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Physical continuity of the perimysium from myofibers to tendons: Involvement in lateral force transmission in skeletal muscle

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Abstract

Advances in muscle physiology suggest that the perimysium plays a role in the transmission of lateral contractile forces. This hypothesis is strongly supported by our recent demonstration of the existence of “Perimysial Junctional Plates” in bovine Flexor carpi radialis muscle (Passerieux, E., Rossignol, R., Chopard, A., Carnino, A., Marini, J.F., Letellier, T., Delage, J.P. 2006. Structural organization of the perimysium in bovine skeletal muscle: junctional plates and associated intracellular subdomains. J. Struct. Biol. 154 (2), 206–216) However, the overall organization of the perimysium collagen network, as well as its continuity and heterogeneity, have still not been described in detail throughout the entire muscle. We used an extension of the standard NaOH digestion technique and scanning electron microscopy to analyze perimysium architecture in bovine Flexor carpi radialis muscle. First, we observed that the perimysium is made of a highly ordered network of collagen fibers, binding the myofibers from tendon to tendon. We identified basic collagen cable structures, characterized by a straight portion (3 cm long) in the direction of the myofibers and a curved terminal portion at 60°. These cables reach the myofiber surface at the level of the previously described “Perimysial Junctional Plates”. At a higher level of organization, these cables stick together to form the walls of numerous tubes arranged in a overlapping honeycomb pattern around the myofibers. At the ends of these tubes, the straight portions of the collagen cables ramify in large bundles that merge with the tendons. Taken together, these observations identify four levels of organization in the perimysium: (i) Perimysial Junctional Plates that constitute the focal attachment between the perimysium and the myofibers, (ii) collagen plexi attaching adjacent myofibers, (iii) a loose lattice of large interwoven fibers, and (iv) honeycomb tubes connecting two tendons. This spatial arrangement of the perimysium supports the view of a complex pattern of lateral force transmission from myofibers to tendons and adjacent muscles.

1. Introduction

Skeletal myofibers are embedded in a complex network of connective tissue consisting of endomysium and perimysium (Borg and Caulfield, 1980; Rowe, 1981). The former can be considered the main component of the extracellular matrix involved in muscle flexibility, while the latter is generally described as simple packing tissue. However, there is a great deal of evidence that the perimysium plays a role in the lateral transmission of contractile forces (Tidball and Chan, 1989; Huijing et al., 1998; Monti et al., 1999). This hypothesis is strongly supported by the recent demonstration of the existence of “Perimysial Junctional Plates” (PJP) (Passerieux et al., 2006).

These adhesive regions connect the myofiber surface with a specific intracellular subdomain. However, our observations were restricted to the vicinity of myofibers and there was no global description of the perimysium...
from tendon to tendon. In particular, the overall organization of the perimysium collagen network, as well as its continuity and heterogeneity throughout the entire muscle had never been investigated.

In fact, perimysium organization is essentially known from the pioneering observations of Rowe (1974), using optical microscopy, describing crimped collagen fibers running through the muscle, some in the direction of myofibers and others at 60°. The first observations using scanning electron microscopy (SEM) on formalized muscle samples were presented by Borg and Caulfield (1980) and Rowe (1981), who demonstrated the presence of collagen fibers in various muscles from different animal species. The existence of these collagen fibers was more recently confirmed by Nishimura et al. (1996), Jarvinen et al. (2002) and Nakamura et al. (2003) using the 2 N NaOH cell-maceration digestion technique, originally introduced by Ohtani et al. (1991). Unfortunately, the contaminating presence of endomysium made it impossible to observe perimysium continuity with the tendons by this method, nor was it possible with 5 N NaOH cell maceration (Passerieux et al., 2006).

In this study, we adapted this technique to digest the myofibers and endomysium of bovine *Flexor carpi radialis* (FCR) muscle selectively, thus making it possible to visualize the perimysium collagen network. Myofibers and endomysium were digested in different concentrations of NaOH (pH) at varying incubation temperatures, and then examined by SEM. This revealed the entire perimysium architecture: a highly ordered network of collagen fibers binding myofibers to the tendon. In particular, we identified perimysium cables that stuck together to form the walls of tubes in a honeycomb arrangement. The ends of these tubes formed the tendons.

2. Materials and methods

All procedures were compliant with institutional guidelines for animal care. All FCR samples were taken just after slaughter.

