Free patellar tendon grafts used for the intra-articular replacement of ruptured anterior cruciate ligaments (ACL) lack perfusion at the time of implantation. The central core of the graft undergoes a process of ischaemic necrosis which may result in failure. Early reperfusion of the graft may diminish the extent of this process.

We assessed the role of peritendinous connective tissue in the revascularisation of the patellar tendon graft from the day of implantation up to 24 days in a murine model using intravital microscopy. The peritendinous connective-tissue envelope of the graft was either completely removed, partially removed or not stripped before implantation into dorsal skinfold chambers of recipient mice.

Initial revascularisation of the grafts with preserved peritendinous connective tissues began after two days. The process was delayed by five to six times in completely stripped patellar tendons (p < 0.05). Only grafts with preserved connective tissues showed high viability whereas those which were completely stripped appeared to be subvital.

The presence of peritendinous connective tissues accelerates the revascularisation of free patellar tendon grafts.


Materials and Methods

Transplantation model. We used adult male DDY mice six to eight weeks old and with a body-weight of 25 to 30 g. The experiments followed institutional guidelines and were approved by the local animal research review board. Surgical procedures were performed under strictly aseptic conditions using a dissecting microscope (type 334790; Wild, Heerbrugg, Switzerland).

Donor and recipient mice were selected randomly from our animal colony. Recipient mice were fitted with a dorsal skinfold chamber (Workshop, Institute for Surgical Research, Klinikum Grosshadern, University of Munich, Germany) which served as the site of implantation.
Under ketamine hydrochloride and xylazine anaesthesia (Ketalar; Parke-Davis, New Jersey; 100 mg/kg body-weight; Xyla-Ject; Pheonix Pharmaceutical, St Joseph, Missouri; 10 mg/kg body-weight) a dorsal skinfold was raised and sandwiched between two symmetrical titanium frames. In one of the two skin layers of this fold, a circular area 15 mm in diameter was removed thus visualising the tissue of the opposite skin layer. To prevent drying of the exposed tissue, the chamber preparation was closed with a sterile transparent removable glass cover slip. A period of two days was allowed to elapse so that the animals could recover from surgery. Thereafter, only chambers meeting the criteria of intact microcirculation were used as sites for implantation.

Before the grafts were removed, donor mice were killed by inhalation of CO₂. After disinfecting the skin with a 70% alcohol solution, both knees were excised in toto by cutting approximately 1 cm proximal and distal to the joint. To avoid drying of the tissue, preparation of the patellar tendons was carried out in approximately 4°C Dulbecco’s modified Eagle’s medium (Seromed; Biochrom, Berlin, Germany). The patella with its tendon was mobilised and the infrapatellar fat pad excised close to the tibia, but retaining its connection to the patellar tendon. The latter was then dissected from its osseous insertion at the tibial tuberosity and held with microforceps at the patella for further steps of preparation thus avoiding mechanical damage to the tendinous tissue. The tendons were assigned to one of the three experimental groups. Five were untreated (PTU), five had the infrapatellar fat pad pulled off but all other structures of the peritendinous connective tissue preserved (PTP) and six were stripped of all connective tissue (PTS).

Each graft was implanted into one dorsal skinfold chamber in which the striated cutaneous muscle served as the site of implantation. The time from killing of the donor animal to transplantation of the graft into the recipient mouse was 25 to 30 minutes.

**Intravital microscopy.** We performed intravital microscopy of grafts in the non-anaesthetised animals every 12 hours for four days and thereafter every 24 hours using an Axioplan microscope (Zeiss, Oberkochen, Germany) which was equipped with a 100W halogen lamp for transillumination and a 100W mercury lamp for epi-illumination. Regions of interest were recorded by a charge-coupled device video camera (CF 8/1 FMC; Kappa Messtechnik GmbH, Gleichen, Germany) and a S-VHS videocassette recorder (AG-7355; Panasonic, Osaka, Japan) for off-line analysis. The variables measured after implantation were the time of first appearance of newly-formed vessels, the onset of their perfusion and the first appearance of haemorrhages in the vicinity of the grafts. Visualisation of newly-formed vessels and their perfusion was carried out by the use of a green filter and transillumination or an adequate fluorescence filter set and epi-illumination after intravenous injection of the plasma marker, fluorescent isothiocyanate (FITC)-labelled dextran (M₄ 150000; ICN Biomedical Inc, Aurora, Ohio; 0.1 ml 5% FITC-dextran solution in 0.9% NaCl).

Macroscopically, the grafts were assessed photographically at a magnification of ×2.8 (Nikon F4, AF 105 mm Micro Nikkor with a teleconverter; Nikon, Tokyo, Japan).

**Histological examination.** After termination of the experiments on the 24th day after transplantation, the grafts were excised in toto including the underlying skin. Fresh non-stripped patellar tendons were removed from donor mice and used as non-transplanted controls. Tissue samples were fixed in phosphate-buffered saline (PBS) with 4% paraformaldehyde and 3% dextran, embedded in paraffin, cut in slices 5 µm thick with a microtome and stained with haematoxylin and eosin for evaluation by light microscopy.

**Statistical analysis.** The results are presented as medians unless otherwise indicated. Using the software program SigmaStat for Windows (Version 2.03; SPSS AG, Zürich, Switzerland), the data were analysed statistically by the Kruskal-Wallis test. Dunn’s method was applied for pairwise multiple-comparison procedures. A p value of less than 5% was considered significant.

