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The remodelling process of allogeneic and autogenous patellar tendon grafts in rats: a radiochemical study

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Abstract In order to study the remodelling of collagen fibres of transplanted tendons, one-half of the patellar tendon of the knee in 54 rats was replaced with a radioisotope (^{3}H-proline)-labelled patellar tendon procured from a donor rat. Three transplantation models were used in this study: fresh-frozen allograft, fresh-frozen autograft (fresh-frozen isograft), and fresh autograft (fresh isograft). The percentage of old collagen was calculated as an indicator of collagen turnover from the amount of hydroxyproline and the radioactivity level of ^{3}H-hydroxyproline in the transplanted tendons at 4, 12 and 24 weeks postoperatively. Histological evaluation was also performed at 2, 4, 12 and 24 weeks. At 4 weeks, the percentage of old collagen in the grafts from the fresh-frozen allograft group was significantly lower than in the autograft groups (20% vs. 48%). Although the percentage of old collagen in the fresh-frozen autograft group had decreased to the same level as in the fresh-frozen allograft group by 12 weeks (approximately 10%), the value was still high in the fresh autograft group at 12 weeks and remained higher (38%). Histologically, at 2 weeks, the cellularity in the fresh-frozen allograft was higher than in the fresh-frozen autograft. After 4 weeks, however, no significant difference was found between these two groups. In the fresh autograft group, the cellularity was lower than in the fresh-frozen groups at all times. In conclusion, the collagen turnover rate in the fresh-frozen allograft was the most rapid of the three transplantation models based on hydroxyproline turnover.

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Introduction

Reconstruction of the anterior cruciate ligament (ACL) is widely performed using autogenous or allogeneic tendon grafts [7, 15, 18]. Previous studies have demonstrated that the transplanted tendons, both allografts and autografts, undergo a process of ischaemic necrosis, revascularization, proliferation of fibroblasts and remodelling of the collagen fibres. These studies have examined the remodelling process from the histological and microangiographic perspectives [1, 3, 17, 18, 20]. However, differences in the metabolic turnover of the collagen fibrils between allografts and autografts have not been studied quantitatively. It is important to know the fate of the residual collagen fibres of transplanted tendons, but previous animal models of ACL reconstruction have not yielded accurate biochemical data because of difficulties inherent to the grafting technique. To mimic intra-articularly transplanted grafts, fresh-frozen autogenous and allogeneic tendons were used in an extra-articular rat model. This model is characterized by two features. First, the model was designed to minimize overstress on the transplanted grafts by replacing only half of the patellar tendon. Second, this model also enabled us to create an autograft model by grafting a tendon of an inbred strain rat to the same strain rat (isograft). Using this model, collagen turnover in tendons transplanted using different techniques (fresh and fresh-frozen) could be investigated. We studied metabolic differences in the remodelling of autogenous and allogeneic tendons with respect to the absorption of collagen fibres.

Methods

Labelling tendons and graft harvest

L-[2,3,4,5,-^{3}H]-proline solution (37.0 MBq per ml of 0.01 N HCL TRK 750; Amersham, UK) was diluted 40 times with phosphate-buffered saline (PBS). Two milliliters of the diluted solution were injected into the peritoneum of 18 4-week-old juvenile male Lewis rats (LEW/crj, obtained from Charles River Japan) twice a week

