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BRIEF REVIEW

Repair of injured skeletal muscle: a molecular approach

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ABSTRACT

RUSSELL, B., D. J. DIX, D. L. HALLER, and J. JACOBS-EL. Repair of injured skeletal muscle: a molecular approach. Med. Sci. Sports Exerc., Vol. 24, No. 2, pp. 189–196, 1992. We review cellular and molecular processes involved in injury and repair of skeletal muscle with regard to the amount and location of damage produced. Discussion is based on advances made by use of newer techniques, including immunochemistry, in situ hybridization, molecular biology, ultrastructural analysis, and cell culture. Damage and repair processes after eccentric work, stretch, overload, chronic stimulation, cold injury, and other models are discussed for cellular and molecular components. Hypertrophy or hyperplasia can occur under certain conditions. After injury, satellite cells are activated by growth factors. These cells can also be activated during fiber-type transformation, probably to provide necessary DNA content rather than to supply cells of a new lineage. Emphasis is given to myosin mRNA studied by in situ hybridization to localize subcellular distribution. Increases in mRNA concentration are found near nuclei in damaged regions and at the subcellular sites being repaired in the middle of skeletal muscle fibers or near the myotendinous junction. The activation of genes for muscle regulatory factors during development is compared with their activation in regeneration and response to injury.

MUSCLE INJURY, IN SITU HYBRIDIZATION, mRNA, MYOFIBRIL, MYOSIN, MYOTUBES, REGENERATION, MUSCLE, REPAIR, MUSCLE, EXERCISE, SATELLITE CELLS, MYOTENDON JUNCTION, MUSCLE REGULATORY FACTORS, HELIX-LOOP-HELIX, MYOGENIN, myoD, GROWTH FACTORS, ECCENTRIC WORK

Skeletal muscle fibers are repeatedly damaged and repaired throughout life. Damage is caused by events inside the fiber such as ischemia, metabolic deficits, or disease. In other situations damage results from external events such as mechanical stress, injection of toxic agents (marcaine), or injury (cold, heat, crush, mince, and transplantation) (6,11). In most cases the damage is widespread and can involve muscle cells, connective tissue, and epithelia. Interruption of the nerve and vascular supplies have secondary effects on the muscle fibers. Simple passive stretch produces injury throughout the fiber and is particularly destructive at the myotendon junction region (18,75).

DAMAGE

Animal models. We stretched the anterior latissimus dorsi muscles of chick by wrapping a shackle equal to 10% of the bird’s weight around the upper wing (39). Tibialis anterior muscles of rabbit were stretched by putting a soft cast around the leg in the fully extended position (19). Constrictions performed by muscle in a lengthened position also cause widespread damage (29,47). Focal injury to a segment of a fiber can be produced by localized freezing (72) or similar localized insults.

Exercise can be mimicked by pacing the motor nerve with an implanted electrode (10,38,44). There has been considerable debate about the significance and extent of damage in long-term chronic stimulation of tissue. Here it is important to distinguish the regeneration processes from other compensatory processes. Pathologists use the central location of muscle nuclei as indicative of regeneration after repair. We have found that in chronically stimulated rabbit muscle there are very few, if any, pyknotic nuclei or centrally located nuclei. There are, however, major increases in the number of myonuclei observed at the fiber periphery. Using an antibody to proliferating nuclear cell antigen to tag dividing cells, we estimate that during the fast-to-slow conversion in chronically stimulated muscle one in three nuclei is new (27). Slow fibers normally have higher numbers of nuclei per fiber volume than fast fibers (24,67). Therefore, increase in nuclear number after fiber type transformation is not necessarily an indication of muscle damage. Instead, the increase may

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189
simply be a consequence of the need for the transformed slow fiber to add nuclei so that the slow fiber can synthesize protein at a higher rate. It is possible that the new nuclei produce a diffuse transcription factor that is able to alter the transcriptional activity in the existing myonuclei (54).

**Anatomical damage.** The extent of damage of muscle tissue can vary markedly from a barely imperceptible change in the molecules of the filament to a gross change in the whole muscle discernible by eye. The damage depends on the nature and degree of the injuring process. The smallest damage probably results in a few cross-bridges being broken off myosin molecules or a few myosin molecules being pulled free from the shaft of the thick filament. These can fairly readily be replaced by newly synthesized myosin molecules available in the cytoplasmic pool (61,78).

