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Ultrastructural studies on myofibrillogenesis and neogenesis of skeletal muscles after prolonged traction in rabbits

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Summary. Little is known about the morphological response of muscle after long term traction. The purpose of this study was to investigate the morphological changes of skeletal muscle during limb lengthening. After application of mini-extraskeletal fixator, the hindlimb of New Zealand white rabbit was osteotomized and then slowly lengthened at the rate of 1 mm/day up to a 20 mm gain in length. The muscles of hindlimbs were perfused and dissected. Morphological studies were performed at electron microscopic level. Transmission electron microscopy revealed foci of microtrauma at the myotendinous junction. The distance between the muscle fibers and tendon parenchyma increased, with numerous primitive mesenchyme-like cells interposed within this gap. The cytoplasmic space of these cells was devoid of myofibril formation at the ends of stretched fibers. Within the satellite near the myotendinous junction myofilament production was observed in various gradations of maturation. It is concluded that myofibrillogenesis with traction neogenesis of skeletal muscle during limb lengthening does exist and occurs mainly near the myotendinous junction. The myotendinous junction in mature skeletal muscle actively participated in the process of limb lengthening.

Key words: Myotendinous junction, Myofibrillogenesis, Neogenesis

Introduction

The work of Ilizarov, if replicated, promises to revolutionize the practice of orthopedic surgery by virtue of new insight into the biology of tissue regeneration (bone, ligament, skin, muscle, vessel, and nerve). He reported a slow, gradual distraction force as a mechanical signal to stimulate tissue growth, even in mature organ systems. His achievements in both experimental and clinical subjects far surpass traditional treatment methods (Paley, 1988).

The ability of adult skeletal muscle fiber to regenerate after injury has been documented since the 18th century (Grounds, 1991). Investigators later showed that adult skeletal muscles did have great powers of regeneration. These excellent investigations describe the regeneration of skeletal muscle after injury (Perth et al., 1966; Allbrook, 1981; Garret et al., 1984; Gutierrez et al., 1986; Grounds, 1987; Papadimitriou et al., 1990) or transplantation (Hall-Craggs, 1978; Gulati, 1986; Jennische, 1986; Roberts et al., 1989); although little is known about the cellular interactions which control this process.

It was first suggested that muscle fibers lengthen by adding sarcomeres at the myotendinous junction (Schmalbruch, 1976). By cast immobilization in lengthened position, sarcomeres were found to be added in series at fiber ends during stretch-induced growth (Williams and Goldspink, 1973; Goldspink, 1985). In recent years, a series of experiments using the Ilizarov technique to lengthen the bones (Aroson et al., 1988) and to investigate the mineralization sequence during distraction osteogenesis (Aroson et al., 1990) have been conduced. However, less is known about the long term effects of traction on muscle morphology and the biology about traction neogenesis of the soft tissue regeneration. In a previous study, it has been elucidated that traction neogenesis of the skeletal muscle during limb lengthening does exist and occurs mainly near the myotendinous junction (MTJ) (Sun et al., 1994). The purpose of our study is to investigate the morphological response of skeletal muscle to chronic traction, at electron microscopic level.

Materials and methods

Twelve mature New Zealand white rabbits of both sexes weighting 2.0 to 2.5 kg were used in the present study. The operation procedure was the same as

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previously reported (Sun et al., 1994). Briefly, after premedication with 0.5 mg atropine and then anesthesia by ketamine (50 mg/kg, i.m.) and rompun [2-(2,6-xylidino-)-5,6-dihydro-4H-1,3-thiazine hydrochloride] (12 mg/kg, i.m), a biplanar external fixator equipped with a lengthening apparatus was transfixed by four transverse K-wires inserted across the tibia. Three days following osteotomy of tibial and fibular shafts mechanical distraction of the hind limb through the external fixator to the osteotomy site was carried out daily at the rate of 1 mm/day (average: 5 mm/week; with 2 days' rest in each week). The traction experiment was completed when a total of 20 mm lengthening was obtained, then the animal was prepared for sacrifice. To prevent the postoperative starvation effect of bilateral osteotomy, the opposite hindlimb was used as non-operative control.

