Summary

Experimental tenotomy induces degenerative changes in muscle fibers, followed by an internal repair process. This study examined the cytoarchitectural changes taking place in muscle fibers due to delayed tension recovery prompted by removal of a segment of tendon. The anterior tibial muscles of Wistar rats were studied using light and electron microscopy at 4, 5 and 6 weeks after tenectomy and compared with the same muscles in controls 4 weeks after tenotomy. Control muscles displayed occasional core-like fibers. Tenectomized muscles, in addition to a larger number of core-like lesions, exhibited myofibrillar disorientation as evidenced by desmin staining and electron microscopy, which enabled identification of ring fibers, snake-coil fibers and occasional lobulated fibers. Whereas core-like lesions tended to disappear, the number of fibers displaying a myofibrillar disorientation increased with the time post-tenectomy. This suggests that extraction of a segment of tendon delays the repair of tenotomy-induced core-like lesions and gives rise to abnormal repair, leading to the formation of ring and snake-coil fibers.

Key words: Muscle fiber – Repair – Tenotomy – Tenectomy – Core-like fiber – Ring fiber

Introduction

The term “muscle repair” refers to the restoration of one or more damaged elements following an injury that does not lead to the death of the muscle fiber or a segment of it. It is thus distinct from regeneration, the process by which – following injury – necrotic muscle fibers are replaced by new fibers (Carpenter, 2001). The morphology of muscle fiber repair will depend on which cytoplasmic components have undergone pathological alteration (Carpenter and Karpati, 2001).

In experimental muscle pathology, tenotomy is a suitable model for the study of myofibrillar regeneration (Karpati et al., 1972). The reversibility of the injuries undergone by tenotomized muscle fibers, which return to histological normality 4-6 weeks post-tenotomy (Baker and Hall-Craggs, 1980; Baker and Poindextor, 1991), provides a rare example of muscle fiber repair (Carpenter and Karpati, 2001). Tenotomy leads to degeneration or breakdown of myofibrils in zones that are sharply demarcated within fibers; these injuries are known as core lesions, due to their resemblance to the injuries seen in central core disease (Chou, 1984; Jamali et al., 2000). However, while the latter are static, tenotomy-induced core lesions undergo a process of dynamic or reparative remodeling (Carpenter...
and Karpati, 2001). Thus, in order to reflect this distinction, they are referred to as core-like lesions (Karpati et al., 1972). Repair of these lesions involves de novo myofibrillar formation, which is evident in basophilia and increased ribosome numbers (Wróblewski and Edström, 1983) and in the expression of desmin, in turn indicative of cytoskeletal reorganization (Kaminska and Szyluk, 1996).

Just as the progress and effectiveness of muscle regeneration may be hindered by impaired revascularization, reinnervation, or tension (Grounds, 1991), muscle fiber repair may be hindered by any modification of the conditions in which it occurs. However, little information is available about the consequences of abnormal muscle fiber repair, although it has been suggested that the presence of aberrant myofibrils, ring fibers, trabecular fibers and sarcoplasmic masses would indicate focal failure of myofibrillar regeneration in pathological human muscle (Del Bigio and Jay, 1992; Niiyama et al., 2002).

Given that tenotomy is an experimental model of myofibrillar repair, the purpose of this study was to identify the cytoarchitectural abnormalities that may arise when normal skeletal muscle fiber repair is modified. Crawford (1975) reported that simple tendon transection in the anterior tibial muscle may facilitate tendon regeneration, whereas prevention of regeneration by gross displacement of the transected tendon may lead to imperfect tendon repair and reduced muscle growth. Tenectomy may thus be reasonably expected to influence muscle fiber recovery following injury, leading to modification of the histological features of the repaired muscle fibers. In light of this, here we were prompted to study the effects of tenectomy; in addition to cutting the tendon, a segment was removed in order to hinder or delay tension recovery and hence modify the conditions under which muscle fiber repair took place.

**Material and Methods**

**Animals**

A total of 16 male Wistar rats weighing roughly 300 g. were used in this study. The experimental procedures were approved by the University of Córdoba Ethical Committee on Animal Experimentation.

