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Tendon and myo-tendinous junction in an overloaded skeletal muscle of the rat

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Summary. Overloading of rat plantaris muscles was produced by aseptic ablation of the synergists. The morphological changes occurring after 1 or 2 weeks were investigated at the light and electron microscopical level in the distal tendon of the plantaris and at the myotendinous junction. Sham-operated rats were prepared as controls. In the tendon, quiescent fibrocytes were replaced by activated fibroblasts displaying a vesicular nucleus with prominent nucleoli and an outstanding increase in cytomembranes, particularly the rough endoplasmic reticulum and the Golgi complex. The plasmalemma of the fibroblasts was modified by the presence of caveolae and the surbsurface cytoplasm contained many membrane-bound vacuoles. In the tendon, the collagen bundles were disrupted, resulting in the formation of empty longitudinally oriented spaces; in these spaces, as in the pericapillary areas, no inflammatory cells were observed. At the myotendinous junction, fibroblast activation was consistently observed in both control and overloaded specimens. At this level, the sarcolemma of the finger-like projections of muscle fibres presented many caveolae close to clusters of large subsurface vacuoles. These observations indicate that, at the beginning of the compensatory hypertrophy, the adaptative changes to overloading include a non-inflammatory reaction of the tendon characterized by enhanced collagen synthesis and intensive membrane renewal and recycling. From the mechanical point of view this reaction can impair the tendon resistance to stretch. At the myotendinous junction the increased membrane turnover of the sarcolemma and the fibroblast activation can be considered permanent phenomena consequent to the increased stress exerted upon the interface connecting the contractile apparatus to the stroma.

Key words: Tendon – Myotendinous junction – Fibroblast – Muscle overloading – Hypertrophy – Rat

Introduction

The protein components of the vertebrate muscle fibres are deeply modified from the beginning of hypertrophy. Many investigations have been devoted to the study of the changes taking place in the muscle fibres, particularly in the contractile apparatus. Changes in the non-contractile components, either inside or outside the muscle fibres, have been investigated much less. Nevertheless, the participation of non-contractile elements in muscle hypertrophy is well known. Jablecki et al. (1973) using quantitative autoradiography showed that work-induced growth of rat soleus muscle was accompanied within a few hours by increased synthesis of new RNA, mainly in proliferating connective tissue cells. The histological observations of Williams and Goldspink (1981) have supported this interpretation, by showing increased density of the endomysium and the perimysium in muscles overloaded after tenotomy of the synergists. In the same material there was a significant increase of the collagen content per unit of dry tissue weight, which was in agreement with the microscopical findings. Similar results were obtained by Suominen et al. (1980) and by Kovanen et al. (1984), who demonstrated an increase in the incorporation of collagen precursors in tendon and in muscle connective tissue during physical training of young mice and of adult rats, respectively.

Since the myotendinous junctions as well as the tendon could be involved in the response to muscle overloading, we have investigated the histological and cytological modifications occuring in these areas in the early stages of the compensatory changes in the rat plantaris muscles after ablation of the gastrocnemius and tenotomy of the soleus muscle. A partial account of this work has been already published (Zamora et al. 1986).

Materials and methods

Experimental design and surgical procedure. Twenty four adult, female Sprague-Dawley rats, 12 weeks old, were assigned randomly to either control or experimental groups. The plantaris muscles were overloaded by removing its synergists under aseptic conditions. Sham-operated rats of the same age, where the muscles were separated but not removed, were prepared as controls. The operation was done bilaterally to compel the animal to use both legs. The rats were anesthetized with sodium pentobarbital (4.5 mg/100 g body weight, intraperitoneally). After the skin incision, tenotomy of the soleus was carried out followed by ablation of the gastrocnemius; this ablation was as complete as possible, taking care to prevent damage to the blood supply and the innervation of the plantaris. Ablation of the gastrocnemius was chosen instead of simple tenotomy since this procedure precludes any postoperative occurence of reattachment or healing (for discussion of the surgical procedure see Armstrong et al. 1979; Vaughan and Goldspink

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1979). After surgery the animals were placed in standardized individual cages, where they were allowed to move and feed freely for 1 or 2 weeks. Identical cages were used to give all the animals similar possibilities of movement.

Microscopy. The rats were anesthetized by an intraperitoneal injection of chloral hydrate (35 mg/100 g body weight), and fixed by transcardiac perfusion of 1000 ml of aldehyde solution containing 1% (w/v) paraformaldehyde, and 1% (v/v) glutaraldehyde in 0.12 M monosodium-dipotassium phosphate buffer adjusted to pH 7.4, at room temperature. Care was taken when pinning the anesthetized animal to avoid any posture that could hinder the blood circulation of the hindlimbs. Immediately before passing the fixative solution, the rats received intracardiac injections of 0.1 ml of heparin and 0.5 ml of 1% sodium nitrite, separately. After perfusion, the hindlimbs were excised from the body, skinned, and immersed in the same fixative for 12 h. Plantaris muscles and tendons were then dissected out. Samples were taken from the distal tendon and the myotendinous region of the plantaris; they were cut in oriented plans to get longitudinal and transverse views. Tissue blocks were post-fixed in 2% solution of osmium tetroxide in phosphate buffer at 4° C for 2 h. After dehydration, the blocks were embedded in epoxy resin. Adjacent semi-serial 1000 nm and 80 nm sections were cut, stained, and observed in the light and electron microscopes, respectively.