2.1. NaOH cell-maceration digestion technique and SEM

The usual NaOH digestion technique consists of macerating muscle samples previously incubated in paraformaldehyde in 2 N NaOH solution at room temperature. This eliminates myofiber components, making it possible to observe connective tissue components. In contrast, macerating non-formolized muscle samples results in the elimination of all the muscle components. This indicates that paraformaldehyde causes intermolecular crosslinking between some muscular proteins, as shown by Metz et al. (2004). The strength and stability of these crosslinks are different according to pH and temperature (Jaenicke, 1998; Fathima et al., 2004). Indeed, after 2 N NaOH cell maceration at room temperature, only the endomysium and perimysium remain (Trotter and Purslow, 1992; Nakamura et al., 2003), while cell maceration in 5 N NaOH at temperatures below 20°C eliminates the endomysium, whereas the myofibers and perimysium remain (Passerieux et al., 2006).

We extended this cell maceration technique to a wider range of NaOH concentrations and temperatures, using muscle samples of different sizes (from mm to cm). For each condition, the procedure was as follows: fixation in 10% paraformaldehyde for 8 days and NaOH maceration at a specific concentration at 15°C for 5 days. The maceration was rapidly heated to the chosen temperature and rinsed in water at this temperature for 2 days. Muscle samples were then treated with 2% tannin and freeze-dried for easy dissection and observed under a binocular microscope.

The results are summarized in Table 1, with NaOH concentrations from 2 to 6 N and temperatures from 5 to 70°C. The results show that perimysium, epimysium, and tendons resisted all NaOH concentrations up to 37°C and gradually disappeared at the same pH when temperatures exceeded 56°C. Myofibers remained at all NaOH concentrations, but only below 15°C. The endomysium was resistant under a set of conditions from 2 to 4 N NaOH at temperatures from 15 to 25°C.

In this study, we applied the three cell maceration conditions:

- 5 N NaOH maceration and a rinsing temperature of 15°C, noted 5–15 for myofiber, perimysium, epimysium, and tendon observations.
- 5 N NaOH maceration and a rinsing temperature of 37°C, noted 5–37 for perimysium, epimysium, and tendon observations.

Table 1

<table>
<thead>
<tr>
<th>NaOH concentrations</th>
<th>5°C</th>
<th>10°C</th>
<th>15°C</th>
<th>20°C</th>
<th>25°C</th>
<th>29°C</th>
<th>37°C</th>
<th>56°C</th>
<th>70°C</th>
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<tr>
<td>Perimysium, Epimysium, Tendon</td>
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<td>Myofibers</td>
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NaOH concentrations from 2 to 6 N and temperatures from 5 to 70°C on preservation of muscular components (myofibers in blue, endomysium in green and perimysium, epimysium, and tendon in brown).
−2 N NaOH maceration and a rinsing temperature of 25 °C, noted 2–25 for perimysium, epimysium, tendon and fine endomysium observations.

Each sample for the three conditions was dissected, then gold-coated and examined using a Philips 515 scanning electron microscope.

2.2. Embedding of the capillary network and immunohistochemistry

2.2.1. Capillary bed

One of the FCR arteries was perfused using successive 2 ml injections of colored ink (black and white). When a myofiber bundle was dissected, it was possible to distinguish between arteries (white) and veins (black).

2.2.2. Immunohistochemistry

FCR muscle samples were rapidly frozen in liquid nitrogen-cooled isopentane and stored at −80 °C. Longitudinal sections (10 µm thick) were obtained using a cryostat (Frigocut 2800, Reichert Jung) at −22 °C. The sections were fixed in a solution of 4% paraformaldehyde (Sigma Aldrich) at 25 °C for 30 min. After two washes with PBS (5 min each), the sections were dehydrated in successive baths of 70%, 90%, and 100% ethanol (2 min each), followed by rehydration in successive baths of 90% and 70% ethanol, then washed twice in PBS (2 min each). Sections were then incubated in a blocking solution [10% fetal calf serum (Hyclone) diluted in PBS] at 25 °C for 2 h. They were then incubated overnight with primary antibody (rabbit polyclonal anti-collagen III, Rockland). After two washes in PBS (10 min each), the sections were incubated in 10% normal goat serum (Sigma) in PBS at 25 °C for 1 h. Subsequently, sections were incubated with the secondary antibody (Alexa Fluor 546 goat anti-rabbit IgG, Molecular Probes) at 25 °C for 90 min, and then washed three times with PBS (15 min each). Finally, sections were mounted with Mowiol 4–88 (Calbiochem). Sections were observed on an epifluorescence microscope (Nikon Eclipse E600), photographed by a digital camera (COHU High Performance CCD Camera), and acquired using Vistiolab software (Biocom).

