Tendon crimps and peritendinous tissues responding to tensional forces

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Tendons transmit forces generated from muscle to bone making joint movements possible. Tendon collagen has a complex supramolecular structure forming many hierarchical levels of association; its main functional unit is the collagen fibril forming fibers and fascicles. Since tendons are enclosed by loose connective sheaths in continuity with muscle sheaths, it is likely that tendon sheaths could play a role in absorbing/transmitting the forces created by muscle contraction.
In this study rat Achilles tendons were passively stretched *in vivo* to be observed at polarized light microscope (PLM), scanning electron microscope (SEM) and transmission electron microscope (TEM). At PLM tendon collagen fibers in relaxed rat Achilles tendons ran straight and parallel, showing a periodic crimp pattern. Similarly tendon sheaths showed apparent crimps. At higher magnification SEM and TEM revealed that in each tendon crimp large and heterogeneous collagen fibrils running straight and parallel suddenly changed their direction undergoing localized and variable modifications. These fibril modifications were named *fibrillar crimps*. Tendon sheaths displayed small and uniform fibrils running parallel with a wavy course without any ultrastructural aspects of crimp. Since in passively stretched Achilles tendons fibrillar crimps were still observed, it is likely that during the tendon stretching, and presumably during the tendon elongation in muscle contraction, the fibrillar crimp may be the real structural component of the tendon crimp acting as shock absorber. The peritendinous sheath can be stretched as tendon, but is not actively involved in the mechanism of shock absorber as the fibrillar crimp. The different functional behaviour of tendons and sheaths may be due to the different structural and molecular arrangement of their fibrils.

Key words: Achilles tendon, sheaths, collagen fibrils, TEM, SEM.

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Joint movements of the body in mammals are generated by skeletal muscle cell activity, but the structures of the muscle-tendon complex able to transmit the forces of muscle contraction to bone are tendons and aponeuroses (Magnusson *et al.,* 2003). Tendons are considered highly flexible but inextensible structures offering a considerable resistance to tension. They also act as mechanical buffers or shock absorbers in protecting tendons to bone attachment during the initial elongation related to rapid muscle contraction (Stolinski, 1995a).

Tendons are dense fibrous collagen structures organized in a hierarchical manner whose main functional unit, strong and stiff in tension, is the collagen fibril (Kannus, 2000; Provenzano and Vanderby, 2006). The particular arrangement and dimensions of the collagen fibrils, together with their interactions with hydrophilic proteoglycans of the extracellular matrix, are responsible for the transmission of forces and resistance to tension. Collagen fibrils run straight and parallel in relaxed tendons, and are always arranged in fibers, fibril bundles and fascicles showing a zig-zag or waveform aspect called *crimping*. During initial stretching the crimps disappear or become more flattened acting as shock absorbers to tension (Diamant *et al.,* 1972; Kastelic *et al.,* 1980; Screen *et al.,* 2004; Franchi *et al.,* 2007). Increasing the tensile strength, the intra- and intermolecular cross-links of collagen fibrils are primarily involved in the transmission of mechanical forces (Kjaer, 2004; Provenzano and Vanderby, 2006). During this phase proteoglycans with their bridges also play a role in absorbing and/or transmitting the tension stress to bone (Cribb and Scott, 1995; Fratzl *et al.,* 1998; Scott, 2003).

Tendons are often surrounded by loose connective sheaths forming the paratenon, epitenon, peritenon and endotenon (Strocchi *et al.,* 1985; Kannus, 2000; Kjaer, 2004). According to Trotter and Purslow (1992) and Kjaer (2004) tendon sheaths

are linked to skeletal muscle sheaths and it is reasonable to think that even these apparently indifferent membranes play a role in absorbing and/or transmitting tensional forces in tendon.

Microscopic and ultrastructural analyses of rat tendons in this study may shed light on the morphologic and functional changes to collagen in tendon and peritendinous tissues when tendon is mechanically stretched *in vivo*.

Materials and Methods

Animals

Twelve female Sprague-Dawley rats (3 months old) were anaesthetized with an intraperitoneal injection of 87 mg/kg ketamine (Ketavet, Farmaceutici Gellini Spa, Italy) and 13 mg/kg xylazine (Rompun, Bayer Italia Spa, Italy). A resin brace, modified to induce foot dorsal flexion, was applied to one posterior leg in order to reach a final 55° angle flexion.

The stretching position was kept for 10 minutes. At the end of the stretching session and still under anaesthesia, the tendon of the gastrocnemius muscle with its sheaths was exposed and fixed in situ (i.e. still connected to the muscle belly and to the bone) in Karnovsky's solution. The tendon of the controlateral leg of each animal was kept relaxed and fixed as with the stretched tendon to be analysed as a control sample. Finally, the rats were euthanized via an intracardiac injection of Tanax (Hoechst, Frankfurt am Main, Germany).