**Tenotomy and tenectomy**

Rats were divided into four groups of four. In the control group, both anterior tibial muscles were exposed and cut (tenotomy), whereas in the other three groups both muscles were exposed and a 5 mm-long section was extract ed (tenectomy). Finally, the skin was sutured and disinfected.

Tenectomized rats were killed at 4, 5 and 6 weeks, while control tenotomized rats were killed 4 weeks post-tenotomy, i.e. the time required for muscle histology to return to normal (Baker and Hall-Craggs, 1980; Baker and Poindexter, 1991). In all cases, rats were decapitated under ethyl ether anesthesia, and the anterior tibial muscles were extracted from their compartments.

**Light and electron microscopy**

The middle third of each muscle was immediately frozen in isopentane precooled in liquid nitrogen. Serial sections 8-10 μm thick were cut on a cryostat and stored at -20°C until examination. Sections were stained with hematoxylin-eosin, NADH-tetrazolium reductase, ATPase at pH 9.4 and acridine orange. For desmin immunostaining, an antidesmin monoclonal antibody was used (1:50, Desmin, DERM-11, Dako, Denmark).

Small muscle samples for electron microscopy were fixed by immersion in 2.5% buffered glutaraldehyde, postfixed in osmium tetroxide, embedded in Araldite and cut on an ultramicrotome. Semithin sections were stained with toluidine blue and ultrathin sections were contrasted with uranyl acetate/lead citrate and examined under a Philips CM10 transmission electron microscope.

**Quantification**

Counts were performed using a Leitz Dialux 20 microscope with a built-in camera, connected to a PC running an image analysis program (IMAGO, Grupo SIVA, University of Córdoba, Spain). Quantitative analysis included abnormal muscle fibers as a percentage of the total fibers per area. Subsequently, each fiber abnormality—core-like fibers and fibers showing myofibrillar disorientation (ring fibers and snake-coil fibers)—was expressed as a percentage of the total abnormal fiber count. These counts were performed on antidesmin-stained sections in four randomly-selected fields. A minimum of 250 fibers was analyzed.

**Statistical analysis**
Results were expressed as mean percentages ± standard errors for the fields analyzed in the eight muscles of each group. Differences were evaluated by simple analysis of variance, followed by multiple comparison using the Newmann-Keuls test (p<0.05).

RESULTS

Light and electron microscopic examination of control muscles 4 weeks post-tenotomy revealed few changes, consisting of the presence of core-like fibers (Table 1). By contrast, in addition to core-like lesions, fibers in the three tenectomized groups displayed other cytoarchitectural abnormalities in the form of myofibrillar disorientation. Quantitative analysis revealed that the percentage of core-like lesions was higher in the tenectomized groups than in controls, with an especially marked difference in the 4-week group; thereafter, the percentage of core-like lesions gradually diminished as the post-tenectomy time increased, while the percentage of fibers displaying myofibrillar disorientation increased (Table 1).

<table>
<thead>
<tr>
<th>% affected fibers</th>
<th>% core-like fibers</th>
<th>% fibers with myofibrillar disorientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 weeks post-tenotomy</td>
<td>0.3 ± 0.04</td>
<td>99.4 ± 0.2</td>
</tr>
<tr>
<td>4 weeks post-tenectomy</td>
<td>19.9 ± 2.3*</td>
<td>51.5 ± 4.2*</td>
</tr>
<tr>
<td>5 weeks post-tenectomy</td>
<td>10.3 ± 1.8#†</td>
<td>38.0 ± 2.8#†</td>
</tr>
<tr>
<td>6 weeks post-tenectomy</td>
<td>5.6 ± 1.9##‡</td>
<td>8.5 ± 1.7##‡</td>
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* significant differences vs. 4 weeks post-tenotomy (p<0.05)
† significant differences vs. 4 weeks post-tenectomy (p<0.05)
‡ significant differences vs. 5 weeks post-tenectomy (p<0.05)

Core-like fibers

Core-like lesions, which affected both type 1 and type 2 fibers, were visible as basophilic areas in muscle fibers, primarily in central locations but also in some peripheral zones. These areas showed no reaction to ATPase and NADH-tr staining, and discrete orange fluorescence in response to acridine orange; granular material stained moderately positive for desmin. The proportion of fibers displaying core-like lesions was greatest at 4 weeks post-tenectomy, thereafter decreasing significantly as the post-tenectomy time increased (Table 1). Ultrastructurally, these areas exhibited myofibrillar disorganization and a loss of alignment, together with the absence of mitochondria. Polyribosomes were visible around the edges of the core area.

