The effect of repeated cycles of muscle strain was studied in the soleus muscle of female rats. Muscle strains were repeated 3×/week for 1 month using two different strain protocols. Striking changes, including marked variability in fiber size, evidence of degeneration and regeneration, and an expanded extracellular matrix were pronounced in the fast-stretched muscles. Using scanning electron microscopy, fibrosis was confirmed in the fast-stretched muscles but not in the slow-stretched muscles. However, the slow-stretched muscles did contain struts of connective tissue joining adjacent myofibers. Therefore, repeated muscle strains at high strain rates produced morphological changes similar to many myopathies, including fibrosis, whereas adaptation occurred in response to the same number of strains at slow strain rates. Such diverse tissue responses have relevance to the understanding of the mechanisms of skeletal muscle dysfunction in cumulative trauma disorders and in the design of preventive actions and treatments.

Key words: skeletal muscle • strains • fibrosis • collagen • matrix
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FIBROSIS AND INTERCELLULAR COLLAGEN CONNECTIONS FROM FOUR WEEKS OF MUSCLE STRAINS

WILLIAM T. STAUBER, PhD, KATHLEEN K. KNACK, DO, GERALD R. MILLER, BS, and JUANITA G. GRIMMETT, BS

Fibrosis is a term usually referring to an increased amount of connective tissue and occurs in a wide variety of tissues in response to injury and inflammation. Fibrosis begins as a normal repair response but apparently requires continued tissue injury to progress to an abnormal fibrotic state.[4] This disease progression is common in a wide variety of pathological conditions involving many diverse organs such as the liver,[19] lung,[18] and kidney.[5]

In skeletal muscle, fibrosis is seen in chronic inflammatory and noninflammatory myopathies[2] and vitamin E deficient animals.[12] Although a relationship between myonecrosis and fibrosis has been proposed,[15] few studies have been conducted to investigate the mechanisms involved in muscle fibrosis. Presumably weakened muscles, in all myopathic disease states, would be expected to be more susceptible to repeated injury, especially during eccentric muscle actions[16,21]; the repeated injury could lead to fibrosis and movement dysfunction.

Following a single bout of damage from the injection of toxin[8] or blunt trauma,[24] complete restoration of muscle structure was observed, whereas repeated bouts of damage from injections of toxin[22] resulted in endomysial fibrosis and muscle fiber splitting. Thus, the development of skeletal muscle fibrosis may require repeated injury similar to that of other organs.

We developed a model to characterize the effects of chronic stretch-induced injury in rat soleus muscles.[26] The present study evaluates the outcome of chronic strain injury produced by two different strain protocols. The hypothesis that chronic strain injuries would produce muscle fibrosis was supported for the high-velocity protocol only. However, matrix changes were noted in the slow-stretched muscles; most important were the intercellular connections (i.e., collagen struts) between the myofibers. If marked connective tissue proliferation is present following repeated muscle injury or muscle disease, complete restoration of normal function may be impaired or even impossible.

From the Departments of Physiology (Drs. Stauber and Knack, Mr. Miller, and Ms. Grimmett) and Neurology (Dr. Stauber), West Virginia University, Morgantown, West Virginia.

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Address reprint requests to William T. Stauber, PhD, Department of Physiology, West Virginia University, PO Box 9229, Morgantown, WV 26506-9229.

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MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats (Hilltop Lab Animals, Scottdale, PA) were 8–12 weeks of age; 13 were in the fast-stretching group and 12 were in the slow-stretching group. The use and care of the animals in this study were approved by and followed the guidelines of the West Virginia University Animal Care and Use Committee.

Forced-Lengthening Model. While under anesthesia (Brevital, 0.10 mg/100 g body weight), repeated muscle strains were performed as described elsewhere.[28] This procedure was performed for 5 bouts of 10 repetitions with a 30-s rest between bouts. The protocol was carried out every other day for 4 weeks (except weekends). Briefly, the left plantar flexor muscles were electrically stimulated and the dorsum of the foot was moved to forcibly stretch the active muscles through the normal range of foot motion. After the last session, the animals rested the 2 days before sacrifice. The soleus muscle of the contralateral limb served as the control.

