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Collagen fibril diameter distribution in patellar tendon autografts after posterior cruciate ligament reconstruction in sheep: changes over time

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ABSTRACT

The alterations in collagen fibril diameter distribution, mean fibril diameter and the area occupied by collagen after posterior cruciate ligament reconstruction using a patellar tendon autograft were estimated 2, 6, 16, 26, 52 and 104 wk postoperatively. Patellar tendons and posterior cruciate ligaments from unoperated animals were used as control tissues. Collagen fibrils were divided into histograms according to their diameter in order to analyse distribution maxima. There was a significant decrease in mean fibril diameter of the grafts in comparison with the control tissues. At 104 wk it was only about 51% of that for control posterior cruciate ligaments. The total area occupied by collagen was significantly reduced at 6 wk postoperatively and was about 57% in comparison with normal posterior cruciate ligaments. A considerable increase of small diameter collagen fibrils together with a loss of large fibrils was responsible for these results. There was no evidence of reestablishment of large diameter fibrils, which are normally found in tendon and ligaments, up to 2 y after transplantation. The total area covered by collagen was still reduced at this stage although the number of fibrils had increased.

Key words: Knee joint; ligament replacement.

INTRODUCTION

The distribution of collagen fibril diameters and the mean fibril diameter of connective tissues have been shown to depend on many different influences. Changes in collagen fibril populations are demonstrable during maturation (Frank et al. 1989), immobilisation (Newton et al. 1990) and by increasing the level of stress on the tissue (Michna, 1984; Williams et al. 1985). It has been suggested that the mean collagen fibril diameter in connective tissues is correlated with the resistance to applied maximum mechanical stress (Parry & Craig, 1984). Other investigators have reported that a completely different collagen fibril size distribution develops in the lesion area of ruptured tendons in comparison with normal tendons. This alteration showed no changes over time (Matthew & Moore, 1991).

The central third of the patellar tendon (PT) is the most commonly used autograft for cruciate ligament replacement because its mechanical properties correspond very closely to those of the normal cruciate ligament (Butler et al. 1979; Noyes et al. 1984). After transplantation the PT autograft undergoes a remarkable histological transformation including necrosis and subsequent restoration. During this transformation process the resistance to mechanical stress is decreased. Even 2 y after operation the mechanical properties of the PT autograft do not reach what is documented for the posterior cruciate ligament (PCL) (Bosch & Kasperczyk, 1992). This finding reflects clinical experience where the posterior cruciate ligament substitute, in particular, is less satisfactory than anterior cruciate reconstructions. The aim of the present study was to investigate whether changes in ultrastructure could explain changes in biomechanical properties.

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MATERIALS AND METHODS

In 30 2-y-old, skeletally mature, purebred sheep the PCL of the left knee joint was replaced in a standard manner by the central third of the PT from the same leg. The surgical technique described by Clancy et al. (1981) was followed in order to achieve a graft position as isometric as possible to avoid overstretching or overloading of the transplanted tissue. The autografts were fixed in bone tunnels in the tibia and femur via threads to cancellous bone screws in 70° of knee flexion, anterior drawer position, and with a tension of 50 N. Postoperatively no protection was given on the operated knee. On the 10th postoperative day the sheep were released into a farm flock.

After 2, 6, 16, 26, 52 and 104 wk, 5 animals from each time period were killed by an intravenous injection of a lethal dose of T 61 (embutramide mebenzonium-iodide tetracainhydrochloride). Samples were prepared from the edge (peripheral) and from the core (central) at midpoint of PT autografts. In addition, PT and PCL from 5 knees of unoperated sheep were prepared in the same way and used for control purposes.