Fibers displaying myofibrillar disorientation

In the three groups of tenectomized rats, a number of muscle fibers exhibited histological, histochemical and ultrastructural features indicative of myofibrillar disorientation. These were identified as ring fibers, snake-coil fibers and lobulated fibers. The number of fibers displaying myofibrillar disorientation increased progressively with the post-tenectomy time, while the number of core-like lesions declined (Table 1).

Examination of H-E stained sections disclosed some small fibers with well-rounded outlines, alongside larger fibers with irregular outlines that in some cases displayed cytoplasmic protrusions arranged between adjacent fibers. The smaller fibers, identified by NADH-tr staining as type 1 fibers, exhibited an intermediate ring showing no oxidative activity (Fig. 1); anti-desmin staining of this ring disclosed a circular arrangement of myofibrils (Fig. 2a). By contrast, type 2 fibers were larger, had less oxidative activity and did not display the pattern disclosed by NADH-tr in the smaller fibers; the circular myofibrillar arrangement observed in type 2 fibers differed from that of type 1 fibers in that the circular myofibrils passed through the center of the fiber (Fig. 2b).

In most ultrastructurally-identified ring fibers, mitochondrial clusters were observed in the subsarcolemmal region, below which polyribosomes and circular myofibrils were visible (Fig. 3). Myonuclei were irregularly-shaped, with dense patches of chromatin lining the nuclear membrane. Small numbers of activated satellite cells were occasionally observed; although they were surrounded by
Fig. 1. Many type 1 muscle fibers have rounded outlines and show a characteristic pattern of oxidative enzyme distribution. NADH-tr. x 250.

Fig. 2. Antidesmin staining. a: Ring fibers show circumferential peripheral myofibrils (arrows). b: Two muscle fibers with ring myofibrils that cross through its center (arrows); their cross-sectional outline is irregular. a: x 400; b: x 400.

Fig. 3. Several myofibrils arranged longitudinally at the edge of the fiber (curved arrow) around the transverse central myofibrils, in the vicinity of a neuromuscular junction (nj). x 5,200.

Fig. 4. Transmission electron micrograph showing a probably activated satellite cell enclosed by the basal lamina (bl), associated with a ring fiber. The peripheral myofibrils lie transversely (arrow) around the central longitudinal myofibrils. x 15,500.
Abnormalities in tenectomized muscle fiber repair

Fig. 5. Muscle fiber showing a disoriented bundle of myofibrils (arrow). Antidesmin. x 400.

Fig. 6. Transmission electron micrograph showing a muscle fiber with an abnormal orientation of myofibrils. x 5,000.

Fig. 7. Muscle fibers with a “lobulated” aspect. NADH-tr. x 250.

Fig. 8. Transmission electron micrograph showing clusters of mitochondria between disoriented myofibrils at subsarcolemmal level. x 6,610.

basement membrane, these protruded onto the fiber surface (Fig. 4).

In some large, deformed fibers, anti-desmin staining disclosed an irregularly-arranged striate pattern; these fibers were identified as snake-coil fibers (Fig. 5). Ultrastructurally, myofibrils displayed a disorientation of the longitudinal pattern (Fig. 6).

In the anterior tibial muscles of two rats killed 4 weeks post-tenectomy, some fibers exhibited characteristic peripheral lobular or trabecular staining to NADH-tr (lobulated fibers); these lobulated fibers were more numerous in only one case (Fig. 7). They were devoid of anti-desmin staining activity.
Ultrastructural examination disclosed poorly-aligned peripheral myofibrils interspersed with clustered mitochondria (Fig. 8).

