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Macrophage Activation and Muscle Remodeling at Myotendinous Junctions after Modifications in Muscle Loading

Barbara A. St. Pierre* and James G. Tidball†

From the School of Nursing and the Department of Physiological Science and Jerry Lewis Neuromuscular Research Center,† University of California, Los Angeles, California*

Modifications in muscle loading have been reported previously to result in increased numbers of mononucleated cells and changes in myofibril organization at myotendinous junctions (MTJs). The goals of this study were to determine the identity of those mononucleated cells and to examine the relationships between changes in their structure, location, and number with structural aspects of remodeling at MTJs experiencing modified loading. Soleus muscles from rats subjected to 10 days of hindlimb suspension were analyzed 0, 2, 4, and 7 days after return to weight bearing. Immunohistochemistry showed that ED1$^+$, ED2$^+$ and Ia$^+$ macrophages were present at the MTJ and microtendon of control muscle. After reloading, ED2$^+$ macrophages increased in number and size at MTJs and microtendons, indicating their activation. ED1$^+$ cells showed no change in size or number whereas Ia$^+$ cells were increased in size at day 7 of reloading. Electron microscopic observations showed that mononucleated cells near MTJs of control or suspended muscle were not highly active in protein synthesis or secretion. However, in reloaded muscle, mononucleated cells were found to be in close proximity to MTJs and to contain a high concentration of organelles associated with protein secretion. During these stages of reloading, extensive remodeling of myofibril-membrane associations occurred and nascent sarcomeres appeared in the MTJ regions of muscle fibers. Immunohistochemistry showed that during these stages of nascent sarcomere formation, there was renewed expression of developmental myosin heavy chain at MTJs, with this heavy chain appearing most prominently at the MTJ at day 7 of reloading. The activation and increased numbers of macrophages at MTJs and the close apposition of secretory cells to the MTJ membrane during remodeling lead us to propose that macrophage-derived factors may influence remodeling of MTJs in muscles experiencing modified loading. (Am J Pathol 1994, 145:1463–1471)

Myotendinous junctions (MTJs) are sites at the ends of skeletal muscle fibers where myofibrils terminate at the muscle cell membrane. MTJs are functionally specialized in that they are the region of the muscle fiber where sarcomeres are added or deleted during muscle growth or after modified use.[1,2] They are also sites where forces are transmitted between myofibrils and the extracellular matrix and the region where muscle injury is predisposed during excessive strains during eccentric contractions.[3]

Previous studies have provided evidence that MTJs undergo remodeling in response to changes in muscle rest length or loading. For example, stretching muscle to a new rest length leads to the accumulation of polysomes and mitochondria at the MTJ, the appearance of nascent sarcomeres, and elevated concentrations of mRNA for myosin heavy chain at the MTJ.[4] Reductions in muscle loading associated with spaceflight result in a substantial reduction in force-transmitting surface area at the MTJ as well as increases in ribosome and mitochondria concentrations at the MTJ.[5]

These changes in muscle structure and protein synthesis that are restricted to MTJs in muscle experiencing modified loading suggest the possibility

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Address reprint requests to Dr. James G. Tidball, Department of Physiological Science, 405 Hilgard Avenue, University of California, Los Angeles, CA 90024-1527.

that these aspects of muscle remodeling are regulated locally in the muscle fiber. The hypothesis that specific regions of muscle fibers are subject to distinct regulatory influences that control gene expression, protein synthesis, and cell structure has been supported amply in investigations of neuromuscular junction formation. Current knowledge concerning the regulation of protein synthesis and cell structure at neuromuscular junctions shows that non-muscle cells can play important regulatory roles. For example, neuron-derived factors are important in determining the level of expression of specific isoforms of acetylcholine receptors, and this influence is restricted to myonuclei located adjacent to the forming neuromuscular junction.[6,7] The possibility that non-muscle cell types may also play a role in regulating myofibril-membrane associations at MTJs is supported by recent findings showing that cytoskeletal organization in cultured skeletal muscle cells is strongly influenced by platelet-derived growth factor.[8] Furthermore, previous investigations have shown that MTJs are surrounded by large populations of mononucleated, secretory cells during periods of rapid muscle growth,[9] modified loading,[5,10] or acute strain injuries.[11,12] In those studies, it was assumed that the mononucleated cells at the growing or remodeling MTJs were fibroblasts[5,9–11] or macrophages[11,12] on the basis of their morphology.

In this investigation, we examined the identity of mononucleated cells at MTJs that were activated after changes in muscle loading and investigated how those changes in mononucleated cell populations related to remodeling at MTJs. The morphological indicators of activation that we used were increase in cell number and increase in cell volume. The model used was hindlimb suspension of rats followed by muscle reloading imposed by return to weight bearing. MTJ remodeling was assayed by immunohistochemical analysis for developmental isoforms of myosin heavy chain (dMHC) expression at the MTJ because recent studies have shown that muscle regeneration and remodeling are associated with renewed expression of dMHC at non-MTJ regions of muscle fibers.[13] MTJ remodeling also was evaluated by electron microscopy to determine whether there is ultrastructural evidence of modified myofibril-membrane associations or synthesis of nascent sarcomeres. Electron microscopy also was used to evaluate changes in the structure and location of mononucleated cells at MTJs over the time course of reloading after suspension and to examine changes in the relationships between these cells and the muscle fibers during this period.

