

Tendon Healing in a Bone Tunnel. Part II: Histologic Analysis After Biodegradable Interference Fit Fixation in a Model of Anterior Cruciate Ligament Reconstruction in Sheep

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Purpose: Tendon-to-bone healing of soft-tissue grafts has been described to progress by the development of a fibrous interzone that undergoes a maturation process leading to the development of an indirect type of ligament insertion. Previous studies used extra-articular models or fixation far away from the joint line; thus, no data are available investigating tendon-to-bone healing of a soft-tissue graft fixed anatomically. Therefore, we studied the tendon-to-bone healing of the anatomic soft-tissue graft interference fit fixation in a model of anterior cruciate ligament (ACL) reconstruction in sheep. **Type of Study:** Animal study. **Methods:** Thirty-five mature sheep underwent ACL reconstruction with an autologous Achilles tendon split graft. Grafts were directly fixed with biodegradable poly-(D,L-lactide) interference screws. Animals were euthanized after 6, 9, 12, 24, and 52 weeks and histologic evaluations were performed. Undecalcified specimens were evaluated under normal and polarized light. Additionally, animals received a polychrome sequential labeling (tetracycline, xylenol orange, and calcein green) to determine bone growth per time under fluorescent light. **Results:** Intratunnel histologic findings at 6 weeks showed a tendon-bone junction with only a partial fibrous interzone between the graft tissue and the surrounding bone. A mature intratunnel tendon-bone junction with a zone of fibrocartilage was found at 9 to 12 weeks. At the tunnel entrance site a wide regular ligamentous insertion site was seen in all specimens after 24 weeks. This insertion showed regular patterns such as the direct type of insertion of a normal ligament with a dense basophilic transition zone consisting of mineralized cartilage. **Conclusions:** A fibrous interzone between the graft tissue and the bone tunnel was only partially developed, which is in contrast to all previous studies in which nonanatomic fixation was used. Thus, it is reasonable to assume that the tendon-to-bone healing in the present study may progress partially by direct-contact healing without the development of a fibrous interzone. To our knowledge, this is the first report describing the development of a direct type of ligament insertion after ACL replacement with a soft-tissue graft. This is in contrast to previous studies reporting the development of an indirect type of insertion when using nonanatomic fixation far away from the joint line. Thus, histologic data strongly indicate that anatomic interference fit fixation is beneficial for tendon-to-bone incorporation by leading to the development of a direct type of ligament insertion. **Key Words:** Anterior cruciate ligament—Tendon healing—Histology—Biodegradable interference screws—Animal model.

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Currently, hamstring tendon graft fixation is performed nonanatomically using spiked washers, transfixation devices, staples, or suture and tape fixation to a button, an anchor, or a post. Nonanatomic soft-tissue graft fixation may create intra-tunnel graft motion,^{1,2} which may impair tendon-to-bone healing.³

To facilitate anatomic soft-tissue graft fixation, recent reports have described the use of direct tendon fixation with metal or biodegradable interference screws.^{4,5} Although clinical data show promising results^{6,7} and biomechanical studies have investigated the initial fixation strength of soft-tissue graft interference fit fixation,⁸⁻¹⁰ there has been no analysis of the progression of tendon-to-bone healing using this fixation. Several animal studies have investigated soft-tissue graft-to-bone healing using nonanatomic graft fixation.¹¹⁻¹⁶ In these studies, tendon-to-bone healing has been described to progress via the development of a fibrous interface between the graft and bone tissue, which is bridged by Sharpey-like fibers and undergoes a subsequent maturation process. By using interference fit fixation, certain graft-tunnel motions may be neutralized, and thus we hypothesized that different healing patterns may be present if anatomic interference fit fixation were used.

Therefore, the goal of the present study was to examine the healing stages of soft-tissue graft anterior cruciate ligament (ACL) reconstruction in a sheep model using direct tendon-to-bone interference fit fixation with biodegradable poly-(D,L-lactide) interference screws. We asked the following research questions: (1) How does tendon-to-bone healing progress histologically with respect to intratunnel and articular surface healing? (2) How do the bone growth and remodeling at the graft fixation site progress? (3) Does screw degradation compromise osseous graft incorporation or graft viability?

METHODS

Study Design and Operative Procedure

Thirty-five skeletally mature female merino sheep (mean weight, 51.4 ± 8.7 kg) were used in this study. Animals underwent replacement of the ACL in an open fashion as described in the part I of the present study.¹⁷ Animals were killed at 6, 9, 12, 24, and 52 weeks.

