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Tendon response to tensile stress: an ultrastructural investigation of collagen:proteoglycan interactions in stressed tendon

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ABSTRACT

Tendons are parallel arrays of collagenous fibres which are specialised to resist and transmit tensile stresses. The response of tendon fibres to tensile stress is age-dependent and complex. Elastic elongation at low stress is accompanied by the disappearance of alternate light and dark bands seen in transmitted polarised light. This region of the stress/strain curve is associated with straightening of fibre 'crimps'. At higher stress, elongation is still elastic and reversible until break point is reached. This behaviour may be associated with straightening of a helical arrangement of collagen fibrils. In addition to the collagen fibrils, there are transverse and longitudinal proteoglycan filaments, many of which bridge and link between the fibrils. We have investigated the effect of various levels of stress from very low up to breaking point on the appearance of the proteoglycan filaments and their relationships with the collagen fibrils. Proteoglycan–collagen fibril interactions in rat and mouse tail and flexor digitorum tendons were visualised by Cupromeronic blue staining, applied to dissected fibres in the resting state and at stresses up to breaking. Proteoglycan filaments were seen to be orthogonally arranged in every D period, probably at the d band in mature tendons. In immature tendons proteoglycan filaments took up more varied orientations, but were mainly orthogonal or axially arranged with respect to the collagen fibrils. Both pictures appeared unchanged after application of stress of any level up to breaking point. Young tendons ruptured at lower stresses than mature tendons. It is suggested that PG bridges between collagen fibrils play a part in transmitting and resisting tensile stresses in tendons, contributing to the strength of the tissue.

Key words: Rat tail tendon; dermatan sulphate; Cupromeronic blue.

INTRODUCTION

Tendon is connective tissue that transmits tensile forces from muscle to bone. It is composed mainly of type I collagen fibrils oriented along the length of the tendon. Proteoglycans (PGs) are seen as filaments regularly attached to the collagen fibrils in electron micrographs of tendon stained with Cupromeronic blue under critical electrolyte concentration (CEC) conditions. CEC methods can ensure that only sulphated PGs are stained (Scott, 1985). In relaxed mature tendon, most PG filaments are arranged orthogonally across the collagen fibrils at the gap zone—usually in the 'd' band, as shown by UO_2^{2+} staining (Scott & Orford, 1981). In mature tendon the PG(s) are predominantly proteodermochondan (formerly dermatan; Scott, 1993) sulphates (PDSs).

When viewed in polarised light at low magnification, alternate light and dark bands are visible along the tendon fibres, probably arising from the zigzag or crimped course of collagen fibrils within the fibres (Gathercole & Keller, 1991). Under tensile stress this pattern changes, until, at higher stress, it is no longer visible. This suggests that extension of the tendon initially involves straightening of the crimp.

The stress–strain curve of tendon (Torp et al. 1974) can be divided into three regions: (1) toe region, in which the pattern of bands in polarised light fades and

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finally disappears with increasing tension; (2) linear region, in which the tendon is reversibly extendable; (3) yield and failure region, in which extension of the tendon fibres damages their structure.

The specific interactions that occur between collagen fibrils and PGs (Scott, 1988), which probably help to organise extracellular matrix (Scott, 1992) may be involved in the behaviour of tendon under tension. We therefore investigated the ultrastructural response to tensile stress in tendon of the interactions between collagen fibrils and PGs, as demonstrated by Cupromeronic blue–CEC staining. At the same time changes in the patterns induced by polarised light were examined.

Since tendons from different sources and stages of development respond differently under tension (Torp et al. 1974), these variables were taken into account.

MATERIALS AND METHODS

Cupromeronic blue of this batch was from Seikagaku Corporation, Tokyo.

Tendon fibres from the tails of rats aged 3 wk (n = 1), 6–7 wk (n = 1) and 10–12 wk (n = 3) (RTT) and 3 adult mice (MTT) (all taken from the midpoint of the tail), and from a single 6–7 wk rat flexor digitorum tendon (RFD) were dissected and handled in phosphate buffered saline (PBS) throughout the experiment. Fibre diameters were measured against a 400 mesh EM grid in a Nikon comparison dual light microscope. One grid square equalled 45 μm.

