



The origin of cells that repopulate patellar tendons used for reconstructing anterior cruciate ligaments in man

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Cryopreserved patellar tendon allografts are often recommended for reconstruction of anterior cruciate ligaments (ACLs) because living donor fibroblasts are thought to promote repair. Animal studies, however, indicate that ligaments regenerate from recipient rather than donor cells. If applicable to man, these observations suggest that allograft cell viability is unimportant. We therefore used short tandem repeat analysis with polymerase chain reaction (PCR) amplification to determine the source of cells in nine human ACLs reconstructed with cryopreserved patellar tendon allografts. PCR amplification of donor and recipient DNA obtained before operation and DNA from the graft obtained two to ten months after transplantation revealed the genotype of cells and showed only recipient cells in the graft area. Rather than preserve the viability of donor cells, a technique is required which will facilitate the introduction of recipient cells into patellar tendon allografts.

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A variety of techniques and tissues has been used to reconstruct the anterior cruciate ligament (ACL), but the patellar tendon (PT) is the favoured tissue because of its inherent

strength and accessibility, and because it can be harvested without destabilising the knee.¹⁻⁵ Histological and biochemical studies have shown that the transplanted PT develops into tissue with ligamentous properties by the process of ligamentisation.^{6,7} The general view is that living fibroblasts in the transplanted grafts can synthesise and maintain collagen fibrils of the appropriate diameter and other extracellular components. Thus, because the fate of living donor fibroblasts in grafts may ultimately affect the clinical result, the use of fresh and cryopreserved allografts is often recommended.⁸

Studies on dog, rabbit, and goat models, however, have shown that the cells repopulating the graft originate from a source other than the native PT graft.^{5,9-11} Jackson et al,¹¹ using DNA-probe analysis in a goat model, showed that in fresh allografts transplanted for the ACL, recipient DNA completely replaced donor DNA. There are no reports, however, of the fate of transplanted living donor fibroblasts in human allografts. In order to investigate the origin of cells populating transplanted ligaments in man, we have used a DNA genotyping method employing the fingerprinting technique with polymerase chain reaction (PCR) amplification to determine whether the DNA was that of the donor or the recipient.

Patients and Methods

Of 45 patients who underwent transplantation of an allogenic PT for reconstruction of the ACL, nine also underwent second-look arthroscopy as volunteers and, thus, were able to provide tissue both during preparation of the graft and after transplantation. Table I gives details of the patients and the times of the post-transplantation biopsies.

Preparation of DNA. Before transplantation, we obtained blood peripheral to the site of the graft from the recipient, and set aside a small portion of the graft in order to extract donor DNA. Taking care not to include the synovium covering the graft, we harvested a small part of the transplanted graft during second-look arthroscopy. We then used short tandem repeat (STR) analysis to compare the DNA in these samples.

Short tandem repeat analysis. We used commercially available STR systems (Gene Print™ STR System, Promega, Wisconsin). All of the STR loci have been well character-

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Table I. Details of the nine subjects who underwent a reconstruction of the ACL with a cryopreserved PT allograft and the results

Case	Age (yrs)	Gender	Biopsy (postoperative month)	Loci*			Gene alteration
				FFv	CTT	DIS80	
1	23	M	3	+	+	-	Yes
2	32	M	2	+	+	-	Yes
3	20	M	2	+	-	-	Yes
4	25	M	3	+	+	-	Yes
5	35	M	2	+	+	-	Yes
6	32	F	6	+	-	-	Yes
7	22	F	3	-	-	+	Yes
8	24	M	10	+	+	-	Yes
9	28	M	4	+	+	-	Yes

*FFU, F13A01, FESFPS, vWA; CTT, CSFIPO, TPOX, THO1, +, DNA in the ACL before transplantation has changed to be the same as the host peripheral blood; -, DNA in the ACL before transplantation is not different from host peripheral blood, meaning it does not have discrimination potential.

Table II. STR locus-specific information

GenePrint STR system locus	Chromosomal location	Repeat sequence 5-3	Allelic ladder size ranges (bases)	STR ladder alleles (number of repeats)
CSFIPO	5q33.3-34	AGAT	299-323	7, 8, 9, 10, 11, 12, 13, 14, 15
TPOX	2p13	AATG	232-248	8, 9, 10, 11, 12
THO1	11p15.5	AATG	179-203	5, 6, 7, 8, 9, 10, 11
FESFPS	15q25-qter	AAAT	222-250	7, 8, 9, 10, 11, 12, 13, 14
vWA	12p12-pter	AGAT	139-167	13, 14, 15, 16, 17, 18, 19, 20
F13A01	6p24-25	AAAG	169-185	4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16

ised with regard to chromosomal location, repeat sequence, and allelic polymorphism (Table II).^{10,12-20} Amplification of DNA by PCR requires the DNA primer pairs, dNTP and *Taq* polymerase. The first stage, denaturation at about 94°C, produces the complementary strands of DNA, then annealing allows the primers to bind to the separated DNA (starting below the melting temperature of the primers and increasing to the annealing temperature) and during extension *Taq* polymerase copies the DNA (optimal temperature about 72°C). This cycle is repeated to produce multiple DNA copies.