All stretched and control tendons with their own sheaths were excised. Ten tendons (five stretched and five controls) were processed for polarized light microscopy (PLM). The other eight tendons (four stretched and four controls) were processed for transmission electron microscopy (TEM) and the last six tendons (three stretched and three controls) were longitudinally cut to be investigated by scanning electron microscopy (SEM).

The experimental protocols were conducted in accordance with Italian and European Laws on laboratory animals use and care.

Polarized light microscopy

Specimens were fixed in 10% buffered formalin, dehydrated in graded concentrations of ethanol, embedded in paraffin and longitudinally sectioned at 6 μm. The sections were stained with 5% Picrosirius Red to enhance the natural birifrangence of collagen fibers when observed under the polarized light microscope (Leitz Ortholux 2, Wetzlar, Germany).

Transmission electron microscopy

Specimens for TEM were fixed in Karnovsky' s solution, rinsed with a 0.1M sodium cacodylate buffer (pH 7.2) and post-fixed in 1% osmium tetroxide. Thereafter, they were dehydrated in graded alcohols and embedded in Araldite resin. The ultrathin sections were stained with lead citrate and uranyl acetate and viewed under a Philips CM-10 electron microscope.

Scanning electron microscopy

For SEM study, the samples were fixed in Karnovsky's solution, dehydrated in a graded ethanol series and then in hexamethyldisilazane. Finally they were mounted on metal stubs and coated with gold using a sputter coater (Emitech K550). Observations were made under SEM (Philips 515 and Philips XL30-FEG) operating in secondary-electron mode.

Results

Relaxed Achilles tendon

Longitudinal sections of relaxed rat Achilles tendon analyzed by light microscopy showed parallel collagen fibers with a wavy course that under polarized light microscope is displayed as alternating dark and light bands corresponding to *tendon crimps* (Figure 1). Flat fibroblast-like cells were interposed between adjacent fiber bundles. The outer surface of the Achilles tendon was covered by a sheath of collagen fiber bundles running in a waveform pattern. At the polarized light microscope the collagen fibers of this sheath showed dark and light bands similar to the tendon crimps (Figure 1).

Other specimens observed at SEM showed the tendon fibers to be composed of large plurimodal collagen fibrils running straight and parallel. At the crimp apex these fibrils suddenly changed their direction showing an evident elbow with knots corresponding to deformations of the fibril shape. In particular, collagen fibrils appeared bent on the same plane like bayonets, or twisted and bent (Raspanti *et al.,* 2005; Franchi *et al.,* 2007) (Figure 2). The tendon sheath appeared composed of thin wavy collagen fibers made up of small unimodal collagen fibrils. No crimps were recognizable

along these fibril bundles (Figure 3).

Other specimens analysed at TEM better showed that tendon collagen fibrils, when changing their direction at the crimp apex, modified their shape (bent on the same plane like bayonets, or twisted and bent) and lost their D-period disclosing their microfibrillar arrangement (Figure 4). As in previous SEM observations, thin sections showed the small collagen fibrils of the sheaths running in a smooth undulating arrangement without any ultrastructural aspects of crimp (Figure 5).

Stretched Achilles tendon

Longitudinal sections of stretched rat Achilles tendons observed at direct and polarized light microscope showed most of the tendon collagen fibers running straight and parallel with a few flattened crimps (Figure 6). The collagen fibers in stretched tendon sheaths ran straight with a slightly wavy course.

In similar specimens observed at SEM tendon fibers showed rare or otherwise completely flattened crimps. In all crimps, including those whose collagen fibrils appeared completely straightened, the fibrils still retained the knots at the apex of the crimps as in relaxed specimens (Figure 7). On the contrary tendon sheath collagen fibrils showed a less undulating path than the relaxed specimens and no ultrastructural knot or fibril size deformation was detectable at ultrastructural level (Figure 8).

At TEM, the same fibril knot described in relaxed specimens were detected even in straightened fibrils of completely flattened crimps (Figure 9). Collagen fibrils of fiber bundles in tendon sheath appeared partially stretched along the main axis of tendon (Figure 10).