Fast-Stretch and Slow-Stretch. The slow-stretch animals were pushed through their range of motion 10 times in 13 s (estimated average ankle velocity of 150°/s). The fast-stretch animals were pushed through the same range of motion 10 times in 5 s (estimated average ankle velocity of 375°/s). These protocols matched the natural movement rhythms of the investigator's wrist supination.

Tissue Preparation. The animals were weighed and exsanguinated while under deep anesthesia. The soleus muscles were removed, weighed, and cut so that the midbelly portion of the muscle was oriented for sectioning. The muscle pieces were mounted, frozen and sectioned at −20°C at a thickness of 10 μm. The frozen sections were collected on glass slides, allowed to air dry, and stored in airtight containers at −20°C until use.

Localization of Marker Proteins. Using indirect immunohistochemistry, desmin, dystrophin, and fast myosin antibodies were used to identify muscle-specific proteins in soleus muscles.[14] Double labeling techniques for desmin and dystrophin were applied to the same slide.[27] Extracellular matrix markers consisted of antibodies specific for collagens (types I and III), fibronectin, proteoglycans (heparan sulfate and chondroitin-6-sulfate PG),[6] and vimentin.

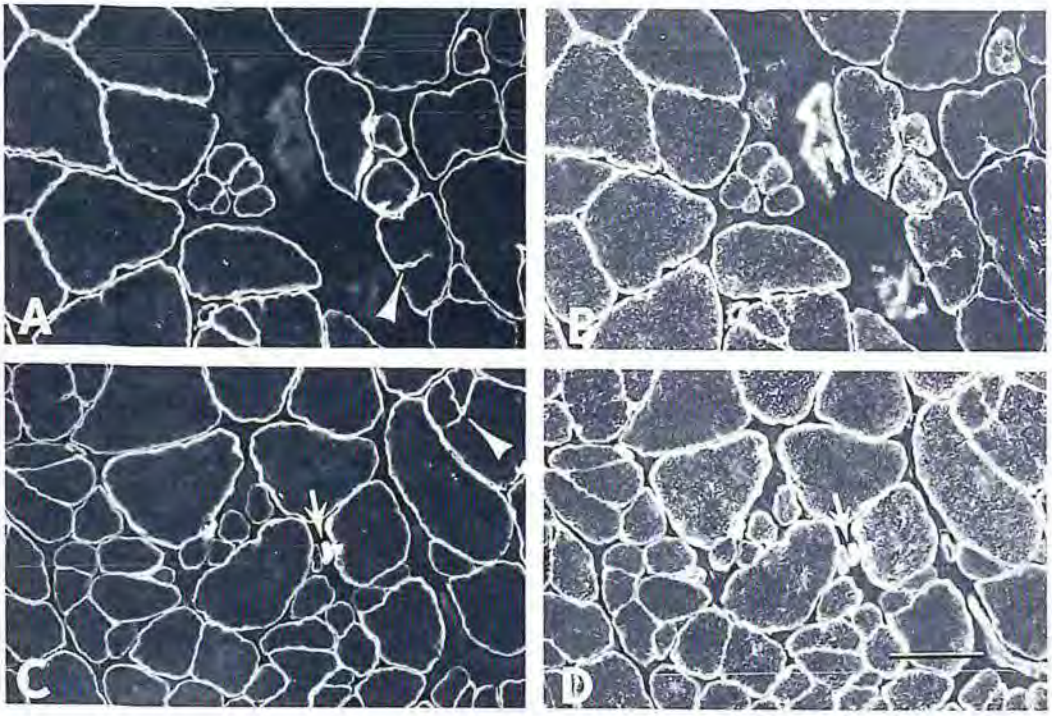

FIGURE 1. Immunohistochemical localization of fast myosin isoforms in rat soleus muscles. Unstretched control muscles are presented in (**A**) and (**C**) and fast-stretched muscles in (**B**) and (**D**). Arrows indicate small regenerating fibers; arrowheads point to fiber grouping. Original magnification 25×; bar = 200 μm.