All animals were sacrificed under sodium pento-barbital (25 mg/kg b.w.) anesthesia by intravascular perfusion via descending aorta with a fixative of 2% paraformaldehyde and 2% glutaraldehyde in 0.067M sodium cacodylate buffer (pH 7.4) for both light and electron microscopy. At completion of perfusion, triceps surae, tibialis anterior, and tibialis posterior muscles were dissected out.

For electron microscopy, portions of tendon, muscles belly and MTJ from each muscle were cut into small pieces (1 mm^3) and fixed for an additional 4 to 6 hours. After buffer rinsing, the specimens were post-fixed in 1% osmium tetraoxide for 1 hour, dehydrated in a graded series of alcohols and embedded in an Araldite-epon mixture. Thin sections were cut, both in longitudinal and in cross-sectional profiles, and collected on carbon-reinforced parllodion-coated 75-mesh grids, doubly stained with uranyl acetate and lead citrate, and examined in a JEOL 2000EXII electron microscope at 150 KV. Semi-thin sections were also cut and stained with toluidine blue for correlative light microscopic study. The length of sarcomeres of each specimen was measured. The data were analyzed by paired Student's t-test.

Results

After lengthening, low power electron microscopic observation revealed gaps between the muscle and tendinous portions with the appearance of numerous mononucleated cells at the MTJ (Fig. 1). These mono-nucleated cells at the MTJ were identified as neogenetic cells because they were rarely observed in the corresponding region of the control specimen. Three types of mononucleated cells were categorized according to their ultrastructural characteristics.

Mesenchyme-like cells

Mesenchyme-like cells (Figs. 1, 2) appeared as small (2x8 µm) with a central, very dense oval nucleus in which patches of heterochromatin were obvious. In their scanty cytoplasm, few relatively small mitochondria, small quantities of polyribosomes and a rather small Golgi apparatus were localized around the nucleus. They were recognized as «primitive mesenchymal cells».

Putative precursor cells of myoblasts

These cells were larger (3x11 µm) than the previously mentioned mesenchyme-like cells and contained a centrally-located vesicular nucleus with a prominent nucleolus. Their cytoplasm was expansive and characterized by the presence of abundant rough endoplasmic reticulum, well-developed Golgi apparatus, numerous microtubules and cytoplasmic microfilaments (Fig. 2). The myogenic identify of this type of cell cannot be confirmed in the absence of sarcomeric arrangement of cytoplasmic filaments; however, it was located between the muscle fiber and its surrounding basal lamina. They were recognized as «putative precursor cells of myoblasts».

Satellite cells

The third type of cells were irregularly-shaped with numerous deep indentations and cytoplasmic projections on their surface. This type of cell contained an eccentric nucleus with patches of heterochromatin (Figs. 1, 3). Their cytoplasm contained numerous polyribosomes and many randomly-oriented cytoplasmic microfilaments. The mitochondria were typical and distributed randomly in the cytoplasm while the well-developed Golgi apparatus were always located in the perinuclear region. At higher magnification, different profiles of thin and thick microfilaments arranged into regular and irregular sarcomeres were observed. These arrangements of cytoplasmic filaments, recognized as different developmental stages of the sarcomere formation, are clearly demonstrated in Figures 3B and 4.

The sarcomere length was 2.27±0.10 µm for the control muscle and 2.16±0.34 µm for the experimental lengthened muscle. The difference was not statistically significant.

Fig. 1. Electron micrographs of myotendinous junction from lengthened triceps surae muscle. Low power view of the myotendinous junction. Note the increased electron-lucent gaps (g) between the muscle and tendinous portion and between the final muscle fiber and neogenetic tissue. In neogenetic tissue, satellite cells (S) and mesenchyme-like cells (Me) can be identified. Bar: 3 µm.

