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Subfibrillar architecture and functional properties of collagen: a comparative study in rat tendons

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INTRODUCTION

As long as forty years ago, early electron microscopy studies on connective tissue indicated a helical pattern within collagen fibrils (Wickoff, 1949). Since then this arrangement has been frequently observed with a wide range of techniques (Reed, Wood & Keech, 1956; Szirmai, Van Raamsdonk & Galavazi, 1970; Bouteille & Pease, 1971; Rayns, 1974; Belton, Michaeli & Fudenberg, 1975; Lillie, McCallum, Scaletta & Occhino, 1977) and has been given different interpretations. The helical aspect has been ascribed to surface structures as well as to helically wound fibrillar components of the collagen fibril. However, while commonly observed, this arrangement was by no means present in all tissues and, furthermore, X-ray diffraction studies consistently showed neither subfibrils nor a helical pattern.

The subfibrillar appearance of several tissues was investigated by TEM and freeze-fracture (Ruggeri, Benazzo & Reale, 1979; Reale, Benazzo & Ruggeri, 1981). Cartilage fibrils, mainly composed of Type II collagen, appeared to represent a class by themselves. The collagen fibrils of fibrous connective tissues, on the other hand, were seen to show two different aspects consistently related to their anatomical site: a 'straight' arrangement, mainly found in the large, parallel fibrils of tendons and ligaments, and a 'helical' arrangement present in small, wavy fibrils often gathered in sinuous bundles or tri-dimensional networks and associated with cells and elastic fibres; this was mainly found in blood vessel walls, interstitial tissues and sheaths. A close correlation was also found between these arrangements and the presence of Type I and Type III collagen.

The X-ray diffraction technique, limited for practical reasons to a single tissue, rat tail tendon (Hulmes & Miller, 1979; Fraser & MacRae, 1981; Lees, Pineri & Escoubes, 1984), was for a long time unable effectively to prove or disprove the correlation between these findings and the inner architecture of the fibril. Recent X-ray diffraction studies of other tissues (Folkhard *et al.* 1987) and biochemical analysis of insoluble cross-links (Mechanic *et al.* 1987) have now further shown that these aspects reflect important structural differences and that the 'straight' fibrils are built of collagen molecules which either lie parallel to the fibril axis or at a slight angle, while in the 'helical' pattern these same molecules appear to be helically wound around the axis at a constant angle of about 17°.

Further research indicates Type I, Type III and Type V collagens as coforming mixed fibrils *in vitro* and *in vivo* (Henkel & Glanville, 1982; Keene, Sakai, Bachinger & Burgeson, 1987, 1988; Adachi, Hayashi & Hashimoto, 1989), that Type III collagen tissue content varies widely but never exceeds 50% of the total collagen (Mays, Bishop & Laurent, 1988) and that Type II coforms cartilage fibrils along with Types IX and

XI (Eyre *et al.* 1987; Vaughan *et al.* 1988). If co-polymerisation of different collagen types within the same fibril can be regarded as the norm, then the fibril architecture in Type I-based fibrils is probably not determined by the fibril composition, as suggested by the former studies, but is rather influenced by tissue functional requirements (Raspanti, Ottani & Ruggeri, 1989).

The present research was undertaken in order to verify that a correlation between the subfibrillar arrangement and functional requirements can be seen *within a single anatomical site*.

The most suitable tissue for this purpose seemed to be the tendon, composed almost exclusively of collagen fibrils, the majority of which run parallel to the tendon axis and are maintained in place by other fibrils (the tendon sheath) not directly subjected to tensile load. The research was carried out on four tendons of very different morphology: the distal tendon of the *flexor digitorum profundus* of the foot, the mandibular insertion of the *masseter* muscle, the vertebral insertion of the *latissimus dorsi* and the tail tendon.

MATERIALS AND METHODS

Specimens were removed from 16 weeks old male albino Wistar rats under ether anaesthesia and fixed in 2·5% glutaraldehyde with 4% paraformaldehyde in sodium cacodylate buffer at 4 °C.

For conventional electron microscopy, the specimens were postfixed in 1% osmium tetroxide in the same buffer, dehydrated in graded ethanol and embedded in Araldite. Thin sections were obtained with a diamond knife on a Reichert–Jung ultramicrotome, collected on Cu-Rh grids and stained with uranyl acetate and lead citrate.