To rule out a possible effect of the biomechanical tests on the eventual histologic evaluations, 3 sheep were treated in the same fashion in a pilot study. Animals were euthanized after 6 weeks and there was

no graft pullout from either the femoral or the tibial tunnel observed. Histologic evaluation of these specimens showed no tissue damage at the screw-tendon-bone interface. Thus we considered biomechanical and histologic evaluation of a single specimen to be appropriate for the main study.

Conventional Histology

After biomechanical testing, distal femoral and proximal tibiae were fixed in 4% phosphate-buffered formalin for 10 days. Specimens were then cut sagittally in the center of the expected screw-tendon-bone interface using a precision saw system (Exakt; Apparatebau GmbH, Norderstedt, Germany). Specimens were dehydrated in graded alcohols (30% to 100%), and embedded undecalcified in polymethylmethacrylate (Technovit 7200; Heraeus Kulzer GmbH, Wertheim, Germany). In the center of the screw-tendon-bone interface, serial sagittal sections ($6 \mu\text{m}$) were cut with a hard-tissue microtome (Polycut SM 2500; Leica GmbH, Bensheim, Germany). For visualization of the different tissue structures, the serial sections were stained using Masson-Goldner's trichrome stain, a modified Safranin O-van Kossa stain, and Alcian blue techniques, respectively. Specimens were evaluated using normal and polarized light.

Fluorescence Microscopy

To determine bony ingrowth within a certain frame of time, all animals received a polychrome sequential labeling with 3 different fluorochromes according to the technique described by Rahn et al.^{18,19} Calcein green (10 mg/kg body weight subcutaneously; Sigma-Aldrich GmbH, Steinheim, Germany), xylenol orange (90 mg/kg body weight subcutaneously; Sigma-Aldrich GmbH), and tetracycline (25 mg/kg body weight intravenously, Supramycin; Grünthal GmbH, Aachen, Germany) were given at different time intervals in order to ensure a continuous labeling over the whole experimental period (Table 1). The fluorochrome activity in later histologic evaluation represents the bone mineralization at the time the fluorochromes were administered, thus allowing determination of bone growth at time points independently from the time animals were sacrificed.

Samples were embedded undecalcified in polymethylmethacrylate as described above and precision grinds ($80 \mu\text{m}$) were made with the precision Exakt grinding system. These specimens remained unstained for fluorescence light microscopy. Sections were evaluated using a digital image analysis system (KS 400

TABLE 1. Polychrome Sequential Labeling in the Different Experimental Groups

	Calcein Green	Xylenol Orange	Tetracycline
6-week group	1	3	5
9-week group	4	6	8
12-week group	7	9	11
24-week group	13	18	23
52-week group	28	38	48

NOTE. Values present the time point of fluorochrome administration after surgery in weeks.

Imaging System, Release 3.0; Carl Zeiss Vision, Echting, Germany) linked with a high-resolution microscope (Leica DMRB, Leica GmbH) and a motorized XY-table (MCX-2eco; ITK Dr. Kassen GmbH, Lahnau, Germany) to create a digitized picture of the whole section without overlapping. Two different filter combinations were used to discriminate the 3 different colors under fluorescent light.

RESULTS

Conventional Histologic Findings

Six-Week Group: At 6 weeks, the tendon graft was in part directly adjacent to the bone and a partially fibrous interzone (FIZ) was found between the graft and the tunnel wall (Fig 1). In those areas where a direct graft-bone contact zone was present, only a moderate osteoblastic activity with osteoid formation was found. The adjacent bone tissue was immature woven bone. Sharpey-like fibers were mainly present in areas where an FIZ developed between the graft and



FIGURE 1. Photomicrograph at 6 weeks showing the interface between the graft and the bone. The graft is immediately adjacent to the bone and a FIZ is only developed in part (arrows) (Masson Goldner's trichrome stain, longitudinal section, original magnification $\times 31.5$).

the bone or at areas of expected high stresses such as at the articular tunnel entrance site (Fig 2). In areas where an FIZ was present, a high osteoblastic activity with immature woven bone and a high amount of noncalcified osteoid was found trying to bridge the FIZ. At the tunnel base, there were many foreign-body giant cells and osteoclasts phagocytosing graft tissue and bone debris. The graft within the tunnel appeared to have a normal crimp pattern and a regular number of cells, which have a tendency to decrease at the graft-screw interface and increase at the graft-bone interface, showing immature fibroblasts and some round cells. In those areas, the cellular morphology

