An immunohistological study of the integration at the bone-tendon interface after reconstruction of the anterior cruciate ligament in rabbits


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We studied bone-tendon healing using immunohistochemical methods in a rabbit model. Reconstruction of the anterior cruciate ligament was undertaken using semitendinosus tendon in 20 rabbits. Immunohistochemical evaluations were performed at one, two, four and eight weeks after the operation. The expression of CD31, RAM-11, VEGF, b-FGF, S-100 protein and collagen I, II and III in the bone-tendon interface was very similar to that in the endochondral ossification. Some of the type-III collagen in the outer layer of the graft, which was deposited at a very early phase after the operation, was believed to have matured into Sharpey-like fibres. However, remodelling of the tendon grafted into the bone tunnel was significantly delayed when compared with this ossification process. To promote healing, we believe that it is necessary to accelerate remodelling of the tendon, simultaneously with the augmentation of the ossification.

Almost all methods for the surgical replacement of the anterior cruciate ligament (ACL) require free tendon grafts to be fixed in bone tunnels. This procedure can be difficult because the bone-tendon interface is mechanically weak immediately after the operation. This interferes with early rehabilitation. In the mid- to long-term there may be enlargement of the bone tunnel. Ideally, the grafted tendon should attach promptly to the bone tunnel and establish a biological bond that has adequate mechanical strength.

Experiments in animals have shown that by three to four weeks after surgery, the attachment of the tendon graft in the bone tunnel occurs by indirect anchoring to the bone through fibrous tissues called Sharpey-like fibres.\(^1,2\) These fibres are still preserved after 52 weeks.\(^3\) Although they are not uniformly present at all sites in the bone tunnel,\(^4\) their number has been shown to be correlated to the pull-out strength.\(^1,3\) Similar results have been shown in humans.\(^5,6\) ACL reconstruction using a bone tunnel does not recreate a normal bone-ligament junction, but it is believed that the mechanical strength is maintained through the formation of fibrous anchors. It is unclear what type of molecular responses cause these distinct histomorphological changes.

In this study, we examined the maturing process of tendon grafts in the bone tunnel after ACL reconstruction using immunohistological methods. Various cells, matrix proteins and cytokines were used as targets for immunostaining. Our objectives were to clarify the timing of expression of these targets between the bone and tendon grafts and to determine how the tissue structures are formed.

Materials and Methods

Ethical approval was obtained from Kurume University’s Animal Experiment Center.

We used the surgical method described by Martinek et al.\(^7\) The animals were anaesthetised intravenously with Nembutal (Hembtal, Osaka, Japan) (0.025 mg kg\(^{-1}\)). A 10 cm median incision was made on the left hind leg in order to harvest a semitendinosus tendon. The tendon was prepared by folding it in two and the ends were sutured together using a Tevdek suture (Akiyama Manufacturers, Baukya-ku, Japan). The ACL was exposed through a medial arthrotomy and was excised. Tibial and femoral tunnels (2.0 mm diameter) were created at the original insertion of the ACL. The tendon graft was placed through the tunnels and tied over extra-articular buttons. Postoperatively, the rabbits mobilised freely in their cages.

Preparation of specimens. Five rabbits were killed at one, two, four and eight weeks after surgery and the tibiae were harvested. They were immediately fixed by 48-hour perfusion in 10% neutral buffered formalin. They were decalcified using a formic acid-sodium citrate solution (29 g citric acid, 18 g trisodium citrate...
dihydrate, and 100 ml formic acid, with distilled water added to yield 1000 ml total volume). Dehydration was performed immediately after the specimens were confirmed to be adequately decalcified and then they were embedded in paraffin. The specimens were sectioned sagitally to the tendon grafts. The sections were approximately 5 µm thick. They were stained with haematoxylin & eosin and examined using a light microscope.

**Immunostaining protocol.** Anti-CD31 monoclonal antibody (DAKO, Code No. M 0823), anti-rabbit macrophage monoclonal antibody (DAKO, Code No. M 0633), anti-VEGF monoclonal antibody (Oncogene, Cambridge, Massachusetts), anti-b-FGF monoclonal antibody (Wako Junyaku, Osaka, Japan), anti-S-100 protein monoclonal antibody (DAKO) and anti-collagen type-I, type-II, and type-III monoclonal antibodies (Daiichi, Toyama, Japan) were obtained. Normal mouse IgG1 (DAKO, Code No. X 0931) was adjusted to the concentrations of the primary antibodies and used as the negative control.