Materials and Methods

Experimental Design

Fifty-eight adult (9 to 10 weeks) female Wistar rats (Charles River Laboratories Wilmington, MA) were housed individually, maintained on a 12-hour light/12-hour dark cycle, fed food and water *ad libitum*, and allowed to acclimate for at least 1 week. Thirty-one animals were suspended for 10 days with the use of a modification of an apparatus used by Morey-Holton and Wronski.[14] The tail was splinted with self-adhesive skin trac tape (Zimmer, Warsaw, IN) and fastened to a swivel hook to allow 360-degree rotation. With this system, the animal can freely move the forelimbs without any weight bearing of the hindlimbs. Animals were assessed routinely for adequate blood flow to the tail, hydration, breathing, and any signs of discomfort. After 10 days, the animals were removed from the suspension and either immediately sacrificed (n = 8) or allowed to recover for 2 (n = 8), 4 (n = 7), or 7 (n = 8) days under normal cage activity, designated as reloading. Twenty-eight weight- and age-matched controls were sacrificed at 10 days of suspension (n = 6) and recovery days 2 (n = 7), 4 (n = 7), and 7 (n = 7). This study was approved by the UCLA Animal Research Committee.

Tissue Preparation

At sacrifice, the animals were overdosed with sodium pentobarbital (100 mg/kg intraperitoneal) and the soleus muscles were excised with the tendons intact. After weighing, one of the soleus muscles was stretched and maintained near resting length by stapling the tendons to balsa wood coated with a thin layer of OCT compound. The tissue was then frozen in melted isopentane cooled in liquid nitrogen. Samples were stored at −70 C. The proximal portion of the muscle was removed and oriented so that MTJs were evident in longitudinal sections. Sections (10 μ thick) were cut on a Reichert-Jung cryostat at −20 C and adhered to slides coated with chromium potassium sulfate and gelatin. Slides were stored at −20 C. The other soleus muscle was prepared for electron microscopy as described below.

Immunohistochemistry

Frozen sections were air dried and fixed in acetone for 10 minutes at room temperature. After fixation, sections were air dried for 30 minutes, washed in 15 mmol/L phosphate-buffered saline (PBS; pH 7.5) and

blocked in 2% bovine serum albumin in PBS for 30 minutes. After rinsing the sections in PBS, the primary antibody was applied for 3 hours at room temperature. Frozen sections were labeled for immunohistochemistry with the following antibodies: 1), anti-ED1 (diluted 1:50; Bioproducts for Science, Indianapolis, IN), which recognizes an antigen specific for subpopulations of monocytes and macrophages; 2), anti-ED2 (diluted 1:100; Bioproducts for Science), which recognizes an antigen specific for a subpopulation of tissue macrophages; 3), anti-Ia antigen (diluted 1:50; Bioproducts for Science), which recognizes class II major histocompatibility antigen present on macrophages; and 4) anti-dMHC (diluted 1:50; Vector Laboratories, Burlingame, CA), which recognizes embryonic and neonatal forms of myosin heavy chain. The sections were washed in PBS and incubated with a biotinylated anti-mouse IgG (Vector) diluted in PBS and 2% rat serum for 90 minutes. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide in methanol for 30 minutes. After rinsing the sections in PBS for 20 minutes, the sections were incubated with the Vectastain ABC reagent (Vector) and then developed with diaminobenzidine tetrahydrochloride diluted in 0.1 mol/L Tris buffer (1 mg/ml), pH 7.2, to which was added an equal amount of 0.02% hydrogen peroxide. For control sections, the primary antibody was omitted. Sections were viewed with an Olympus light microscope with Nomarski optics.

Electron Microscopy

Soleus muscles were tied to an applicator stick at rest length and then immersed in 2.4% glutaraldehyde in 0.2 mol/L sodium cacodylate (pH 7.2) for 1 hour. Muscles were then dissected into smaller pieces (approximately 3 mm^3) and fixed for an additional 30 minutes on ice. Samples were then fixed in 1% osmium tetroxide for 30 minutes on ice and dehydrated in a graded series of ethanols. Samples were embedded in epoxy resin and then sectioned longitudinally through the muscle fibers at the MTJ for viewing by electron microscopy.