The samples were fixed by immersion for transmission electron microscopy. Fixation was with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate/HCl buffer (pH 7.2–7.4), followed by rinsing in the same buffer and postfixation with 1% osmium tetroxide (1 h), still in the same buffer. The samples were dehydrated in graded alcohols and embedded in epoxy resin. Ultrathin transverse sections were stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 101 electron microscope. At a magnification of $\times 30000$, 20 randomly selected micrographs (10 peripheral and 10 central tissue samples) from each tissue sample were made to analyse individual collagen fibril diameters in a representative field corresponding to 1 μm^2 of tissue.

For morphometric analysis, an interactive image processing system (Kontron IBAS) was used. To avoid errors due to obliquity of sectioning the minimum fibril diameter was always measured. Within the standardised field the point counting method (Weibel, 1979) was employed. On every sample between 700 and 3500 collagen fibrils were digitised and divided into histograms containing 5 classes at intervals of 40 nm with a 6th open class. For better illustration of the main collagen portions within the tissue the total area occupied by collagen was determined and divided in the fraction for each diameter class. The mean collagen fibril diameter from each structure was calculated and used for statistical comparison. Significant differences were sought by using the Kruskal–Wallis test with a significance level of $P < 0.05$.

RESULTS

The intrafascicular collagen fibril distribution of the PT was homogenous over the whole width of the tissue and contained fibrils of various diameters (Fig. 1). After a transformation process characterised by a heterogeneous collagen fascicle architecture at wk 6 (Fig. 2), PT autograft specimens 16 wk postoperation

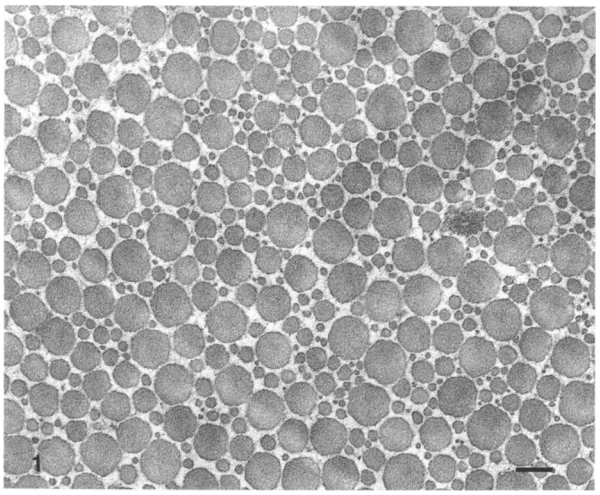

Fig. 1. Transverse section through control patellar tendon showing a homogeneous collagen pattern with fibrils of various diameters. Bar, 200 nm.

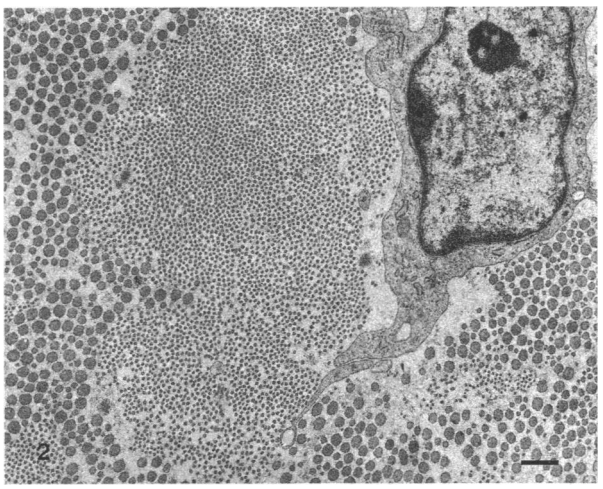

Fig. 2. At wk 6 after transplantation the patellar tendon autograft was characterised by a heterogeneous collagen fibril pattern. Intrafascicular regions made up of small diameter fibrils alternate with regions of large diameter fibrils. Bar, 600 nm.