3. Results

3.1. Anatomy of the FCR muscle and associated connective tissue

The belly of the FCR muscle is about 20 cm long (Fig. 1A). Myofibers are about 6–8 cm long and join the two tendinous sheaths at an angle ranging in 15°–30°: the proximal tendinous sheath (pts) on the back of figure is directly attached to the humerus and the distal tendinous sheath (dts) ends in a long tendon joining the carpe. The pennated architecture of the FCR is summarized in Fig. 1B, to facilitate understanding of the indications of the main directions shown on the following figures.

The general architecture of perimysium was observed in large sections of FCR, cut along the myofibers, and treated at 5–37 (Fig. 2A and B). Parallel perimysial structures (arrow) are visible on both figures. In Fig. 2A, these structures form sections across large tubes (tu) joining the proximal tendon (t) at a 30° angle. These tubes are apparently filled with a thin network of collagen fibers. Observation of a cross-section of a muscle sample treated at 5–37 (Fig. 2C) confirmed that the perimysium consisted of tubes in a honeycomb arrangement, with a maximum diameter of 3 mm. This is consistent with the sections observed in Fig. 2A and B. These tubes show a large amount of thin lateral strips (ts) from their walls scattered through the internal space, but also large lateral collagen strips (ls) from their walls that resemble the ends of tubes or junctions with adjacent tubes. Examination of samples of 2–25 treated muscle revealed the arrangement of walls (w) and strips (s), as well as thin honeycomb structures of endomysium (en) embedding myofibers (Fig. 2D). The tube walls consisted of large bands of thick accumulations of collagen fibers embedding honeycomb structures of endomysium, while the strips were thinner bands of collagen fibers between the thin honeycomb structures of the endomysium.

3.2. Perimysium tube arrangement and wall structure

Muscle sample sections along the myofibers treated at 5–15 showed the direction of the tubes (tu), myofibers (mf), and tendon (t) (Fig. 3). Fig. 3A shows that the tube walls are parallel with the myofibers and in continuity with tendon collagen bundles. Fig. 3B shows that the tube walls consist of parallel bundles of collagen fibers (cf) crossing the myofibers at a 60° angle (arrow).

Tubes from muscle samples treated at 5–37 were dissected to investigate the arrangement of their walls.
Fig. 4A, where the thin lateral strips were removed, presents a lateral view of a longitudinal tube section (tu1) at the junction with the tendon (t) and the continuity with the other two tubes (tu2 and tu3). It reveals details of the perimysium tube walls and large lateral strips (ls) which form the end of tube walls near the tendon or the junction with the two adjacent tubes. This figure shows also that the tube walls consist of cables made up of collagen-fiber bundles, some aligned with the tubes, others oblique. A dissection of the walls at the junction between two tubes (Fig. 4B) shows two layers (---) of crimped collagen-fiber bundles (arrow), the upper layer clearly showing the change of direction in the same collagen-fiber bundles (—/). This preparation made it possible to dissect a set of collagen-fiber bundles (Fig. 4C) from the tube walls. The figure shows that a set of stuck collagen-fiber bundles forms large cables consisting of a 3 cm straight portion (sp) parallel with the direction of the tubes and, therefore, the myofibers and numerous 2 cm curved portions (cp) at a 60° angle in different planes. The larger cables produced the large lateral strips (ls) on the tube walls and the thinner ones formed thin lateral strips (ts) inside the tube, as seen in Fig. 2C.

This arrangement of perimysium cables (ca) made up of tubes (tu) is present throughout the muscle and near the myotendinous junction. The straight portions connect to the tendon (t) (Fig. 5A) or epimysium (e) around the belly of the muscle (Fig. 5B).

3.3. Junction between perimysium tube and myofibers

Dissection of thin lateral strips (ts) from the internal spaces of tubes from 5 to 37 treated muscle samples (Fig. 6A) shows that each parallel collagen-fiber bundle ramifies into oblique branches (b) on the myofibers (mf), as seen in Fig. 6B, a dissection of 5–15 treated muscle sample. At higher magnification (Fig. 6C and D), the composition of these bundles and branches (b) becomes clearer: the bundles are crimped and produce terminal non-crimped branches (tb) that spreading (arrowheads) all along the myofibers (mf) (Fig. 6C). The junction with the myofibers consists of a small plexus (p) of collagen fibers attaching the surfaces of adjacent myofibers (Fig. 6D). This organization corresponds to the three hierarchical levels of perimysium network described in a previous work (Passerieux et al., 2006).