For freeze-fracture, the tissue was cryoprotected in 30% glycerol, frozen in solidifying Freon 22 and stored in liquid nitrogen until transfer to a Balzers BAF 301 freeze-etching unit fitted with two electron beam sources and a quartz crystal thin film monitor. The specimens were fractured at −100 °C, shadowed with 2 nm platinum-carbon from an elevation angle of 40° and replicated from 90° with 20 nm pure carbon. The tissue was removed with a commercial bleach solution and the replicas collected on Formvar-coated Cu-Rh grids.

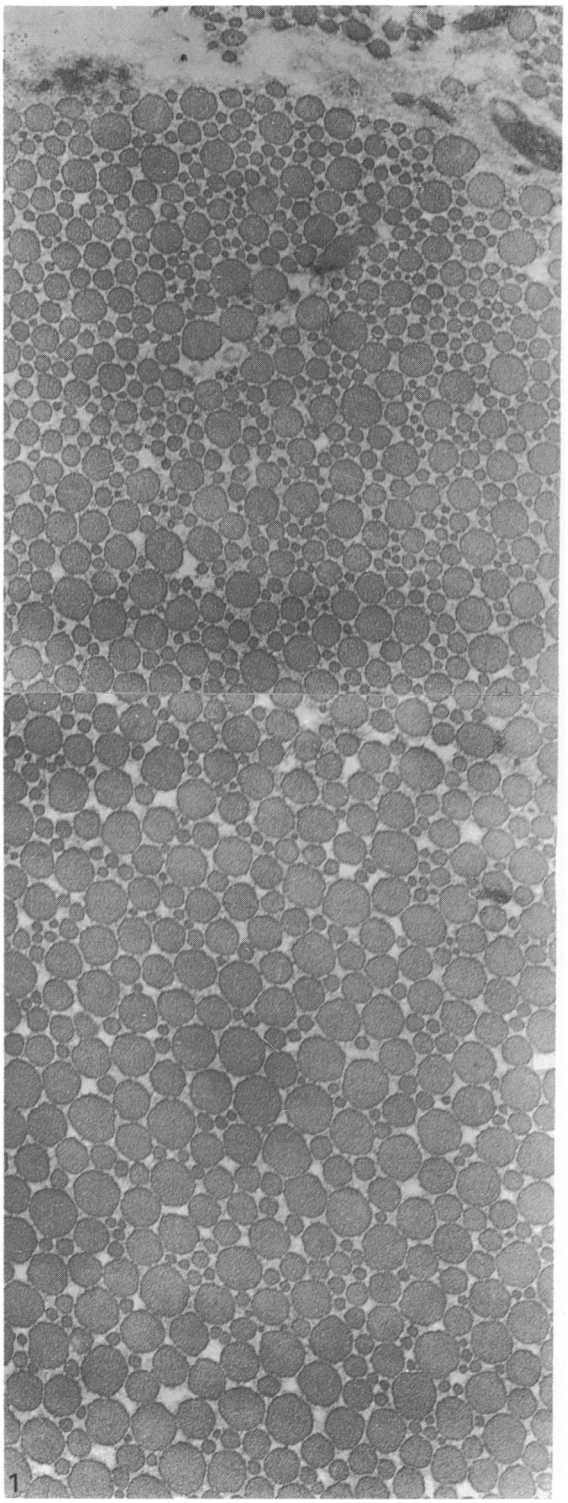
All replicas and thin sections were observed with a Siemens Elmiskop 101 electron microscope.

RESULTS

In all the tendons observed, the vast majority of the collagen fibrils are gathered in large bundles aligned to the tendon axis. As was the case with previous observations, the diameter of these fibrils is widely variable, ranging from 30 to 220 nm or more (Fig. 1), even if, in terms of mass distribution, almost all the collagen tissue content is found in the largest fibrils. Fibril volumetric density also varies within and among the tendons, being in some cases very high, as in the flexor digitorum profundus. In longitudinal sections these fibrils run noticeably straight and in parallel and only occasionally a whole bundle shows a steep change in direction ('crimp').

The tendon fibrils are contained and delimited by a system of sheaths, composed of incomplete layers of flattened fibroblasts interspersed with thin collagen fibrils and rare elastic fibres; this predominance of the cells over the matrix is unique to the sheath (Fig. 2).