Fig. 2. Electron micrographs of myotendinous junction from lengthened triceps surae muscle. Note that there are putative muscle precursor cells (PM) adjacent to mesenchyme-like cells (Me). Note the abundant rough endoplasmic reticulum (ER) and ribosomes within thin cell. M: normal muscle cell; bl: basal lamina; cf: collagen fiber. Bar: 3 µm.

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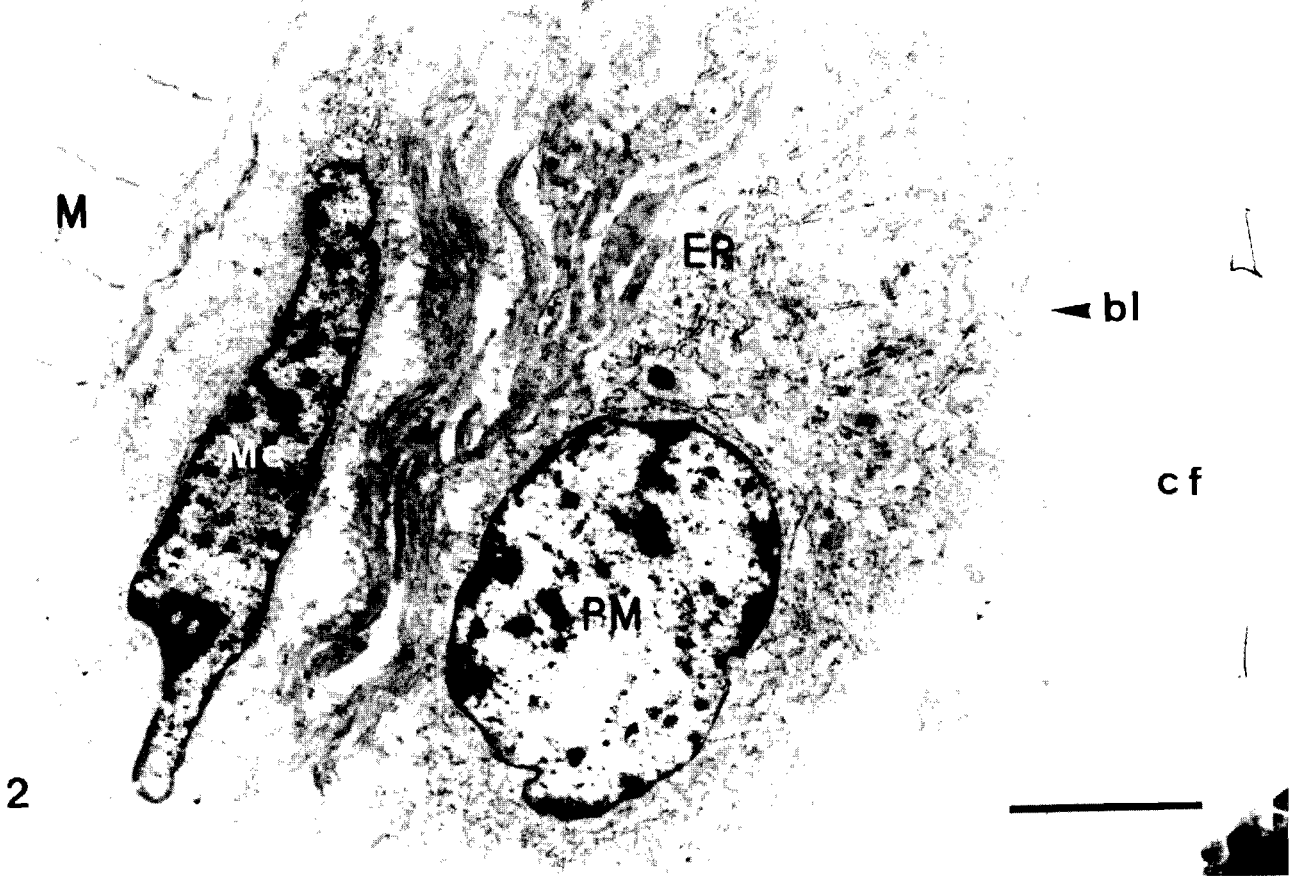