An oblique view of a transverse section of non-dissected 5–15 muscle samples (Fig. 7A) indicates that the distribution of collagen-fiber bundles between myofibers (mf) is heterogeneous, with denser collagen regions approximately
200–300 μm apart (Fig. 7A). This heterogeneity is confirmed by the distribution of type III collagen, a component of the perimysium, observed on the immunohistochemistry FCR longitudinal section (Fig. 7B). The dense regions of collagen bundles in Fig. 7A may correspond to the position of thin lateral strips and less dense regions to their branches. This corresponds to the vascular bed distribution observed after ink injection of FCR muscle (Fig. 7C): arteries (white) and veins (black) are evenly spaced, approximately 200–300 μm apart. This correspondence between the distribution pattern of the collagen network and vascular bed supports previous observations (Bosman and Stamenkovic, 2003) highlighting the correspondence between capillary beds and collagen networks in muscle. This led to the conclusion that thin lateral strips ramify with great regularity from the tube walls, which is consistent with the apparently regular distribution of branches (b) in Fig. 6A.

3.4. Model of perimysium organization from myofibers to tendons

From the above detailed observations, we constructed a model of perimysium organization in FCR. This summarizes the perimysium architecture and the way it is built from an assembly of basic structures: perimysium cables. These cables are stuck together to form the walls of tubes (Fig. 8A) that, in turn, form honeycomb structures arranged alternately throughout the muscle, from tendon to tendon (Fig. 8B), thus constituting the fourth level of perimysium organization (Passerieux et al., 2006).

In this manner, myofibers are embedded in finely organized, three-dimensional connective tissue.

4. Discussion

The objective of this work was to clarify the perimysium organization of FCR bovine muscle, with a particular emphasis on its continuity from tendon to tendon. To achieve this, we adapted the standard NaOH cell-maceration digestion technique to eliminate specific elements: (i)
Fig. 5. SEM views of dissections of FCR after 5–37 NaOH cell maceration allowing the removal of myofibers and endomysium. (A) Junction of cables (ca) with the tendon (t). Note the continuity of the straight portion of the collagen bundles with the tendon. (B) The tube (tu) walls connecting to the epimysium (e) at the junction of the belly and tendon (t).

Fig. 6. SEM views of dissections after (A) 5–37 allowing the removal of myofibers and endomysium and (B, C and D) 5–15 NaOH cell maceration allowing the removal of endomysium. (A) Thin lateral strips (ts) from cables ramifying into branches (b), forming obliquely oriented collagen-fiber bundles. (B) Thin lateral strips (ts) from the tube walls (w) ramify into collagen-fiber branches (b) between myofibers (mf). (C) Crimped collagen-fiber-bundle branches (b) from strips lead into numerous terminal non-crimped branches (tb) spread (arrowheads) on a myofiber (mf). (D) Detail of the end of a branch (b) producing a plexus (p) at the surface of adjacent myofibers (mf).
myofibers and endomysium, to observe the arrangement of cables of collagen-fiber bundles through the muscle, (ii) the endomysium alone, to observe the direction of the perimysial tubes and their attachment to tendons and myofibers. Morphological criteria were sufficient to identify the perimysium as crimped type I collagen fibers and the endomysium as a network of thin type IV collagen fibrils. This digestion technique under 5–37 conditions also eliminated the proteoglycans that assemble perimysial fibrous elements, i.e., cables, making it possible to examine them specifically. However, the results of this digestion technique vary considerably according to muscle type, due to the great diversity in the ECM components of individual muscles and different muscle types (for review, see Purslow, 2002).

In this article, we clarified the perimysium organization, consisting of tubes arranged in a honeycomb structure, composed of the collagen cables connecting the tendons. Elimination of the proteoglycans from the perimysium revealed the fundamental shape of perimysium cables, made up of collagen-fiber bundles. They have an unusual configuration, characterized by a long 3 cm portion parallel to the myofibers, and a large curved portion at a 60° angle. Dissections showed that these cables were sometimes stuck together in opposite directions (as shown in Fig. 5A), so that they enter in opposite tendons in the same direction. Our findings highlight the complexity of perimysium organization in the FCR muscle: multi-directional orientation of crimped collagen bundles and their attachment along the full length of myofibers as well as to a large tendinous sheath... This complexity certainly reflects that of muscular mechanics.