Of the fibers displaying myofibrillar disorientation, most were ring fibers (75%, 80% and 87% at 4, 5 and 6 weeks post-tenectomy, respectively), whereas snake-coil fibers accounted for 25%, 20% and 13% over the same intervals. Trabecular or lobulated fibers were not counted, since they were observed only occasionally.

DISCUSSION

The results of the present study indicate that cutting and extraction of a section of tendon (tenectomy) leads to a delay in muscle fiber repair and the appearance of cytoarchitectural abnormalities consisting in abnormal orientation of myofibrils, giving rise to ring fibers, snake-coil fibers and, occasionally, lobulated fibers. Since in normal conditions tenotomized muscle returns to normality, the cytoarchitectural abnormalities observed here must be the result of a tenectomy-induced tension deficiency.

The virtual absence of core-like lesions in the control (tenotomy) group was indicative of a return to histological normality, previously reported at 4-6 weeks post-tenotomy (Baker and Matsumoto, 1988; Baker and Poindexter, 1991). By contrast, the core-like fibers observed under light and electron microscopy in the tenotomized groups displayed the features reported by other authors for tenotomized muscles (Kamińska and Szyluk, 1996; Abou-Salem et al., 2001), and were indicative of a process of dynamic or reparative remodeling (Carpenter and Karpati, 2001). Other changes reported in tenotomized muscle, such as nemaline bodies (Engel et al., 1966) and target fibers (De Reuck et al., 1977, 1978) were not observed here; this may be due to the varying susceptibility of different muscles to tenotomy (Karpati et al., 1972).

The persistence of core-like fibers 4 weeks after tenectomy would suggest that repair was delayed; however, it was not completely halted, since the proportion of core-like fibers decreased as the post-tenectomy time increased. The delay was in all probability prompted by the extraction of a tendon segment; delayed tendon repair has been reported to lead to significant sarcomere loss, implying that more time is required to regain normal status (Abrams et al., 2000). This would account for the presence of core-like lesions in the post-tenectomy group, but not the post-tenotomy one, in the present study.

The progressive decrease in the number of core-like fibers was matched by an increase in the number of fibers displaying myofibrillar disorganization, suggesting that the latter may result from the development of the former. A number of studies have reported late-stage abnormal myofibrillar arrangement following tendon injury in some muscle fibers (Tomanek and Cooper, 1972; Jósza et al., 1978; Gerber et al., 2004). By contrast, in the present study, myofibrillar disorientation was very marked, indicating that the cutting and extraction of a section of tendon (tenectomy) leads to a delay in muscle fiber repair and the appearance of cytoarchitectural changes consisting of an abnormal orientation of myofibrils. This gives rise to ring fibers, snake-coil fibers and, occasionally, lobulated fibers, which appear to be due to impaired recovery from tenectomy-induced muscle injury.

In human skeletal muscle, snake-coil and ring fibers may be the consequence of a cytoskeletal disorder (Carpenter and Karpati, 2001); in cross-section, ring fibers display a circular arrangement of myofibrils in the subsarcolemmal region, while snake-coil fibers characteristically exhibit a much more striking myofibrillar disorientation affecting the inside of the fiber. Aberrant myofibrils and a poor distribution of the intermyofibrillar mitochondria are also observed in lobulated or trabecular fibers, caused by malfunction of a putative anchoring mechanism (Weller et al., 1999). Since the repair of tenotomy-induced lesions is known to involve the formation of new myofibrils (Baker, 1983; Wróblewski and Edström, 1983) and an increased expression of desmin, which is essential for the maintenance of the internal structure of the fiber (Kamińska and Szyluk, 1996), tension deficiency may lead to a failure of the new myofibrils to become attached to cytoskeletal components, leading to abnormal myofibrillar alignment. The appearance of three distinct types of cytoarchitectural alteration in our study raises the possibility that all of them might have derived from the same pathogenic mechanism, and is evidence of deficient repair. However, it
cannot be concluded from these experimental findings that there is a common pathogenic mechanism with cytoarchitectural changes similar to those observed in human muscle pathology. Although some authors have reported that muscle regeneration processes in several myopathies may favor the appearance of abnormalities such as ring fibers, trabecular fibers and sarcoplasmic masses (Del Bigio and Jay, 1992; Niiyama et al., 2002), recent studies suggest no involvement of an abnormal regeneration process in the pathogenesis of trabecular fibers (Weller et al., 1999) or sarcoplasmic masses (Vattemi et al., 2005).