Results

Control animals

Longitudinal semi-thin sections of the tendon shaft showed compact, regular bundles of collagen fibres running parallel to the lenght of the tendon. Embedded in the collagen, the fibrocytes appeared as slender cells with thin processes (Fig. 1). In transverse sections, the tendon appeared as a dense amorphous structure, in which multipolar spider-shaped cells with scanty perinuclear cytoplasm were lodged. In the electron microscope the densely packed collagen fibrils displayed a 70 nm longitudinal periodicity. The multipolar fibrocytes presented nuclei with dotted chromatin concentrated at the periphery; nucleoli were small or absent. The perinuclear cytoplasm was scarse and contained few cisternae of rough endoplasmic reticulum (RER) no thicker than 180 nm and filled with finely granular material (Fig. 2). The organization of the tendon shaft of sham-operated animals resembled that described by Merrilees and Flint (1980) in the tensional zone of the tendon of the flexor digitorum profundus of the rabbit.

The histology of the myotendinous junction of control animals corresponded to previous descriptions of this region (Mair and Tome 1972; Trotter et al. 1985). The muscle fibres were separated from the dense tendinous territory by clear spaces traversed by multiple microtendons (Moore 1983). The fibroblasts found between the microtendons contained a vesicular nucleus with one or two prominent nucleoli (Fig. 3) and large Golgi complexes and dilated cisternae of RER which spread also in the cytoplasmic projections (Fig. 4). The finger-like projections of the muscle fibres displayed large numbers of subsurface vacuoles and plasmalemmal caveolae (Fig. 5).

The extracellular spaces of the tendon and that of myotendinous junctions were not infiltrated with inflammatory cells.

Experimental animals

The most striking feature observed in the tendon of overloaded plantaris muscles, one or two weeks after surgery, was the transformation of quiescent fibrocytes into active fibroblasts. In fact, the entire population of slender spider-shaped fibrocytes was replaced by large fibroblasts, stacked against each other to form longitudinally oriented columns located in lacunae of extracellular space (Fig. 6). Like the cells found at the myotendinous junctions of control animals, the fibroblasts in the overloaded tendon had a vesicular nucleus, which frequently contained one or two prominent nucleoli. Not all the fibroblasts were enlarged to the same degree, but only a minority of them retained the shape and volume observed in control animals. Propitious sections showed hypertrophied fibroblasts in which the cytoplasm was split over several microns by a slender wedge of extracellular material oriented longitudinally. Such arrangements strongly recall the bundle-forming compartments postulated by Birk and Trelstad (1986) as intervening in tendon morphogenesis (Fig. 6, 11).

At the myotendinous junction, the active fibroblasts developed a high degree of polymorphism, projecting thick, undulating cytoplasmic processes between the end-processes of the muscle fibres (Fig. 7). Thus, by comparison with the control, the fibroblasts of the early overloading period developed a higher degree of activation of cytoplasm, nucleus, and nucleolus.

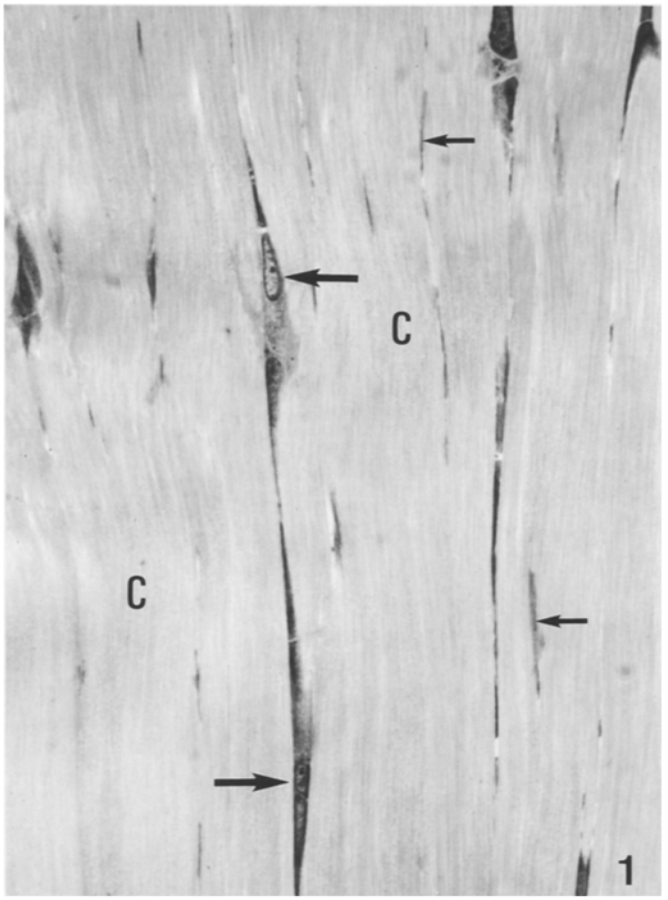
Fig. 1. Control, sham-operated rat, 7 days. Photomicrograph of longitudinally sectioned distal tendon of plantaris muscle. Collagen bundles (*C*) and fibrocytes (*large arrows*) form a compact structure. Narrow lacunae lodge slender cell projections (*small arrows*). × 640

