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Collagen structure in human anterior cruciate ligament and patellar tendon

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Human anterior cruciate ligaments (ACL) and patellar tendons (PT) were examined using scanning and transmission electron microscope to determine collagen architecture and morphology. Morphometric measurements were performed using an image analyser. Similarities and differences were found between the ACL and the PT. In both tissues, the anterior band is more collagenous than the posterior band giving rise to a material heterogeneity. In addition, a considerable variation in the collagen fascicle cross-sectional area was found. There was no significant difference between the ACL and PT fibre diameters, except for the elderly subjects.

The collagen wave pattern was found to be helical in the PT while in the ACL it may be helical as well as planar. In addition, the waviness period was significantly larger in the PT than in the ACL. Finally, transverse fibrils, the nature of which is unknown, were found only in the ACL.

1. Introduction

In clinical orthopaedics, a number of surgical procedures have been devised in order to repair or replace injured ligaments. In the case of an anterior cruciate ligament (ACL) rupture, the substitute material varies from biological grafts to synthetics such as carbon fibres [1–5]. Over the years, the one-third of the patellar tendon (PT) auto-graft has become a favoured ACL replacement procedure because of its superior mechanical properties [6–10]. It is known that the success of ligament reconstructive procedures depends on several factors including remodelling of tissue–fibre microgeometry. Previous studies of this remodelling were made by histologic examination of biopsy specimens [11]. Recently, Amiel *et al.* [12] developed a rabbit model for the ACL reconstruction using the PT and concluded on a histological and biochemical basis that a process of "ligamentization" occurred on the grafted tissue. In an earlier paper, the same authors showed that although the ACL and PT are predominantly composed of highly oriented type I collagen fibrils, they are histologically and biochemically different [13]. However, significant architectural and morphological factors, not seen at the histological level, have been reported to characterize the ACL and PT collagen [14].

The purpose of this paper is to examine the collagen structure in human ACL and PT, making use of both scanning (SEM) and transmission electron microscopy (TEM). The results presented here provide further evidence for the organization of the ACL and PT collagen. Hopefully, they will provide a structural basis not only for the selection of ACL biological substitutes but also for follow-up studies of the substitute remodelling.

2. Materials and methods

Twelve human ACL and PT obtained from the right and left knees at autopsy (3–23 h post-mortem) were used in this study. The age of the subjects varied from 47 to 77 years, and their weight ranged from 59 to 86 kg. These tissues came from uninjured normal-looking joints with no evidence of articular disease. Table I lists the data of the autopsy material investigated.

Immediately after excision, the specimens were cut transversely and longitudinally and then processed for microscopical observation.

Two preparation procedures were used for the SEM studies.

(i) *Standard method.* Specimens were fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide, and dehydrated in a graded series of distilled water–ethanol and ethanol–amyl acetate solutions. As a transitional solvent, carbon dioxide was used to remove the alcohol and specimens were critical point

TABLE I Data of the specimens investigated

Specimen	Sex	Age	Postmortem time (h)	Cause of death
54M	M	54	3	Lung cancer
77F	F	77	18	Ovary cancer
62M	M	62	19	Cardiac failure
62F	F	62	15	Cardiac failure
57M	M	57	18	Cardiac failure
73F	F	73	5	Cardiac failure
58M	M	58	12	Cardiac failure
51M	M	51	16	Cardiac failure
61F	F	61	23	Leukemia
52M	M	52	13	Cirrhosis of the liver
63M	M	63	3	Cardiac failure
47M	M	47	7	Leukemia