We do not know the basis of the molecular mechanisms for subcellular muscle damage. It is, however, well documented that contraction of a lengthened muscle is unusually destructive. This kind of work is termed eccentric by exercise physiologists. (6,9,29,47). At long sarcomere lengths a fiber has high passive tension, and only a few cross-bridges are able to form in the overlap zone between the thick and thin filaments. Activation of cross-bridge cycling in this situation apparently results in an unstable production of force and subsequent breakage of the titin filaments that normally prevent the thick filament from moving too far away from the Z-band (37). Stretched and immobilized muscles in rabbit suffer damage mostly in slow fibers (18–20) perhaps because slow fibers are recruited more often than fast fibers in the immobilized state. However, in eccentric work on normal muscle, it is the fast fibers that are selectively damaged (43), as is the case for dystrophic muscle. The deficiency in dystrophin leads to a lowered threshold of work-induced injury and a concomitant increase in fibrosis to protect the fiber from being overstretched (71).

More extreme damage results from increased loads, greater stretching, longer periods of eccentric work, or numerous forms of stress discussed above. In serious damage the fiber can be torn apart. The weakest zone in a mechanically stretched muscle is near the myotendon junction (18,75). When the muscle membrane breaks, the muscle enzymes leak into the blood. Myofiber necrosis, pyknotic nuclei, exploded mitochondria, and contraction bands are seen inside the fiber and an inflammatory reaction with macrophage and eosinophil infiltration is seen in the interstitial spaces within 24 h (35).

**REPAIR**

**Satellite Cells**

**Satellite cell activation.** Cells with some features of embryonic muscle cells, called satellite cells (45), have been shown to be the source of new nuclei in growing muscle (51). Satellite cells are defined as the cells that sit dormant under the basement membrane of a multinucleated muscle fiber. They have little cytoplasm and express no muscle proteins. Adult muscles can increase their mass or repair an injury by recruiting satellite cells as the source of new nuclei (66,69). Slow fibers have more satellite cells per fiber than fast fibers (68). Satellite cells can fuse with existing fibers (hypertrophy) or with themselves to form new myotubes (hyperplasia) (65,68). After injury the satellite cells take several hours to be mobilized (39), and cell division is highest 2–4 d later in rat (68).

Growth factors appear to be involved in activation of satellite cell proliferation and availability for the repair process. Fibroblast growth factor, FGF, stimulates cell proliferation (12); but transforming growth factor-β, TGF-β, has the opposite effect and depresses proliferation of cultured satellite cells in vitro (2). Insulin growth factor, IGF, can override TGF-β effects and stimulate proliferation of satellite cells.

Once the satellite cells have divided, the muscle differentiation program is turned on to make contractile proteins. This means that the contractile protein genes are transcriptionally activated by DNA binding proteins, such as the muscle regulatory factors. These muscle regulatory factor genes are themselves regulated by yet unidentified factors in the serum (8). Thus, like many other regulatory processes of molecular biology, there is a cascade of several stages to provide both positive and negative control of the cell. Growth factors regulate in a complex way: alterations in the concentrations, and combinations of factors can either block or permit satellite cell division, muscle differentiation, and myotube formation. The molecular biological basis of this complex regulation is a topic of current research (8). It is clear that the interaction of growth factors and DNA binding proteins plays a role in regulation of satellite cell division and of the expression of muscle proteins. Some DNA binding proteins, such as myogenin, belong to the family of muscle regulatory factors that are discussed below.

The responses of satellite cells to growth factors may be mimicked in vivo as growth factor levels change in the region of injury immediately after cellular damage and alter progressively at different rates during the repair process. The growth factor receptors are located on the surface of satellite cells and terminally differentiating myoblasts. There is regulation of these receptors, which also may play a role in repair processes (53). Satellite cell activation appears to fail in dystrophic muscle perhaps because the extracellular matrix becomes too thick to be crossed by growth factors (15).