Fig. 2. Control. Electron micrograph of a transverse section of the tendon. The fibrocytes (*F*) lie among compact collagen bundles (*C*), and send fine cytoplasmic processes (*small arrows*) containing narrow cisternae of RER (*large arrow*). × 13500

Fig. 3. Control. Photomicrograph of a longitudinal section of the myotendinous junction showing activated fibroblasts (*arrows*). *M*, muscle fibre. × 1000

Fig. 4. Control. Electron micrograph of a transverse section of the myotendinous junction. An activated fibroblast (*F*) displaying expanded cisternae of RER (*large arrows*) occupies the space between the microtendons (*mt*). *g*, Golgi complex. × 37000

Fig. 5. Control. Electron micrograph of a serial section of the myotendinous junction illustrated in Fig. 3. Multiple caveolae and clusters of vacuoles are shown (*large arrows*). *mt*, collagen fibres of the microtendon; *small arrows*, basal lamina. × 50000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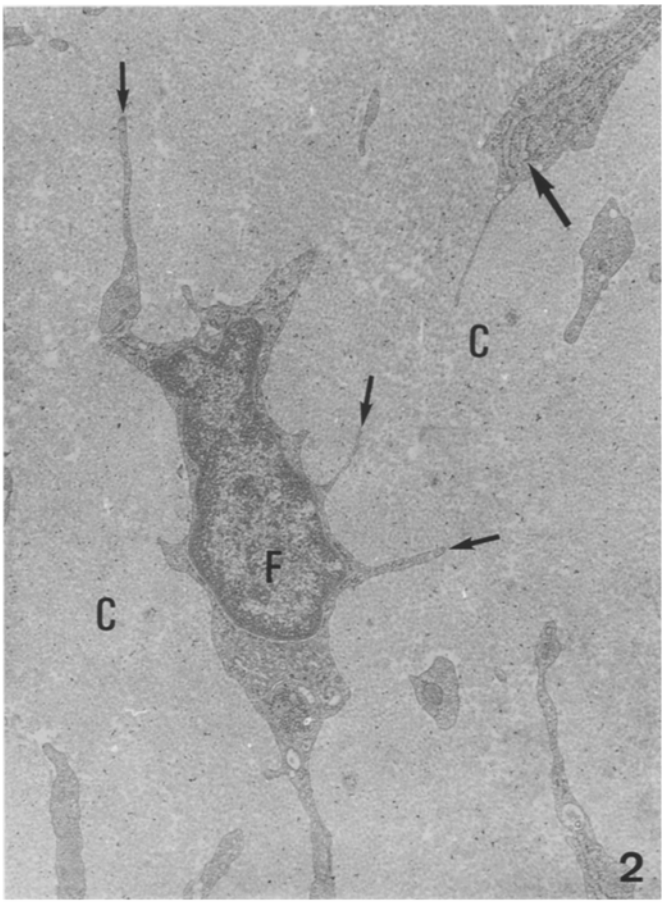
