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A quantitative ultrastructural study of rat tendon from birth to maturity

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INTRODUCTION

Mature mammalian tendon is a regular dense connective tissue consisting of tenoblasts (fibroblast-like cells) surrounded by large bundles of collagen fibrils (Bloom & Fawcett, 1975). In mature tendon the cellular component is very sparsely distributed by comparison with the collagen fibrils of the extracellular space (Squier & Magnes, 1983). In contrast, during development both before and after birth, tendon is a relatively cellular tissue (Greenlee & Ross, 1967).

Within the tendon the dispersion of tenoblasts that occurs soon after birth is accompanied by changes in the collagen fibril diameters which at birth are distributed over a narrow range, but as maturity is reached over a much broader range (Parry & Craig, 1977; Ippolito *et al.* 1980).

We have sought to confirm and extend many of the qualitative observations described above by undertaking a rigorous quantitative analysis of the age changes in (i) cellular and extracellular volume and surface densities, (ii) collagen fibril diameter distributions and (iii) cytoplasmic volume density of rough endoplasmic reticulum as an indicator of protein synthetic potential in the tenoblasts. In addition we have compared these structural variables in rat tail tendon and extensor digitorum longus tendon in the same animals.

MATERIALS AND METHODS

The material for this study was taken from 24 Hooded Lister rats. Three groups of six rats were killed by an ether overdose at two, six and twelve weeks, whilst a further group of six rats was killed by decapitation on the day of birth (newborn group). From each animal both the extensor digitorum longus tendon (EDLT) to the middle toe and a complete fascicle (Rowe, 1985) of the tail tendon (RTT) were removed, after random choice of right or left side. Tissue was fixed in 2% paraformaldehyde and 2·5% glutaraldehyde in 0·1 M phosphate buffer (pH 7·2). Whilst in the fixative, the tendons were diced into tiny fragments. Following postfixation in 1% osmium tetroxide in distilled water, six to eight of these fragments were embedded, randomly orientated as a cluster (Stringer, Wynford-Thomas & Williams, 1982) in the tip of an Eppendorfer pipette. After polymerisation of the resin (Epon 812), the plastic pipette was discarded and the resin tip with contained fragments was cut off and re-embedded in a BEEM capsule for ease of handling.

A single thin section (pale gold interference colour) was taken from both EDLT and RTT for each rat and stained with uranyl acetate and lead citrate. Using the corners of the copper support grid as convenient reference points, six to nine micrographs were