For amplification of F13A01, FESFPS, vWA, CSFIPO, TPOX, and THO1 loci we used the Gene Print STR system (Promega) and the Perkin Elmer 2400 thermal cyclor, and for amplification of the DIS80 locus, the GeneAmp PCR System 9600 (PE Applied Biosystems, Foster City, California). All amplification was according to the manufacturer's instructions. The cycling protocol for F13A01, FESFPS, vWA, CSFIPO, TPOX, and THO1 was 2 minutes at 94°C, ten cycles of 94°C for 1 minute, 64°C for 1 minute, and 70°C for 1.5 minutes, followed by 20 cycles of 90°C for 1 minute, 64°C for 1 minute, 70°C for 1 minute, and extension at 72°C for 10 minutes. For DIS80, after denaturing at 94°C for 2 minutes, according to heat-soaked PCR, the samples underwent a 30-cycle protocol (94°C for 1 minute, 65°C for 1 minute, and 72°C for 8 minutes), with final extension at 72°C for 10 minutes.

Using a model SA-32 system (Life Technologies, Rockville, Maryland), samples associated with F13A01, FESFPS, vWA, CSFIPO, TPOX, and THO1 loci underwent

denaturing polyacrylamide gel electrophoresis in a pH 8.4 buffer (50 mM Tris, 45 mM borate, 0.5 mM EDTA), at a constant 80 W for five minutes and then at 40 W at room temperature. Electrophoresis of amplified DIS80 was by a polyacrylamide gel (Gelbond film, FMC Corporation, Philadelphia, Pennsylvania) discontinuous buffer method using the Hoefer model SE620 system (Amersham Pharmacia Biotech, Bjorkgatan, Uppsala, Sweden) and a running buffer at 250 V and 10°C.

Genotype determinations were carried out by comparison with an allelic ladder cocktail (Perkin Elmer Cetus). Visualisation of the amplified PCR products was by silver staining. Archival copies of stained polyacrylamide gels were made using Kodak Electrophoresis Duplicating Film (Eastman Kodak Company, Rochester, New York).

Evaluation. Comparison of the band-patterns in the grafts with those in the pure donor and recipient samples allowed specific identification of DNA and discrimination between the DNA of the donor and that of the recipient.

Results

PCR amplification successfully unravelled the genotype of cells obtained from each graft during postoperative arthroscopy. In all nine patients, it identified DNA from the recipient in the repair site and no DNA from the donor. More specifically, in eight of the nine patients, there were differences in the F13A01, FESFPS and vWA (FFv) system (Fig. 1). In six of the nine patients, there were differences in the CSFIPO, TPOX, and THO1 (CTT) system. In six of the

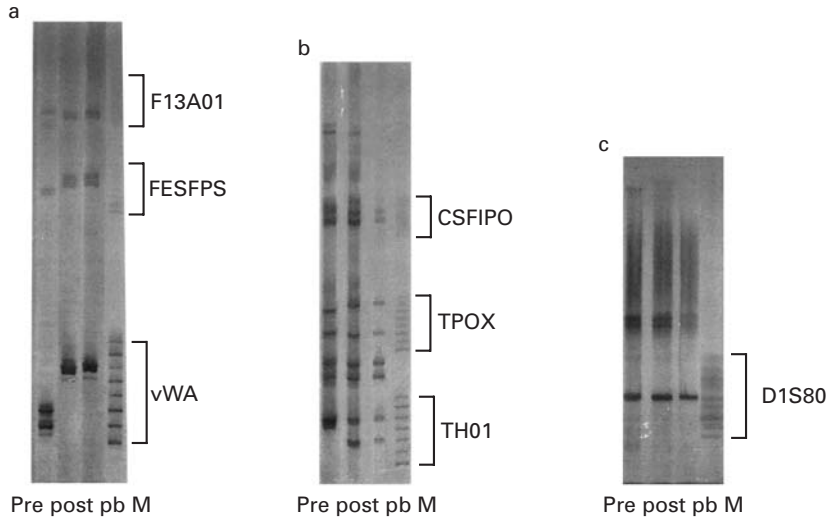


Fig. 1

Allelic profiles of cells from donor peripheral blood (pb), PT (pre) and reconstructed ACL (post) two months after operation (M, marker). The DNA in the reconstructed ACL is different from that in the PT only in the FFv system. It became identical to that in the host blood. The CTT and D1S80 system show no difference.