**Lineages.** There has been recent discussion about the origin and dynamic range of satellite cells in different fiber types (48). It has been suggested that these myogenic cells belong to distinct myogenic lineages.
with limited plasticity for regulation of the contractile isoform expression for each. Specific lineages are thought to be established during development by the pattern of innervation, activation, and hormone exposure. Thus, in chick, satellite cells from the "fast-class" can never express slow contractile proteins while those in the "slow class" cannot express fast isoforms. A third class has a greater dynamic range of expression and can be transformed from one class to the other in the adult (73). In mammal, we find convincing evidence for a separate lineage for embryonic muscle in mouse (77) and for the "superfast" myosin expressed exclusively in muscle of the head, especially the masseter muscle of the jaw (36). Fibers can be transformed from slow-to-fast and vice versa (25,26,55), but no direct evidence shows that this is a result of recruitment of satellite cells from another lineage. Experimentally it is found that fiber-type transformation is often incomplete (3), and this has led some authors to conclude that myonuclei from a particular lineage have a restricted range of plasticity (55).

**Anatomical Nature of Repair**

**Hypertrophy.** Increase in fiber cross-sectional area is termed hypertrophy (62). Existing adult muscle fibers hypertrophy when overloaded (70). Increases in fiber size are thought to consist of increases in the number of myofibrils by a fission process occurring throughout the whole fiber cross-section (31). In our laboratory we have overloaded muscles by addition of a weight to the chicken wing or by casting the lower hind limb of rabbits in full plantarflexion (18–21,39,40). The immediate anatomical consequence of stretch or load is a distortion of the alignment of the A- and I-bands, an irregular Z-band and slippage of the thick filaments out of the thin filament lattice. The area affected can be focal and involve only a few myofibrils and sarcomeres, leaving the fiber essentially in a good functional state. A day after the injury, fibers generally have an enlarged cytoplasmic space between the sarcolemmas and the first myofibrils. This subsarcolemmal space varies in width from a few nanometers in control fibers to several microns across in rapid hypertrophy. We also noted increased spacing between the deeper myofibrils in overloaded fibers. Passive stretching results in fibers with greater concentrations of myosin mRNA around the nuclei and in the sarcolemmal annulus (Fig. 1). The cytoplasmic space just under the sarcolemma is not filled with myofibrils but is enlarged and filled with myosin mRNA, polysomes and desmin (see refs. 59,60).

**Hyperplasia.** Increases in muscle fiber number (hyperplasia) in adult have been reported, for example, in exercising cats (34), in weight-overloaded chicken muscles (39,70), and in compensatory hypertrophy of the rat plantaris muscle (65). A good review of earlier literature is given by Saltin and Gollnick (62). The nascent fiber population first expresses a myosin phe-

Figure 1.—Tibialis anterior muscle from a rabbit 2 d after stretching. Tissue is frozen, sectioned longitudinally, and hybridized with a labeled RNA probe for detection of myosin mRNA. Densest areas of stained reaction product (arrows) indicate that the myosin mRNA is greatest around the empty nuclei under the sarcolemma. Note longitudinal streaks between myofibrils (arrowhead) and faint transverse 1-banding near the nuclei. Bar 10 μm.

**GENE EXPRESSION OF MUSCLE NUCLI**

**Myosin**

In situ hybridization in normal muscle. Messenger RNA molecules leave the nucleus via pores and are distributed in the cytoplasm (Fig. 1). Localization of a specific mRNA can be a good indication of the site of translation and assembly of new protein (59). A specific mRNA has a sequence that can be hybridized to form a duplex with a labeled complementary nucleotide probe. Subsequently the label can be detected anatomically in the cell by the method called in situ hybridization. The complementary nucleotide may be either single-stranded RNA or DNA and the label incorporated for later detection is often isotopic, but chemical moieties, such as biotin, give higher spatial resolution (16). Suitable detection methods reveal the cellular location of the hybridized probe. Nonspecific binding of the labeled nucleotide to the tissue can cause artefactual images; for example, all nucleotides stick well to connective tissue and intermediate filaments. Therefore, excess unbound label must be removed by use of digestive enzymes and extensive washes. Controls can be run using labeled sense-strand RNA probes that cannot hybridize to form duplexes and should result in very little background staining.