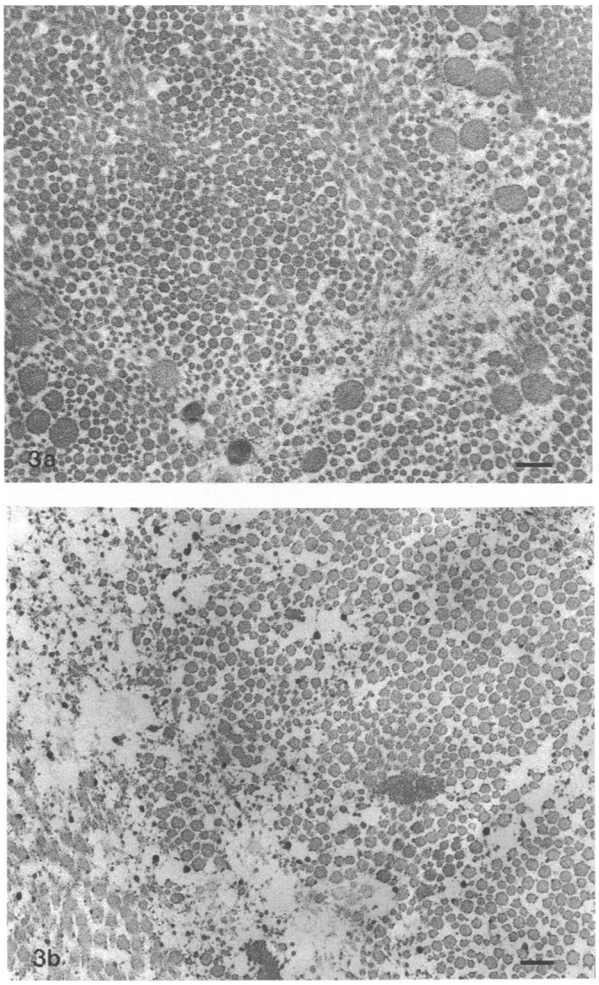

Fig. 3. Later stages after cruciate replacement show a collagen fibril pattern dominated by small fibril diameters. (*a*) 16 wk; (*b*) 104 wk. Bar, 200 nm.

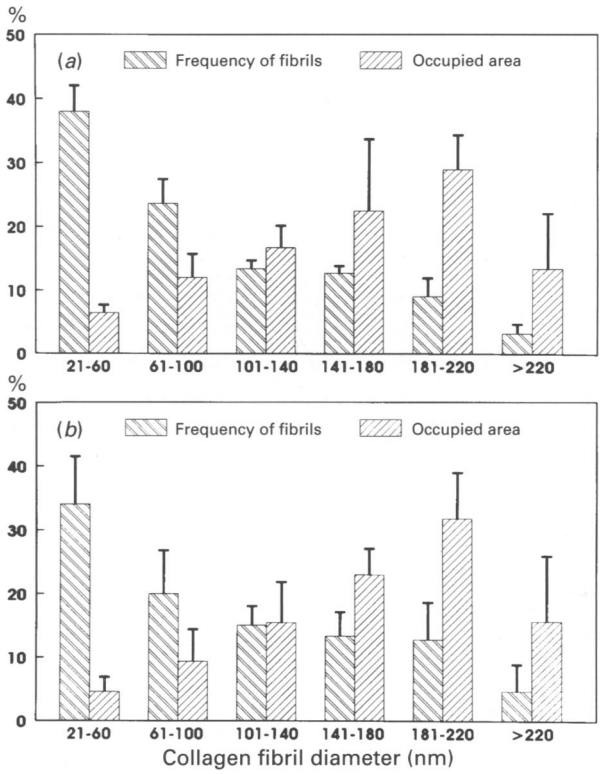

Fig. 4. Histograms of collagen fibril diameter distribution and the area occupied within each class in unoperated animals. (*a*) Patellar tendon; (*b*) posterior cruciate ligament.

(Fig. 3*a*) again showed a uniform collagen fibril pattern. This pattern did not change until wk 104 (Fig. 3*b*). It was characterised by small diameter collagen fibrils.