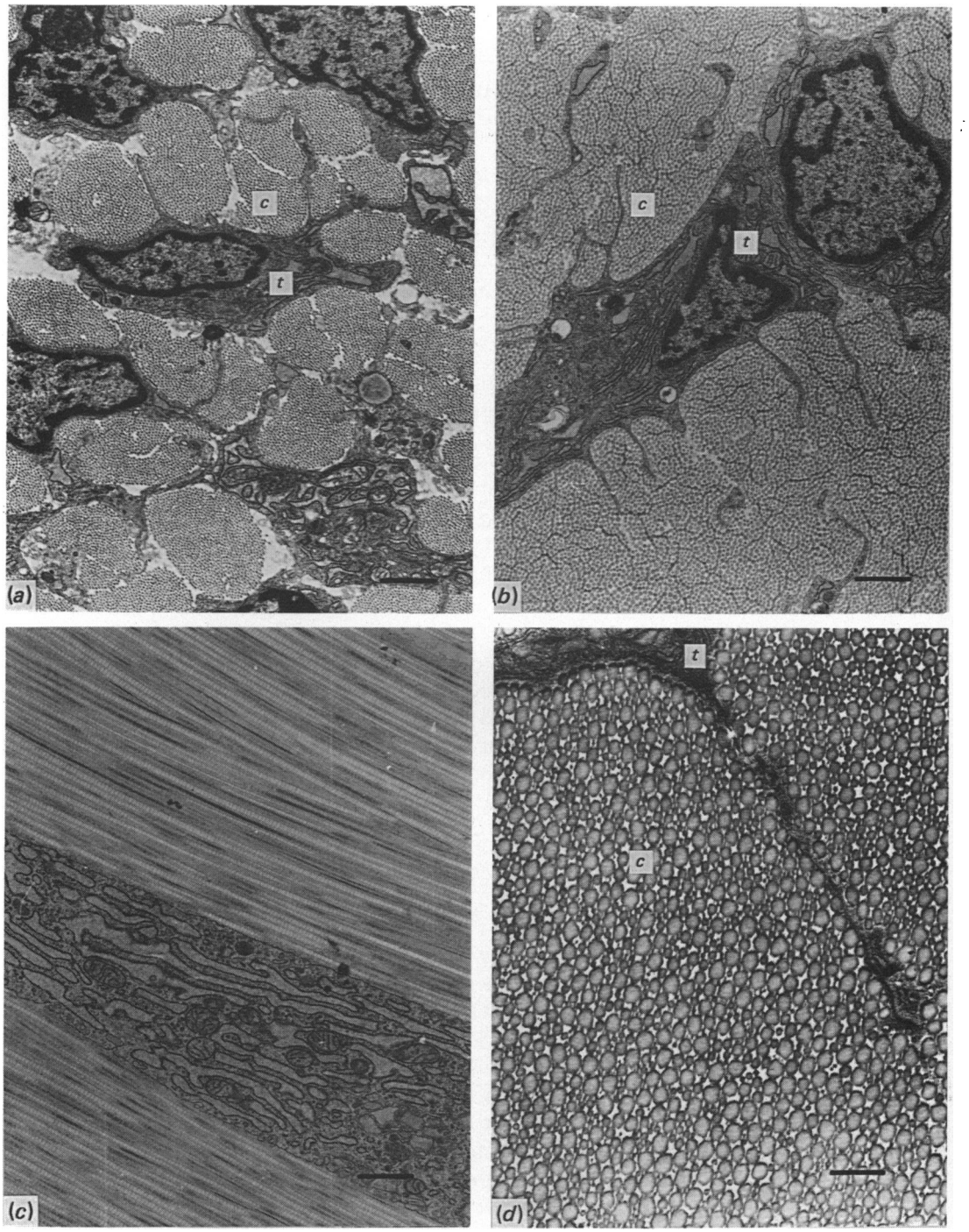

Fig. 1(*a–d*). Electron micrographs of sections of newborn (*a*), two weeks old (*b*), six weeks old (*c*) and 12 weeks old (*d*) tendon. (*a*) and (*d*) are rat tail tendon whilst (*b*) and (*c*) are extensor digitorum longus tendon. These micrographs depict the changes which occurred over the time period analysed. *t*, tenoblast; *c*, collagen; bar, 1 μm.

Table 1. *Morphometric analysis of rat tail and extensor digitorum longus tendons*

(Group means ± standard error of means ($n = 6$))

Age		Newborn	2 weeks	6 weeks	12 weeks
Percentage collagen volume fraction	RTT	45±3·2	62±1·5	82±1·5	95±1·1
	EDLT	52±3·0	70±2·0	86±0·4	97±0·4
Percentage tenoblast volume fraction	RTT	54±3·2	38±1·5	18±1·5	5±1·1
	EDLT	48±3·0	30±2·0	14±0·4	3±0·4
Percentage r.e.r. volume fraction	RTT	40±1·1	44±2·1	35±3·1	37±3·4
	EDLT	37±1·9	41±2·0	38±2·0	34±2·4
Plasmalemmal surface density ($cm^2\ mm^{-3}$)	RTT	14±0·6	9±0·6	6±0·2	2±0·3
	EDLT	13±0·4	10±0·5	4±0·3	1±0·1
Mean collagen fibril diameter (nm)	RTT	31±0·8	48±0·8	139±5·9	185±6·5
	EDLT	33±1·3	49±0·5	111±3·7	130±2·8

RTT, rat tail tendon; EDLT, extensor digitorum longus tendon.

Table 2. *Morphometric analysis of rat tail and extensor digitorum longus tendons*

(Results of two-way analysis of variance. Values are variance ratios.)