**In situ** hybridization of normal adult rabbit skeletal muscle shows myosin mRNA concentrated around the nucleus with a fairly steep density gradient so that by 8
there is an exponential decline (Fig. 1; 17,19). The myosin mRNA is also found throughout the subsarcolemmal and in smaller amounts in the intermyofibrillar spaces. At present there is some uncertainty about the relative distribution of the myosin mRNA in the A- and I-bands between the myofibrils. We originally described a striated appearance in the heart (17). However, we and others do not routinely see periodicity in longitudinal sections of skeletal muscle (16,18,19,28,57); therefore, we now think that the striations were an artefact perhaps arising from contraction banding or technical problems (see Discussion, 59,60). In contrast, presence of myosin mRNA in the I-band of skeletal was recently reported (1). Our opinion is that myosin mRNA is nonperiodically distributed, with the possible exception of some I-band concentration just under the sarcolemma (Fig. 1). Banding distributions could conceivably vary with adaptive states in muscle, particularly if translational control is a factor in myofibril assembly (59,60). Further experimentation will be needed to elucidate this issue.

**Midregion of stretched muscle.** Transcription rates are thought to be a major factor determining myosin gene expression in muscle and have been shown to increase in developmental and hypertrophic models (46,76). The increased amount of myosin mRNA in stretched fibers could be due to increased nuclear density or increased transcription per nucleus. DNA content per gram of tissue in the stretched fibers of mouse (79) and rat (32) did not change appreciably. Using anatomical techniques, we confirmed that stretched rabbit fibers have no significant change in nuclear densities (19). Presumably nuclei from activated satellite cells add to the myonuclei so that the ratio between nuclear number and myofibrillar mass is maintained constant during hypertrophy.

Myosin mRNA concentration increases during rapid growth and was explored by *in situ* hybridization (18–20). We found most of the extra mRNA concentrated around the nucleus and under the sarcolemma, with some increase also occurring between the myofibrils. Some fibers had undergone segmental damage and regeneration marked by central migration of nuclei. A marked increase of myosin mRNA was found in the region corresponding to disruption of the normal myofibrillar architecture. These features of myosin mRNA distribution are summarized in Figure 2. Concentrations of myosin mRNA create a favorable environment for increase in myofibril assembly. So it is not surprising that we found evidence compatible with myofibrillogenesis and assembly in regions of high mRNA concentration (59).

**Myotendinous junction stretch.** Fibers lengthen in response to stretch and create a need for rapid contractile protein synthesis and assembly into myofibrils at the myotendinous junction. It has long been known that muscle fibers lengthen by adding sarcomeres in this region. Protein synthesis is high at the ends of normal fibers, and this accelerates when fibers are lengthening in response to stretch (32,33,80). Indeed, the region close to the tendon insertion is particularly vulnerable to stretch injuries for athletes (75). Remarkable ultrastructural changes are seen near the termination of the existing adult fiber (18). A large cytoplasmic space containing polysomes opened up between the myofibrils and the sarcolemmata of the myotendon junction of lengthening fibers, and many developing myofibrils were found (Fig. 3). The accumulation of slow myosin mRNA at the end of the muscle in stretched fibers was greater than in control fibers (18). Some of the excess polysomes located at the myotendinous junction of elongating fibers were synthesizing myosin for assembly into new sarcomeres.

**Myotubes in regenerating muscle.** A few days after damage caused by stretching muscle, myotubes of newly forming fibers are seen within the basement membrane of the severely damaged adult fibers. Myotubes are also seen between healthy fibers. *In situ* hybridization of myosin mRNA shows that the intracellular distribution is much more diffuse than seen in

![Figure 2](image-url)
adult fibers (20). The myotubes have a mottled appearance that corresponds to heavy staining for myosin mRNA everywhere except in the nuclei and myofibrils. A more homogenous distribution of myosin mRNA was also reported in cultured myotubes (57) and in developing rabbit fibers (20). It seems the organization of the myofibrils plays a large role in determining myosin mRNA distribution because mRNA is effectively excluded from the orderly myofilament lattice.

We mentioned above that the myotendon junction is most susceptible to damage in muscle. Four to six days after stretch, nascent myotubes were common in this zone beyond the end of the existing fibers. All of these myotubes expressed large amounts of myosin mRNA. It is expected that these myotubes will fuse with and become extensions of existing fibers, similar to the development process (51,79). The anatomical events at the myotendon junction are depicted in Figure 2.