Morphometric analysis demonstrated that the distribution of collagen fibril diameters of the PT and PCL was non-Gaussian being skewed to the right and showing a maximum frequency for fibrils in the smallest diameter class. However, the main portion of the area occupied by collagen was represented by large diameter fibrils (Fig. 4*a*, *b*). At each time point examined, the PT autografts showed a similar distribution for fibril frequency with a maximum in the smallest diameter class (Fig. 5*a–f*). At 2 wk after transplantation an increase in small diameter collagen fibrils was found, combined with a decrease of large diameter fibrils. This process of change reached a maximum at 16 wk, when 82.7% of the collagen fibrils had a diameter less than 60 nm (Fig. 5*c*). After 52 wk an increase of fibrils with a diameter between 61 and 100 nm was identified (Fig. 5*e*). At 104 wk the proportion in this class was 36.3% (Fig. 5*f*). However, there was no reestablishment of collagen fibrils with a diameter over 100 nm. The area occupied by collagen at 2 wk after transplantation was quite similar to the PT and its main part was occupied by fibrils of large diameter (Fig. 5*a*). At 6 wk the area occupied by collagen was represented by collagen fibrils of all diameter classes (Fig. 5*b*). From wk 16 until wk 104 the area occupied by collagen was made up of fibrils measuring less than 100 nm (Fig. 5*c–f*).

Mean fibril diameter (Fig. 6) of the PT (100.7 ± 5.1 nm) showed a significant decrease (49.5 ± 4.9 nm) up until wk 16. Up to 104 wk mean collagen fibril diameter in the PT autografts showed a smooth but nonsignificant increase (55.9 ± 3.9 nm). Calculation of total area occupied by collagen within the standard field of 1 μm^2 demonstrated that in PT ($54.2 \pm 4.4\%$) and PCL ($58.0 \pm 2.0\%$) more than half the cross-section was occupied by collagen fibrils. At every time point the area occupied by collagen in PT autografts was significantly smaller and was about $37.5 \pm 4.4\%$ at wk 104 (Fig. 7). Finally the mean number of collagen fibrils counted was double at 16 wk after transplantation in comparison with the early stages after replacement. But at this time point