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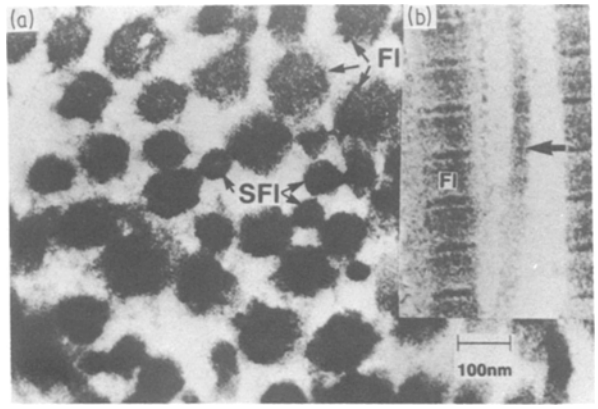

Figure 1 Electron micrographs of specimens obtained from the midpoint of the ACL (58-years-old female). (A) collagen fibrils (FI) and subfibrils (SFI) in cross-section (original magnification, × 100 000). (B) longitudinal section showing the periodic striation of the fibrils (original magnification, × 100 000).

dried in a LADD apparatus. The dry specimens were mounted on SEM stubs and sputter coated with gold using a Hummer II coating apparatus.

(ii) *Enzymatic digestion.* In order to study the micro-architecture of the ACL and PT collagen, two digestive enzymes were used to remove the non collagenous material surrounding the collagenous entities. The specimens were exposed, prior to the fixation step, to the action of elastase (10 mg/100 ml sodium carbonate buffer, pH 8.8, 0.05 M, 37° C), and hyaluronidase (15000 units/150 ml sodium acetate buffer, pH 5.4, 0.1 M, 37° C) for 6 to 20 h. The specimens were then treated following the standard method and examined with a Jeol JSM-840 scanning electron microscope set at an accelerating voltage varying from 10 to 20 kV.

The specimens for transmission electron microscopy were prepared as follows. Fragments of tissues were fixed immediately after excision in 2 % glutaraldehyde in a 0.15 M phosphate buffer with a pH of 7.20, and followed by a post-fixation in 1% osmium tetroxide. The specimens were then embedded in resin (Araldite 502). Thin sections were cut with a LKB III ultramicrotome, and double-stained with uranyl acetate and lead citrate. The thin sections were studied and photomicrographed with a Jeol 100 Cx transmission electron microscope using an accelerating voltage ranging from 40 to 60 kV.

Morphometric measurements of the collagenous entities were performed using a Quantimet 720 image analyser (Imenco). Negatives of scanning electron micrographs were obtained at magnification varying from 30 to 60 000. For collagen subfibrils, fibrils, and fibres whose cross-sectional areas are roughly circular, diameter measurements have been chosen. The average values were evaluated by measuring about 10–30 entities in each ligament. For collagen subfascicles and fascicles whose cross-sectional areas are irregular, surface measurements were used to obtain their range of variation. The collagen waviness period was measured for about 5–40 wavy units per specimen. Microstructural measurements were expressed as means standard deviations. The results were statistically evaluated using Student's t-test. The differences were regarded as significant at the level of $p < 0.05$ (p value).

3. Results

In both the ACL and PT, the collagen is arranged in a hierarchical structure as in the other fibrous collagenous tissues [15–18]. The collagen fibrils constitute the smallest structure of the collagen organization which could be observed by electron microscopy. The transverse and longitudinal sections of the ACL shows a considerable variation in diameter of the fibrils (Fig. 1). The typical cross-banding pattern of collagen fibrils is seen on the longitudinal sample (Fig. 1b); the