A single fibre was placed in the apparatus (Fig. 1) containing 12.5 ml PBS, securing first with the static Spencer–Wells forceps then at the clamp on the pivot. The ends of both the metal forceps and clamp were coated with plastic to prevent them coming into contact with the Cupromeronic blue, which is chemically degraded in the presence of ferrous metal.

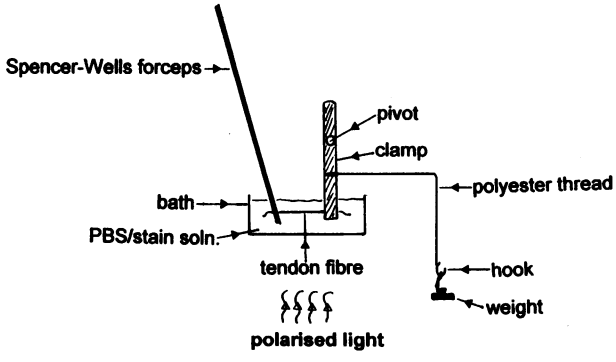


Fig. 1. Diagram of apparatus in which tension was applied to tendons of various kinds and ages. The tendon fibre was held between Spencer–Wells forceps and a clamp in the staining bath. Addition of weights stretched the fibre. The appearance of the fibre in polarised light was monitored through a low power binocular eyepiece (not shown).

The tendon fibre was examined under polarised light (from polaroid plastic filter HN32) to observe the pattern of light and dark bands. The length of the relaxed tendon fibre between the forceps and clamp was measured using dividers. Loads were then applied to the fibre by placing weights on a hook attached to the clamp (Fig. 1). At 2 min after applying the stress the pattern of light and dark bands under polarised light was reexamined. 2.5 ml of stain solution was then added to the 12.5 ml of PBS in the bath, giving final concentrations of 0.12 M NaCl; 0.016 M NaAc buffered at pH 5.8; 2.5% glutaraldehyde and 0.05% w/v Cupromeronic blue (Haigh & Scott, 1986). The 0.12 M Na^+ (from PBS) was sufficient to ensure that only sulphated PGs stained. The solutions were mixed thoroughly using a Pasteur pipette. Cover slips were placed on either side of the clamp and forceps, partially covering the bath to prevent evaporation of the solution. The tendon fibre was left overnight under stress in the stain solution at room temperature, to allow Cupromeronic blue staining to reach equilibrium.

The Cupromeronic blue salt of monohydrogen phosphate (HPO_4^{2-}) is poorly soluble. Fine dark blue deposits were seen in PBS solutions at the end of the staining periods, but the greater part of the dye was still in solution.

After releasing the stress, the fixed, stained tendon fibre was cut into 1–2 mm lengths, washed in PBS, treated with aqueous 0.5% w/v sodium tungstate and then with 0.5% w/v sodium tungstate in 50% v/v ethanol, dehydrated in graded ethanols, embedded in Spurr resin (4 h 50% v/v resin:ethanol; overnight 75% v/v resin:ethanol, then 3 × 1 h in 100% resin) and heated at 70 °C for 8 h.

Silver ultrathin sections were cut on an LKB V Ultratome and examined in a Philips 400 transmission electron microscope.

Calculations

$$\text{Stress (N/mm}^2) = \frac{\text{actual load (N)}}{\text{cross-sectional areas (mm}^2)}$$

Cross-sectional area of fibre = πr^2, where $2r$ = diameter measured before application of stress.

$$\text{Actual load} = \text{weight added (N)} \times \left(\frac{\text{length (mm) from fulcrum to weight attachment}}{\text{length (mm) from fulcrum to tendon}}\right).$$

RESULTS

Response to stress

As stress was applied to the tendon fibres, the strain increased. This was accompanied by an (age-related) decrease in cross-sectional area of the fibre. The 3 wk RTT was unable to withstand as high a stress as the mature (10–12 wk) RTT. The Table shows the highest nonbreaking stress applied to each tendon.