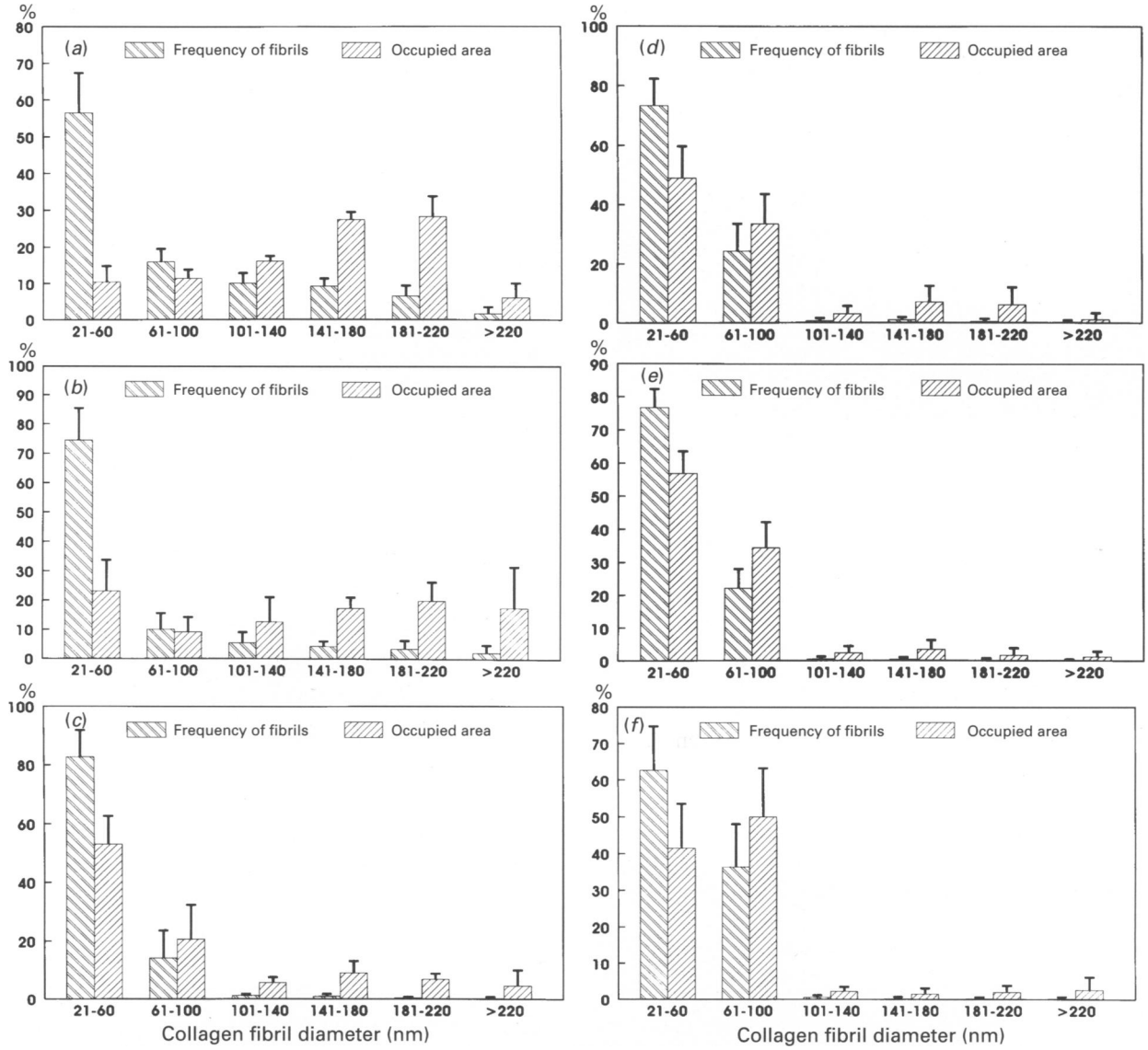

Fig. 5. Histograms of collagen fibril diameter distribution and the occupied area within each class for the patellar tendon autograft over time. (*a*) 2 wk; (*b*) 6 wk; (*c*) 16 wk; (*d*) 26 wk; (*e*) 52 wk; (*f*) 104 wk.

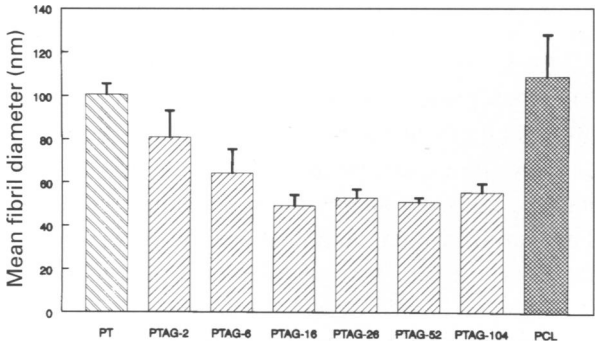

Fig. 6. Mean fibril diameter for the control patellar tendon (PT) and posterior cruciate ligament (PCL) in comparison with the patellar tendon autograft (PTAG) over time.

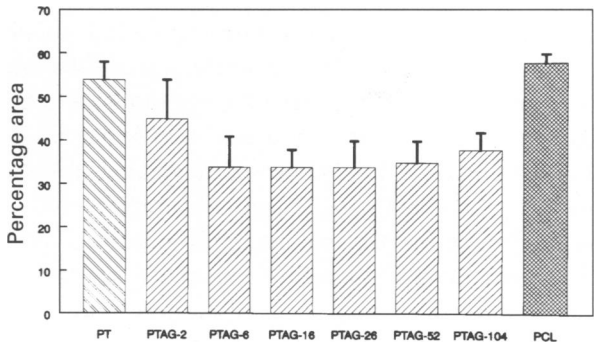

Fig. 7. Total area occupied by collagen for the control patellar tendon (PT) and posterior cruciate ligament (PCL) in comparison with the patellar tendon autograft (PTAG) over time.