Localization of concanavalin A (Con A) was performed by direct application of fluorescein-conjugated Con A lectin[7] as described elsewhere.[6]

Scanning Electron Microscopy. Thick frozen sections (500 μm) were cut from the same samples used for immunohistochemistry. The thick muscle slices were immersed in a fixative containing 2.0% glutaraldehyde in 0.2 mol/L cacodylate buffer, pH 7.4, for 24 h and rinsed with buffer. Some samples were macerated by the method of Ohtani et al,[17] leaving only the connective tissue. The samples were dehydrated in graded ethanol and dried using a fluorocarbon compound, Peldri II (Ted Pella, Inc., Redding, CA). The samples were coated with gold and prepared for scanning electron microscopy by conventional techniques.

RESULTS

After 1 month of repeated stretching, the soleus muscles were slightly larger but with no evidence of scarring between the soleus and adjacent muscles. Histological examination revealed that the fast-stretched soleus muscles contained a variety of different fiber sizes (Fig. 1) ranging from normal size fibers to small muscle fibers (Fig. 1B, arrows), with regions of type II myofiber grouping (Fig. 1D, arrowheads). The slow-stretched soleus contained normal sized or slightly larger myofibers with no apparent grouping (Fig. 1A and C). Many of the very small fibers in the fast-stretched soleus stained for myofiber-specific proteins: dystrophin (Fig. 2C, arrow), desmin (Fig. 2D, arrow), and fast myosin (Fig. 1B, arrow). Using a double-labeling technique for dystrophin and desmin staining (Fig. 2), it was apparent that fiber splitting was common (Fig. 2A and C, arrowheads) and small presumptive myoblasts were present (Fig. 2C and D, arrows).

In the fast-stretched muscles but not the slow-stretched muscles, the extracellular matrix (ECM) was expanded and contained a number of typical ECM markers. The expanded matrix can best be characterized by the localization of fibronectin (Fig. 3) and Con A staining (Figs. 4 and 5). Since the overall morphology of other ECM markers was not qualitatively different, they are summarized in Table 1.

It was apparent that the endomysium, perimysium, and epimysium were thickened in the fast-stretched muscles (Fig. 3B and D). In order to eliminate the possibility that the enlarged matrix was a result of swelling and inflammation, especially in the region of the endomysium, the collagen fibrillar net-