Antigen activation was performed for CD31, VEGF, and b-FGF stainings by reacting the sections with proteinase K (DAKO) at room temperature for seven minutes. Antigen activation was performed for S-100 protein and collagen type-I, type-II, and type-III stainings by reacting the sections with 0.1% trypsin solution (0.2 g trypsin, 0.2 g calcium chloride, 200 ml 0.05 M Tris-Cl buffer) at 37°C for 30 minutes. Subsequently, endogenous peroxidase was blocked by reaction with 0.3% H₂O₂ in methanol at room temperature for 30 minutes. The primary antibodies were then diluted appropriately using Anti Diluent [Buffer] (DAKO, S 3022). Each solution was allowed to react overnight at 4°C. For immunostaining, the EPOS method was used for the anti-S-100 protein antibody and the Envision method was used for all the other antibodies. For the sec-
Secondary antibodies in the Envision method, anti-mouse enzyme labelled dextran polymers (DAKO, K4000) were allowed to react for 30 minutes at room temperature. We used 3,3’-diaminobenzidine tetrahydrochloride as the chromogenic substrate in the EPOS and Envision methods. The sections were prepared by counterstaining with haematoxylin, dehydrating and clearing. The wash solution used in each process was 0.1% Tween 20 in 0.05M Tris-HCl buffer with 0.3 M NaCl. The sections were examined using a light microscope.

A positive internal control for immunostaining was used for each section. Expression of the following antibodies was observed: anti-CD31 antibody in the vascular endothelia of the periosteum, anti-VEGF antibody in the vascular endothelia, osteoblasts, chondrocytes, fibroblast-like cells; anti-b-FGF antibody in vascular endothelia, osteoblasts, osteocytes, anti-S-100 protein antibody in articular cartilages and the matrices of cartilaginous material around the bone plates. Furthermore, expression of the following antibodies was observed: anti-collagen type-I antibody in the tendons and lamellar bones, anti-collagen type-II antibody in articular cartilage surroundings and intercellular matrices of the growth plate cartilage and anti-collagen type-III antibody in the vascular adventitia and periosteal fibres.

Results

One week. In some areas (Fig. 1a), the gaps between the lamellar bone and the tendon were stained darkly with basic stain. The enlarged cells were densely arranged and their surroundings were composed of matrix that stained lightly with acidic stain. Vascular tissues were seen. CD31 expression (Fig. 2a) was observed and coincided with the presence of granulation tissues between the lamellar bone and the tendon. The stained form indicated a luminal structure. RAM-11 expression (Fig. 2b) was seen to coincide with relatively large round cells in the granulation tissue and the bone marrow margins. VEGF expression was uniformly observed, coinciding with the presence of dense and enlarged fibroblast-like cells in the granulation tissue. However, it was not seen in the intercellular matrices. The expression of b-FGF was observed in the enlarged fibroblast-like cells, consistent with the granulation tissue and in the spindle-shaped fibroblast cells in the peripheral region of the tendon. After staining for S-100 protein, positive cells were present at the interface on the side of the bone tunnel. However, we did not observe the expression of every cytokine in the grafted tendon.

From the collagen type-I staining, minimal expression was seen in the granulation tissue at the interface. From the collagen type-II staining, expression was not observed in the granulation tissue at the interface whilst from the collagen type-III staining, expression coincided with the presence of the intercellular matrix fibres of the granulation tissue at the interface.

Two weeks. At the interface (Fig. 1b), we observed chondroid cells which had relatively large nuclei and vacuoles in their amorphous cytoplasm. The intercellular matrix was stained uniformly lightly with acidic stain, and the orientation of the matrix fibres was indistinct.

CD31 and RAM-11 expression declined with the loss of granulation tissue at the interface. There was no significant expression in the chondroid cells and intercellular matrices. VEGF expression was seen in the chondroid cells, but it was not observed in the grafted tendon. The expression of b-FGF was observed in the chondroid cells formed at the interface and the spindle-shaped fibroblast-like cells found in the central region of the grafted tendon. S-100 (Fig. 3a), was expressed coinciding with the presence of the chondroid cells at the interface.
Type-I collagen was expressed in the chondroid cells, in the intercellular matrices at the interface and in the bone tunnel. Collagen type-II expression was confined to the matrices around the chondroid cells (Fig. 3b) and collagen type-III expression was observed in the intercellular matrices around the chondroid cells in the marginal tendon.

**Four weeks.** The boundary between the lamellar bone and tendon was ill-defined. In some areas (Fig. 1c), the fibres extended in a perpendicular direction, from the lamellar bone to the tendon. These fibres appeared to be orientated to resist the shear stress developed by pulling out the graft. The fibre density of the tendon was sparse compared with that at two weeks and spindle-shaped fibroblast-like cells were observed in the peripheral region.

Significant expression of CD31 and RAM-11 was not observed at the ill-defined boundary between the lamellar bone and the tendon and the internal areas of the tendon. The pattern of VEGF expression was similar to that at two weeks. There was strong expression of b-FGF in the fibroblast-like cells in the central tendon (Fig. 4). The S-100 protein expression was not observed at the interface or in the tendon. Its expression appeared to have diminished with the decline of the chondroid cells.