Interestingly, most ring fibers were type 1, while snake-coil fibers were type 2. It is not clear why this should be so, but it may be linked to a differing susceptibility of red and white fibers to tenotomy (Tomanek and Cooper, 1972; Reuck et al., 1977, 1978; Luque et al., 2002), or to a different cytoskeletal response to different types of aggression. Slow-twitch and fast-twitch fibers are reported to display a significantly different cytoskeletal response to denervation, which would account for their differing susceptibility to denervation-induced atrophy (Boudriau et al., 1996). A recent report has shown that plectin, a cytoskeletal protein, is involved in the formation of certain cytoarchitectural abnormalities such as lobulated fibers (Vita et al., 2003). This protein is expressed in type 1 fibers, but much less so in type 2 fibers (Schröder et al., 1999). This suggests that type 2 fibers would be more susceptible to cytoskeletal alterations, which might account for the deformity of some type 2 fibers encountered here. The differing repair process observed here for type 1 and 2 fibers may also be due to differences in the cytoskeletal response to tension deficiency.

In a previous study it was found that tenotomized regenerating fibers developed into ring fibers (Peña et al., 2001). In that study, however, there was no evidence of snake-coil or trabecular fibers. In contrast with the results observed in the present study, this would suggest that regenerating muscle fibers may be less predisposed than those undergoing repair to cytoarchitectural changes in response to tension deficiency. This could explain the morphological and functional immaturity of regenerating muscle fibers as compared to adult fibers undergoing repair. It is well known that structural changes in tenotomized muscles appear with intact innervation (Karpati et al., 1972; De Reuck et al., 1977); regenerating fibers, by contrast, grow and develop – at least initially – in the absence of innervation (Gulati, 1988; Grounds, 1991). This may protect regenerating fibers from tenotomy-induced injury, but not from disorientation of peripheral myofibrils, since during regeneration myofibrillogenesis largely takes place at subsarcolemmal level (Dix and Russell Eisenberg, 1991). Nevertheless, the present study focused on muscle fibers with intact innervation that were affected by injuries due to sectioning of the tendon, and whose repair involved both central and subsarcolemmal myofibrillogenesis (Kamińska and Szyluk, 1996).

The role of satellite cells in muscle regeneration is well documented; however, their involvement in muscle fiber repair processes remains obscure. Tenotomy in mouse anterior tibial muscle has been shown to prompt a significant degree of cell proliferation (also involving satellite cells) after 7 days (McGeachie and Allbrook, 1978), which is no longer evident at 21 days post-tenotomy, suggesting that myonuclei and satellite-cell nuclei may have been extruded to the extracellular space (McGeachie, 1985). However, in a study of tenotomized rats, Koishi et al. (1995) found that muscle fiber nuclei did not stain to antiMyoD in the anterior tibial, soleus and extensor digitorum longus muscles at 3 days and 2 weeks post-tenotomy. Here, although satellite cells were not counted they were very scarce; those that were observed showed signs of activation and were attached to ring fibers. Satellite cells have occasionally been found to be attached to ring fibers in the muscles of human patients with myotonic dystrophy (Klinkerfuss, 1967). Chou and Nonaka (1977) noted that in diseased human skeletal muscle a delayed fusion of activated satellite cells presenting myofibrils arranged perpendicularly to the parent fiber may give rise to the formation of ring fibers. No satellite cells with these characteristics were observed in the present study. Since these cells play a role in regeneration and repair, the observation here of satellite cells in ring fibers supports the view that this cytoarchitectural abnormality would be linked to an abnormal muscle fiber repair process.
To summarize, prolonged loss of tension following tenectomy, as distinct from tenotomy, prompted a delay in muscle fiber repair and the appearance of cytoarchitectural abnormalities consisting of fibers displaying several patterns of myofibrillar disorientation. It appears that skeletal muscle repair may remain incomplete in tenectomized rats.

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REFERENCES