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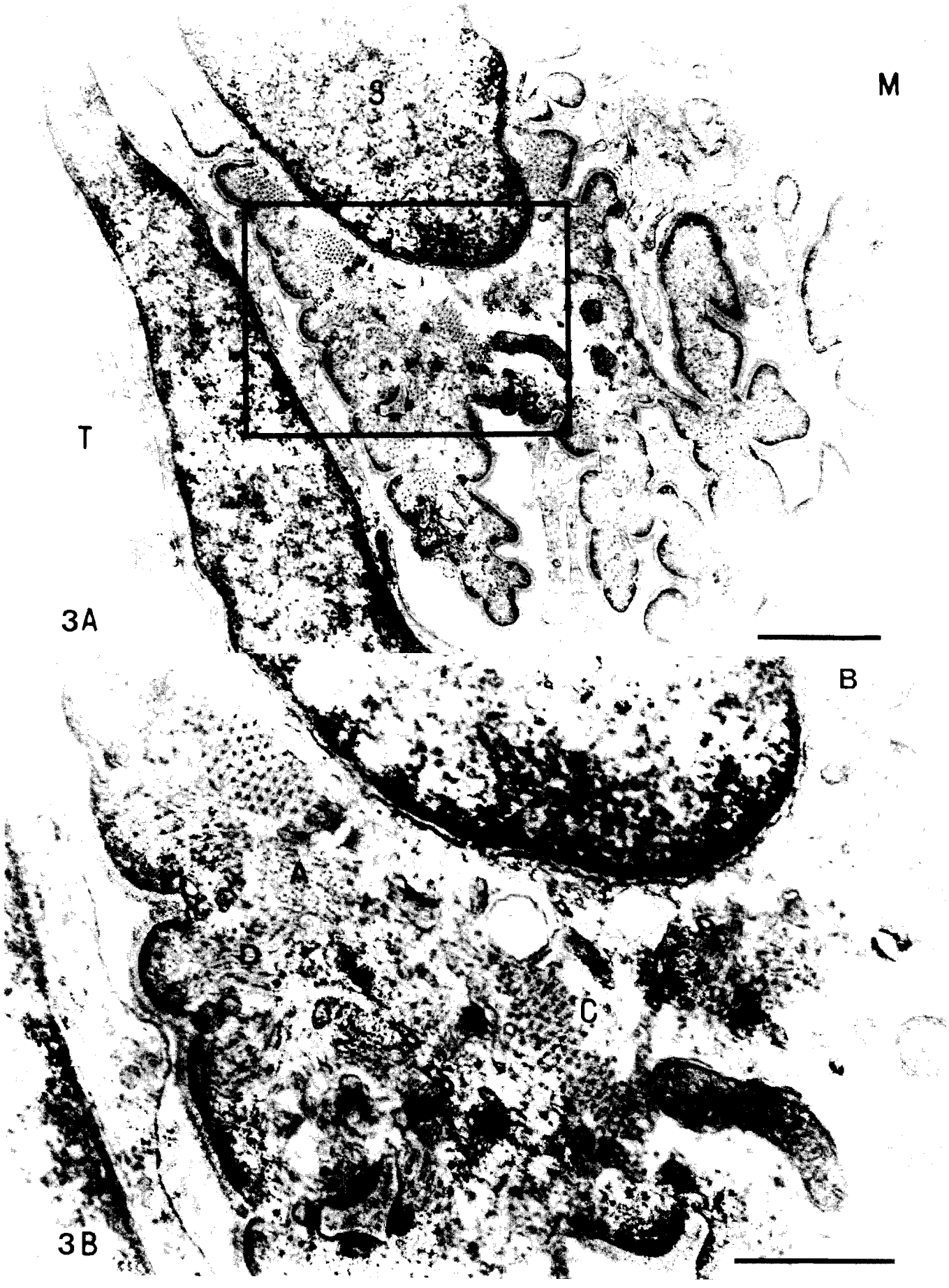
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Discussion