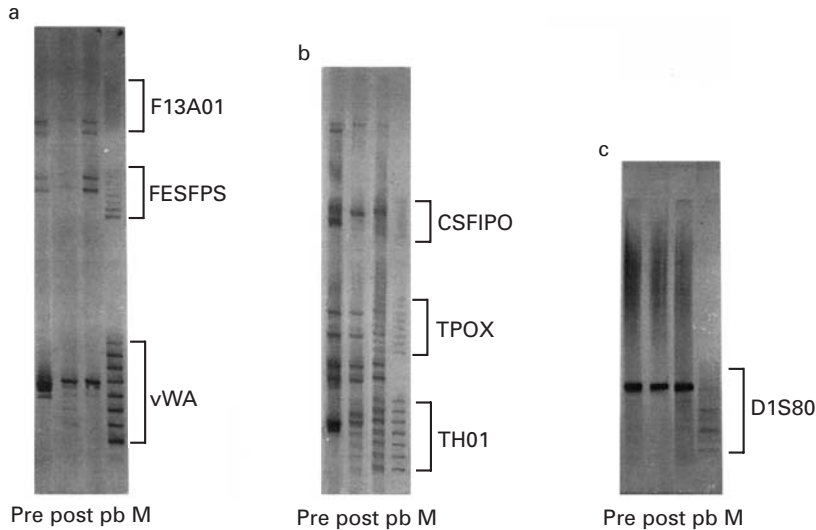


Fig. 2

Allelic profiles of cells from donor peripheral blood (pb), PT (pre) and reconstructed ACL (post) two months after operation (M, marker). The DNA in the PT is completely different from that in the reconstructed ACL in both the CTT and FFv systems even although both originated from the same tissue. The DNA in the reconstructed ACL is completely the same as that in the recipient's peripheral blood.

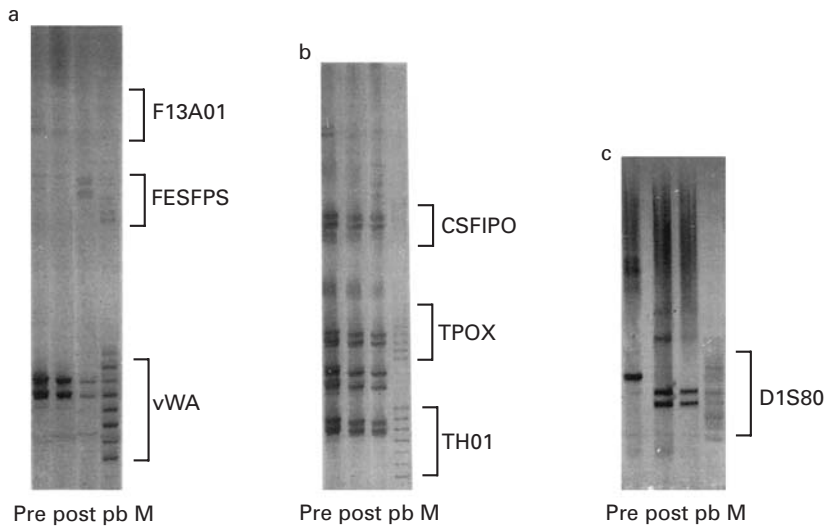


Fig. 3

Allelic profiles of cell from donor peripheral blood (pb), patellar tendon (pre) and reconstructed ACL (post) three months after operation (M, marker). The DNA in the PT is different from that in the reconstructed ACL only in the D1S80 system. The FFv and CTT systems show no differences.

nine patients, discrimination was apparent in both FFv and CTT systems (Fig. 2). In one patient, the D1S80 gene was identical to that of the recipient's preoperative DNA (Fig. 3). These findings indicate that genes were altered in the DNA of the grafted tendon and matched those obtained from the DNA in the recipients' blood. The results are summarised in Table I. These changes were apparent in the samples obtained as early as two months after surgery, and the length of the postoperative period made no difference.