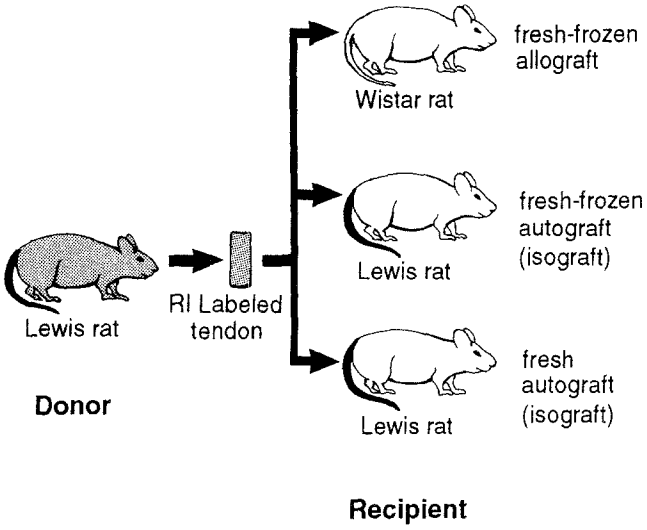

Fig. 1 Experimental models. Fresh-frozen allograft model: The radiolabelled patellar tendon of a Lewis rat was transplanted to the knee of a Wistar rat. Fresh-frozen and fresh autograft models: The radiolabelled patellar tendon of one Lewis rat was transplanted to another Lewis rat

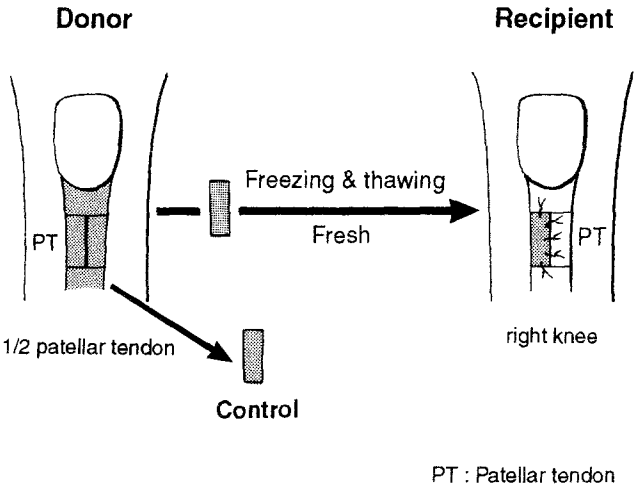

Fig. 2 Operative procedures. Under general anaesthesia, a 2.5 × 6 mm defect was made in the lateral half of the patellar tendon of the right knee in each recipient rat. For the fresh-frozen allograft and autograft models, a radiolabelled tendon strip frozen at –80° C for at least 2 weeks was transplanted. After thawing at room temperature, the graft was sutured firmly to the surrounding patellar tendon using 6-0 nylon suture. In the fresh autograft model, a radiolabelled tendon strip was grafted immediately after harvest

for 5 weeks (total dose 18.5 MBq of ^{3}H-proline). Injected ^{3}H-proline solution was incorporated into collagen fibres throughout the rat's body in the form of ^{3}H-hydroxyproline. Twelve weeks after injection, the animals were killed (average weight 399 ± 46 g). Both patellar tendons were harvested and split in half longitudinally. Therefore, 72 radiolabelled patellar tendon grafts were obtained from 18 Lewis rats. Each graft was approximately 2.5 mm wide and 6 mm long. Of the four patellar tendon strips from each rat, one was used as a control and three for experimental studies.

Experimental models

Three experimental models were used in this study (Fig. 1). (1) For the fresh-frozen allograft model, 16-week-old male Wistar rats (Crj: Wistar, obtained from Charles River Japan) were used (n = 19). The labelled tendons were frozen at –80° C for at least 2 weeks immediately after harvest from the Lewis rats. After thawing at room temperature, these were transplanted to the Wistar rats. (2) In the fresh-frozen autgraft model, the thawed radiolabelled frozen tendons from the Lewis rats were transplanted to 16-week-old male Lewis rats (n = 19). (3) In the fresh autograft model, the labelled grafts from the Lewis rats were transplanted to 16-week-old male Lewis rats immediately after harvest (n = 19).

Operative procedures

Under general anaesthesia, a 2.5 × 6 mm defect was made in the lateral half of the patellar tendon of the right knee in each recipient rat (Fig. 2). For the fresh-frozen allograft and autograft models, radiolabelled strips frozen at –80° C for at least 2 weeks were transplanted. After thawing at room temperature, the grafts were sutured firmly to the surrounding patellar tendon with a 6-0 nylon suture. In the fresh autograft model, the graft was transplanted immediately after harvest. After surgery, the limb was not immobilized, and cage activity was allowed in all three groups.