Results

Morphology of Macrophage Subpopulations

Immunostaining revealed that fusiform $ED2^+$ macrophages were distributed within and subjacent to the main tendon region of control and unloaded muscle and were found in especially high concentrations in the loose connective tissue surrounding the tendon, called the epitendineum (Figure 1). Fusiform Ia^+ cells were fewer in number but distributed similarly to the $ED2^+$ macrophages (Figures 1 and 2). A few small $ED1^+$ cells were present subjacent to the main tendon region but not within the main tendon and rarely near the MTJ (Figure 1). $ED2^+$ and Ia^+ macrophages were present in the microtendon and commonly observed in close opposition to the MTJ (Figures 1 and 2). Neither of these macrophage subpopulations appeared within the muscle fiber at the MTJ in any of the control or unloaded muscles. No difference in the size, number, or distribution of $ED1^+$, $ED2^+$, or Ia^+ macrophages was observed by light microscopy after 10 days of unloading when compared with ambulatory controls.

The number of $ED2^+$ cells was increased in the microtendon, endomysium, and near the MTJs of muscles after recovery days 2, 4, and 7 compared with controls (Figure 3). $ED2^+$ macrophages in recovery muscles were much larger than those observed in control or 10-day suspended muscles. No difference in $ED1^+$ cell size, distribution, or number was observed at any of the time points studied during reloading. The Ia^+ macrophages did not demonstrate

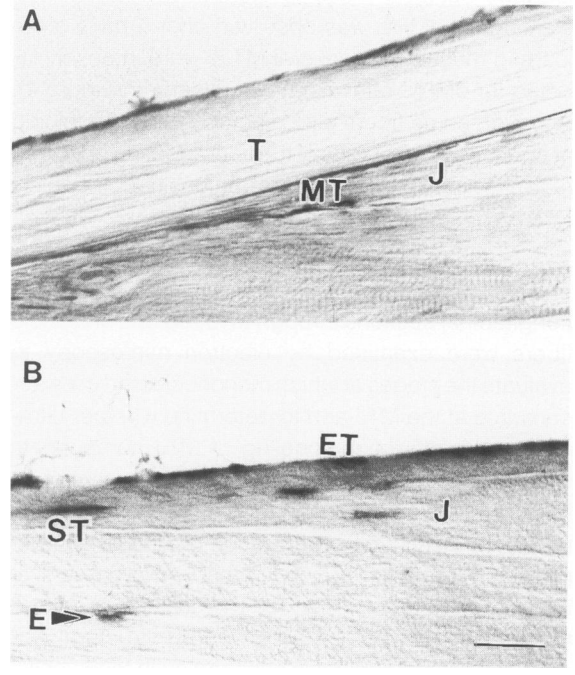

Figure 1. *Longitudinal sections of control rat soleus muscle stained with anti-ED1 and anti-ED2.* A: *Few $ED1^+$ macrophages are present in the microtendon (MT).* B: *$ED2^+$ macrophages are distributed in the epitendineum (ET), subjacent to the main tendon (ST), near the MTJ (J), and within the endomysium (E, double arrowheads). T, main tendon. Bar = 30 μ.*

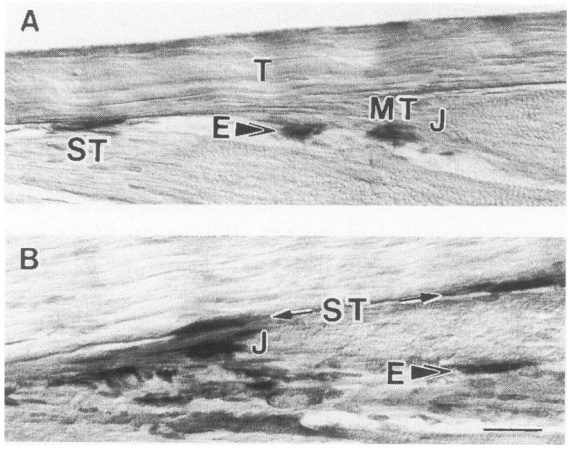

Figure 2. *Longitudinal sections of rat soleus muscle stained with anti-Ia.* A: *In control soleus, Ia$^+$ macrophages are localized subjacent to the main tendon (ST), within the endomysium (E, double arrowheads), in the microtendon (MT), and near the MTJ (J).* B: *After 7 days of reloading, Ia$^+$ cells appear to increase in size at several sites. Arrows indicate Ia$^+$ macrophages present subjacent to the main tendon. T, main tendon. Bar = 30 μ.*

any evidence of morphological change after 2 or 4 days of reloading but exhibited increases in cell size after 7 days of reloading (Figure 2).

dMHC Expression

dMHC was not observed at the MTJ in control or unloaded muscles or muscles reloaded for 2 days. Faint staining for dMHC was observed after 4 days of reloading muscle and several MTJs were intensely labeled for dMHC after 7 days of reloading (Figure 4). Although some fibers also displayed dMHC staining at nonjunctional regions of the fiber, labeling occurred more frequently and at greater intensity at the MTJ.