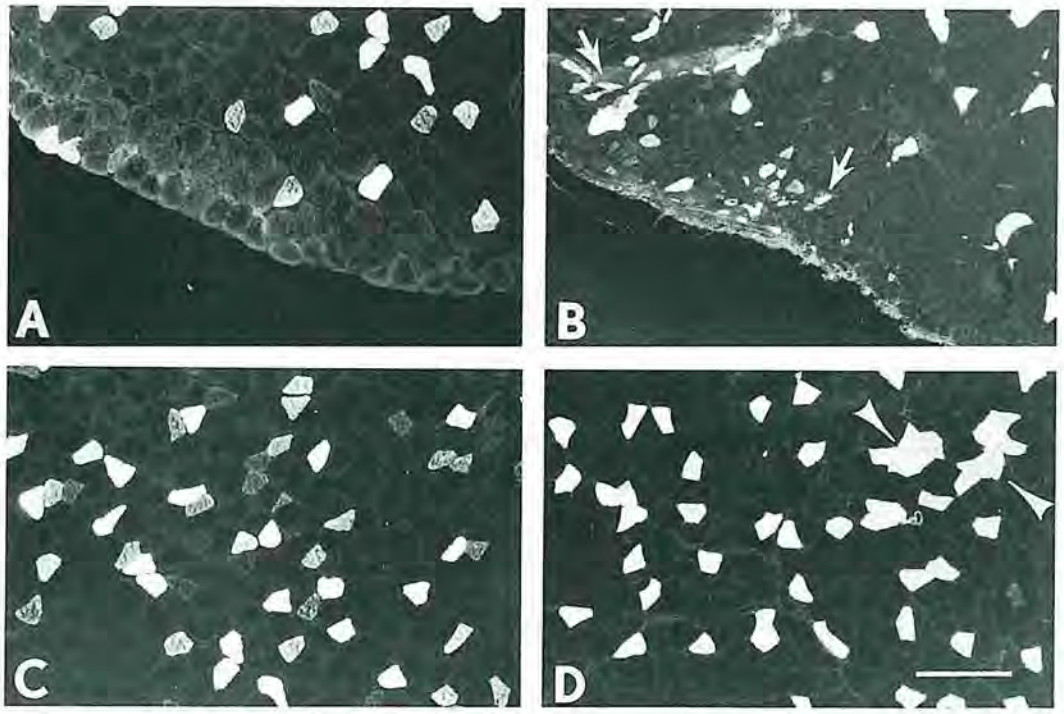

FIGURE 2. Immunohistochemical localization of dystrophin [(**A**) and (**C**)] and desmin [(**B**) and (**D**)] in fast-stretched rat soleus muscles. Arrows [(**C**) and (**D**)] indicate small regenerating fibers or myoblasts; arrowheads [(**A**) and (**C**)] point to myofiber splitting. Original magnification 100×; bar = 50 μm.

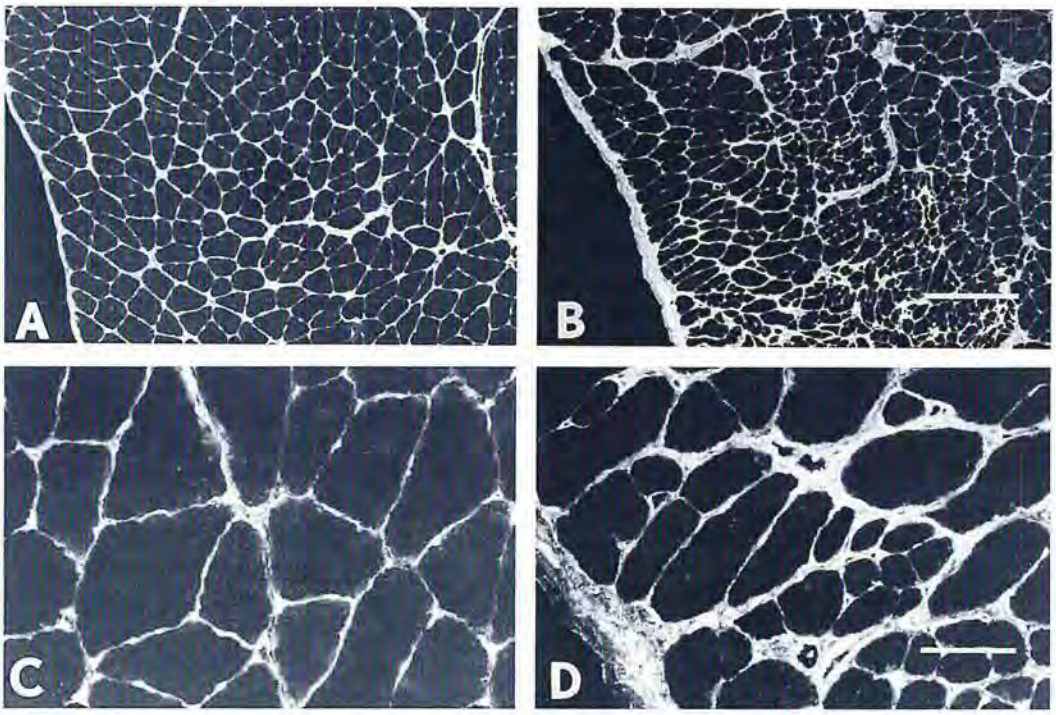

FIGURE 3. Immunohistochemical localization of fibronectin in rat soleus muscles. Unstretched control muscles are presented in (**A**) and (**C**) and fast-stretched muscles in (**B**) and (**D**). Original magnification: (**A**) and (**B**) = 25×, bar = 200 μm; (**C**) and (**D**) = 100×, bar = 50 μm.

work was visualized using cell maceration and scanning electron microscopy (Fig. 6). The endomysial connective tissue of the fast-stretched muscle (Fig. 6B and D) was thickened around both small and normal size myofibers. Perimysial thickening was also apparent in these muscles. Because the ECM changes were less noticeable in the slow-stretch muscles, conventional scanning electron microscopy was performed on whole muscle slices. A small increase in the amount of connective tissue was observed in these muscles (Fig. 7B, arrow). On close examination, collagen struts were present in the endomysium connecting adjacent individual myofibers (Fig. 7C and D). These struts have not been described for skeletal muscle, nor were they seen in the control muscle samples (Fig. 7A). Thus, connective tissue changes occurred in all muscles subjected to chronic stretch protocols and were most dramatic for the fast-stretched muscles.