Sheath thickness differs from tendon to tendon, being greatest in the rat tail, where four different layers with characteristic morphology and relationships have been



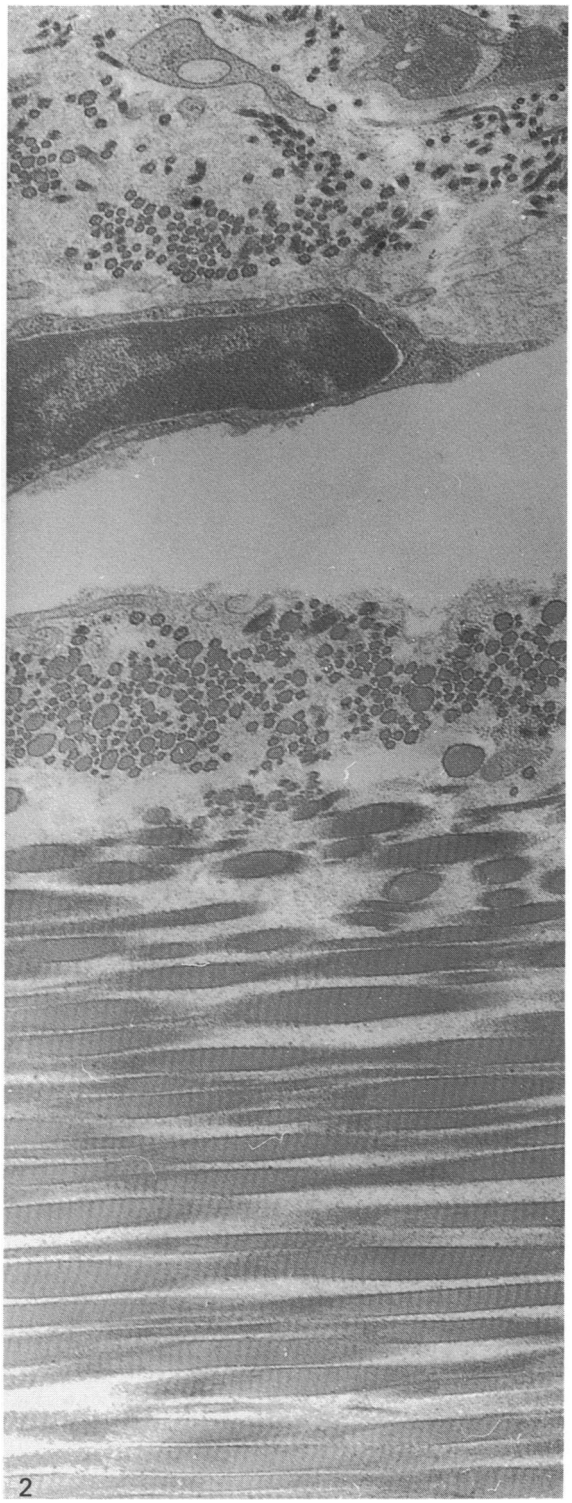


Fig. 1. Cross section of flexor digitorum profundus tendon. Heterogeneity of the collagen fibrils size is evident. × 30000.

Fig. 2. Longitudinal section of masseter muscle tendon (below) and sheath. Collagen fibrils of the sheath appear to be circularly wound around the tendon. × 20000.

described (Strocchi *et al.* 1985). Other tendons show much simpler sheaths, roughly corresponding to the innermost layers of rat tail tendon sheaths (peritendineum and endotendineum). Their collagen fibrils are organised in small, wavy bundles more or less circularly wound around the tendon or randomly arranged like felt fabric. Their distribution varies widely even in the same specimen and in some situations, particularly in the latissimus dorsi tendon, fibrils may indeed be absent. A distinctive feature of sheath fibrils is their consistently small and prevalently uniform diameter.

When visualised by freeze-fracture after glycerol treatment, all tendons appear basically similar. Collagen fibrils are readily detectable in replicas and can be easily attributed to tendon or to sheath by size and homogeneity; when observed at low magnification their course is consistent with the TEM observations, tendon fibrils appearing as long, straight, parallel rods aligned with the tendon axis (Fig. 3), while sheath fibrils show a typical multidirectional disposition (Fig. 4). At higher magnification, the fibrils consistently show the fine superficial texture of subfibrillar particles ('microfibrils'), characteristic of the two fibril architectures: the large tendon fibrils are frequently split longitudinally by the fracture plane and invariably show a 'straight' subfibrillar arrangement (Fig. 5) while sheath fibrils, mainly found in small bundles, always maintain their cylindrical shape and their subfibrils exhibit a right handed helical arrangement (Fig. 6) with an angle of 17–18°, consistent with previous observations in other tissues.