Light and dark banding pattern

Unstressed tendon fibres showed a clear pattern of light and dark bands in polarised light. The dark bands became less visible with increasing stress, until banding could no longer be seen. Complete loss of the banding pattern occurred at different stresses in different tendons. The stress at which the pattern was lost was always much less than the highest non-breaking stress (see Table).

Ultrastructure

The tendons from a total of 6 rats and 3 mice were examined before and after stress was applied at varying levels, from low to almost-breaking. All fibrils showed associated Cupromeronic blue-stained PG filaments (Fig. 2). These filaments were evenly spaced along the fibrils, at about 1 per D period, probably at the 'd' band (Scott, 1988). The PGs are 'small' dermatan sulphate-containing PGs in mature animals (Scott, 1988).

In unstressed 6–7 wk RTT, 10–12 wk RTT, 6–7 wk RFD and adult MTT, most PG filaments were arranged orthogonally with respect to the collagen fibrils. In 3 wk RTT, however, although most PG filaments were orthogonally and axially (parallel to the fibrils) oriented, some randomly oriented filaments were present. Similar changes in ultrastructure with age in young tendons have been reported previously (Scott et al. 1981). No similar data are available from MTT, so data were obtained only from mature animals.

The ultrastructural arrangement of PG filaments along the collagen fibrils did not change, even under high stress, in tendon fibrils of any age.

Table. *Effect of stress on tendon fibres*

Sample age	Highest non-breaking stress (N/mm^2)	Range of stresses at which the light/dark bands disappeared (N/mm^2)
	*	*
RTT 3 wk	3.0	0.3–0.6[10]
RTT 10–12 wk	8.8	4.9–5.9[5]
RFD 6–7 wk†	~ 8.8	1.0–3.0[4]
MTT adult (2)	36.3[5]	2.0–7.8[9]

* Calculated on the cross-sectional area measured before applying stress.

† Fibre cross-sections were elliptical rather than cylindrical, not amenable to exact calculations. Superscripts denote number of samples dissected from tendons on which results were obtained. Results are reported on 1 animal, except for the mouse tail tendons (MTT) when 2 animals were used.

DISCUSSION

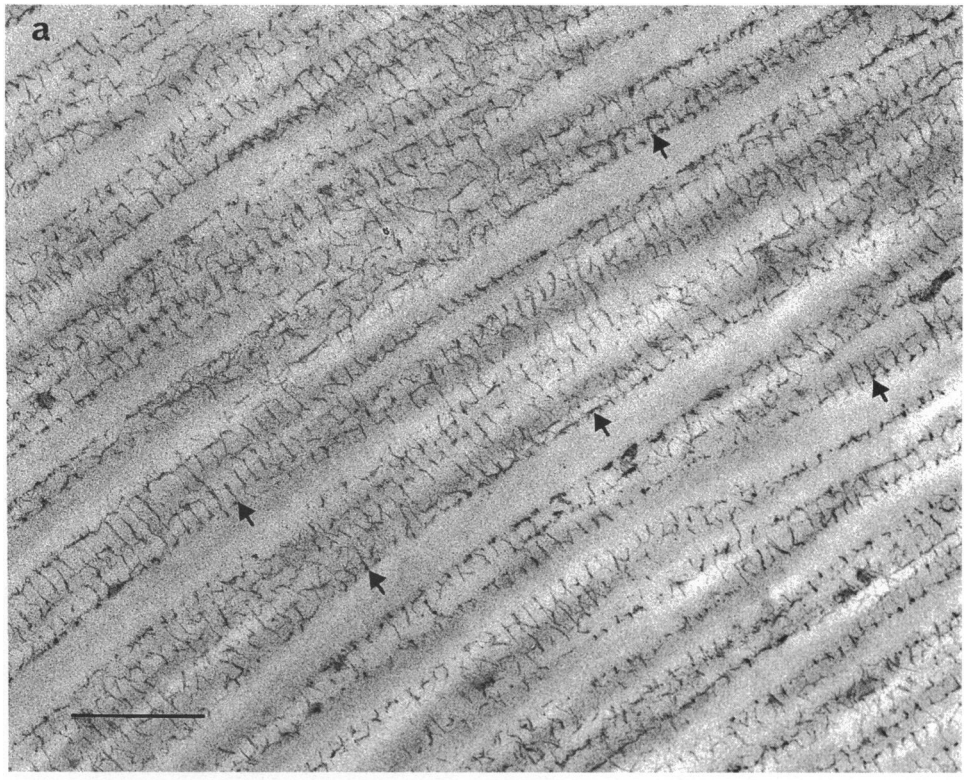
It is highly likely that PDS organises and orients collagen fibrils in relation to each other in extracellular matrix (Scott, 1992) and hence it would not be surprising if they also constituted a part of stress-transmitting structures. To understand the response of tendons to stress, interactions between PGs and the collagen fibrils therein were examined at stresses from zero up to breaking point. Our results show that ultrastructural relationships between PG filaments and collagen fibrils were visibly unchanged at all tensile stresses short of breaking point.