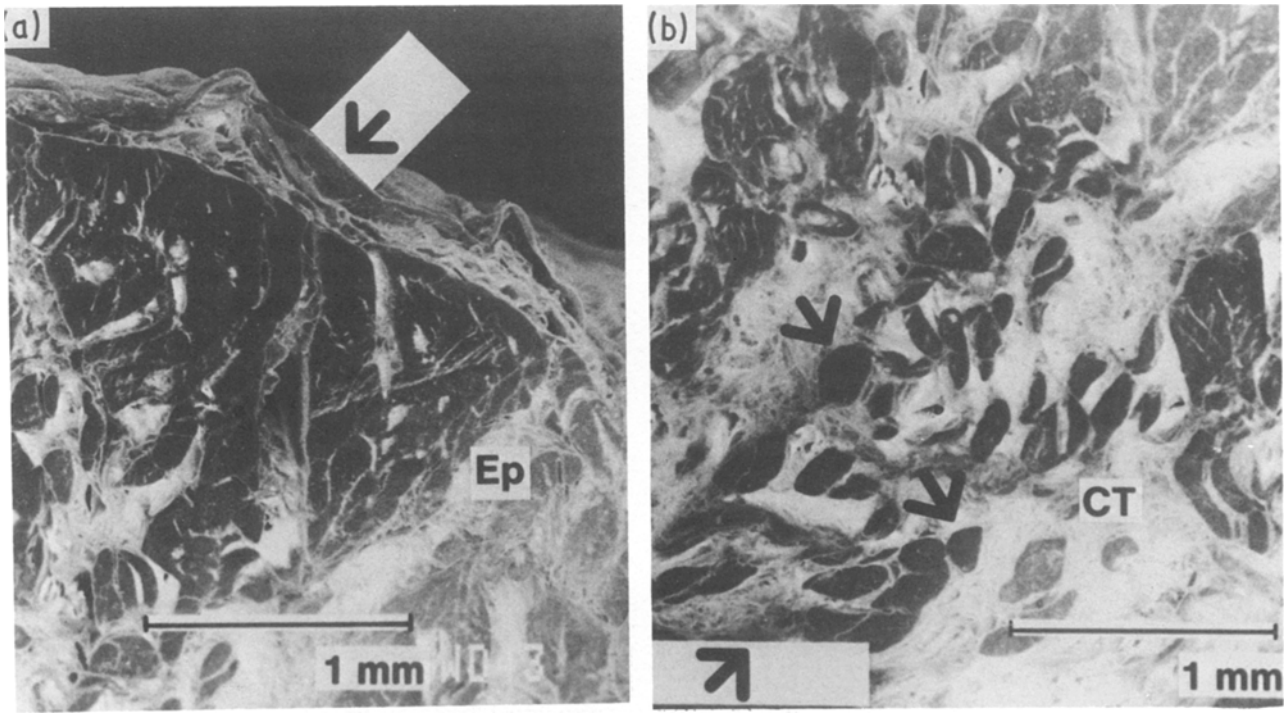

Figure 2 Transverse sections of the ACL (47-years-old male) obtained near the midsection. (A) large fascicles delineated by the epitenon (Ep) are seen in the antero-medial band (arrow) (× 19). (B) single fibres and subfascicles (arrows) embedded in connective tissue (CT) are found in the postero-lateral band (× 17).

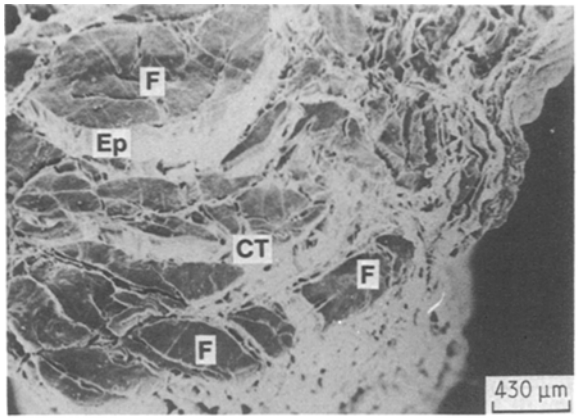

Figure 3 SEM micrograph of a PT (62-years-old female) cross-sectioned at its midpoint. Collagen fascicles (F) of different sizes are seen to be enclosed in the epitenon-connective tissue complex (Ep, CT) (× 16).