Many of the important factors which control skeletal growth or atrophy have been shown since the time of the early Greeks. Later, investigators showed that adult skeletal muscle did have great capability of regeneration in response to chemical (Basson and Carlson, 1980; Gutierrez et al., 1986) and physical (Perth et al., 1966; Snow, 1976; Garrett et al., 1984; Grounds, 1987) injury. The extent and success of regeneration varied with genetics, exercise, the nature of the injury, nutritional status, innervation, and hormonal factors (Vandenburg, 1987). In all situations the process involves injury and necrosis of mature skeletal muscle fibers, cellular infiltration and phagocytosis of necrotic myofibril debris, revascularization, proliferation of muscle precursor cells and their fusion and finally, re-innervation

Jennische (1986) reported that there is a rapid regeneration in postischaemic skeletal muscle with undisturbed microcirculation: mitotic figures were obvious in satellite cells of damaged as well as of uninjured muscle cells within 48 hours after the ischaemic insult. According to his study, all debris of injured cells were phagocytized and removed within 72 hours and replaced by clusters of myoblasts; small

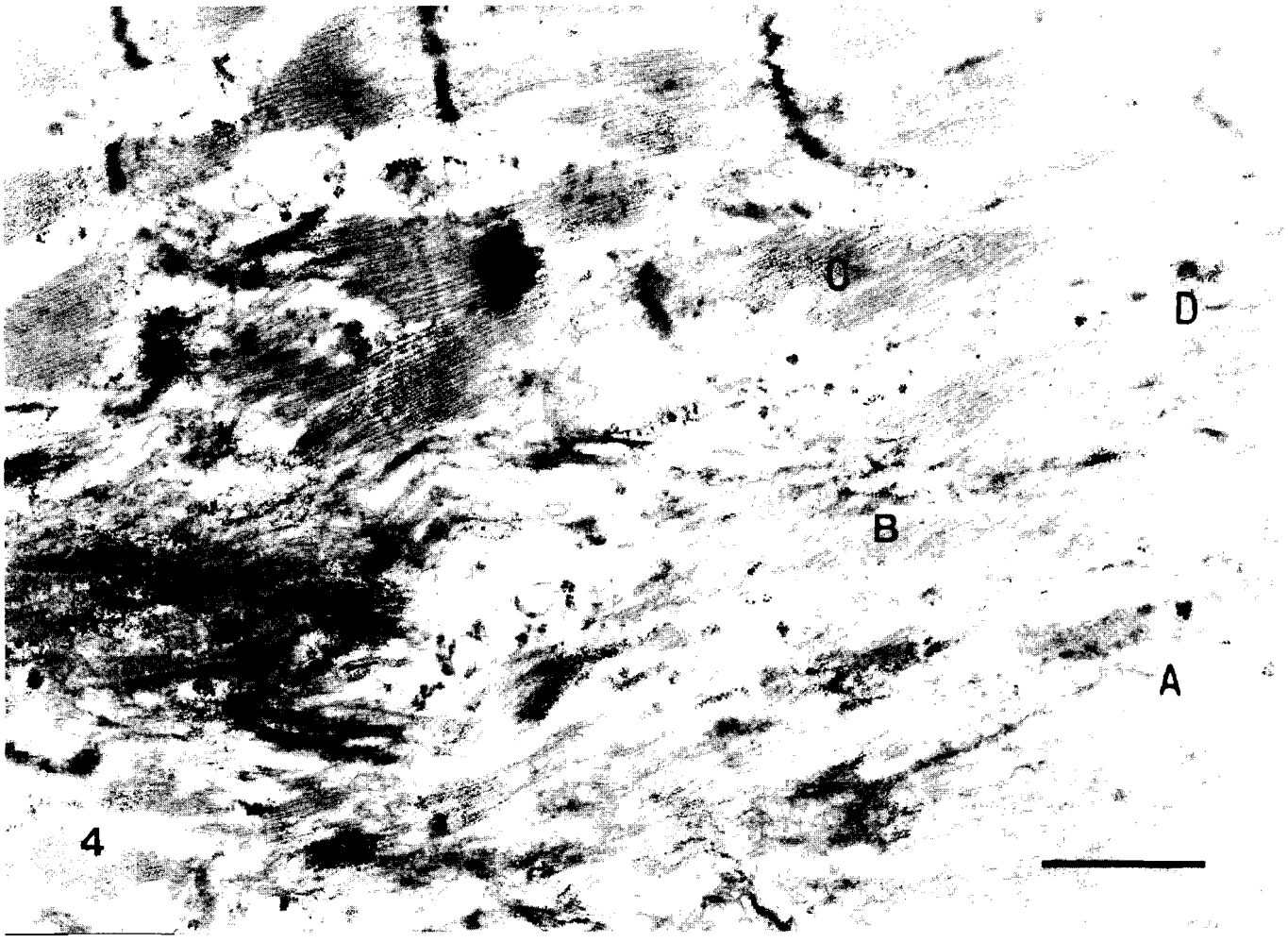

Fig. 4. Myofibrillogenesis at the myotendinous junction (MTJ) of a lengthened fiber. Within this fiber examples of various stages of longitudinally-assembled filaments are presented (labeled A-E). A: Clustering of a few thin filaments at a sarcolemmal cleft. B: At a later stage of development, thin and thick filaments directed longitudinally from a membrane cleft. Note the developing membrane triads within both of these nascent myofibrils (arrowheads). C: Larger accumulation of thin and thick filaments without sarcomeric register. D: Z-body attached to thin with thick filaments still not in sarcomeric register. E: Z-body within an aligned segment of thin (I-band) and thick (A-band) filament. Bar: 1 μm.

Fig. 3. A. Electron micrograph of a satellite cell (S) near the junction of a lengthened muscle (M) and its tendon (T). A centrally-located nucleus of A mesenchyme-like (Me) cell is seen. Bar: 1 μm. **B.** A higher magnification of a portion of the satellite cell showing developing myofilaments (Labeled A-D). A: Clustering of a few thin filaments. B: Loosely-packed thin and thick filaments. C:. Larger accumulations of thin and thick filaments. D: Obliquely oriented packed thin and thick filaments. Note the marked convolution with numerous projections of sarcolemmae. Bar: 0.5 μm.

immature muscle cells could be demonstrated within 96 hours after injury. During the entire regeneration process the basic architecture of the muscle was preserved and surviving muscle cells were present among the regenerating ones. Based on these data, we started traction of osteotomized limb on the 4th postoperative day in our experimental model, since the damage to its circulation was minimal.