Effect (degrees of freedom)	Age (3,32)	Tendon (1,32)	Interaction (3,32)
Percentage collagen volume fraction	240·5***	9·5**	0·0
Percentage tenoblast volume fraction	240·5***	9·5**	0·0
Percentage r.e.r. volume fraction	4·2*	0·3	0·2
Plasmalemmal surface density	248·8***	8·0**	2·1

* $0{\cdot}02 < P < 0{\cdot}05$; ** $0{\cdot}001 < P < 0{\cdot}01$; *** $P < 0{\cdot}001$.

obtained for each tendon by systematic random sampling (Weibel, 1979; Mayhew, 1983). All micrographs were calibrated by reference to a grating ruled at 2160 lines/mm and printed to a final magnification of ×168000 for stereological estimations. For collagen fibril diameter distributions, final magnifications were ×120000 (newborn and two weeks) or ×60000 (6 and 12 weeks). Fibrils were selected by casting a 7 cm diameter 'hole' on to the individual micrographs. Those fibrils whose centres were judged to fall within the hole (Miles, 1978) were measured across their smallest diameter using a sonic digitiser interfaced to a Commodore PET microcomputer.

Stereological estimations were carried out using a test lattice of side length 2 cm superimposed on each micrograph, so as to be independently random in location and orientation (Weibel, 1979; Gundersen, 1980). Volume densities (V_v) were estimated by counting test points (P) which landed on: (i) tenoblasts – P_{ten}; (ii) extracellular space (hereafter referred to as collagen) – P_{col}; (iii) rough endoplasmic reticulum within tenoblasts – P_{rer}.

The total number of points P_{tot} falling on tendon was taken to represent the reference volume for the volume densities of tenoblasts (V_{vten}) and collagen (V_{vcol}) and was equal to $P_{ten}+P_{col}$. For the volume density of the rough endoplasmic reticulum (V_{vrer}) the reference volume was the tenoblast and the point total was the number of points falling on tenoblasts (P_{ten}). Then: (i) $V_{vten} = P_{ten}/P_{tot}$ (volume density of tenoblasts); (ii) $V_{vcol} = P_{col}/P_{tot}$ (volume density of collagen); (iii) $V_{vrer} = P_{rer}/P_{ten}$ (volume density of rough endoplasmic reticulum).

To obtain the volume densities per animal, the above point totals were summed over all micrographs from all blocks from that animal. Surface density of plasmalemma in