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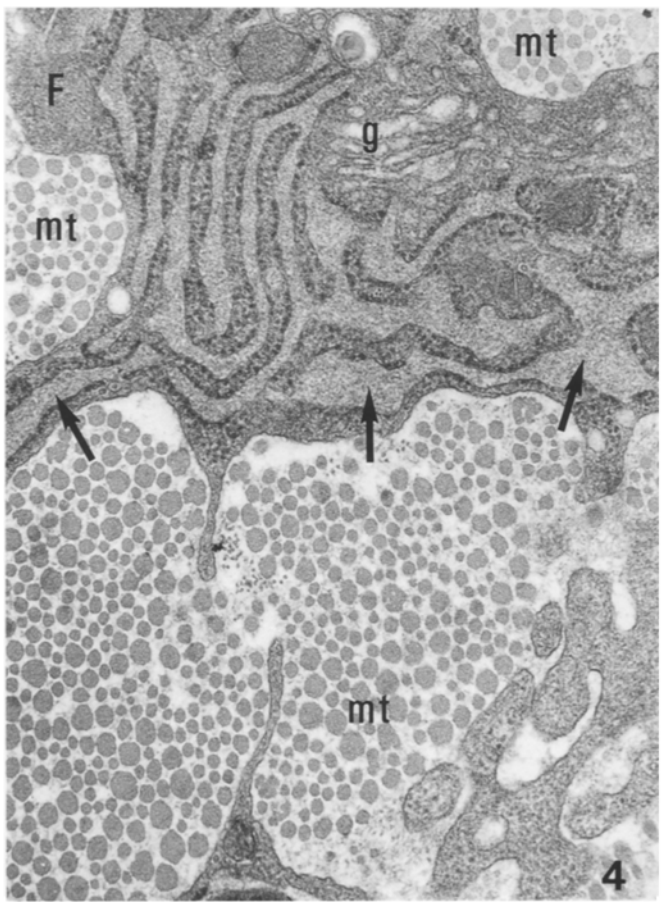
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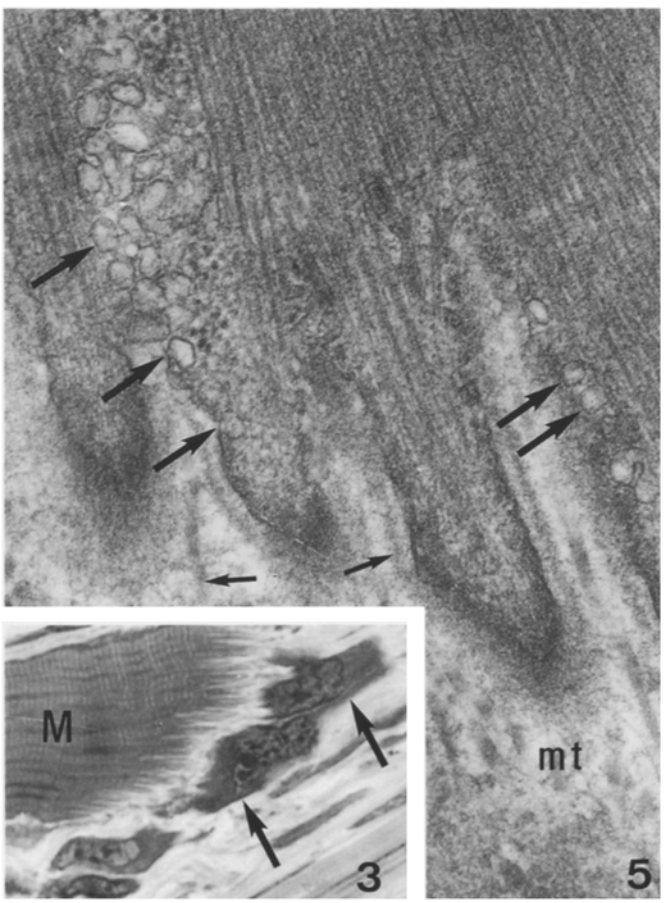
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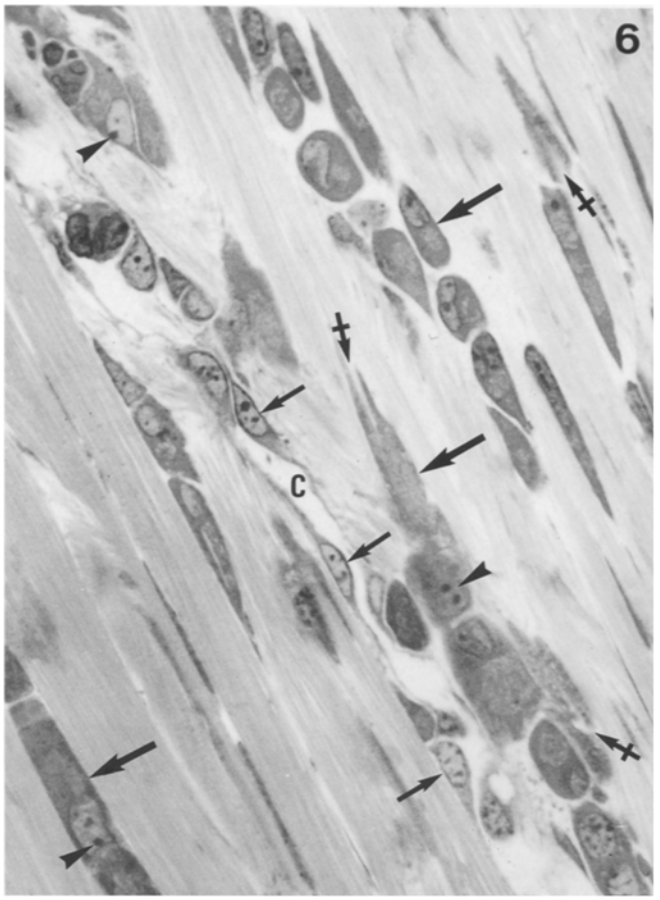
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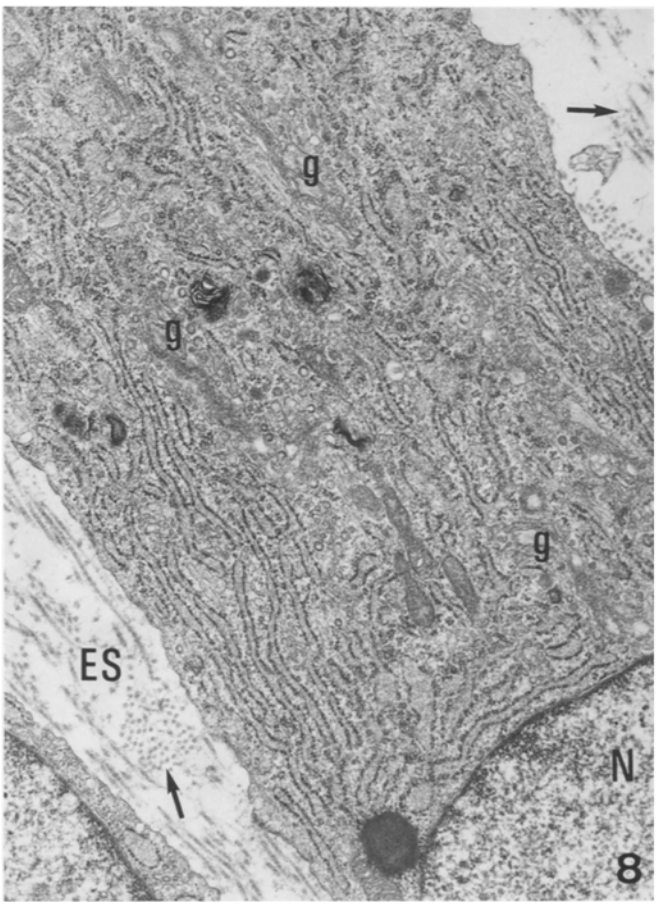
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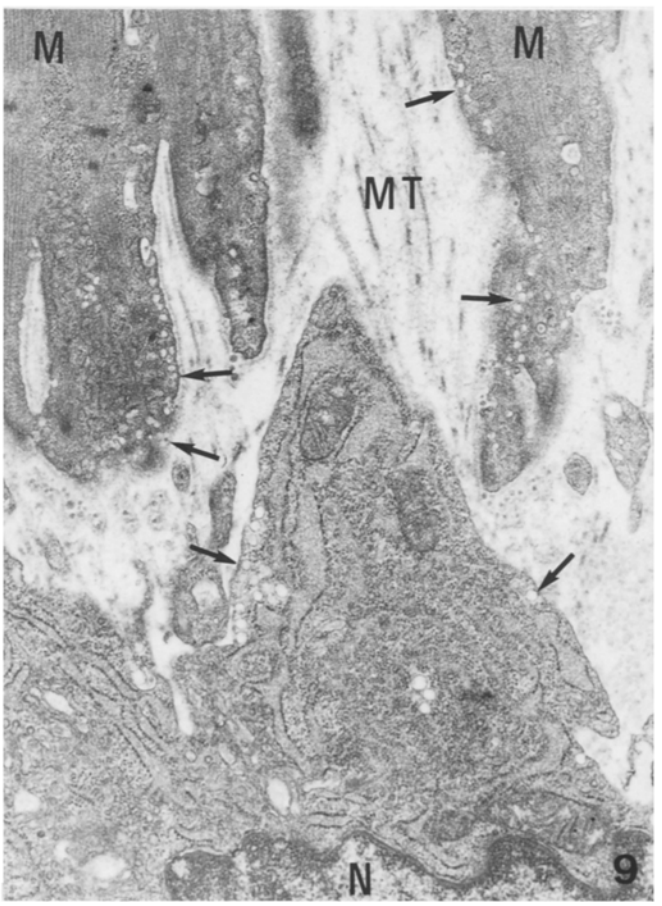
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Electromicroscopically, the enlarged lacunae of the extracellular space of the tendon and the spaces between microtendons were occupied by fibroblasts with nuclei showing fine chromatin granules concentrated mainly under the karyolemma. The cytoplasm displayed numerous cisternae of RER and ribosomes (Fig. 8). The cisternae contained fine electron-dense material, and many of them were enormously expanded (Fig. 6, 9, 10); these huge expansions of RER were preferentially observed in the fibroblasts of the tendon proper, in which multiple Golgi complexes were found (Fig. 8). The cytoplasmic processes contained microtubules and many intermediate filaments (Fig. 11) and their plasma membrane was associated with numerous caveola and vacuoles; the outer surface of the plasma membrane was coated by a fine web of filamentous structures connected to neighboring collagen fibrils (Fig. 12). At the myotendinous junction fibroblast projections intertwined with the muscle fibre end-processes (Fig. 7, 13). The latter displayed caveolae, coated and uncoated vacuoles, either isolated or forming racemose aggregates (Fig. 13). Capillaries were observed both in the tendon proper and at the myotendinous junction; neither around the capillary nor in the extracellular space at large were blood-borne or inflammatory cells found.