No differences are observed between different tendons by the freeze-fracture technique.

DISCUSSION

All four tendons examined in the present study show two distinct classes of fibrils differing in their (a) tissue distribution, (b) prevalent orientation, (c) average diameter, (d) diameter distribution and (e) subfibrillar architecture.

As far as mechanical properties are concerned, the large stiff central fibrils are subjected only to tensile stress along their axis. Inextensibility is of paramount importance here and since a helical subfibrillar arrangement would convert part of the tensile load into radial compression and axial elongation, the straight arrangement is mandatory for these fibrils. Furthermore, such an arrangement sets a preferential direction for fracture, so that the same fibril could be easily split longitudinally if bent or subjected to lateral compression (as happens in the freeze-fracture process). In other words, these fibrils are exclusively suited to their *in vivo* functional requirement.

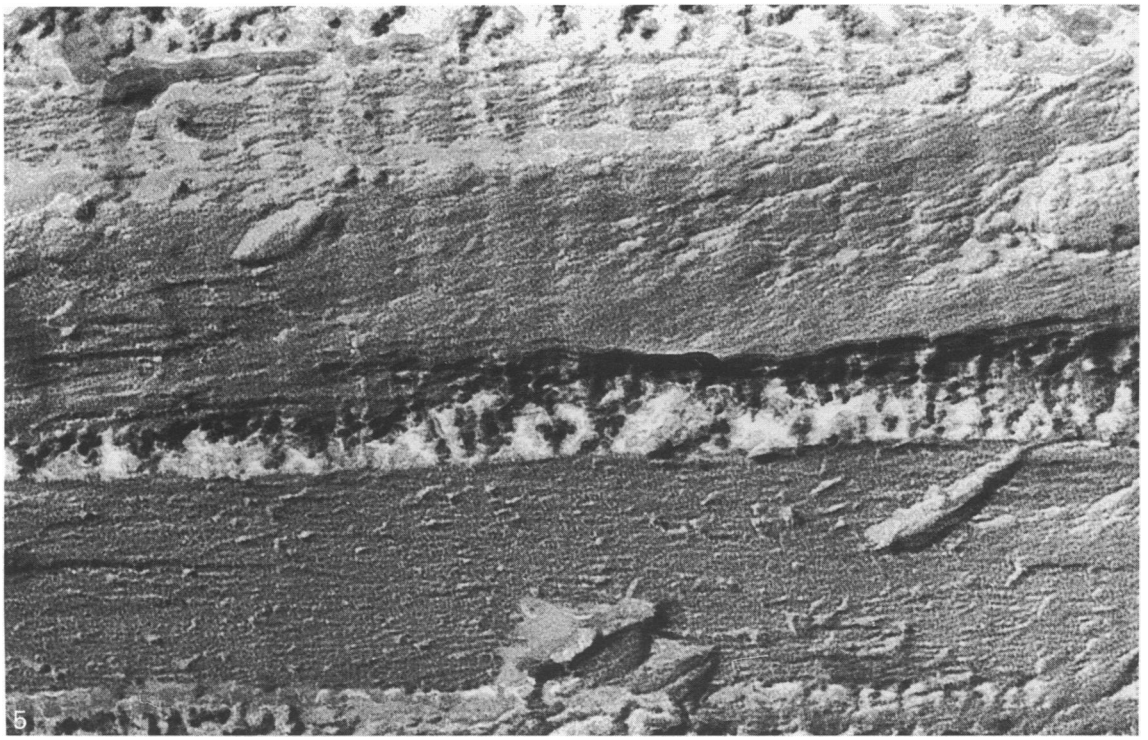
Helical fibrils are better suited to resist flexion, lateral compression or multidirectional deformation, but because of their inherent extensibility they are less suitable to withstand axial loadings: each type of structure appears thus to have its own specialised role.

It is therefore not surprising to find that, independently of the macroscopic aspect of the tendons and the amount of stress, all the fibrils involved in energy transmission show, without exception, the straight arrangement. Tendons may nevertheless show different fibril diameter distributions and different fibril volumetric density; further research is now under way on those parameters.