DISCUSSION

Muscle adaptation to repeated muscle lengthening (strain) has only recently been explored in animals with the major emphasis of the research on myofiber adaptation.[15,28,29] However, little is known about nonmyofiber or connective tissue adaptation to chronic strains. Although many observations have documented fibrotic changes in muscles sampled from animals and humans with chronic myopathies,[2] the emphasis has generally been on myofiber changes such as fiber splitting, hypertrophy, degeneration, and regeneration.[2,9,22] However, the striking similarity between muscle from chronic myopathies and chronic exposure to muscle strains has been noted previously,[15,24,28] but detailed analysis of fibrosis has been lacking.

The morphology of the fast-stretched muscles following 4 weeks of repeated strains was similar to that described for repeated injections of the myotoxin, bupivacaine, including marked variability in fiber size, fiber type grouping, internal nuclei, and fiber splitting.[22] In contrast, no unusual morphology could be observed in the myofibers of the slow-stretched muscles repeatedly strained for the same time period. The pathological response reported for the bupivacaine-treated muscles has been described as an inability of the muscle to fully regenerate before the next bout of injury.[22] We have shown that it takes about 7 days to recover from a single bout of stretch injury, which is consistent with reports of regeneration in other types of muscle injury.[22,25] Thus, if we subjected the animal to repeated strains three times

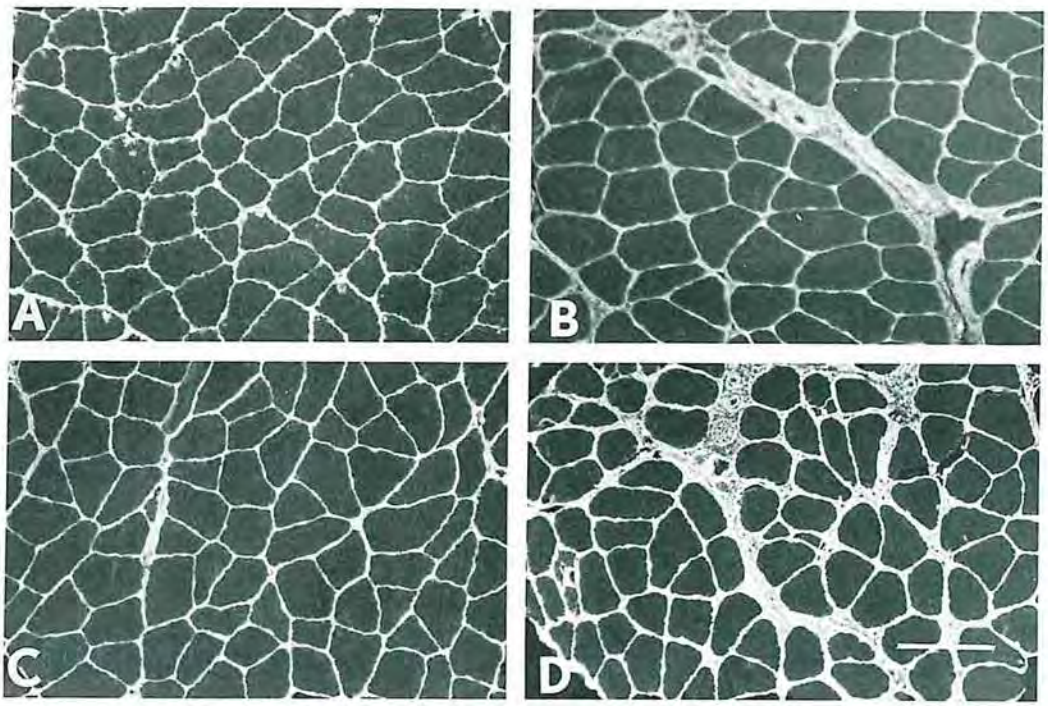

FIGURE 4. Histochemical localization of concanavalin A in rat soleus muscles. Unstretched control muscles are presented in (**A**) and (**C**), slow-stretched muscles in (**B**), and fast-stretched muscles in (**D**). Original magnification 40×; bar = 100 μm.