Discussion

We have previously published that one week of functional overload of the rat plantaris muscle resulted in a significant increase in muscle mass, and a quantitative analysis of type-identified single fibres in the deep region of the muscle showed that both the type I and the type IIe fibres had larger cross-sectional areas (Roy et al. 1987). These results contrast with others obtained in the soleus muscle. For instance, Vaughan and Goldspink (1979) showed that, in spite of an increment in muscle mass, the mean diameters of the fibres do not change one week after tenotomy of the synergists. One explanation for this discrepancy may be related to the fact that soleus and plantaris muscles are involved in a different manner in postural and locomotor activity, and are thus subject to a different overload during compensatory hypertrophy. As a matter of fact, no statically significant difference was observed in the electromyographical activity of overloaded soleus as compared to the contralateral muscle, two weeks after removal of the synergists (Hnik et al. 1986); furthermore, after a similar period of overloading of the plantaris there is a doubling of muscle fibre recruitment (involving type II fibres) (Gardiner et al. 1986). In this context it is most likely that "healing" of the small remaining stump of the myectomysed gastrocnemius onto the plantaris exerts very little influence on the actual degree of overloading undergone by the plantaris, particularly on its distal segment. So we can reasonnably postulate that tenotomy and myectomy of synergists entail overloading of the plantaris mainly by increasing the contractile activity and not by stretching the muscle.

In the present work, we analysed the changes occuring at the beginning of muscle hypertrophy, in which the quiescent fibrocytes normally present in the tendon underwent morphological changes, namely conspicuous development of RER, vesiculation of the nucleus, presence of multiple nucleoli and Golgi complexes; the peripheral cytoskeleton was reinforced and the plasmalemma became the site of accelerated recycling activity. These changes can be interpreted as the morphological correlates of the intensification of biochemical processes linked to hypertrophy, such as membrane biogenesis, synthesis of proteins and enhanced cell motility, which result in the increased secretion and deposition of both collagen fibres and ground substance. Jablecki et al. (1977) have demonstrated that the earliest biochemical change occurring in muscle hypertrophy is a rapid increase in phospholipid metabolism associated to membrane neoformation. Increase of the total protein synthesis in plantaris muscle compensatory hypertrophy has been reported by Noble et al. (1984), and Watt et al. (1982) demonstrated the same phenomenon in the soleus and the extensor digitorum longus after weight-pulling exercice. Moreover, the increased synthesis of RNA observed at the beginning of work-induced muscle hypertrophy is the expression of the proliferation of connective tissue cells (Jablecki et al. 1973). In tendons overloaded with enhanced physical exercice in the mouse (Michna 1984) a significant increase in the number, diameter, and cross-sectional area of collagen fibrils is already detectable after one week.

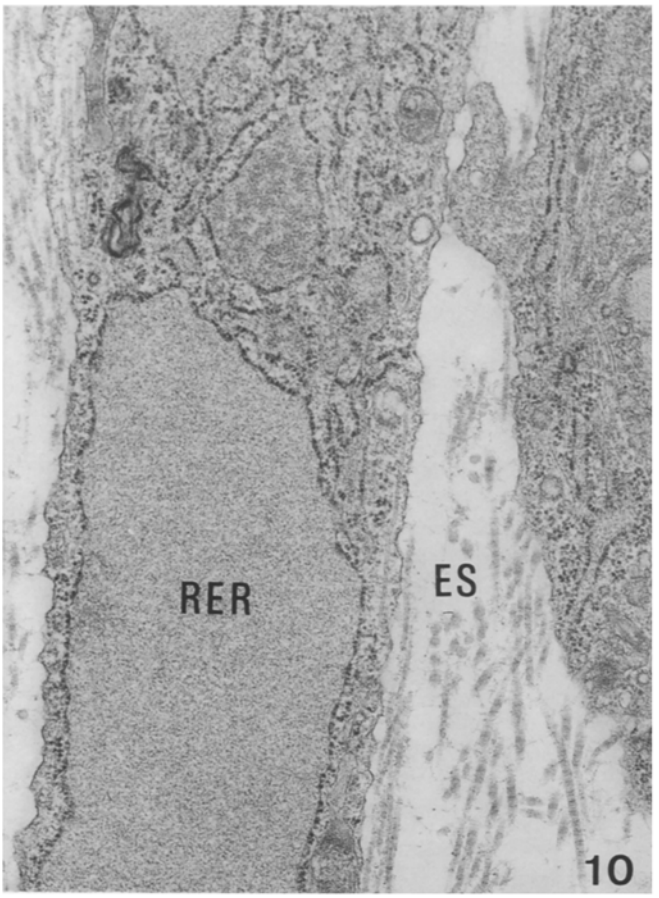
Induction of the synthesis of stromal components has been observed in other cases of functional overloading. Gabella (1984) has described an increase in collagen as well as in amorphous extracellular matrix in compensatory smooth muscle hypertrophy of the ileum. In this case, the phenomenon takes place in the absence of fibroblasts, the stromal components being produced mainly by the muscle cells themselves. Vascular smooth muscle is also able to synthesise collagen (Layman and Titus 1975) and matrix elements (Leung et al. 1976, 1977). Striated muscle fibres

Fig. 6. One-week overload. Photomicrograph of a longitudinally sectioned distal tendon, showing columns of activated fibroblasts (*large arrows*); their nuclei are vesicular and contain nucleoli (*arrow heads*). Some fibroblasts show splits (*crossed arrows*). In the middle of the picture a longitudinally sectioned capillary (*C*) shows endothelial cells (*small arrows*). The pericapillary area is not infiltrated with inflammatory cells. × 760