Six animals from each group were killed at 4, 12 and 24 weeks postoperatively. After harvesting the grafts from the recipient rats, the midportions of the grafts were used for the radiochemical analysis, and the medial parts were used for histological evaluation. One animal from each group was killed at 2 weeks for histological evaluation.

Radiochemical analysis

The transplanted and control strips for the radiochemical study were hydrolysed by heating at 110° C in enclosed tubes with 2 ml of 6 N HCL. The amount of hydroxyproline incorporated into the collagen and the radioactivity level of the ^{3}H-hydroxyproline remaining in the strips were analysed using the following methods.

The amount of hydroxyproline was assayed by the Prockop method [16]. After oxidizing the hydroxyproline with chloramine-T into the form of a pyrrole, the pyrrole nucleus of the product was reacted with Ehrlich reagent under acidic conditions. Colorimetric analysis of the product was carried out at an absorbance of 562 nm wave lenght using a spectrophotometer (UV240; Shimadzu, Kyoto, Japan). Since the factor for converting hydroxyproline weight to collagen weight is 7.5 [2], the amount of collagen can be estimated from the amount of hydroxyproline by the following formula:

$$\text{Collagen wt} = \text{hydroxyproline wt} \times 7.5$$

where wt = weight.

To measure ^{3}H-hydroxyproline, 0.5 ml of the hydrolysed solution from the tendon strip was dissolved in 5 ml of a liquid scintillation cocktail (Clearsol; Nakarai Tesque, Kyoto, Japan). The radioactivity level (DPM: disintegrations per minute) of the ^{3}H-hydroxyproline solution was measured using a liquid scintillation counting system (Mark III; TmAnalytic, IL, USA).

^{3}H-hydroxyproline is not re-incorporated once it is absorbed [9]. Therefore, unmetabolized original radioactive collagen within the transplanted tendons could be distinguished from newly incorporated collagen as old collagen. Based on quantification of hydroxyproline and measurement of the radioactivity level of ^{3}H-hydroxyproline, the percentage of old collagen could be calculated by the formula:

$$\text{Old collagen (\%)} = \frac{^3\text{H - collagen wt/collagen wt (graft)}}{^3\text{H - collagen wt/collagen wt (control)}} \times 100$$

$$= \frac{^3\text{H - hypro.wt/hypro.wt (graft)}}{^3\text{H - hypro.wt/hypro.wt (control)}} \times 100$$

where hypro. = hydroxyproline, ^{3}H-hypro = ^{3}H-hydroxyproline.

The data were analysed by a one-way analysis of variance (ANOVA). Multiple comparisons among groups were made using Fisher's PLSD test.

Histological examination

The medial part of the transplanted tendon in each specimen was used for histological study. After fixation in 10% neutral formaldehyde, each specimen was stained with haematoxylin and eosin and examined by light microscopy.

Results

Macroscopic findings

Five animals in the fresh-frozen allograft model, six in the fresh-frozen autograft model, and three in the frsh autograft model died prior to the planned end of the study. The reason for the death was not associated with the transplantation. All the remaining animals had a normal gait. At death, all the transplanted tendons appeared to be continuous with the surrounding tissue (Fig. 3).

Radiochemical analysis

The amount of hydroxyproline and the radioactivity level of ^{3}H-hydroxyproline in the 18 control strips were closely correlated (correlation coefficient: 0.929, coefficient of determination: 0.863) (Fig. 4). The mean amount of hydroxyproline and the radioactivity level of ^{3}H-hydroxyproline in the three groups are shown in Table 1. At four weeks, the percentage of old collagen in the fresh-frozen allograft group was significantly lower than in the autograft groups (fresh-frozen allograft, 20% ± 8%; fresh-frozen autograft, 49% ± 22%; fresh autograft, 48% ± 14%; $P < 0.05$; Fig. 5; Table 2). The values in the fresh-frozen autograft group had decreased significantly by 12 weeks postoperatively (12% ± 10%, $P < 0.05$), and the old collagen levels in both the fresh-frozen allograft and autograft groups were significantly lower than that in the fresh autograft group (fresh-frozen allograft, 14% ± 8%; fresh autograft, 38% ± 19%; $P < 0.05$). However, no significant differences in the percentage of old collagen existed between the three groups 24 weeks after transplantation (fresh-frozen allograft, 10% ± 7%; fresh-frozen autograft, 13% ± 14%; fresh autograft, 28% ± 20%).