Muscle Regulatory Factors

Development. Muscle-specific regulatory factors are expressed in the embryo during determination, differentiation, and maturation of muscle tissue. Advances in embryology have been rapid in recent years, with identification of numerous genes coding for regulatory factors. Tissue grows or replaces damaged cells by recruiting stem cells of the appropriate tissue type. Understanding these early events of cell determination, differentiation, and maturation have great significance to general cell biological problems of both embryology and repair processes.

A proposed "master regulator" activates the myogenic program in embryonic precursor cells, thus initiating skeletal muscle differentiation. This hypothesis was suggested when demethylation of a stem cell line, C3H10T1/2, with 5-azacytidine resulted in conversion from stem cell fibroblasts to myoblasts at a frequency consistent with the transfer of one or a few closely linked genes (42). One myogenic factor, MyoD1, was subsequently isolated (13,56) that could induce expression of muscle-specific genes when transfected into fibroblasts. A single master regulator may still be possible, but the hypothesis is complicated by the discovery of several other members of the MyoD family that can also turn on the myogenic program (reviewed in ref. 48). The molecular biological approach has been very successful in identifying the myogenic proteins as members of the helix-loop-helix family (HLH), which includes some proto-oncogenes (myc), immunoglobulin enhancers (E12/E47), and others. The proteins appear to function by binding to DNA and being able to form homo- or heterodimers with other members of the HLH family (52). Also, the myd protein, which has not yet been characterized, is thought to act upstream of these HLH myogenic regulatory factors in the cascade of developmental regulation (56).

Some members of the HLH family that positively regulate muscle-specific genes are the proteins: myogenin (22,81), MRF4 (also called herculin or Myf-6, 49,58), and Myf-5 (7). The first negative regulator, Id, forms a nonfunctional heterodimer with the other HLH protein members (4). The mechanism of negative regulation appears to be the lack of an adjacent basic region necessary for transcriptional activation. When present at high levels, Id and c-myc proteins are thought to bind to the positive muscle regulatory factor proteins (such as myogenin) and effectively sequester them from forming functional dimers with each other or with E12/47 (50). Id itself is regulated by changes in some unknown factor in the serum but not by transforming growth factor-β (8).

Cotransfection experiments in various cell lines have further confused the simple "master regulator" scheme because all family members appear to activate their own endogenous transcription and other myogenic factors (74). Thus, the transfected gene product amplifies its own expression and turns on other members of the family that maintain muscle differentiation. The host cell line used for these transfection assays plays an important role; muscle proteins cannot be turned on by the myogenic factors in all cell lines. For example, one liver line can only be activated to express the myogenic program when fused into a heterokaryon with a fibroblast (64). The tissue-specific regulation promises to be a tricky area to resolve and, although fascinating
logical, structural, and molecular biological approaches. The information obtained from novel experiments incorporating these techniques give valuable insight into the mechanisms of injury, repair, and compensation during functional adaptation.

The role of satellite cell activation is discussed for various models of muscle damage. Growth factors and their receptors in the cells are involved in activation of satellite cells. The role of growth factors in developmental stages of muscle shows effects on proliferation and maturation of adult myoblasts. We discuss how satellite cell nuclei can be included into existing fibers (hypertrophy) or used to create of new fibers (hyperplasia). A distinction is made for satellite cell activation during regeneration and for the other compensatory processes, such as fiber-type transformation.

Emphasis is given to linkage between mRNA distributions and regional zones of repair. The regions where myosin mRNA is most concentrated are the same as the places with the highest rates of protein synthesis and myofibril assembly. These sites of high activity are the subsarcolemmal spaces, myotubes, myotendinous junction, and focally damaged regions in the midregions of the fiber.

We discuss the role of muscle regulatory factors in development and possible derepression in regeneration and repair. An understanding of the mechanisms surrounding altered gene expression in the repair model is key to being able to map the complex regulatory events involved. The current studies on expression of DNA binding proteins of muscle regulatory factors and growth factors at various time points during muscle repair will permit insight into how these events interact with each other in the repair process. Material presented in discussion of gene regulation will form a basis for future research in the area of control of skeletal muscle gene expression.

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