Due to their low density and to the multidirectional orientation of their fibrils, tendon sheaths, on the other hand, can hardly have any importance in transmitting mechanical energy. Rather they are subjected to the multidirectional forces arising as a consequence of the functional deformation of the tendon, their sinuous bundles of helical fibrils associated with elastic fibres and cellular elements providing the necessary compliance and deformability.

Fig. 3. Freeze-fractured rat tail tendon. ×15000.

Fig. 4. Freeze-fractured rat tail tendon sheath. ×15000.



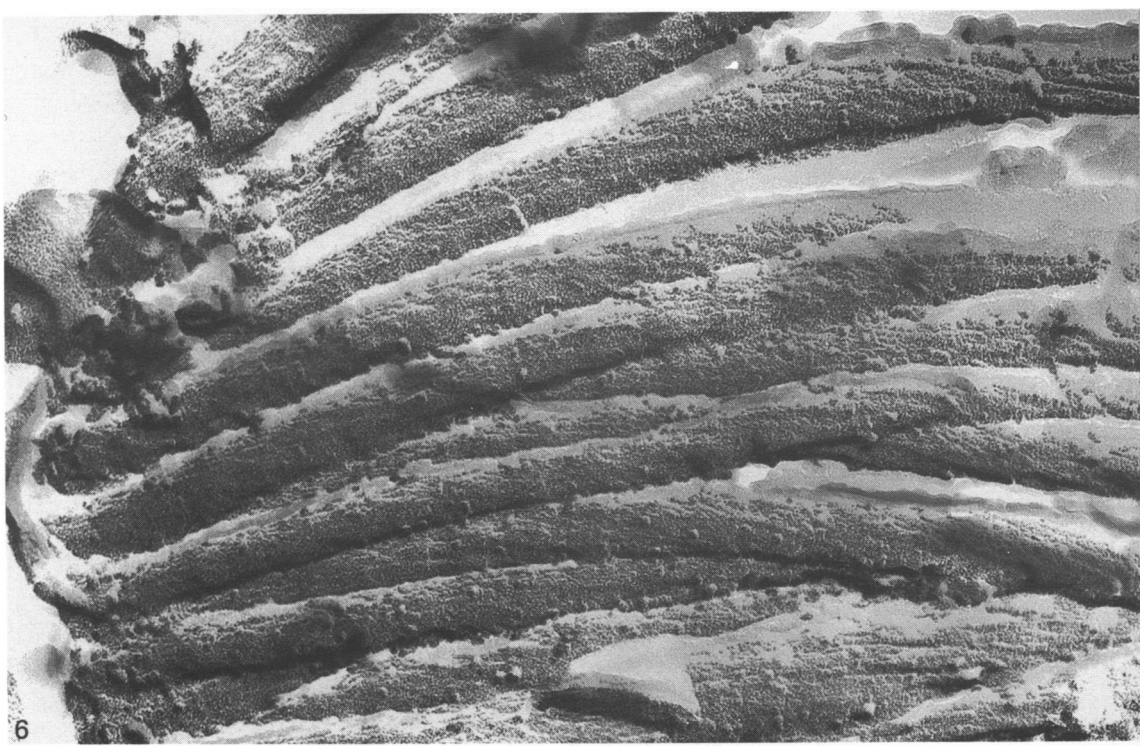


Fig. 5. Freeze-fractured masseter muscle tendon showing the 'straight' subfibrillar arrangement. Collagen fibrils appear to have been split by the fracture plane. × 120000.

Fig. 6. Freeze-fractured sheath of the latissimus dorsi vertebral insertion showing the small uniform diameter of the fibrils and their 'helical' subfibrillar arrangement. × 120000.

These data are also consistent with the arrangement observed in other tissues subjected to the same functional requirements, such as skin, blood vessel wall and interstitial tissue of parenchymal organs. In particular, the differences in the fibril diameter distribution are consistent with the differences reported in a comparative study (Craig, Eikenberry & Parry, 1987) between 'active' and 'passive' skin.

SUMMARY

Collagen fibrils from different rat tendons have been investigated by freeze-fracture and transmission electron microscopy. In all cases, marked differences in both fibril morphology and subfibrillar organisation have been consistently found between the tendon core (composed of large and heterogeneous fibrils comprising tightly-packed, straight, parallel molecules) and sheath (showing small, uniform collagen fibrils with a helical arrangement of the molecules). The bio-mechanical requirements to which these tissues are subjected suggest, as do previous observations on other tissues, that a causal correlation exists between substructure and collagen fibril function.

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