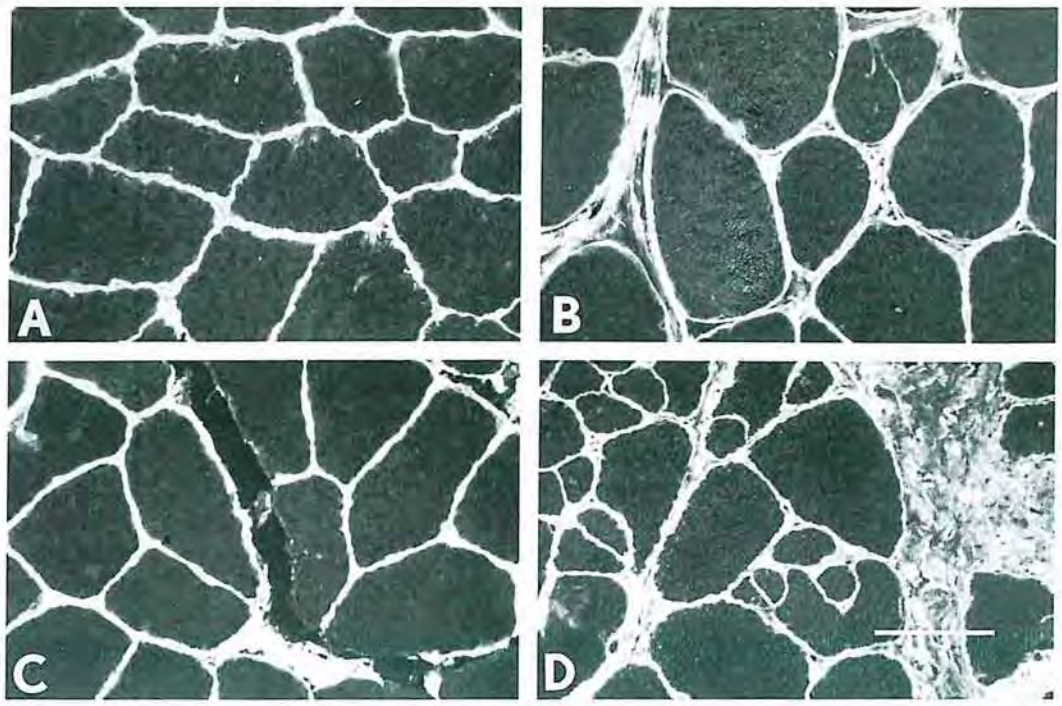

FIGURE 5. Histochemical localization of concanavalin A in rat soleus muscles. Unstretched control muscles are presented in (**A**) and (**C**), slow-stretched in (**B**), and fast-stretched in (**D**). Original magnification 100×; bar = 50 μm.

Table 1. Relative staining of extracellular matrix markers.

Antigen	Control soleus	Slow-stretch soleus	Fast-stretch soleus
Fibronectin	+	++	++++
Vimentin	+	++	++++
Heparan sulfate proteoglycan	+	+	++
Collagen I	+	+	++
Collagen III	+	+	++

a week, the strained muscle would not have an opportunity to repair completely any cellular damage.