Table. *Mean fibril diameter, area occupied by collagen within the standardised field of 1 μm² and the mean number of measurements for control patellar tendon (PT), control posterior cruciate ligament (PCL) and the patellar tendon autograft (PTAG) over time**

Tissue	Mean diameter (nm)	Occupied area/μm² (%)	Mean no. of measurements
PT	100.7±5.1	54.2±4.4	1144±79
PCL	109.5±19.1	58.0±2.0	1038±281
PTAG 2	81.3±12.1	44.9±8.9	1361±444
PTAG 6	64.6±11.4	33.5±7.4	1375±258
PTAG 16	49.5±4.9	33.5±4.2	2879±406
PTAG 26	53.2±3.6	33.9±5.5	2575±238
PTAG 52	51.3±2.2	35.3±5.0	3056±399
PTAG 104	55.9±3.9	37.5±4.4	2744±94

* Means±S.D.

there was no increase in the total area occupied by collagen (see Table): this remained at the decreased level of wk 6.

DISCUSSION

The use of the central third of the patellar tendon for cruciate ligament replacement is based on the close similarity in mechanical properties. The collagen fibril patterns of these different anatomical structures are quite similar. The comparable mean diameters, the nearly identical distribution of collagen fibrils in diameter classes and the proportion of the total area occupied by collagen support the biomechanical findings obtained by Butler et al. (1979) and Noyes et al. (1984). From its collagen fibril pattern the PT autografts might also be a very suitable graft for cruciate ligament replacement.

After transplantation, however, the results of the present study demonstrate a dramatic change in the collagen fibril diameter pattern in PT autografts. Similar findings have been reported for anterior cruciate ligament replacements. In human grafts collagen fibrils less than 100 nm in diameter are the major contributors to the collagen fibril cross-sectional area up to 6 y after transplantation (Frank et al. 1988). Based on these findings and according to morphological changes in PT autografts (Bosch et al. 1992) we suggest that ultrastructural changes in collagen fibril pattern after PCL reconstruction are characterised by two phases. The early phase, which is dominated morphologically by a necrotic reaction, seems to combine the disappearance of large diameter collagen fibrils with a smooth increase in the number of small diameter fibrils. In the following phase restoration becomes the dominant tissue reaction. At this time an obvious increase in collagen fibrils takes place. Morphological findings support the suggestion that this more obvious second increase is due to the synthesis of fibrils. These newly formed fibrils seem to have a limited tendency to grow, which is reflected by a slow increase in collagen fibrils with a diameter of 61–100 nm from wk 16 to 104. The mean collagen fibril diameter, however, did not increase significantly within this period.

Biological reasons for the development of small diameter collagen fibrils in connective tissue have been investigated by several authors and will be related to the results of the present study. At the early stages after transplantation the avascular PT autograft is confronted with necrosis and the new function as a PCL substitute. This is a situation where overloading stresses could occur. High stress imposed on connective tissue induces alterations of the covalent crosslinks between collagen molecules. Subsequent enzymatic attack splits thick fibrils into thinner ones with different diameters (Michna, 1984; Parry & Craig, 1984). This could be a possible explanation for the disappearance of large fibrils combined with a smooth increase of small diameter fibrils up until 6 wk. Another reason for the increase of thin fibrils would be the synthesis of type III collagen. Tendons and ligaments contain mainly intrafascicular type I collagen (Lapiere et al. 1977), forming fibrils with diameter of 100–500 nm depending on the tissue. Type III collagen fibrils do not grow beyond a diameter ~ 60 nm (Fleischmajer et al. 1981). They are normally found as a part of the repair process following tissue injury (Viidik, 1973). By using immunoelectron microscopy the existence of intrafascicular type III collagen in the PT autografts has been detected (Bosch et al. 1992).