Tidball and Chan (1989) reported that: (I) whole muscles loaded to failure frequently ruptured at or near the MTJ (II) site and stress at failure were independent of strain and strain rate over a biologically relevant range; and (III) the failure occurred at MTJ unless the muscle had suffered previous compression injury due to failure within the muscle. In the present study, increased distance with interposed primitive muscle precursor cells between the muscle fibers and tendon parenchyma (Fig. 1) may present a manifestation of microtrauma focusing upon the MTJ.

Bischoff (1989) reported that the mitotic inhibition imposed on satellite cells resulted from contact, with the myofiber plasmalemma and not the basal lamina. As noted in our study, there is an increased gap between the muscle and tendon near the MTJ, with many isolated primitive muscle precursor cells, including mesenchyme-like, putative myoblast and satellite cells, located within this space without any contact with adjacent sarcoplasm (Figs. 1, 2). It is reasonable to postulate that under chronic stretch, the mitotic inhibition on interposed primitive muscle precursor cells will be removed and the cells will be activated. This postulation may also partially explain the observations in a previous study that there was increased cellularity and presence of myoblast syncytia concentrated near the MTJ (Sun et al., 1994). Numerous reports have demonstrated that various cells of mesodermal (particularly fibroblasts and adipocytes) and neuroectodermal origin can give rise to muscle precursor cells under certain conditions (Tapscott et al., 1989; Wright et al., 1989). Changes in extracellular matrix *in vivo* might be involved in converting mononucleated cells within damaged muscle (other than satellite cells) into muscle precursor cells. Greeburg and Hay (1986) reported that differentiated epithelial cells are transformed into mesenchyme-like cells in the presence of purified collagen. Kuhl et al. (1986) also reported that fibronectin binding can change the phenotypic appearance of myoblasts into fibroblast-like cells. From these view-points, it seems possible that cells of non-muscle origin (particularly mesodermal) might well develop into muscle precursor cells *in vivo*, or the silent muscle precursor cells might be activated in situations where normal tissue architecture and cell communication are disrupted as in this study. In the present study, a gap present at MTJ may reflect a disruption of cell communication, and therefore, it is reasonable to suggest that this gap may induce the production of muscle cells or activate the population of silent cells.