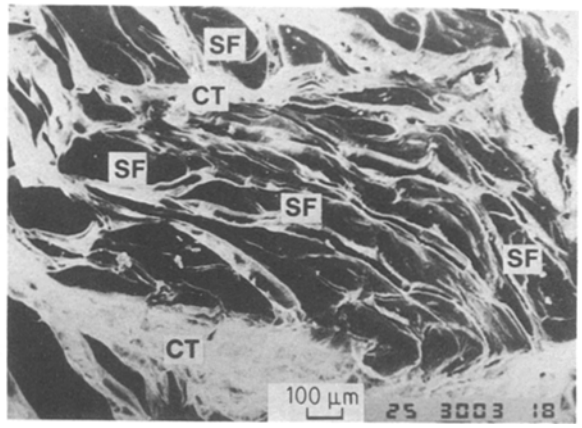

Figure 4 SEM micrograph of a medial cross-section of the PT (52-years-old male) showing numerous subfascicles (SF) elongated in radial direction and enclosed in a fascicle by the epitenon-connective tissue complex. (× 34).

TABLE II Fibre diameter (µm)

Specimen	ACL	PT
57M	10.25 ± 4.55 (22)	10.71 ± 6.70 (12)
62M	5.68 ± 4.04 (25)	5.54 ± 4.47 (26)
62F	4.94 ± 3.50 (8)	6.14 ± 3.82 (7)
73F	7.05 ± 5.77 (14)	2.56 ± 1.12* (21)
77F	11.91 ± 6.7 (20)	5.32 ± 5.10* (5)

( ): number of measurements.
Values are means ± SE.
*Significant ($p < 0.05$).

smallest fibrils correspond to the subfibrils (SFI). The diameter of the subfibrils of human ACL and PT is found to vary respectively from 40 to 140 nm and from 80 to 170 nm, while that of the fibrils ranges from 150 to 840 nm and 210 to 910 nm. The collagen fibrils are grouped into bundles called respectively fibre, subfascicle and fascicle [19]. Our observations show that, in the same section, single fibres and subfascicles could be seen (Fig. 2). These single fibres are surrounded by a loose connective tissue and the single subfascicles are enclosed in a sheath known as the endotenon [15]. Fascicles appear to be composed of subfascicles having different sizes and enclosed together by a connective tissue sheath called the epitenon. Fascicles of different sizes separated by an areolar connective tissue could be observed in a given tissue section (Fig. 3).

Examination of the whole cross-section of the ACL reveals two distinct regions: one mainly composed of thick fascicles having high subfascicular density and the other containing sparse single fibres and subfascicles embedded in a loose areolar connective tissue (Fig. 2). These two distinct regions are also found in the human PT suggesting a non-uniform distribution of collagen density in both tissues. Human ACL and PT subfascicles and fascicles often exhibit a random cross-sectional shape. Fig. 4 shows ACL fascicles radially elongated in the postero-anterior direction.

Morphometric measurements are performed from sections obtained near the midpoint of the ACL and PT. Mean values for the collagen fibre diameter are reported in Table II. It can be seen that there is no significant difference between the ACL and PT fibres of the 57–62-year-old subjects. However, a significant intertissular difference was found in the fibres diameter of elderly individuals (73F and 77F). The average fibre diameter of the ACl is significantly larger than that of the PT.

In Table III, values are presented for the cross-sectional area of the subfascicles and fascicles. There is a considerable range of variation for this parameter: 8 to 140 × 10^{-3} mm^2 for the subfascicles and 100 to 1740 × 10^{-3} mm^2 for the fascicles. This variation is related to the topographo-anatomical dependence of the collagen bundle size observed in the radial direction as well as in the axial direction.