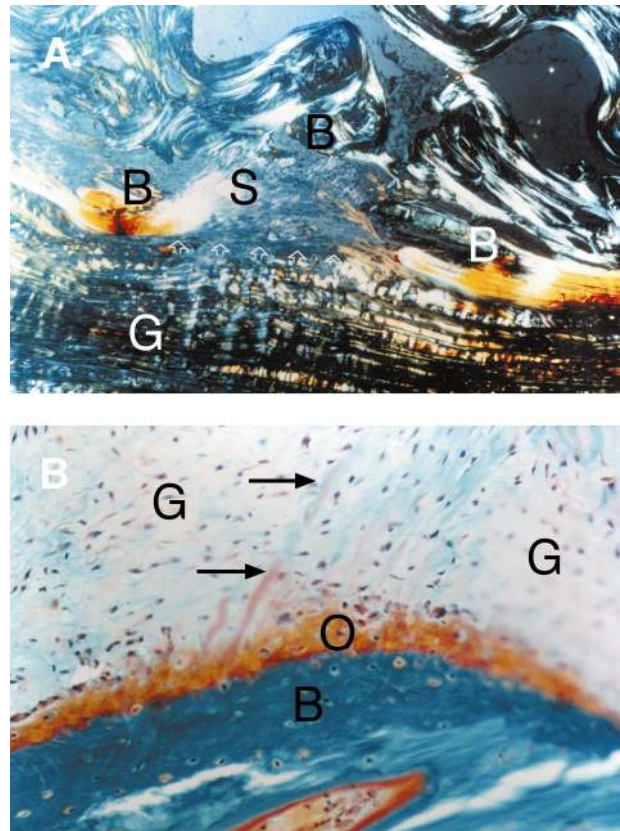


FIGURE 2. (A) Photomicrograph at 6 weeks under polarized light showing the development of a FIZ (arrows) in an area where the graft (G) is not in direct contact with the adjacent bone tissue (B). In the area where a FIZ has been developed, a high number of Sharpey-like fibers (S) extend into the bone (polarized light, Masson Goldner's trichrome stain, longitudinal section, original magnification $\times 31.5$). (B) Photomicrograph of a longitudinal section from the tibial tunnel aperture site at 6 weeks showing Sharpey-like fibers (arrows) bridging the newly formed woven bone (B) and the graft (G). There is a broad seam consisting of nonmineralized osteoid (O). The graft tissue is unstructured and hypercellular with plump and polygonal cells as was found at the midsubstance part of the graft (Masson Goldner's trichrome stain, original magnification $\times 252$).

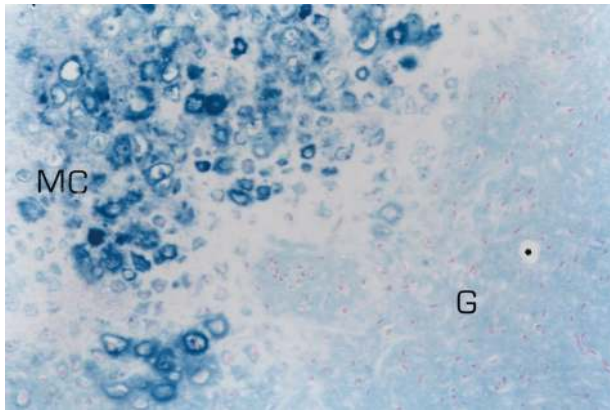


FIGURE 3. Photomicrograph of a transversal section from the tibial tunnel aperture site at 9 weeks showing a blending between the graft tissue (G) and mineralized cartilage (MC). The graft tissue is organized with fascicles and vessels (*) (Alcian blue stain, original magnification $\times 200$).

with plump and polygonal cells was similar to that of the intra-articular part of the graft.

Nine-Week Group: At 9 weeks, osteoblastic activity had decreased markedly and almost all gaps along the graft-bone interface were filled with woven bone that still showed some remodeling activity. At the graft-bone contact zone, fewer osteoid seams were found. A high osteoblastic activity and new woven bone formation were found to narrow the articular tunnel entrance site. In these areas, islets of calcifying cartilage tissue were found blending with the graft tissue (Fig 3). At the tunnel ground, a high remodeling activity with osteoclasts and foreign-body giant cells was still present. The graft itself showed a reduced number of cells in its center, with acellular parts and degenerative changes such as pyknotic nuclei and loss of nuclear material. Close to the graft-bone interface, a high number of immature fibroblasts could be found.