The ability of MTJ to possess the power of regeneration after tenotomy was demonstrated by study of Baker and Poindextor (1991). There is evidence that the skeletal muscle is not only capable of regeneration after a second injury, but the rate of this regeneration is much faster (Gulati, 1986). This increased rate and recovery may be due to a conditioning effect of the first injury (Gulati, 1986). In our previous study, after repetitive microtrauma exerted upon the stretched limb, neogenesis of myotubules was evidenced at light microscopic level by the presence of numerous mononucleated cells at various stages of differentiation (Sun et al., 1994). This is further supported by the ultrastructure evidence that there are (1) gradual changes of maturation of the cells from mesenchyme-like cells toward putative and satellite cells (Figs. 1, 2) and (2) various stages of synthesis of myofilament within the satellite cells (Fig. 3).

It has been reported that new fibers might originate through proliferation then fusion of satellite cells (Allbrook, 1981), and that satellite cells have myogenic potential in damaged muscle (Schmalbruch, 1976). Tension is an important regulator of skeletal muscle hypertrophy *in vivo*. Fiber lengthening in response to stretch creates a need for rapid contractile protein synthesis and assembly into myofibrils at the MTJ. It was first suggested that muscle fibers lengthen by adding sarcomeres at the MTJ (Schmidt, 1927). It has also been reported that during stretch-induced growth, sarcomeres are added in series at fiber ends (Williams and Goldspink, 1973; Goldspink, 1985). The distance between the membrane and the bulk of the myofibrillar mass was greater in chronic stretch model (Dix and Eisenberg, 1990) than in developmental (Tidball and Lin, 1989) and cultured cardiac cell model (Lin et al., 1989). The great distance spanned by the elongating myofibrils provided for expression of various stages of sarcomere assembly within individual fibers (Hill et al., 1986).

In the present study, the sarcomere length was not increased significantly after prolonged traction. However, a significant increment in length of muscular portion in the experimentally-lengthened limbs was detected (Sun et al., 1994). As in the report of Simpson et al. (1993), we could expect that the number of sarcomeres in the experimentally-lengthened limbs would be increased significantly. The small «neogenetic» cells designated as satellite cells containing developing myofilaments were observed in stretched muscle (Fig. 3). Since muscle fiber regeneration was not observed in control muscle (Sun et al., 1994) we postulate that long term, chronic stretched skeletal muscle, as in the present study, may stimulate muscle fiber neogenesis.

Gulati (1988) reported that denervation resulted in progressive atrophy of muscle marked by a reduction in the size of myofibers and an increase in endomysial-perimysial connective tissue. He also proposed that the presence of intact innervation is crucial for the terminal differentiation and maturation of regenerating muscle. In

this series, the nervous function of the experimental animal seemed intact as evidenced by the well preserved motor function in the stretched limb; and this intact nerve function may support the development and differentiation of myofibrils (Figs. 3, 4).

In conclusion, we found that a large cytoplasmic space devoid of myofibrils forms at the ends of stretched fibers. Regional cells with synthesis of contractile proteins, sarcomere assembly, and extension of the myofibrils occur. Further studies concerning the molecular bases of myogenesis and myofibrillogenesis should be carried out to elucidate the mechanism of this event.

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