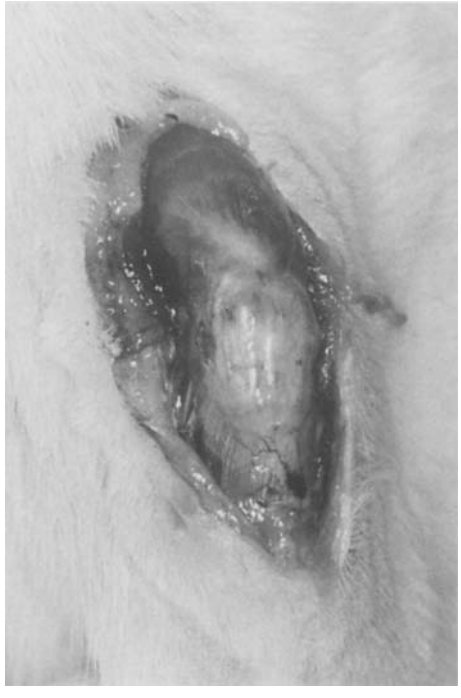

Fig. 3 Macroscopic findings of the fresh frozen allografts 24 weeks post-transplantation. The grafted tendon appeared to be continuous with the surronding tissue, and the boundary between the graft and the recipient tissue was indistinct

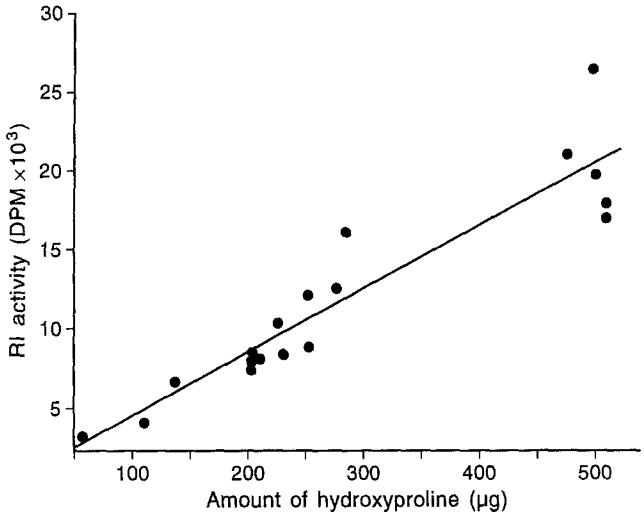

Fig. 4 Correlation between the amount of hydroxyproline and the radioactivity level of ^{3}H-hydroxyproline in the control strips. Correlation showed an excellent linear regression (correlation coefficient: 0.929, coefficient of determination: 0.863)

Histology

Fresh-frozen allograft group (Fig. 6A). No histological evidence of an immunological rejection was obtained at any time. At 2 weeks post-transplantation, cellularity was high, and the collagen fibres were not aligned regularly. Although cellularity was still higher than in a normal tendon at 4 weeks, the collagen fibres were aligned more regularly in a longitudinal pattern. At 12 weeks, fibroblasts and collagen bundles within the graft were aligned regularly, as in a normal tendon, and the nuclei of the fibroblasts were spindle-shaped.

Fresh-frozen autograft group (Fig. 6B). The changes in histology in the fresh-frozen autograft group were similar to those in the fresh-frozen allograft, in that hypercellularity gradually subsided and the collagen fibres became more aligned in a regular pattern over time. However, at 2 weeks, the cellularity clearly was less than in the fresh-frozen allografts.