The expression of collagen type-I and type-III was observed at the interface. In addition, the Sharpey-like
fibres were composed mainly of type-III collagen. Type-II collagen expression was observed around the chondroid cells that remained in some areas. However, the expression reduced with the decline of chondroid cells.

**Eight weeks.** Many relatively large basophilic cells were seen between the lamellar bone and the tendon. The matrix fibres between these cells were arranged as if bridging to the lamellar bone, making the boundary between the lamellar bone and tendon ill-defined. Fibroblast-like cells were seen in the central region of the tendon (Fig. 1d).

The expression patterns of anti-CD31, macrophage, VEGF and b-FGF were almost unchanged from those at four weeks. The distribution of collagen was also similar to that at four weeks. However, there appeared to be increased expression of collagen type-III around the fibroblast-like cells in the centre of the tendon (Fig. 5).

**Discussion**

In order to improve the results of ACL reconstruction, it is necessary for the tendon graft in the bone tunnel to attach rapidly to the surrounding bone and to attain sufficient mechanical strength. Animal studies have been conducted to examine biological acceleration of the anchoring of the tendon graft to bone. Rodeo et al. reported that the healing process could be accelerated and the mechanical strength improved by introducing recombinant BMP-2. There have been reports suggesting introducing FGF-2, TGF-β1 and bone marrow-derived mesenchymal stem cells, as well as gene transfer, such as of BMP-2. However, all these were limited to histomorphological examinations and did not examine the changes in the molecular response.

We used immunostaining to investigate the types of molecular response occurring in the bone tunnel.

In summary, in the initial post-operative stages, the gap between the bone and tendon graft is filled with granulation tissue containing a large amount of type-III collagen. VEGF and b-FGF are expressed in the granulation tissue, migration of enlarged fibroblasts, vascular endothelia and macrophages are seen. However, these histological changes are the same as those seen in the initial stages of wound healing and are not specific to this location. Subsequently, chondroid cells that are S-100 protein positive appear from the side of the bone tunnel. The chondroid cells degrade the fibrous vascular granulation tissue proceeding towards the tendon graft side and deposit type-II collagen into the matrix. However, the degradation is arrested without the destruction of the tendon graft and the number of S-100 protein positive cells decline as they mature into lamellar bone. The histological changes in the wall of the bone tunnel at this time are very similar to those of the endochondral ossification process, as described by Sandberg, Aro and Vuorio. The environment in the bone tunnel is analogous to that of a fracture. The form and behaviour of S-100 protein positive cells resemble those of hypertrophic chondrocytes seen transiently in endochondral ossification. Finally, when these changes begin to subside, Sharpey-like fibres appear between the tendon graft and the front line of ossification. The Sharpey-like fibres are composed of type-III collagen and their orientation opposes the direction of the shear stress. This change is consistent with findings of Liu et al. They indicated that adhesion between the bone and tendon graft involves collagen metabolism similar to that in fracture healing with the final anchoring by the fibres composed of type-III collagen.

The tendon graft itself becomes temporarily hypocellular after surgery. By two to four weeks post-operatively, b-FGF is expressed from the margins of the tendon and gradually proceeds towards the centre. Spindle-shaped fibroblast-like cells start to appear. At eight weeks post-surgery, collagen type-III begins to be deposited around the fibroblast-like cells which infiltrate towards the centre of the tendon and finally, remodelling begins in the tendon. It is believed that changes similar to those in the remodelling of the intra-articular tendon graft, occur in the tendon graft in the bone tunnel. However, the changes in the tendon itself are significantly delayed, compared with the histological changes in the bone, and this delay causes the loss in mechanical strength. Tomita et al. reported that at six weeks, a core of the tendon was pulled away from the tunnel, but the superficial portion remained attached on the tunnel wall. The granulation tissue that fills the interface becomes stronger than the intraosseous portion of the tendon graft at this time.

The histological changes seen in our study were consistent with the theories of normal wound healing. If the objective is to accelerate the anchoring between the bone tunnel and the tendon graft, we believe that an effective strategy would be to augment the ossification mechanism from the bone. However, this would not resolve the problem of the delay in tendon graft modelling. Therefore, it is also necessary to augment and accelerate the remodelling of the tendon graft in the tunnel. The means and timing for the augmentation are topics for future studies. From our experimental results we are unable to determine whether a normal ligamentous attachment is achievable by this surgical method. It is possible that the bone tunnel technique needs to be abandoned in favour of an entirely new surgical approach.

**Supplementary Material**

More figures showing immunostainings at one, two and four weeks are available with the electronic version of this article on our website at www.jbjs.org.uk

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

**References**


