MB. Muscle regeneration: enhancement by ethylene inhalation. Proc Soc Exp Biol Med 1969; 130: 714–7.
23. Przybylski RJ, Davidheiser SJ, Szigeti V, Kirby AC. Calcium regulation of skeletal myogenesis. II.Extracellular and cell surface effects. Cell Calcium 1994; 15: 132–42.
24. Toti P, Villanova M, Vatti R, Schuerfeld K, Stumpo M, Barbagli L, Malendrini A, Costantini M. Nerve growth factor expression in human dystrophic muscles. Muscle Nerve 2003; 27: 370–3.

Z. Mackiewiczius, A. Rimkevičius, E. Dudek, Y. T. Konttinen

SKELETO RAUMENŲ REGENERACIJA ESANT CHRONINEI KRITINEI ISCHEMIJAI

S a n t r a u k a

Histologiškai ištyrėme dešimties ischemijos pakenktų amputuotų kojų raumenis: taikytas standartinis hematoksilino-eozino metodas, histocheminiai ir imunohistocheminiai dažymai. Raumenų ischemijos priežastis buvo aterosklerozė ir/ar cukrinis diabetas. Kontrolei pasirinktos penkių ligonių atitinkamų raumenų biopsijos po galūnės traumų.

Nustatėme, kad kartu su raumeninių skaidulų atrofija, distrofija ir degeneracija rasta ir raumenų regeneracijos židinių. Regeneruojančių miogeninių tubulių zonose nebuvo uždegimo požymių. Regeneracija dažniausiai likdavo neužbaigta. Didesni ir mažesni fibrozės židiniai pakeisdavo neužbaigtos regeneracijos procesą.

Tyrimo rezultatai parodė, kad net tais atvejais, kai yra griežtos indikacijos kojos amputacijai, ischemijos pakenktuose raumenyse išlikdavo biologiškai aktyvių raumenų regeneracijos židinių. Tai leidžia daryti prielaidą, kad chirurginei revaskuliarizacijai galėtų būti nustatomos platesnės indikacijos.

Raktažodžiai: skeleto raumenys, ischemia, regeneracija