Table 1 Mean value of the amount of hydroxyproline and radioactivity level of ^{3}H-hydroxyproline in the three models [Hypro., amount of hydroxyproline (μg); ^{3}H-hypro., radioactivity level of ^{3}H-hydroxyproine (DPM)]

[a] Interval post-transplantation

	Fresh-frozen allograft		Fresh-frozen autograft		Fresh autograft	
Duration[a]	Hypro.	^{3}H-hypro.	Hypro.	^{3}H-hypro.	Hypro.	^{3}H-hypro.
4 weeks	650	5065	118	2455	128	2462
12 weeks	388	1885	347	1382	101	1546
24 weeks	836	2499	434	2316	138	1401

Table 2 Percentage of old collagen in the three models (mean ± SD; number of samples in parentheses)

[a] Interval post-transplantation

Duration[a]	Fresh-frozen allograft	Fresh-frozen autograft	Fresh autograft
4 weeks	20.2 ± 7.8 (5)	48.8 ± 21.5 (5)	48.4 ± 13.5 (4)
12 weeks	13.5 ± 7.8 (4)	11.7 ± 9.6 (4)	37.5 ± 19.1 (5)
24 weeks	9.8 ± 7.3 (4)	13.1 ± 14.3 (3)	27.7 ± 19.8 (6)

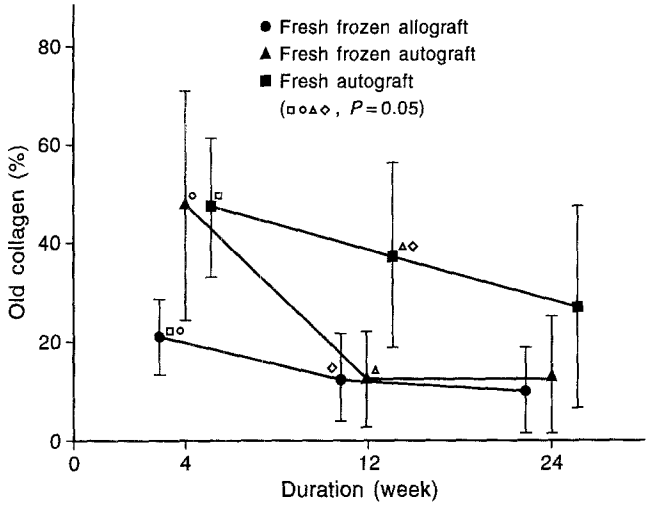

Fig. 5 Changes in the percentage of old collagen over time. At 4 weeks post-transplantation, there was a significant difference in the percentage of old collagen between the allograft and autografts ($P < 0.05$). At 12 weeks post-transplantation, a significant difference was still found between the fresh-frozen grafts and the fresh graft ($P < 0.05$). This difference was no longer noted after 24 weeks

Fresh autograft group (Fig. 6C). Compared with the first two groups, the cellularity in the fresh autograft group was less, with few spaces between the collagen bundles. Spindle-shaped cells were observed in all study periods.

Discussion

The remodelling process of transplanted tendons has been studied by several investigators using radiolabelled tendons [6, 10, 11]. In these experiments, rats' tail tendons were transplanted to the Achilles tendon after complete excision of the whole Achilles tendon to create an autograft model. However, these experiments had several problems: (1) different grafting techniques were used in the allograft and autograft models; (2) differences exist in the structure of the rat tail tendon and the Achilles tendon; and (3) the load on the transplanted tendon may have disrupted the suture line, impairing healing and graft incorporation. To solve these problems, we used a rat model which avoided the sacrifice of autogenous tissues and maintained graft uniformity in the autograft model. Moreover, only half of the patellar tendon was replaced to minimize stress on the suture lines and distribute it equally. In fact, no rupture of the transplanted patellar tendons was found at the time of deeth.

Effect of graft material on collagen turnover

The fresh-frozen allograft and autograft models in this study were intended to simulate intra-articular ACL reconstruction, in which the grafts undergo ischaemic necrosis and the donor cells die. At 4 weeks, the percentage of old collagen in the fresh-frozen allografts was significantly lower than in the fresh-frozen autografts. After 12 weeks, however, the values for the fresh-frozen autografts were similar to those for the fresh-frozen allografts. Rapid progression of collagen turnover within the fresh-frozen allografts could be attributed to the response of the recipient tissue to the allograft [5, 14]. Allografts seemed to accelerate new collagen formation by the surrounding tissue rather than inducing rejection. Our results show that the cellularity in the fresh-frozen allograft was higher than that in the fresh-frozen autograft at 2 weeks, which may reflect a lower percentage of old collagen.