In some ACL specimens, we found fine fibrils between longitudinally oriented collagenous units. This fibrils bind the sheaths of the neighbouring units similar to a bridge, and run perpendicularly to the long axis of the ligament. Fig. 5 shows the collagen fibres connected by transverse fibrils. At higher magnification, direct attachment of these linking fibrils to the fibres' sheath is revealed (Fig. 5b). It was also observed that these transverse fibrils provide structural links for the subfascicular and fascicular units. Although these fibrils, with diameters ranging from 180 nm to 650 nm, were resistant to the elastase

TABLE III Cross-sectional area of subfascicles and fascicles (× 10^{-3} mm^2)

Specimen	Subfascicle		Fascicle	
	ACL	PT	ACL	PT
52M	8.33–19.77	17.72–87.65	108–229.50	355.40–796.40
57M	81.93–102.93	16.85	376.40–947	153.67–306.28
62M	26.37–64.60	104.27–132.57	269.40–517	890–1740

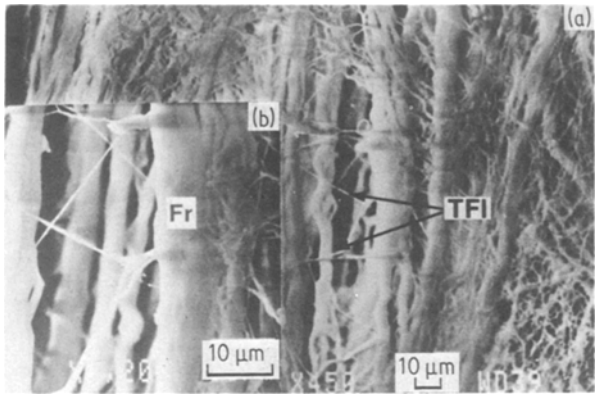

Figure 5 SEM micrographs of the medial longitudinal section of the ACL (47-years-old male). (A) the collagen fibers are seen to be connected by small transverse fibrils (TFI). × 200. (B) higher magnification showing a direct attachment of the transverse fibrils to the fibres' sheath. × 540.

TABLE IV Waviness period (μm)

Specimen	ACL	PT
47M	23.4 ± 4.4 (14)	40.5 ± 2.4* (17)
54M	15.1 ± 5.5 (12)	37.6 ± 4.2* (9)
57F	30.8 ± 3.5 (8)	48.3 ± 3.4* (4)
61F	12.3 ± 2.4 (10)	17.9 ± 1.4* (5)
62M	21.8 ± 3.5 (25)	25.8 ± 4.2† (9)
62F	15.1 ± 3.8 (11)	45.5 ± 4.4* (4)
77F	25.6 ± 5.8 (41)	56.4 ± 6.3* (22)

( ): number of wavy units.
Values are means ± SE.
*Significant ($p < 0.01$).
†Not significant ($p < 0.01$).

and hyaluronidase treatment, their nature remains unknown.

One of the most significant morphological properties of both the ACL and PT is the waviness occurring in their collagenous units. Fig. 6 shows surface undulations of numerous adjacent fascicles of a human ACL. At higher magnification, the waviness pattern appears regular along each fascicle length (Fig. 6b). This regular waviness is seen to occur in ACL and PT collagenous units of width varying from 20 to 640 μm, i.e. from fibre level to fascicular level. Mean values for the collagen waviness period of the ACL and PT are

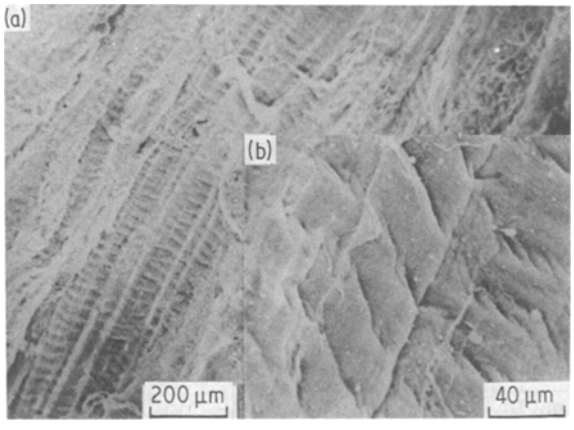

Figure 6 SEM micrographs obtained near the femoral attachment of the ACL (62-years-old female). (A) a surface waviness is seen over the adjacent fascicles. × 34. (B) higher magnification illustrating the details of the waviness. × 157.