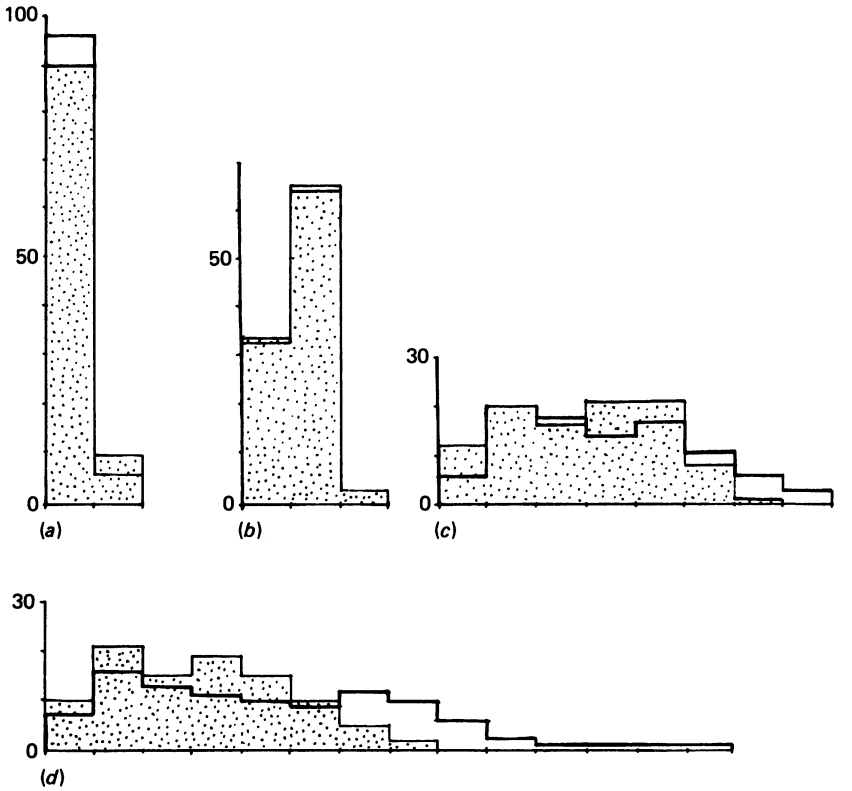

Fig. 2(*a–d*). Histograms of collagen fibril diameter distributions for newborn (*a*), two weeks old (*b*), six weeks old (*c*) and 12 weeks old (*d*) tendons. Solid lines, rat tail tendon; stippled, extensor digitorum longus fibril diameter (class interval 40 nm).

Table 3. *Morphometric analysis of rat tail and extensor digitorum longus tendons*

(Statistical analysis of changes in collagen fibril diameter distributions between time periods.)

	Age	0–2 weeks	2–6 weeks	6–12 weeks
RTT	Test statistic D	0·0573	0·0634	0·0665
	Maximum unsigned difference	0·0614**	0·7096**	0·2359**
EDLT	Test statistic D	0·0672	0·0659	0·0655
	Maximum unsigned difference	0·5611**	0·6155**	0·1428**

** $0{\cdot}001 < P < 0{\cdot}01$; RTT, rat tail tendon; EDLT, extensor digitorum longus tendon.

tendon (S_{vten}) was estimated by assuming isotropic uniform random encounters between sections and membranes. The physical randomisation of tissue described above (Stringer *et al.* 1982) was an attempt to achieve this. The intersections between the horizontal (I_h) and vertical (I_v) grid lines and membranes were counted on the same micrographs as those used for point counting. Then $S_{vten} = I_h + I_v / P_{tot} \times L$, where L = the side length of a grid square in absolute units, namely 1·19 μm. Intersection counts and point counts were summed over all micrographs from all blocks from that animal.

Table 4. *Morphometric analysis of rat tail and extensor digitorum longus tendons*

(Statistical analysis of differences in collagen fibril diameter distributions between tendons at each time period.)

Age	Newborn	2 weeks	6 weeks	12 weeks
Test statistic D	0·0601	0·0648	0·0647	0·0674
Maximum unsigned difference	0·0411	0·0118	0·01738**	0·2613**

** $0{\cdot}001 < P < 0{\cdot}01$.

Values for each tendon from each animal were used to provide group means and standard errors of the means. For comparisons of the tenoblast data a two-way analysis of variance was employed, with age and tendon type as the primary effects. Since the raw data for volume densities did not meet the assumptions required by the analysis of variance the data were first subjected to an arcsin transformation (Sokal & Rohlf, 1981). The interaction term generated by this test indicates whether or not age affects both types of tendon in the same way. For collagen fibril diameter distributions the two-sample Kolmogorov–Smirnov statistic 'D' was employed to test for differences with time and between EDLT and RTT (Sokal & Rohlf, 1981).

RESULTS

The period of growth investigated in the present study was one of substantial change for both EDLT and RTT (Fig. 1), and except where indicated the nature of the change was the same for both tendons.

Results are summarised in Tables 1 (Group means $\pm$ S.E.M.) and 2 (two-way analysis of variance).

Since the tendons were analysed on the basis of only two volume densities, namely those of tenoblast and collagen, the values for these two variables have a reciprocal relationship. It can be seen that there was a significant fall (rise) in tenoblast (collagen) volume density. In line with the fall in tenoblast density there was also a marked decline in plasmalemmal surface density. The fraction of tenoblast cytoplasm occupied by rough endoplasmic reticulum was nearly constant, but there was a small increase at two weeks and a decline thereafter by twelve weeks.

In the analysis of variance the absence of significant interaction terms indicated that the two types of tendon changed in essentially the same way with time. In addition the analysis revealed small but significant differences between EDLT and RTT for all variables except the volume density of rough endoplasmic reticulum. EDLT tended to have a smaller volume density of tenoblasts, a smaller surface density of plasmalemma and a greater volume density of collagen than RTT.