Electron Microscopy

Longitudinal sections through MTJ regions of muscle fibers were examined by electron microscopy to evaluate the stages at which mononucleated cells accumulate at the MTJ and to determine whether ultrastructural signs of remodeling of MTJ components were evident. Comparisons of ambulatory control rat MTJs to MTJs of rats suspended for 10 days showed no differences in the organization of junctional components. However, mononucleated cells present at the MTJ of 10-day suspended rat muscles appeared to be quiescent in that there was no structural evidence of protein synthesis or secretion in those cells. Mononucleated cells at the MTJs of control muscles typically displayed rough endoplasmic reticulum, polyribosomes, and structural evidence resembling fusion of secretory vesicles with the cell membrane

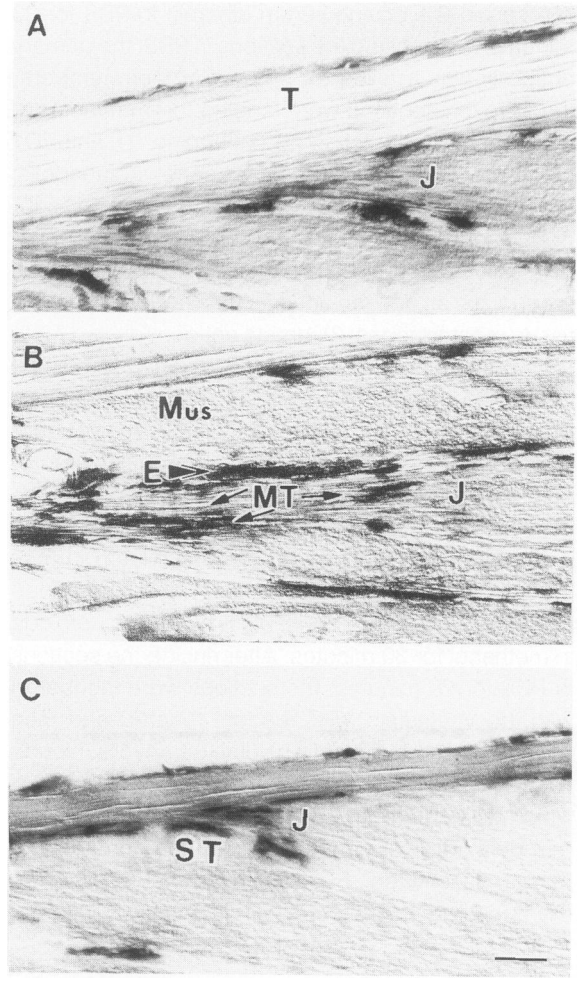

Figure 3. *Longitudinal sections of reloaded soleus muscle stained with anti-ED2. ED2$^+$ macrophages accumulate near the MTJ (J), in the endomysium (E, double arrowheads), within the microtendon (MT, arrows), and subjacent to the main tendon (ST) after 2* (A), *4* (B), *and 7* (C) *days of reloading. T, main tendon; Mus, muscle fiber. Bar = 30 μ.*

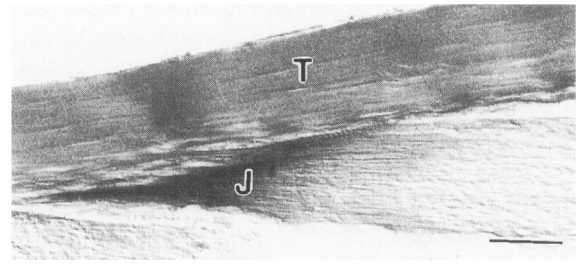

Figure 4. *Longitudinal section of reloaded rat soleus muscle stained with anti-dMHC. dMHC is evident at the MTJ (J) on day 7 of recovery. T, main tendon. Bar = 30 μ.*

(Figure 5). The cytoplasm of mononucleated cells at MTJs of suspended rats was commonly reduced to a thin strand in which the nucleus was the only distinct organelle (Figure 6).

Perturbations in normal MTJ structure were pronounced in some fibers after 2 days of reloading after