The pattern of Cupromeronic blue-stained PG filaments changed with tendon age (Fig. 2). In the 3 wk RTT, both axial and orthogonal arrays of PG filaments along the collagen fibrils were visible; in 10–12 wk RTT predominantly orthogonal arrays were observed. The nature of the PG also changes with age. At birth the RTT PG(s) are mainly chondroitin sulphate-rich, whereas in mature tendons PDS is preponderant (Scott et al. 1981).

The amount of tensile stress that the tendon fibre withstood depended on age: 3 wk RTT broke at much lower stress than mature (10–12 wk) RTT. Three wk RTT has a higher proportion of thin collagen fibrils than mature tendon, in which they may reach 450 nm in diameter (Torp et al. 1974; Scott et al. 1981). The increased number of thicker fibrils is due to an increased deposition of new collagen and also to fusion of the fibrils as the tendon ages (Scott & Parry, 1992). Very little fusion occurs in young tendon.

There are three levels of organisation, in order of decreasing size, at which mechanisms for coping with tensile stress are evident, of which at least two may involve PG:collagen fibril interactions.

(1) At the macro-level (100 μm) the gradual disappearance of the light and dark bands under increasing tensile stress corresponds to a straightening of collagen fibrils within the fibre. Torp et al. (1974) proposed that this was how tendon responds to low stress in the toe region of the stress–strain curve.