Effect of freezing on collagen turnover

At 4 weeks, there were no significant differences in the turnover rates of old collagen between the fresh-frozen and fresh autografts. However, the amount of old collagen in the fresh-frozen autografts was lower than in the fresh autografts at 12 weeks. This difference was attributed to the effect of freezing. Cells within a fresh-frozen graft are destroyed by freezing and thawing. Furthermore, ice crystals within cells and intercellular spaces produced during freezing may have expanded the spaces between collagen bundles in the grafts while the collagen fibres themselves remain intact [19]. Therefore, new tissue induction from the surrounding tissue of the fresh-frozen grafts may have been facilitated by this more open structure. On the other

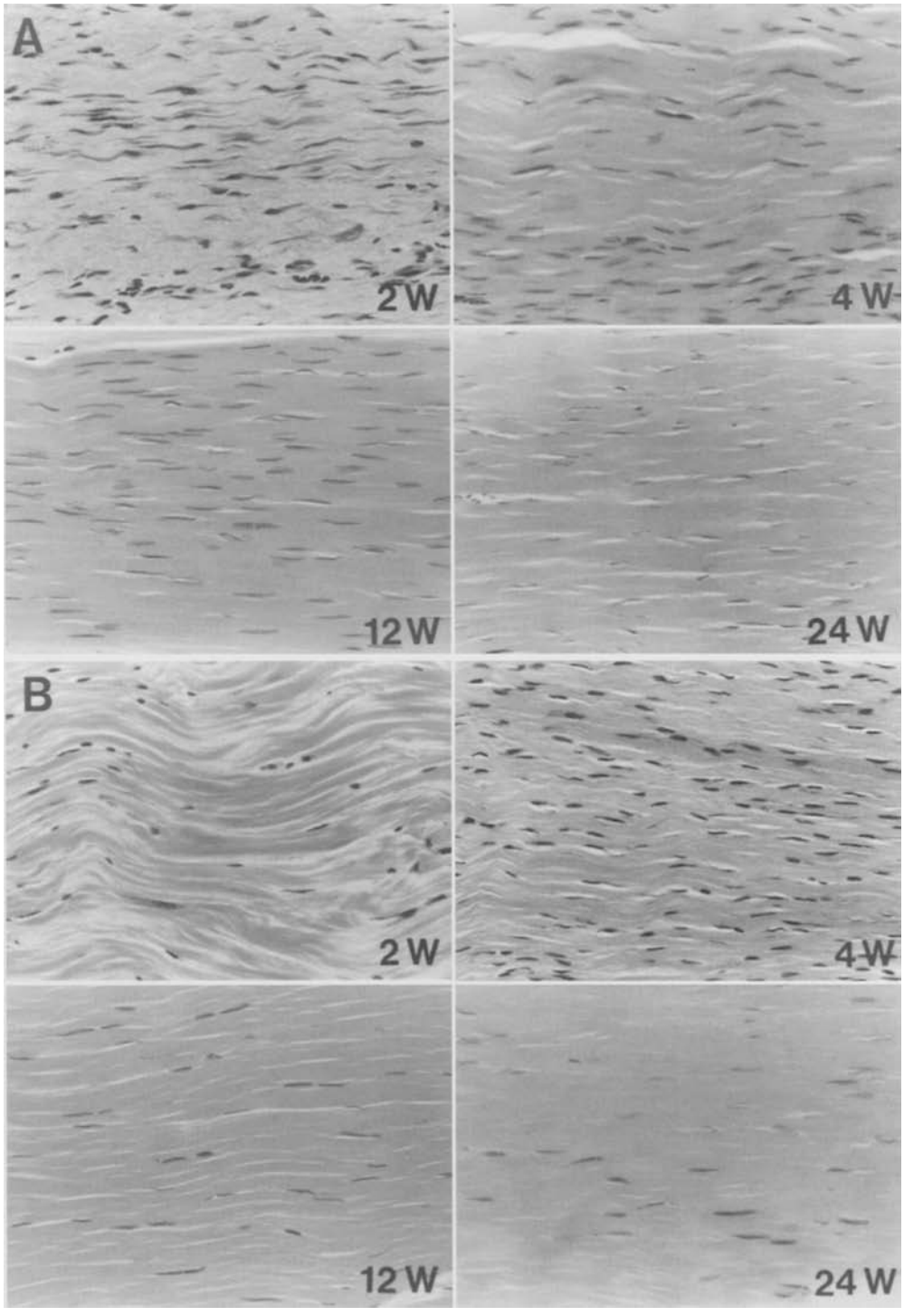

Fig. 6 A, B Histological findings. **A** Fresh-frozen allograft. There was no histological evidence of an immunological response at any time period. At 2 weeks post-transplantation, the cellularity was high, and the collagen fibres were not alinged regularly. Spaces between the collagen bundles were observed. Although cellularity was still higher than in a normal tendon at 4 weeks, the collagen fibres were aligned more regularly, in a longitudinal pattern. At 12 weeks, fibroblasts and collagen bundles within the graft were aligned regularly as in a normal tendon. The nuclei of the fibroblasts were spindle-shaped. By 24 weeks, the hypercellularity had subsided. **B** Fresh-frozen autograft group. There was a similar trend as noted in the fresh-frozen allograft, in that the hypercellularity subsided gradually and collagen fibres became aligned in a more regular pattern over time. Spaces between the collagen bundles were obvious. However, at 2 weeks, the cellularity was less than in the fresh-frozen allograft. Although cellularity was still high at 4 weeks, it had subsided by 12 weeks

hand, it is still unclear whether viable cells within the fresh autogenous grafts survive after extra-articular transplantation. Some investigators have reported that all transplanted tendon tissue undergoes necrosis and is replaced by cellular proliferation derived from surrounding tissues [4, 13], while others demonstrated that the cells do not disappear and that bundles of compact collagen fibres remained intact in fresh autografts [12]. In our study, the percentage of old collagen in the fresh autografts was still high 24 weeks post-transplantation, and histologically, spindle-shaped cells survived without fragmentation of the collagen bundles within the fresh autograft. On the other hand, spaces between collagen bundles were seen in the fresh-frozen autografts. Therefore, we believe that some cells in fresh autografts remain viable and do contribute to the remodelling process of the collagen fibers in extra-articular tendon transplantation.