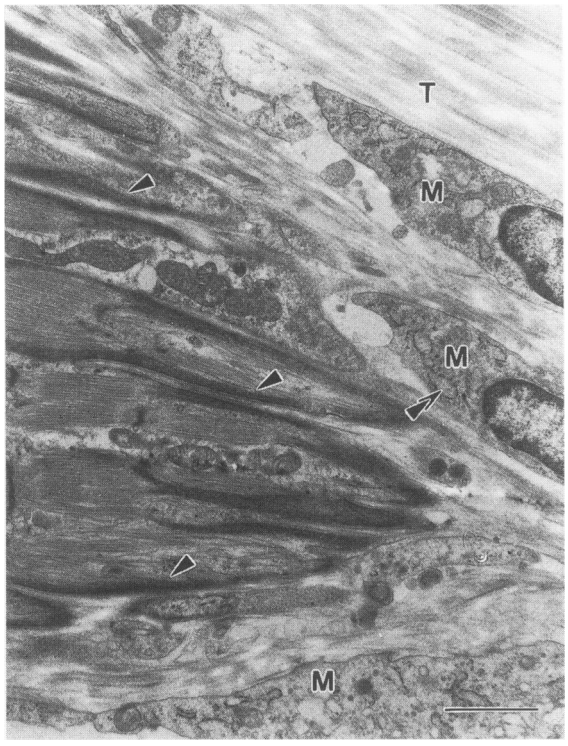

Figure 5. *Electron micrograph of longitudinal section through MTJ of ambulatory control rat. Myofibrils terminate in dense, subplasmalemmal material at the folded MTJ membrane (arrowheads). Mononucleated cells (M) containing inextensive rough endoplasmic reticulum (double arrowheads) lie between the MTJ and the tendon (T). Bar = 1.0 μ.*

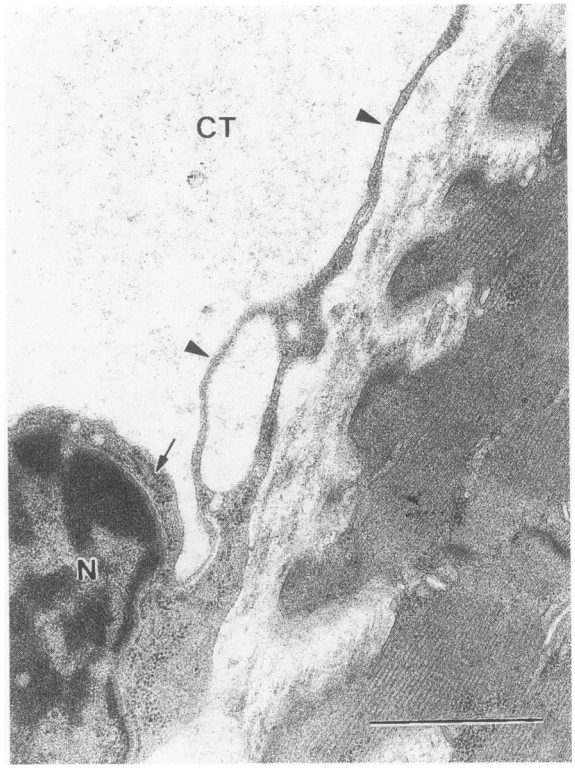

Figure 6. *Electron micrograph of a section through the MTJ region of a rat muscle after 10 days of hindlimb suspension. The mononucleated cell is greatly reduced in volume, compared with mononucleated cells present at MTJs of ambulatory control animals. The majority of the cell is reduced to a thin strand (arrowheads) that separates the muscle cell from surrounding connective tissue (CT). Scant rough endoplasmic reticulum (arrow) is present in regions surrounding the cell nucleus (N). Bar = 1.0 μ.*

hindlimb suspension. Those fibers exhibiting structural reorganization were surrounded by elevated concentrations of mononucleated cells that contained extensive enlarged rough endoplasmic reticulum. None of these mononucleated cells were observed to invade the muscle fiber, although they were observed to lie within approximately 300 nm of the MTJ membrane (Figure 7). Those muscle fibers surrounded by elevated populations of mononucleated cells commonly exhibited a reduction in MTJ membrane folding, indistinct basement membrane at the MTJ, and disruption of normal myofibril organization (Figures 7 and 8). In those fibers, myofibrils in the terminal approximately 50 μ of each cell appeared as diffuse, unorganized assemblages of myofilaments. Distinct subsarcolemmal densities, normally containing proteins capable of mediating myofibril associations with the cell membrane, appeared greatly reduced in size, thickness, and frequency of appearance (Figure 8). Many polyribosomes were present, indicating protein synthesis, and myofilaments occasionally appeared in I-Z-I complexes.

Electron microscopic evidence of MTJ remodeling was rarely observed, and mononucleated cells were no longer closely applied to the MTJs of muscles reloaded for 4 days. In muscles from animals reloaded for 4 or 7 days, myofibrils at the MTJ appeared regularly organized and parallel to the longitudinal direction of the muscle fiber, although many myofibrils had diameters less than 1 μ characteristic of fully formed myofibrils. Myonuclei near the MTJ frequently occurred in rows, subjacent to the muscle cell membrane, and occasionally appeared highly lobulated. The cytoplasm surrounding the rows of nuclei was rich in polyribosomes, mitochondria, smooth endoplasmic reticulum, and Golgi bodies (Figure 9) indicating these regions are active sites of synthesis of secretory or membrane proteins.