It is also common observation that fibrosis accompanies chronic myopathy and chronic muscle injury from myotoxin treatment.[2] The fast-stretch but not the slow-stretch soleus muscle contained an expanded extracellular matrix which could be classified as fibrotic based on morphology. The expanded matrix contained the usual markers observed for tissue healing such as fibronectin, collagens I and III, and proteoglycans. Similar markers have been observed in muscles recovering following a single protocol of fast stretch.[6] However, it is common experience for human muscles to become swollen after a single bout of damaging eccentric muscle actions; some of this swelling is the result of extracellular matrix damage.[23] In order to verify that the expansion of the matrix was due to the proliferation of connective tissue and not swelling, scanning electron microscopy was performed on tissue slices and macerated samples. The expanded matrix proved to contain collagen, which surrounded all the myofibers including the small fibers that were presumed to be regenerating. Thus, incomplete regeneration of myofibers with replacement of some missing muscle cells with connective tissue appears a likely explanation for the myopathic state following repeated fast stretch. A similar mechanism has been proposed for the myotoxin-injured muscles.[22]

It is more difficult to interpret the results of repeated slow stretch. If repeated injury produced the myopathic and fibrotic muscle seen in the fast-stretch muscles, then it must be assumed that the injury was either minor or involved different cellular or extracellular components in the slow-stretch muscles. Nevertheless, changes in connective tissue were observed. The formation of struts between myofibers in skeletal muscle was unique but is characteristic of cardiac muscle.[3,20] In cardiac muscle, the function of the struts is to provide a greater passive stiffness to oppose the dilation of the heart during volume loading (i.e., stretch).[20] If a similar function of intermyofiber struts occurred in soleus muscles, the resulting

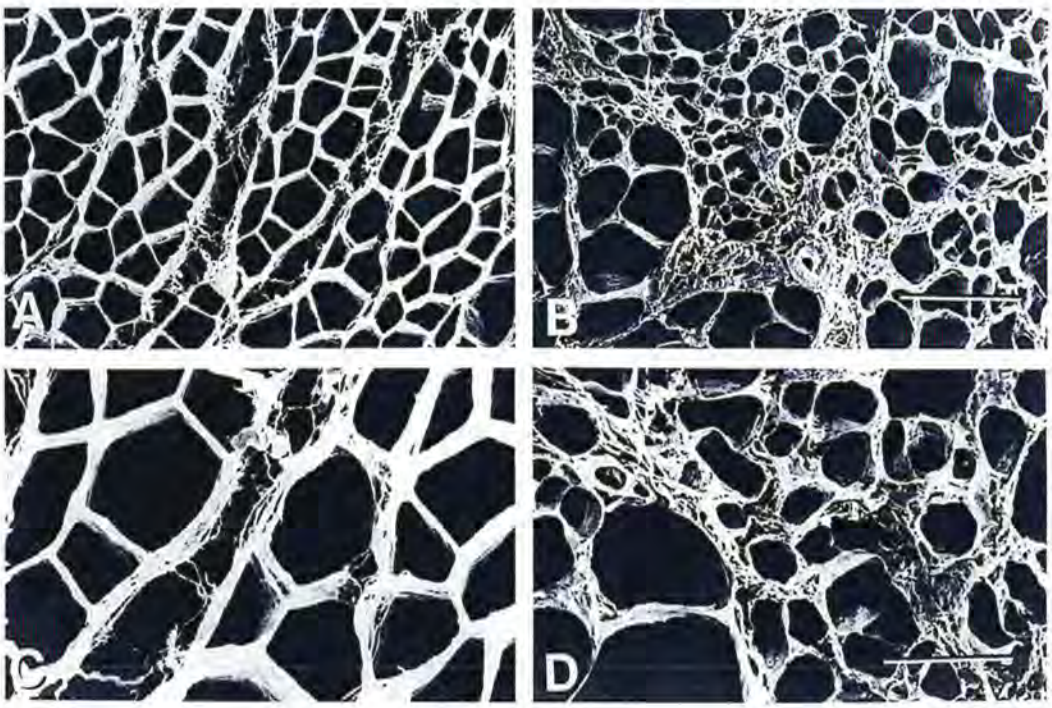

FIGURE 6. Scanning electron microscopy of rat soleus muscles after cell maceration. Unstretched control muscles are presented in (**A**) and (**C**) and fast-stretched muscles in (**B**) and (**D**). Bar = 100 μm [(**A**) and (**B**)]; 50 μm [(**C**) and (**D**)].