The mean collagen fibril diameter increased steadily between birth and twelve weeks (Table 1). Collagen fibril diameters displayed a narrow range of diameters at the two earlier time periods, but by six weeks and twelve weeks the diameter ranges had increased considerably (Fig. 2), especially in RTT. The statistical test confirmed the obvious changes with time (Table 3), which were significant between time periods. In addition, there were significant differences between the two types of tendon in the diameter distributions at six and twelve weeks (Table 4). At these ages, collagen fibril diameters showed a greater range in RTT compared with EDLT.

DISCUSSION

The principal objectives of this study were (i) to obtain a rigorous confirmation and extension of information which was already present in previous reports, (ii) to perform the cellular and collagen fibril diameter analyses on the same experimental animals and (iii) to compare tendons from two different locations.

We have confirmed the rapid fall in tenoblast volume density and the reciprocal rise in collagenous extracellular material which have been reported on a qualitative basis by several authors (Greenlee & Ross, 1967; Torp, Baer & Friedman, 1975; Squier & Magnes, 1983). The quantitative methods used here allowed expression of component planar sections as volume fractions. Since three to four months postpartum represents the mature state for RTT (Torp *et al.* 1975; Squier & Magnes, 1983), the 12 weeks values given here are probably close to the 'endpoint' for both types of tendon.

The major change in growing tendon is the reduction in the ratio of tenoblasts to collagen. Since the volume density of rough endoplasmic reticulum within the tenoblasts falls slightly between birth and 12 weeks, there must be a reduced capability to synthesise protein per unit volume of tissue. This implies that the rate of collagen synthesis required for replacement during maintenance is lower than that required for growth (although the use of tenoblast rough endoplasmic reticulum as a measure of collagen synthesis may not be entirely accurate). A slight rise in the volume density of rough endoplasmic reticulum was observed between birth and two weeks; this may be related to the changes taking place in the collagen fibril population (see below). The amount of tenoblast rough endoplasmic reticulum was observed qualitatively to increase in the period between birth and 30 days in developing rat flexor digital tendon (Greenlee & Ross, 1967) but the increase was not commented upon.

The distribution of collagen fibril diameters has been studied before (Fitton-Jackson, 1956; Eikenberry, Brodsky, Craig & Parry, 1982; Ippolito *et al.* 1980; Parry & Craig, 1977). Briefly stated there is a period in tendon development during which collagen fibrils of nearly uniform diameter increase steadily in size. At some time, usually soon after birth, the variability of diameters increases considerably. This must require an increased quantity of synthesised collagen and could account for the rise in the volume fraction of rough endoplasmic reticulum around that time. Our values for the distribution of fibril diameters in RTT are close to those of Parry & Craig (1977). However, in the EDLT, a tendon not previously analysed, there is a significantly smaller range of diameters by 12 weeks. This may represent a real difference in fibril populations, which presumably is established because of differences in function. It is known that fibril diameters are related to levels and duration of transmitted tension (Parry, Craig & Barnes, 1980). According to the hypothesis of the latter authors the broader range of fibrils in the RTT would suggest that, per unit cross sectional area, this tendon transmits higher levels of tension than EDLT. Confirmation of this must await a better understanding of the relationship in connective tissue between force transmission and collagen fibril diameter distribution.

SUMMARY

In a morphometric analysis of rat tail (RTT) and extensor digitorum longus (EDLT) tendons in rats from birth to 12 weeks of age it was found that the volume fraction of tenoblasts and the surface density of their plasma membranes fell sharply. The collagenous extracellular fraction rose in a reciprocal manner over the same period. The volume fraction of the tenoblast rough endoplasmic reticulum stayed more or less constant from newborn to 12 weeks.

The collagen fibril diameters displayed a sharp unimodal distribution at birth, but at 12 weeks became bimodally distributed and of greater range, especially in RTT.

Generally, observations were similar to data previously published in qualitative studies, but there were small differences between RTT and EDLT which may be accounted for by differences in function.

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