Discussion

In the present study, we have examined the response of MTJs and neighboring mononucleated cells to changes in muscle loading by subjecting rats to hindlimb suspension followed by return to weight bearing. Data presented here show that $ED1^+$, $ED2^+$, and Ia^+ cells are normally present at the MTJ region and that

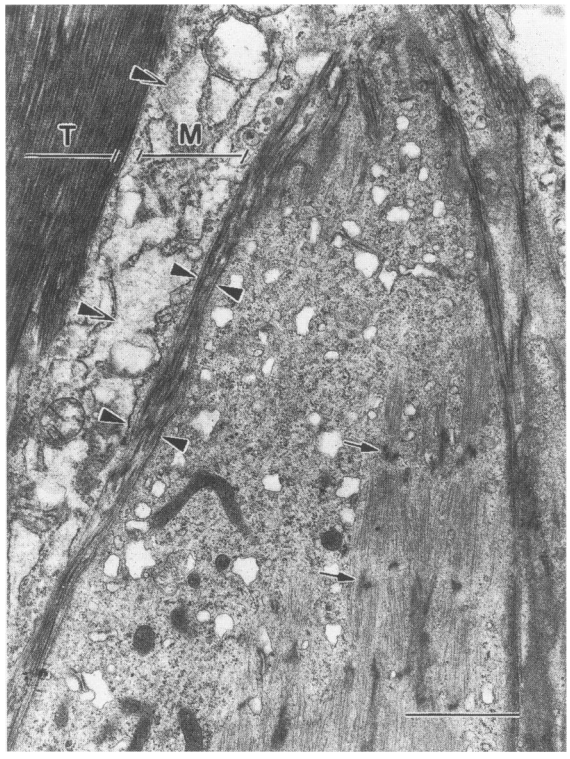

Figure 7. *Electron micrograph of a longitudinal section through the MTJ of rat muscle reloaded for 2 days after hindlimb suspension. Myofibrils lying near the MTJ membrane are smaller in diameter than those in ambulatory controls, and Z-disks (arrows) appear as dense bodies rather than completely formed Z-disks. Much of the muscle cytoplasm at the MTJ contains ribosomes. Membrane folding is greatly reduced, and the space between the MTJ membrane and surrounding mononucleated cells (M) is reduced to approximately 300 nm (between arrowheads). Mononucleated cells lying between the MTJ and tendon (T) are greatly enlarged with much of their cytoplasm occupied by swollen rough endoplasmic reticulum (double arrowheads). Bar = 1.5 μ.*

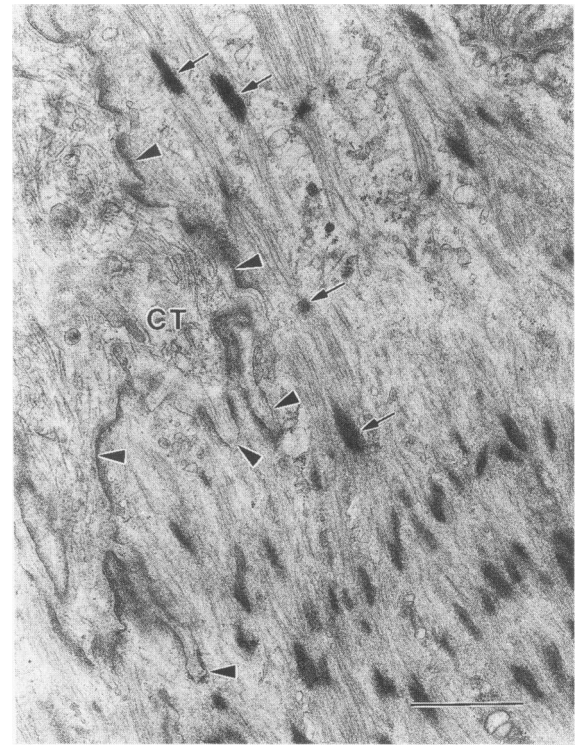

Figure 8. *Electron micrograph of a longitudinal section through the MTJ of rat muscle reloaded for 2 days after hindlimb suspension. Connective tissue (CT) lies to the left of the MTJ (arrowheads); myofibrils are located to the right of the membrane in the micrograph. Myofibrils are thin and poorly organized and Z-disks (arrows) appear as dense bodies. Subplasmalemmal material at the MTJ membrane is reduced in density and little membrane folding is apparent. Bar = 1.0 μ.*

ED2$^+$ macrophages are the most common of the three cell types. The presence of these cells near the MTJs of normal muscle suggests that macrophages may play a role in regulating normal processes occurring at MTJs. These macrophage subpopulations are also shown to respond differently to hindlimb suspension followed by reloading and to differ in their response between MTJ and non-MTJ regions of the fiber. Previous findings[13] have shown that at non-MTJ regions, ED1$^+$ cells reach highest concentrations after two days of reloading and are involved in invasion of damaged fibers. Reloading can result in increases in ED1$^+$ cell concentrations at non-MTJ regions by a factor of twenty-five[15] to >forty,[13] relative to control muscle. ED2$^+$ cells reach highest concentrations in non-MTJ regions at 4 days and remain elevated at 7 days after reloading. In the present investigation, we detected no morphological response of ED1$^+$ macrophages at MTJs after muscle unloading/reloading. Furthermore, no cells were observed to invade MTJ regions of reloaded muscle fibers, although previous studies have shown that ED1$^+$ cells invade non-junctional regions of muscle fibers by day 2 of reloading.[13,15] MTJ and non-MTJ regions also differed in their response to reloading in that the numbers of Ia$^+$ cells were found to increase significantly at non-MTJ regions,[11] although no increase in Ia$^+$ cells was observed at the MTJ. ED2$^+$ cells were found to increase in number, size, and proximity to the muscle fibers at both MTJ and non-MTJ sites.