**Results**

**Intravital microscopy.** As shown in Figure 1a, both in PTU and PTP tendons, the median first appearance of newly-formed vessels was 48 hours after transplantation whereas it was approximately five to six times later in PTS tendons (276 hours). One of the PTS grafts showed no new formation of blood vessels during the entire investigation period of 24 days. For this animal the censored observation ‘576 hours’ (24 days) was introduced for statistical reasons (Fig. 1a). Data were not normally distributed. There was a statistically significant difference in the median values of the treatment groups (Kruskal-Wallis test, p = 0.003). All pairwise multiple comparison procedures (Dunn’s method) showed statistically significant differences for PTS v PTP and PTS v PTU tendons only. There was no statistical difference between PTU and PTP tendons.

In all three groups, the onset of perfusion of newly-formed vessels (Fig. 1b) occurred on average after 24 hours after the first appearance of these vessels. Data were not normally distributed. There was a statistically significant difference in the median values of the treatment groups (Kruskal-Wallis test, p = 0.003). All pairwise multiple comparison procedures (Dunn’s method) showed statistically significant differences for PTS v PTP and PTS v PTU tendons only.

During the investigation period of 24 days, haemorrhages in the vicinity of grafts did not occur in all animals. In PTU tendons only, all grafts induced haemorrhages (Fig. 1c).

Figure 2 shows representative macroscopic photographs of PTU (Figs 2a and 2b) and PTS (Figs 2c and 2d) tendons...
at four (Figs 2a and 2c) and 17 days (Figs 2b and 2d) after implantation. On day 4, newly-formed vessels were seen along with a distinct haemorrhage only in PTU tendons (Fig. 2a). PTS tendons had neither newly-formed vessels nor haemorrhages (Fig. 2c). Thirteen days later, in PTS tendons only solitary newly-formed vessels were occasionally seen (Fig. 2d) whereas in PTU tendons a well-established vascular network was observed, pronounced in the periphery of the graft (Fig. 2b). In addition, PTU tendons (Fig. 2b) appeared to have taken more compared with PTS tendons (Fig. 2d) which had also lost their natural silver-white shine; the fibrous structures of the tendon appeared to be oedematous leading to a diffuse increase in the surface area (Fig. 2b).

Photographs of PTU (a and b) and PTS (c and d) tendons at 4 (a and c) and 17 (b and d) days after implantation (PTU, non-stripped patellar tendon; PTS, completely stripped patellar tendon; the photographs represent an area of 4.7×4.7 mm each).
Histological examination. Depending on the presence or absence of peritendinous tissues, histological sections of patellar tendons showed varying degrees of revascularisation and viability 24 days after transplantation. Both PTU (Fig. 3b) and PTP (Fig. 3c) tendons had intense staining and a normal density of fibroblast nuclei as signs of their viability similar to freshly excised non-transplanted control patellar tendons (Fig. 3a). Collagen fibres in general appeared to be normal in shape and arrangement, but were partially infiltrated by granulation tissue (Figs 3b and 3c). In PTS tendons the density of the fibroblast nuclei was reduced and their staining was less intense (Fig. 3d). The morphology of the tendinous portion of the graft appeared to be degenerated and almost amorphous (Fig. 3d). Although no necrosis was observed, the level of viability in PTS tendons was judged as ‘subvital’ (Fig. 3d). Only in PTS tendons were there no signs of revascularisation; PTU (Fig. 3b) and PTP (Fig. 3c) tendons were well vascularised.

In general and in accordance with the intravital microscopic findings, histological assessment did not detect any differences between PTU and PTP tendons.

Discussion

The model of the dorsal skinfold chamber preparation is well established for the study of angiogenesis and the microcirculation in a wide variety of non-neoplastic and neoplastic tissues by means of intravital microscopy. The dorsal skinfold chamber in mice was developed for the transplantation of patellar tendon grafts to assess the role of preserved peritendinous connective tissues on the time course of revascularisation and reperfusion of the graft. The effect of early reperfusion on the ultimate success of ACL reconstructions has been studied using vascularised (pedicled) tendon grafts, which were implanted into the knee as replacement for the ACL. Promising results...
were reported by Lambert and Benedetto and Klima whereas van Rens et al, Butler and Butler et al did not find any significant improvement in the ultimate mechanical properties of the grafts. No conclusive information on the early phase of reperfusion can be drawn from these studies because of their design. Also, mechanical factors such as orientation, fixation, and tensioning of the graft could not be excluded as factors influencing the ultimate outcome of ACL reconstructions.

In freshly transplanted bone the first signs of angiogenesis can be detected within 17 hours of transplantation, indicating the potency of bone to induce this process. In our study, all patellar tendon grafts were completely separated from their sites of osseous insertion before transplantation. In other studies the revascularisation of patellar tendon grafts originated from the soft tissue of the infrapatellar fat pad and posterior part of the joint. Sites of osseous insertion have been found not to contribute to the physiological blood supply of ligaments and tendons.

In various studies many aspects of the techniques of graft harvesting have been described, but no emphasis has been placed on the handling of the peritendinous soft-tissue envelope. In particular, the role of connective tissue of the preserved graft on the revascularisation pattern of ACL substitutes has not been addressed.

In our study, only grafts which had preserved connective tissues (PTP and PTU) showed a rapid onset of revascularisation within two days after transplantation. In these grafts, the time of revascularisation was almost identical with that of other freely transplanted non-neoplastic or neoplastic tissues. Since in PTS tendons the first appearance of newly-formed vessels occurred four to five times later and was distinctly less pronounced, it is assumed that cells of the preserved connective tissues are able either to produce angiogenic factors or to stimulate host cells to generate and release these factors.

Conclusions. This is the first report of the role of peritendinous connective tissues on the early patterns of revascularisation of free patellar tendon using intravital microscopy. The first appearance of newly-formed vessels and perfusion occurred significantly later in stripped patellar tendon grafts.

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References