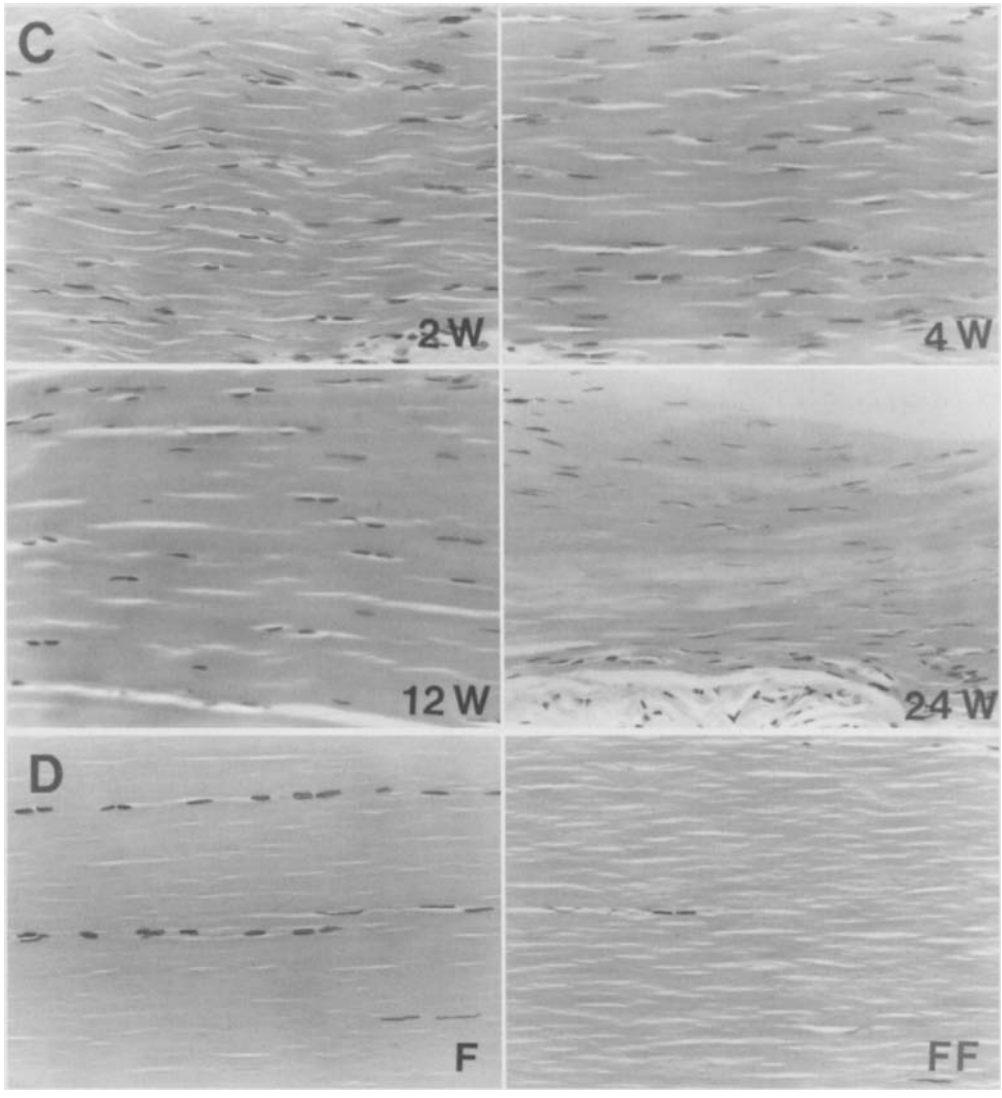

Fig.6 **C** Fresh autograft. Compared with the first two groups, cellularity was less, and the collagen fibres were aligned more regularly at all times. Although the number of cells diminished gradually, spindle-shaped cells were observed at all times. **D** Normal tendon. Collagen fibres are arranged regularly in a longitudinal pattern without fragmentation. The nuclei of the collagen cells are spindle-shaped (a fresh graft). There are more spaces between the collagen fibres than in the fresh graft (a fresh-frozen graft)

Clinical relevance

Since an intra-articularly transplanted fresh autograft undergoes ischaemic necrosis, revascularization, proliferation and remodelling [3], it can be assumed that viable cells within a fresh autograft do not contribute to the remodelling of collagen. The fresh-frozen autograft model simulates intra-articular ligament reconstruction in that its cells all die. Therefore, the comparison between fresh-frozen allografts and autografts parallels the comparison of allogeneic and autogenous grafts used in ACL reconstruction. The greater decrease in the amount of old collagen in the fresh-frozen allografts during the early post-transplantation period suggests that the remodelling process in allografts is more rapid than in autografts. Our radiochemical studies suggest that a fresh-frozen allograft may be more suitable than a fresh-frozen autograft for an intra-articular ligament reconstruction. However, differences in the mechanical properties of transplanted allografts and autografts change with time and may be more important in determining the clinical outcome. Studies are under way to investigate this point using the same rate model.

In conclusion, the collagen turnover in the fresh-frozen allograft was the most rapid based on hydroxyproline turnover. More rapid graft incorporation and remodelling may be possible with fresh-frozen allografts, although at 24 weeks there were no significant differences between the three graft types. Fresh autografts were found to maintain their cellular architecture and extracellular matrix architecture, thus supporting the possibility of graft-cell survival after transplantation.

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