In the second phase the newly synthesised collagen fibrils fail to grow above a diameter of 100 nm. This could be related to many interactions within the revitalised tissue. First, the synthesis of collagen fibrils from fibril segments along the fibroblast surface in fibril forming channels can lead to the formation of small diameter fibrils when other extracellular components inhibit the fusion of the segments (Birk & Zycband, 1994). The composition of extracellular matrix glycosaminoglycans (GAG) is one of the determining factors of radial growth in collagen fibrils (Scott, 1988). Following a hypothesis developed by Parry et al. (1982), hyaluronic acid is the predominant GAG during fetal development, connective tissue regeneration and remodelling after wounding. They postulated that radial growth of collagen fibrils is limited by a hyaluronic acid-rich matrix so that only

fibrils below 60 nm in diameter are formed. During maturation the hyaluronic acid content of connective tissue decreases rapidly whereas chondroitin sulphate and dermatan sulphate tend to increase. In a second stage of fibril development initiated by cells which synthesise chondroitin sulphate, in response to mechanical loading or the microelectrical environment, fibrils with larger diameters appear. Tissues which are subjected to high tensile stresses are characterised by a high percentage of dermatan sulphate and collagen fibrils with diameters around 200 nm. But the dominance of dermatan sulphate does not automatically favour the development of large diameter collagen fibrils. In connective tissues, dermatan sulphate has been discussed as an inhibiting factor for radial growth (Scott, 1984).

The changes in GAG content and their distribution for the PT autografts after cruciate ligament replacement are available in the literature (Decker et al. 1994; Gaessler et al. 1994). In the early stages after transplantation a dominance of hyaluronic acid has been reported. In the extracellular matrix of PT autografts, chondroitin sulphate increases continuously after transplantation. The content of dermatan sulphate shows an early peak at 6 wk and a maximum peak half a year after PCL replacement. The early increase of hyaluronan could be due to the repair process. However, the maximum peak for dermatan sulphate could be a good reason for failure in radial fibril diameter growth by inhibiting the newly synthesised fibrils at this stage. Consequently the mean fibril diameter and the fibril distribution between 26 and 52 wk are almost the same. The non-significant increase of collagen fibrils at the end of this study could be related to the continuous increase in chondroitin sulphate after the inhibitory influence of dermatan sulphate has disappeared.

Another influence on the radial growth of young collagen fibrils is the interaction between different collagen types and their proportion in the extracellular matrix. Keene et al. (1987) and Fleischmajer et al. (1990) demonstrated the existence of heterotypic fibrils containing both type I and type III collagen molecules within an individual fibril. Both collagen types can be synthesised simultaneously by fibroblasts (Gay et al. 1976). In such fibrils a steric inhibition by aminopropeptides of type III procollagens has been suggested and could be responsible for reduced fibril diameters in fibrillogenesis (Fleischmajer et al. 1990). Furthermore, the proportion of types I and V collagen within a heterotypic fibril has been found to determine the growth of fibrils (Adachi & Hayashi, 1986). The average diameter in fibrils with an equal proportion of both collagen types was between 30 and 50 nm. Bosch et al. (1991) demonstrated marked deposits of type V collagen, mainly cell associated, 1 and 2 y after implantation of the PT autograft.

Finally, consideration must be given to the fact that PT autografts fail to gain a collagen fibril diameter distribution and collagen density as are found in the normal PCL. Connective tissues designed to resist high mechanical loading are characterised by collagen fibrils with large diameters and high collagen density. A tissue with small diameter fibrils has a lesser number of intrafibrillar covalent crosslinks and the ultimate tensile strength is reduced (Parry et al. 1978). Based on this and the results of the present study, mechanical properties comparable to those of the PCL cannot be expected from the PT autografts.

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