Discussion

After transplantation, a change in the milieu of the PT graft from extra-articular to intra-articular may account for the gradual biochemical and histological metamorphosis which we observed during which the tendon acquires ligamentous properties. Compared with tendons, ligaments are more metabolically active, and have more plump fibroblast nuclei, a higher DNA content (more cells), more type-III collagen, more proteoglycans, less total collagen, and a different proportion of reducible intramolecular collagen bands.⁶ Amiel et al⁶ found that the percentage of proteo-glycans in PT autografts increased from 3.9% to 11.4%, which was not statistically different from the percentage in the ACL. They also reported that PT grafts had increased levels of type-III collagen, approaching the normal level in the ACL, and the type and distribution of collagen cross-links in the grafts also began to resemble those of the normal ACL.⁷

Implanted grafts also undergo considerable biomechanical changes. After implantation, material properties decrease precipitously and generally do not return to normal levels. As the graft undergoes biological transformation, studies show that the bone-tendon-bone unit becomes less stiff, less strong, and absorbs less energy to failure than either control graft material or control ACLs.^{2,3,21} In autografts, Newton, Haribe and Woo²¹ found that the stiffness remained at 13% and 45% of the control level at 52 to 104 months, and ultimate load ranged from 11% to 52% of the control value during the same period.

All of these histological and biomechanical changes may contribute to the cellular events in the graft, such that they may all be explained by the 'window of cell absence' interval from the death of the cell in the reconstructed tendon immediately after surgery to subsequent repopulation by the recipient cell.

In studies using animal models, the fibroblasts in donor grafts were no longer evident three days after transplantation, and all donor-derived DNA had disappeared within four weeks.²² In addition, many of these studies found that cells in the transplanted ligaments repopulated not by the multiplication of donor cells, but by the introduction of recipient cells into the graft site.¹¹ If these observations are a true reflection of the natural course of cellular events in the reconstitution of a transplanted ligament, then the viability of donor cells is less important than has been previously thought. Consequently, the storage of ligaments for trans-

plantation can become more convenient and less costly. Furthermore, treatment with recipient cells before transplantation could facilitate remodelling into a normal ACL.

Our study of human subjects posed some difficulties not encountered in animal DNA analyses. First, for ethical reasons, post-transplantation arthroscopy was only possible if a patient had a complication which could be resolved with arthroscopy. Thus, our study sample was small. Similarly, because we were able to harvest only a tiny amount of tissue from the implanted graft, DNA multiplication by PCR was necessary. Another limitation was that tests for discriminating human DNA are commercially available for only certain genes. In animal studies, Southern blot analysis could compare all the DNA, but since this was not possible in our study, we used the STR technique. Approximately 25% of the human genome consists of repetitive DNA sequences the inherent polymorphic characteristic of which provides the ultimate molecular bank of information for clinical diagnosis and forensic identification. STR loci consist of tandemly repeated sequences, two to six base pairs in length, which have a high degree of length polymorphism because of variation in the number of repeat units.

The use of amplified fragment-length polymorphisms (AMP-FLPs) and STRs for DNA typing offers several advantages over conventional analysis. First, minimal amounts of template DNA, even if degraded, can be used as a source of starting material for the analysis. Secondly, as with the other variable number of tandem repeat approaches, it allows the bypass of the long autoradiological exposure times of the conventional technique; a brief silver-staining protocol easily detects the genomic allele. Thirdly, both AMP-FLPs and STRs are amenable to automation, making them simple and accurate to use.²³ Therefore, it may play a significant role in transplantation medicine by elucidating the source of cells in various tissues. Taken together, the triplexes of FFv and CTT used in our study has discrimination power ranging from 1 in 125 729 280 to 1 in 46 845 540, depending on the racial group being tested. The matching probability of D1S80 is approximately 1 in 388 800.²⁴

As shown in Table I, in all nine of our patients the DNA has changed. The Clopper-Pearson lower 95% bound for probability that DNA changes is 0.72. Therefore, we have enough statistical evidence to show that DNA changes two to ten months after surgery.²⁵

Our finding that the DNA of the grafted tendon became the same as that of the recipient DNA, agrees with the results obtained in animal studies by Jackson et al.¹¹ Our data suggest that the cells remaining in the grafted tendon after surgery originate from the recipient as early as two months after transplantation. Therefore, the viability of cells in the grafts stored for ligament reconstruction surgery appears to be unimportant, and thus simpler and less expensive methods of storage of grafts may emerge. In addition, a certain length of time seems to be necessary for the recipient cells to invade the grafts and become active. In biomechanical terms, weakening of the graft and its inability to

regain its original strength, even after a long period, may be due to the absence of cells within the graft which should overcome mechanical wear. The introduction of highly viable cells into the recipient during grafting could theoretically shorten the period of weakness of the graft and restore the original strength of the ligament. Therefore, rather than trying to preserve and maintain cell viability in PT allografts, a technique is needed to facilitate the introduction of recipient cells into these allografts.

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