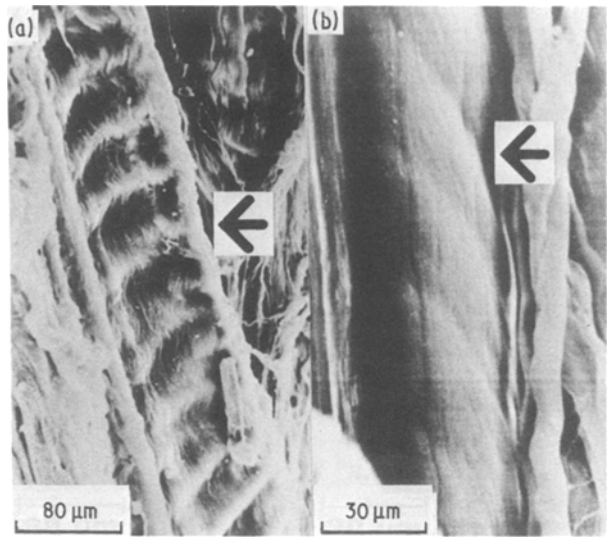

Figure 7 SEM views of the wave fascicles. (A) an ACL fascicle (47-years-old female) showing the typical planar waveform. Arrow indicates that the minima and maxima of the adjacent wave fibrils are at right angles to the fascicle axis. × 116. (B) a PT fascicle (62-years-old female) showing the helical waveform (arrow). × 290.

reported in Table IV. It can be seen that there is a significant intertissular difference ($p < 0.01$). For the same subject, the waviness period in the PT is significantly larger than in the ACL.

Our results show that two kinds of wave patterns – planar and helical waveforms – could be observed at the fascicular surface (Fig. 7). In planar waveform, the collagen fibrils undulate in the same plane, as shown in Fig. 7a. In Fig. 7b, the wavy surface of the fascicle appears helical but it is masked by the epitenon which closely contours the fascicular surface. Once the epitenon is removed by enzymatic treatment, a three-dimensional view of the architecture of the internal fibrils is revealed. Fig. 8 shows peripheral fascicular fibrils following a helical waveform pattern around the fascicle axis.

Examination of the internal structure of fascicles shows that two types of arrangement could be observed depending on the surface waviness. In planar waveform, the internal fibrils appear parallel to the longitudinal axis while in helical waveform, they appear

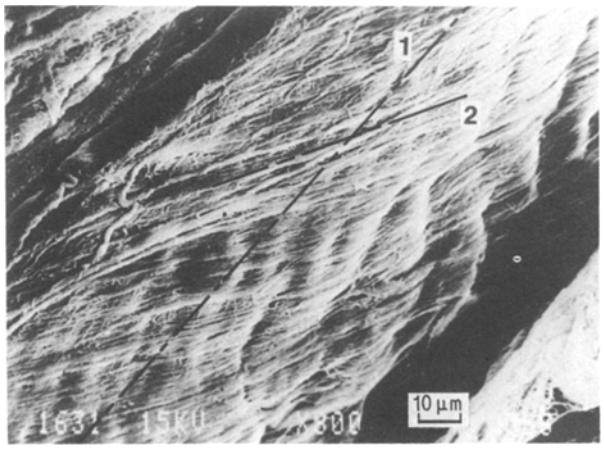

Figure 8 SEM micrograph of the wavy PT fascicle (77-years-old female). After enzymatic treatment, the helical wavy pattern of the peripheral fibrils is revealed. × 392.