Discussion

A waveform configuration of collagen fibers in tendon was first described in polarized light microscopy investigations. The authors correlated the periodic crimping pattern to tendon functions observing that crimping disappeared when tendons were slightly stretched *in vitro* (Rigby *et al.,* 1959; Elliot, 1965; Viidik and Ekholm, 1968; Stromberg and Wiederhielm, 1969; Viidik, 1972; Hess *et al.,* 1989). Some authors (Diamant *et al.,* 1972; Atkinson *et al.,* 1999; Hansen *et al.,* 2002) suggested that the alignment of collagen fibers during stretching of the tendon might correspond to the toe region of the stress-strain curve of tendon. Ultrastructural studies were also carried out to improve the morphological or functional meaning of tendon crimps, but no new functional data were reported (Rowe, 1985a,b; Gathercole and Keller, 1991; Stolinski, 1995a; Magnusson *et al.,* 2002; Hurschler *et al.,* 2003). Recently Franchi *et al.* (2007) described a morphological deformation of collagen fibrils in tendon crimps and named it *fibrillar crimp*. They also observed that fibrillar crimps did not disappear when the Achilles tendon was physiologically stretched *in vivo*, suggesting a modification of the fibril structure at the level of fibrillar crimps.

The study of tendon stretching may help to shed light on the mechanism of force transmission during muscle contraction.

According to Kjaer (2004) tendon sheaths are in continuity with the peri- and intra-muscular collagen sheaths thereby ensuring a functional link between the skeletal muscle and bone. In particular the perimysium seems to play a role in transmitting tensile force (Trotter and Purslow, 1992). It has been suggested that the connective tissue of skeletal muscle and tendon is like a lively structure with a dynamic protein turnover, highly able to adapt to changes in the external environment such as mechanical loading or inactivity and disuse (Kjaer, 2004). As tendon is tightly connected to the skeletal muscle via connective tissue of tendon and muscle sheaths it is probable that the peritendinous collagen fibers might be involved in transmission of forces from muscle to tendon.

Morphological flattened waves of collagen fibers comparable to those described in tendons were also observed in nerve sheath as in the epineurium (Stolinski, 1995b). The pattern was observed in cut or relaxed fascicles *in situ* as well as in isolated and split layers of the nerve sheath. It is interesting that the pattern was not observed on nerve fascicles under tension. The nature of the wavy structure suggested that the sheath length might change on stretching or contraction to accommodate the displacement and movement of nerve fibres (Stolinski, 1995b).

At polarized light microscope the present study disclosed a waveform pattern of collagen fibers both in tendon and tendon sheaths. However, while the waveform pattern of tendon crimps is due to a peculiar structural characteristic of the collagen fibrils (a structure specifically acting as a shock absorber and named *fibrillar crimp*), the waveform