Fig. 7. One-week overload. Photomicrograph of the myotendinous junction. Many activated fibroblasts with vesicular nuclei (*large arrows*) send cytoplasmic projections (*small arrows*) that interdigitate with the end-processes of the muscle fibre (*M*). × 1000

Fig. 8. One-week overload. Electron micrograph of a semi-serial section of the tendon shown in Fig. 6. The cytoplasm of the fibroblast is rich in cytomembranes, particularly RER and Golgi complexes. The extracellular space (*ES*) is enlarged and contain dispersed collagen fibrils (*arrows*). *g*, Golgi complexes. *N*, nucleus. × 20000

Fig. 9. Two-weeks overload. Electron micrograph of the myotendinous junction (*MT*). End-processes of muscle fibres (*M*) interdigitate with projections of an activated fibroblast, whose nucleus (*N*) is at the bottom of the figure. Many subplasmalemmal vacuoles and caveolae are present in the fibroblast and in the muscle fibre (*arrows*). Compare to Fig. 7. × 15700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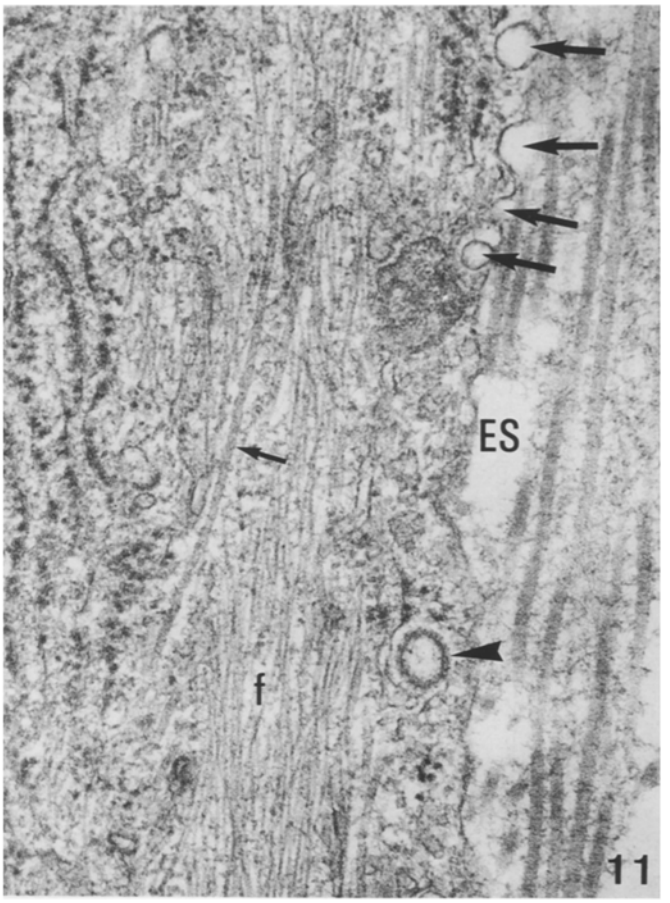
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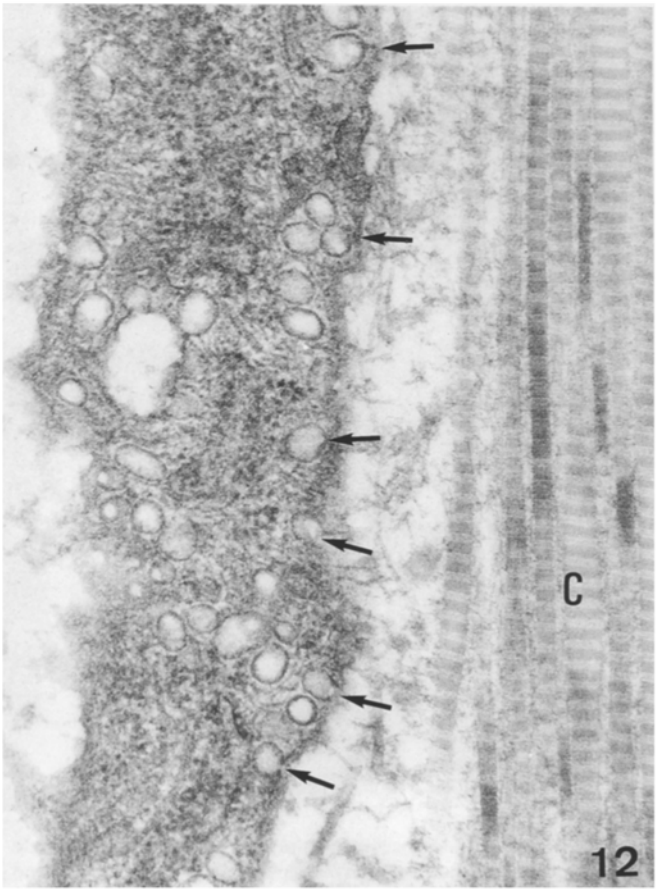
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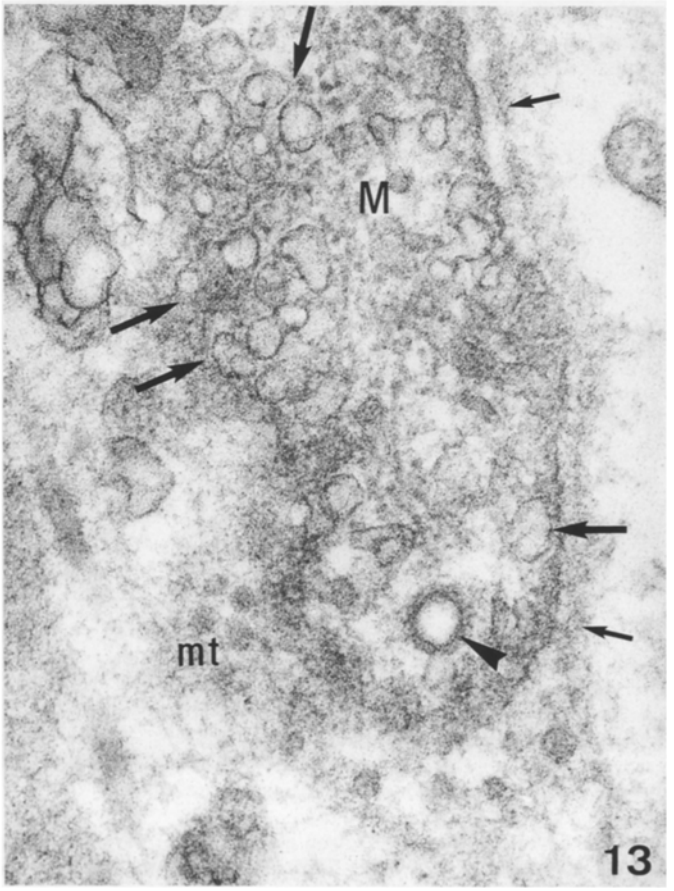
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are highly specialized cells and because of their inability to synthesize connective tissue macromolecules their adaptative hypertrophic response is coordinated with an activation of the fibroblasts.