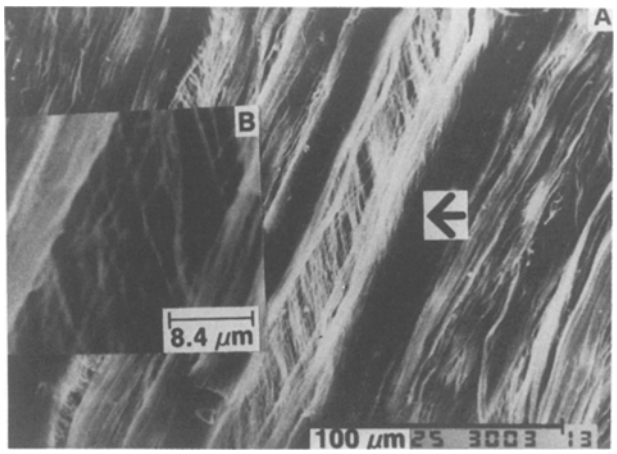

Figure 9 SEM micrographs of the PT (61-years-old female) in longitudinal section. (A) arrow indicates a fascicle cut longitudinally showing internal fibrils oriented obliquely with respect to the fascicle axis. × 240. (B) higher magnification showing the non-undulating fibrils. × 2400.

to make an angle with respect to the fascicle axis (Fig. 9).

Finally, according to our observations, it seems that in the ACL both waveforms could be observed depending on the anatomical site. However, in the PT only the helical pattern has been found.

4. Discussion

The present study shows that two structurally distinct regions could be observed, posterior and anterior, over the whole cross-section of the human ACL and PT. The posterior band is composed of small single fascicles embedded in a loose areolar tissue while the anterior band is formed of dense and thick collagenous fascicles. This introduces some degree of material heterogeneity in the radial direction. The generality of the above statement for all ligaments and tendons needs to be tested, but recently, Arms *et al.* [20] observed differences in the strain patterns of the anterior and posterior border of the medial collateral ligament which could be correlated to this topographo-anatomical heterogeneity.

Morphometric data showed that the diameter of ACL fibre is of the same order than that of PT fibres for the 57–62-year-old subjects, while it is significantly larger for the elderly subjects. It is likely that this decreasing in the PT fibre diameters may be related to the reduction in the muscle loading that could occur with ageing.

Itoh *et al.* [21] suggested that the subfibril is the growing unit in the collagen fibril. Although it is difficult to ascertain this hypothesis from only static micrographs, our results agree with such growth mechanism since considerable variation have been found in the subfibril and fibril diameter, as well as in the subfascicle and fascicle cross-sectional area (Table III). Following Itoh *et al.* [21], it is tempting to suggest that the subfascicle could be the growing unit in the fascicle development.

As far as the transverse fibrils are concerned, they have also been observed in the canine ACL [17, 18] and seem to provide structural links for the load-bearing collagenous entities. Although these fibrils are not destroyed by elastase and hyaluronidase, their nature is still unknown. Amiel *et al.* [13] found that the rabbit ACL contains more type III collagen than does the PT, and immunohistochemical studies are needed to assess whether these linking fibrils are type III collagen.

The surface morphology of the fascicles is characterized by a regular waviness. Our results show that two wave patterns could be observed in the ACL, planar and helical, depending on the anatomical site. In addition, our observations suggest a possible difference between the periphery and interior of the ACL; the peripheral fascicles follow often a helical pattern while the internal fascicles appear straight and parallel to the longitudinal axis. It was found that in the PT, the collagen waviness period is significantly larger than in the ACL. This is an important finding which could be used as a morphometric parameter in comparative studies.

In the PT units we observed only the helical conformation. This architectural difference, in agreement with previous studies where the helical pattern was often associated with tendons [22, 23] may be related to the specific role of the PT.

The morphological and architectural properties obtained in this study provide a structural basis to assess the collagen remodelling of the PT occurring postoperatively in the PT auto-graft. Comparison between the ACL and PT requires further ultrastructural studies on collagen as well as on elastin and proteoglycans.

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