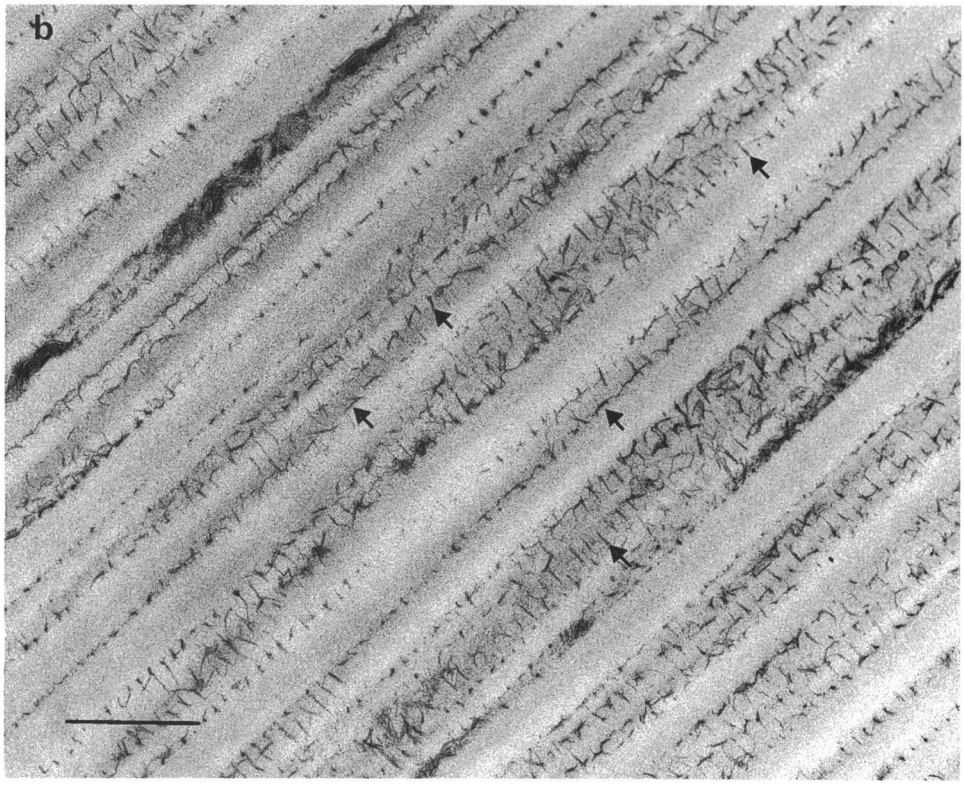


Fig. 2. For legend see opposite.