One interesting aspect of the present study is the finding that the cells normally lying at the myotendinous junction of control specimens have the cytological characteristics of activated fibroblasts. One explanation for this condition may be given if one postulates that the magnitude of mechanical loading imposed to the junctions during ordinary activity is sufficient to stimulate a sustained synthesis of fibroblastic products; on the muscle side of the junction we have also found signs of plasmalemmal activity indicating membrane recycling. Although Tidball and Daniel (1986) have pointed out that caveolae and vacuoles can be the expression of diverse cellular activities, there is evidence from various tissues suggesting that these membrane specializations correspond to exo-endocytotic activity linked to maintenance of the integrity of the plasmalemma (Besterman et al. 1981; Steinman et al. 1983). Therefore, if one assumes that the sarcolemma at the myotendinous junction is constantly supporting heavier mechanical stresses than the rest of the non-contractile apparatus (Tidball 1984; Tidball and Daniel 1986), it is likely that at both sides of this interface there is an intense demand for the renewal of cell membranes and adhesion macromolecules.

The modifications observed in the stroma of the muscle in the early stages of compensatory hypertrophy have been attributed to inflammation caused by surgery (Armstrong et al. 1979). According to these authors, the inflammatory signs become negligible by 16 days after the operation. However, in the present study, no leukocyte infiltration was detected in the tendon nor at the myotendinous junction. On the other hand, the activation of non-contractile elements entails deep modifications of the tendinous extracellular materials, including the enlargement of the intertrabecular lacunae that lodge the fibroblasts, and disaggregation of collagen bundles; these changes make the hypertrophying tendon resemble the developing tendon (Birk and Trelstad 1986). While these structural changes constitute histological and mechanical adaptations to overloading, the remodeling of the tendon architecture may involve a transient period of mechanical weakness of the locomotive apparatus.

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Fig. 10. One-week overload. Cytoplasmic processes of an activated fibroblast with a large cisternae of RER (*RER*). Note the enlarged extracellular space (*ES*) containing scattered collagen fibrils. ×23000

Fig. 11. One-week overload. Activated fibroblast forming a narrow slit as shown by *crossed arrows* in Fig. 6. Many intermediate filaments (*f*) and microtubules (*small arrow*) occupy the peripheral cytoplasm. The plasmalemma shows uncoated caveolae (*large arrows*), and one large subsurface coated vacuole (*arrow head*). *ES*, extracellular space containing collagen fibrils. ×40000

Fig. 12. Two weeks overload. The plasmalemma of a projection of an activated fibroblast (to the left) shows many caveolae and vacuoles (*arrows*). A fine trabecular web projects from the plasma membrane into the pericellular space. *C*, collagen bundle. ×46000

Fig. 13. Two weeks overload. End-process of a muscle fibre (*M*) showing caveolae and vacuoles (*large arrows*). At the lower extremity, a large coated vacuole is present (*arrow head*). *Small arrows*, basement membrane; *mt*, microtendon. ×85000

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