Although the present and previous investigations[16,17] have shown that ED1$^+$ and ED2$^+$ macrophages are found in healthy, fully differentiated muscle, little is known concerning the functional significance of the differential expression of these ED antigens by macrophages. Generally, ED1$^+$ cells[18] are viewed as circulating populations of monocytes or macrophages that increase in number in injured or pathological tissues, possibly as a result of migration into the injured site from the circulatory system[19,20] or as a result of proliferation after onset of disease.[21] ED2$^+$ cells reside in major tissues throughout the

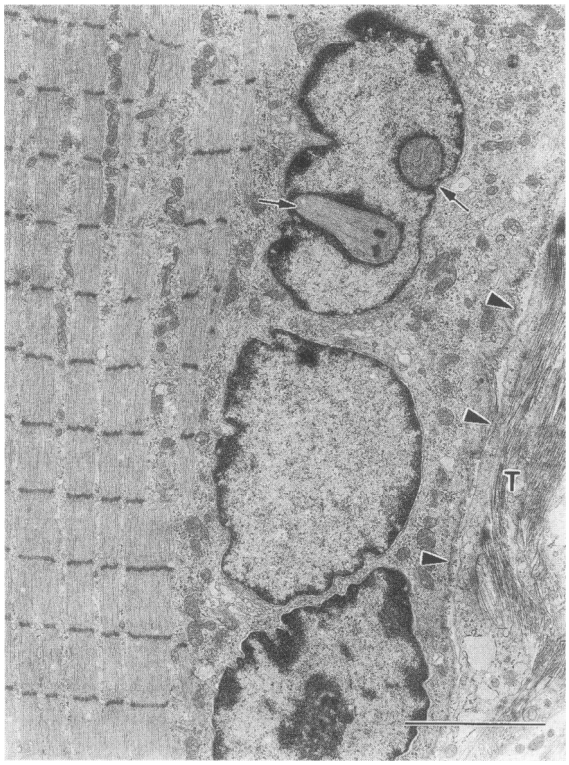

Figure 9. *Electron micrograph of muscle near the MTJ of an animal reloaded for 7 days after hindlimb suspension. Myonuclei observed at this stage of muscle reloading occasionally appeared lobulated and exhibited inclusions (arrows) not observed in control muscle. The cytoplasm in these regions and subjacent to the cell membrane (arrowheads) was enriched in polyribosomes, endoplasmic reticulum, and Golgi bodies. Bar = 3.5 μ.*

body where they can be stimulated to proliferate after the onset of disease or injury.[21,22] ED1$^+$ macrophages in diseased or injured tissue can contribute to the repair response by phagocytosing cellular debris and particulate material[16,23,24] and, at least in some tissues, by expressing monocyte chemoattractant protein-1.[25] Less is known of the function of ED2$^+$ cells in diseased or injured tissue, although ED2$^+$ perivascular cells in brain can phagocytose particulate material.[26] ED1$^+$ and ED2$^+$ cells differ in their time of appearance in developing muscle, with ED1$^+$ cells being most prevalent in embryonic muscle[17] and ED2$^+$ cells predominating in neonatal and adult muscle.[16,17] After muscle injury, ED1$^+$ cells invade damaged fibers, although no fiber invasion by ED2$^+$ cells has been reported.[27] Thus, the present investigation shows that reloading muscle after hindlimb suspension results in an increase at MTJs in macrophages that are normally found in well differentiated muscle and do not invade muscle fibers, whereas previous studies[13,15,27] have shown that reloading and injury are associated with an increase at non-MTJ sites in both noninvasive macrophages as well as phagocytic, invasive macrophages characteristic of embryonic and injured muscle.

Our findings also show that the MTJ region of muscle fibers subjected to hindlimb suspension and reloading undergoes extensive remodeling during reloading. Electron microscopic observations provide evidence that myofibril-membrane associations undergo remodeling that is most apparent on day 2 after reloading. At this stage of reloading, myofilaments appear disorganized or in I-Z-I bodies in the MTJ region of some of these fibers. These morphological rearrangements of myofibrils at MTJs resemble those seen during embryonic formation of MTJs[9] and after muscle stretching.[4] Results of the present investigation also indicate that the modification in myofibril organization observed here involves the synthesis of sarcomeres that contain thick filaments consisting of developmental isoforms of myosin heavy chain. Previous investigations have shown that muscle stretching[4] and recovery from muscle shortening[2] are associated with synthesis of new thin and thick filaments and increased mRNA concentrations for myosin heavy chain at the MTJ[4] and that expression of myosin isoforms is affected differentially by reloading after hindlimb suspension.[28] However, it has not been previously demonstrated that nascent thick filaments at the remodeling MTJs are formed by dMHC. Thus, MTJ remodeling involves renewed expression of genes expressed normally during development, at least in the model used in the present study.