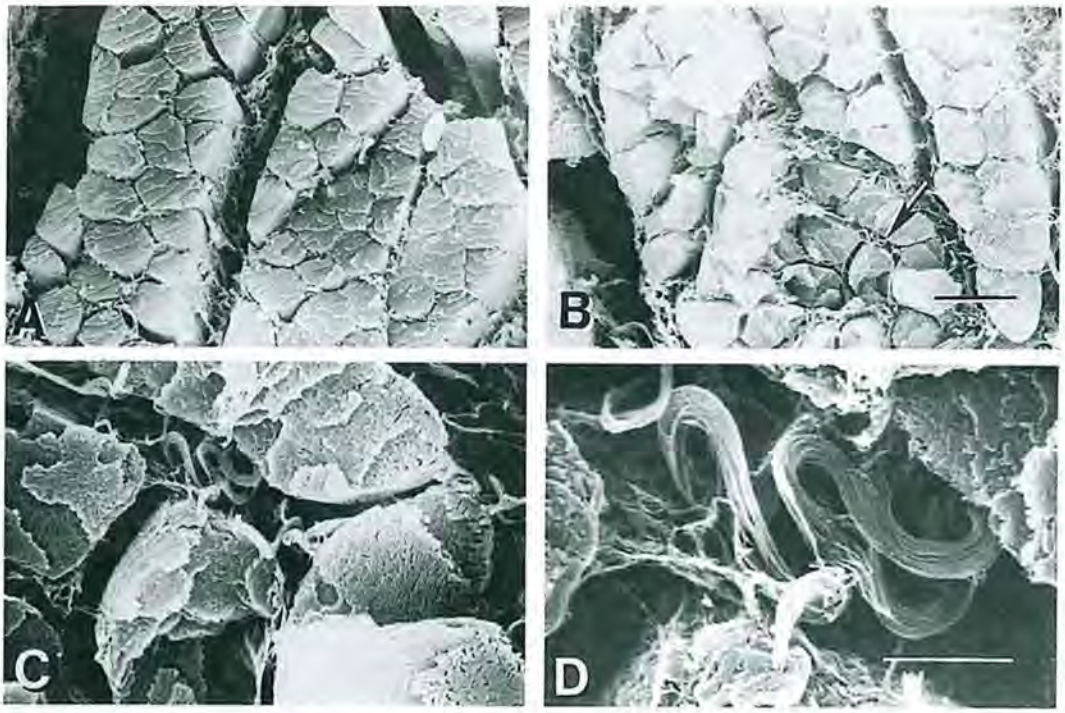

FIGURE 7. Scanning electron microscopy of rat soleus muscles. Unstretched control muscles are presented in (**A**) and slow-stretched muscles in (**B**), (**C**), and (**D**). Arrow points to an intermyofiber collagen strut shown at higher magnifications in (**C**) and (**D**). Bar = 50 μm [(**A**) and (**B**)]; 5 μm (**D**).

increase in passive stiffness would serve to protect myofibers from strain injury. In addition, it would keep the myofibers in register so that uniform force output would be insured. However, increased stiffness might require higher forces for normal movements and result in an increased energy cost for simple tasks.

The addition of intermyofiber struts could also account for some aspects of exercise training (i.e., strength increases do not carry over to all activities of the trained muscle). Jones and colleagues[11] proposed that connective tissue attachments would increase isometric force but reduce the effective length of the muscle fibers. Since stretch-induced injury is related to strain (force)[13] and the length that a muscle contracts,[10] this adaptation of connective tissue attachments might be advantageous.

Two major questions remain: (1) Is the connective tissue change a continuum beginning with struts and progressing to fibrosis or are they separate adaptations to different stimuli? (2) Is fibrosis irreversible in skeletal muscle as it appears to be in other fibrotic organs? Bradley[2] observed increased endomysial fibrosis persisting 1 month after a series of bupivacaine injections; observations have not been reported for fibrotic changes over longer times. The answers to these questions are of great importance to clinicians treating patients with chronic injury to skeletal muscles from occupations requiring repetitive motions where deceleration of loads is common (i.e., eccentric muscle actions). If fibrosis resulting from cumulative muscle damage is irreversible, then repeated muscle injury must be prevented. In addition, the use of drugs that impair the regenerative processes in muscle, such as nonsteroidal anti-inflammatory medication,[1] should be avoided.

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