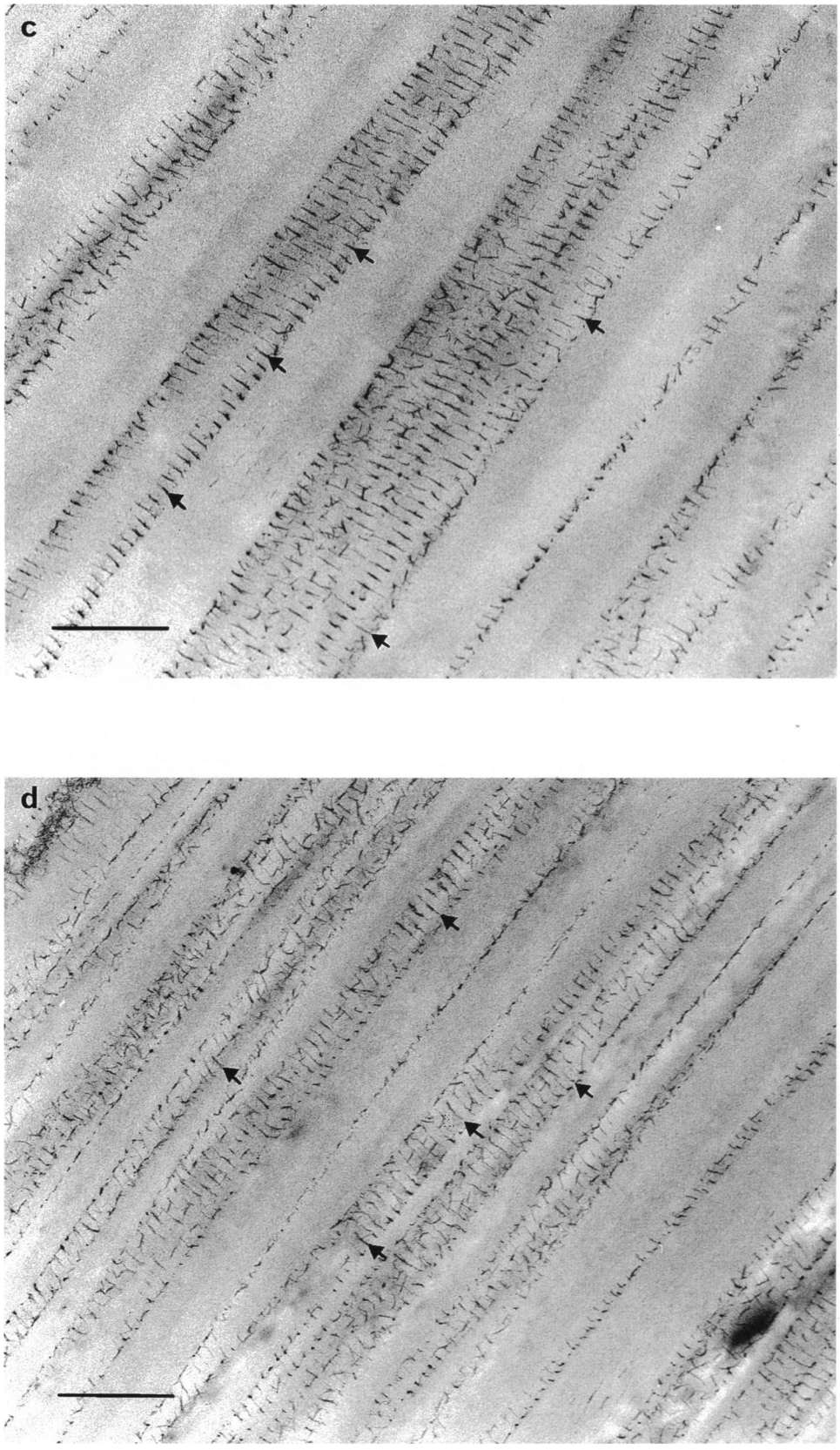


Fig. 2. Electron micrographs of 3 wk and 10 wk rat tail tendons (RTT) showing longitudinal sections through collagen fibrils with associated Cupromeronic blue-stained PG filaments (arrowed). 3 wk RTT (*a*) unstressed and (*b*) after 11.8 N/mm^2 stress show both orthogonal and axial arrays of PGs while 10–12 wk RTT (*c*) unstressed and (*d*) after 34.3 N/mm^2 stress show only orthogonal arrays of PG filaments. Bar, 500 nm.

Reversible extension, which occurs in the larger, linear, part of the stress–strain diagram must involve other mechanisms, probably including the following.

(2) On a smaller but still large scale, the tendon is modular, composed of protofibrils (10–15 nm) which, in unstressed tendon, follow a helical path along the axis of the fibril (Scott, 1990). Part of the extension of the tendon fibre under stress may be due to the straightening of this helix (Castellani et al. 1983), as in the straightening of a spring. This could account for reversible extension in the linear region of the stress–strain curve. It is relevant that sometimes there is axially oriented PG between the protofibrils (Scott, 1990), which could act as both lubricant and elastic deformation space to facilitate movement among the protofibrils. When helical straightening is complete, further extension may entail damage to the tendon.

Both mechanisms 1 and 2 would give thinner fibrils under tensile stress, as was observed.

(3) On a still smaller scale the collagen fibril:PG complexes may be directly involved.

In many tissues the PG anionic glycosaminoglycans (AGAGs) are frequently seen to extend from one collagen fibril to another, like the rungs in a ladder (Scott, 1992) at intervals of $\sim$ 60 nm. Providing they are not slack, these ties would be able to transmit forces from one collagen fibril to another. It seems very probable that in the cornea, for example, where the collagen fibrils are not in contact with each other, and yet they remain in very regular relationships, the PG ties are responsible for maintaining the structural regularity (Scott, 1992).

The PG$\rightarrow$collagen fibril and AGAG$\rightarrow$AGAG interactions in the interfibrillar bridge are both noncovalent and therefore in principle reversible (Scott, 1988, 1992). In particular, the AGAG:AGAG interactions easily form and dissociate (Scott, 1992). It is therefore possible that a ratchet type movement along the direction of the collagen fibril axes could occur with new bridges being broken and reformed every 60 nm or so of mutual displacement. This would allow elongation of the tendon fibre, and at the same time a decrease in fibre diameter, providing only some of the fibrils moved relative to the others.

In our experiments we could see the PG:collagen interactions only before and after stress, not during the onset of the stress. Thus, we cannot say if a given PG bridge was between the same collagen fibril binding sites after stress as in the resting state.

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