At first view, electron microscopic observations do not perfectly coincide with observations of dMHC accumulation at the MTJ, in that muscles reloaded for 2 days show the most prominent structural evidence of remodeling whereas muscles reloaded for 7 days show the most prominent dMHC labeling at the MTJ. A possible explanation for these observations is that the structurally apparent remodeling on day 2 after reloading primarily involves removal or modification of previously existing MTJ components with little synthesis of new sarcomeric components. Two days of reloading may represent the stage of highest catabolic change at the MTJ, during which myofibrils appear disorganized at junctional regions and there is a reduction in MTJ membrane folding and loss of subsarcolemmal densities. This stage appears to be followed by a stage in which new sarcomeres are synthesized, incorporating dMHC and restoring normal MTJ structure.

The coinciding appearance of increased concentrations of ED2$^+$ macrophages and remodeling of myofibril associations with the cell membrane at MTJs suggests the possibility that these two responses could be functionally related, perhaps through the re-

lease of macrophage-derived products that are capable of causing cytoskeletal reorganization in target cells. Although the repertoire of cytokines secreted by $ED2^+$ cells has not been examined, macrophages in general are capable of secreting many factors that may influence cytoskeletal organization. In particular, macrophages can induce cytoskeletal reorganization in target cells by secreting: 1), interleukin-8 (IL-8), which can cause actin polymerization in receptor-bearing cells;[29] 2), IL-1α, IL-1β, γ-interferon, and tumor necrosis factor, all of which cause changes in the structure of contact sites between cells and the substratum *in vitro* and modifications in cytoskeletal structure;[30] and 3), platelet-derived growth factor, which causes rapid remodeling of the cytoskeleton of muscle cells.[8,31] Many of these potential effectors of MTJ cytoskeletal remodeling are known to be present at higher concentrations in injured or pathological skeletal muscle. For example, eccentric muscle loading can result in elevated concentrations of IL-1β in muscle for periods up to 5 days after exercise.[32]

Previous investigations that have demonstrated increased concentrations of cytokines in injured muscle have not tested whether there were specific regions within the muscle in which the cytokines were enriched and mediating site-specific effects. Findings presented here show that there are regional differences in macrophage populations in muscle experiencing increased loading, which is consistent with the possibility that different regions of the muscle fiber are exposed to different macrophage-derived factors and thereby mediate local effects on muscle fibers. An alternative mechanism by which site-specific effects could be mediated would be by regional differences in the distribution of cytokine receptors on muscle fibers. Currently, platelet-derived growth factor receptors are the only cytokine receptors known to be enriched at MTJs.[8] Receptors for IL-1β have also been shown concentrated at focal contacts,[33] which are sites of cell-substratum adhesion that are structurally and functionally similar to MTJs.[34] Evidence from *in vitro* studies indicates that platelet-derived growth factor or IL-1 stimulation leads to remodeling of focal contacts and the actin cytoskeleton concomitant with talin phosphorylation,[8,35] suggesting that talin phosphorylation may be a regulatory event in these episodes of cytoskeletal remodeling. The enrichment of talin at MTJs[34] indicates that similar regulatory mechanisms may be in place at these *in vivo* sites.

These regional differences in macrophage activation in response to modified muscle loading suggest the hypothesis that muscle loading may cause local release of factors from muscle fibers that selectively activate macrophage subpopulations. Although it is still feasible that the changes in fluid distribution in animals upon return to horizontal position after suspension may account for an increase in $ED1^+$ cell populations in muscle,[15] the present results that show regional differences in $ED1^+$ cell distribution in muscle indicate active regulatory processes also play a role. This conclusion is supported by recent findings that show that increased muscle loading causes disruption in the plasma membrane of muscle fibers[36] and loss from the muscle cytoplasm of basic fibroblast growth factor[37] a known macrophage mitogen. Injured muscles also release factors that are chemotactic for macrophages.[38] Together, these findings could indicate that the macrophage response to increased muscle loading results from membrane disruptions and basic fibroblast growth factor loss from the muscle fiber, followed by macrophage chemotaxis or proliferation. However, these findings do not provide an explanation for the selective activation of macrophage subpopulations or the regional (MTJ *versus* non-MTJ) differences in macrophage response. Whether the site-specific macrophage responses merely reflect regional differences in muscle fiber injury or whether they reflect an active response by the fibers in which the release of activating factors from the MTJ region differs from non-MTJ regions remains to be investigated.

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