The high number of collagen terminations stuck on the myofibers may transmit forces from contracted myofibers to tendons. These terminations may collect a fraction of the contractile forces in a system parallel to myofibers, reducing the level of force transmission at the myotendinous junction in the same proportions, as proposed by Tidball (1991). This view is also supported by the specific multi-directional organization of collagen-fiber bundles from myofibers to tendons and epimysium shown in our study. Tension from contracted myofibers may be collected by the perimysium terminations in various loci along the entire muscle, and then distributed to the two tendinous
sheaths and epimysium. This may support the transmission of forces to bone junction and contiguous muscles, as proposed in our model (Fig. 9). This distribution of forces at the muscle periphery was recently confirmed by Meijer et al. (2006). It was also proposed by Young et al. (2000) in the case of the in series-myofiber junctions. Moreover, Yucesoy et al. (2002) hypothesized the existence of an elastic link between myofibers. Our results indicate that this may correspond to the numerous crimped bundles of collagen fiber cables. According to Cribb and Scott (1995), decrimping collagen fibers lengthens them considerably, which is unlikely as myofibers are linked together by a short network of endomysium type IV collagen fibers. To investigate this issue, we dissected bundles of fresh FCR muscle and added markers (indicated by circles) to two different bundles of myofibers (noted mf1 and mf2 in the Fig. 10A). When a relative displacement up to 5 mm was imposed on one bundle under slow strain (Fig. 10B), the second bundle did not move. This demonstrated that the myofiber linkage via the endomysium is not constant through the muscle belly, so there is the possibility of large displacement of portions of the muscle. The displacement of the collagen-fiber bundles shown in Fig. 10 also suggests that there may be significant modifications in the direction of the curved portion of the cables when the length of the muscle is modified. In the case of passive stretch, Rowe (1974) showed that the angle between the collagen fibers and the myofibers decreased. However, it is difficult to predict the variation in this angle in case of contraction, as modifications in the length of collagen fibers under stress may interact with displacement in the muscle: changes in myofiber angle and length in contracted muscle (Huijing et al., 1989), modifications in tendinous sheath length during contraction (Rack and Westbury, 1984), and increase in diameter of contracted muscle, etc.

Fig. 9. Model of diffusion of forces through the perimysium network. A contracted myofiber (––) pulls on perimysium branches at the PJP. This creates tension (▶) in the curved and straight portions of the cables. Tensions diffuse through the entire perimysium network, affecting non-contracted myofiber, as well as the various portions of the tendons and epimysium.

Fig. 10. Photographs of a dissection of two myofiber bundles (mf1 and mf2) from FCR muscle with surrounding connective tissue. (A) Note the parallel arrangement of collagen fibers at 60° (arrowhead) (B) Low stress (arrow) applied to the bundle at the top of the dissection pulled the markers (circles) 5 mm apart, whereas the second bundle did not move.
The last property of the perimysium organization, suggested by our observations in FCR muscle, concerns the possible increase in tube-wall tension during muscle elongation or contraction. During stretching, the elasticity of the curved portion of the cables creates tension in the tube walls, compressing the myofibers inside. This may explain the increase in intramuscular pressure demonstrated by Davis et al. (2003). However, the behavior of the perimysium network during contractions is still unknown, so no conclusions can be drawn on this aspect.

One interesting question is whether other muscles have equivalent structures. We performed first investigations on the rat Peroneus digiti quadratus muscle. An SEM view of a transverse section of 5–56 NaOH-macerated muscle shows a similar tubular structure (tu) in the perimysium (Fig. 11A). In addition, a muscle fracture procedure described by Passerieux et al. (2006) shows that collagen-fiber bundles of perimysium (p) connect myofibers (mf) to form a similar PJ to that described in FCR muscle (Passerieux et al., 2006). These results indicate that the lateral force transmission may be a general mechanical property of muscles. This view is also supported by the indirect evidence of lateral force transmission in the rat Extensor digitorum longus muscle (Huijing et al., 1998). However, the consequences of lateral force transmission by the perimysium may be expected to vary among muscles depending on the content of perimysial connective tissue, the density of PJPs, and tube direction, which differ according to the muscles’ general architecture (pennation angle, etc.).

To conclude, these observations from FCR, together with our previous work (Passerieux et al., 2006), distinguish four levels of organization within the perimysium: (i) Perimysial Junctional Plates that constitute the focal attachment of the perimysium to the myofibers, (ii) numerous collagen plexi attaching adjacent myofibers, (iii) a loose lattice of large interwoven fibers, and (iv) a honeycomb arrangement of tubes connecting the two tendons. Hence, the perimysium can be considered a complex elastic structure may transmit contraction forces and produce tension at the PJPs, with their underlying myonuclei and subsarcolemmal mitochondria (Passerieux et al., 2006). Thus it constitutes a good candidate for participating in mechanosensing and associated control of the skeletal muscle metabolism.

Acknowledgments

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References