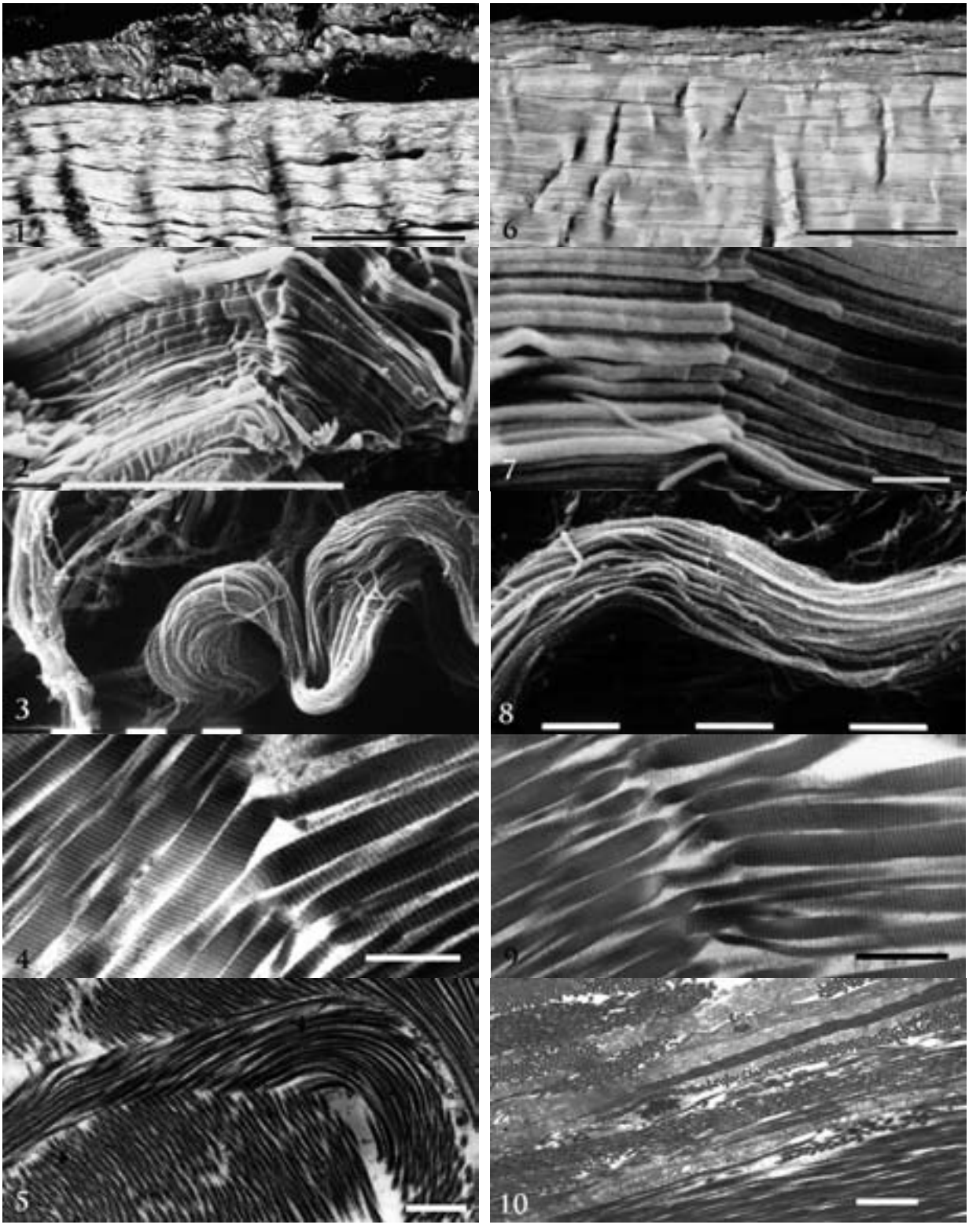

Figure 1. Relaxed rat Achilles tendons at PLM. Crimped fibers of tendon sheath (top) and crimped tendon fibers (bottom). Scale bar = 100 µm. Figure 2. Relaxed rat Achilles tendons at SEM. Fibrillar crimps in a tendon crimp. Scale bar = 10 µm. Figure 3. Relaxed rat Achilles tendons at SEM. Undulating fibrils in a tendon sheath fiber. Scale bar = 1 µm. Figure 4. Relaxed rat Achilles tendons at TEM. Fibrillar crimps in a tendon crimp. Scale bar = 1 µm. Figure 5. Relaxed rat Achilles tendons at TEM. Undulating collagen fibrils of tendon sheath. Scale bar = 100 µm. Figure 6. Stretched rat Achilles tendons at PLM. Straightened tendon sheath (top) and straightened tendon fibers (bottom). Scale bar = 100 µm. Figure 7. Stretched rat Achilles tendons at SEM. Fibrillar crimps in straight fibrils. Scale bar = 1 µm. Figure 8. Stretched rat Achilles tendons at SEM. Straightened fibrils of tendon sheath. Scale bar = 1 µm. Figure 9. Stretched rat Achilles tendons at TEM. Fibrillar crimps in straight fibrils. Scale bar = 1 µm. Figure 10. Stretched rat Achilles tendons at TEM. Straightened fibrils in tendon sheath. Scale bar = 100 µm.

configuration of tendon sheath appears as a simple undulating arrangement of collagen fibrils with no fibrillar crimps. Therefore, the straightening of the sheath collagen fibrils should be interpreted as a passive morphological adaptation to changes in tendon length.

Transmission of forces from skeletal muscle to bone involves different phases in tendon elongation. During initial tendon stretching crimps disappear or become more flattened acting as shock absorbers to tension with no local tissue strain increase (Diamant *et al.,* 1972; Kastelic *et al.,* 1980; Screen *et al.,* 2004; Franchi *et al.,* 2007). Increasing the tensile strength, the intra- and intermolecular cross-links of collagen fibrils are then involved in the transmission of mechanical forces (Kjaer, 2004; Provenzano and Vanderby, 2006). Some authors suggest that short proteoglycan bridges linked to collagen fibrils, like decorin, may also absorb and then transmit the tension stress to bone (Cribb and Scott, 1995; Fratzl *et al.,* 1998; Scott, 2003). Our results may suggest that during the passive static stretching of tendon, and presumably during tendon elongation in muscle contraction, the peritendinous sheath can be stretched like tendon, but is not actively involved in the shock absorber mechanism like the fibrillar crimp. The different functional behaviour of these two structures (tendons and sheaths) is also due to the different structural and molecular arrangement of the fibrils: tendon fibrils are large in diameter, parallely tightly packed and with a straight microfibrillar arrangement; fibrils in tendon sheaths are small and uniform in diameter, run in thin wavy bundles and have an helicoidal microfibrillar arrangement. Attending to the distribution in the connective tissue of the body, tendons are prevalently submitted to unidirectional tensional forces while sheaths undergo multidirectional loading (Ottani *et al.,* 2001).

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