Twelve-Week Group: At 12 weeks, a mature and continuous graft-bone interface with Sharpey-like fibers was present. Slight osteoid formation was still visible and was comparable to that of the regular cancellous bone close to the graft fixation site. Between the collagen fibers, extending into the bone of the intra-tunnel part, chondroid cells were aligned representing a zone of fibrocartilage. Alcian blue and Safranin O–van Kossa staining confirmed the absence of mineralized cartilage adjacent to the fibrocartilage (Fig 4). At the articular tunnel entrance site, osteoblastic activity was lower compared with that at 9 weeks. The graft tissue blended with the articular cartilage and a layer of fibrous connective tissue. The

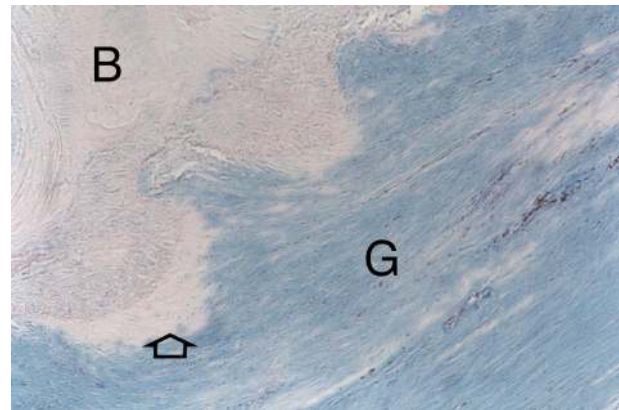


FIGURE 4. Intratunnel part of a specimen at 12 weeks showing a high number of Sharpey-like collagen fibers in continuity with the newly formed woven bone. The Alcian blue staining shows the absence of mineralized cartilage tissue at the site of tendon attachment to bone. Note the zone of fibrocartilage with chondroid cells (arrow) between the bone (B) and the graft (G) (Alcian blue stain, longitudinal section, original magnification $\times 31.5$).

layer of mineralized cartilage of the articular cartilage was in continuity with the mineralized cartilage adjacent to the graft tissue (Fig 5). The intratunnel part of the graft itself showed more mature fibrocyts and less active fibroblasts. Some small acellular parts with round cell invasion were found at the tunnel base.

Twenty-Four-Week Group: At 24 weeks, the implant site was filled with irregular hypervascular fibrous tissue, and remnants of screw material were detectable within macrophages and foreign-body giant cells (Fig 6). At the former screw-graft interface and at



FIGURE 5. Photomicrograph of a specimen at 12 weeks showing the articular tunnel aperture site. The graft tissue (G) blends with the articular cartilage (AC). The layer of mineralized articular cartilage is in continuity with the layer of mineralized cartilage close to the graft insertion (arrow). (Alcian blue stain, longitudinal section, original magnification $\times 25$).

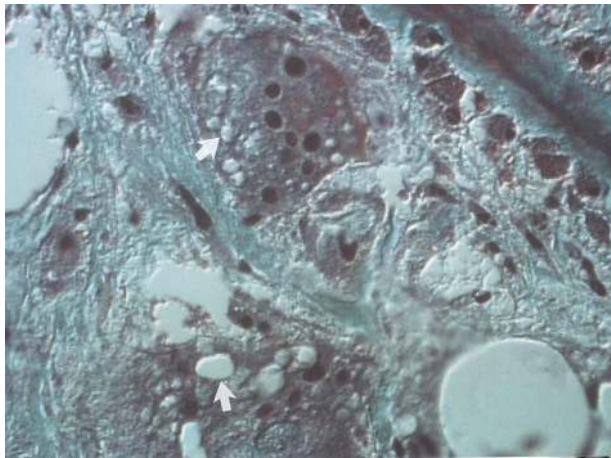


FIGURE 6. High-magnification photomicrograph of an implant site at 24 weeks. There are polyactide particles (arrow) deposited in foreign-body giant cells (Masson Goldner's trichrome stain, interference contrast, original magnification $\times 400$).

the graft-bone interface, the graft appeared comparable to what was found at 12 weeks. There were predominantly mature fibrocysts and vessels to be found in the ligament within the tunnel. At the former graft-implant interface, there was granulomatous tissue and the graft appeared with a normal number of cells and some vessels (Fig 7). In some cases, the intra-tunnel part of the graft was osseously separated from its insertion site at the joint surface and the graft tissue appeared unstructured and partially resorbed. At the articular tunnel aperture site, there was an almost complete bony overgrowth of the former implant site

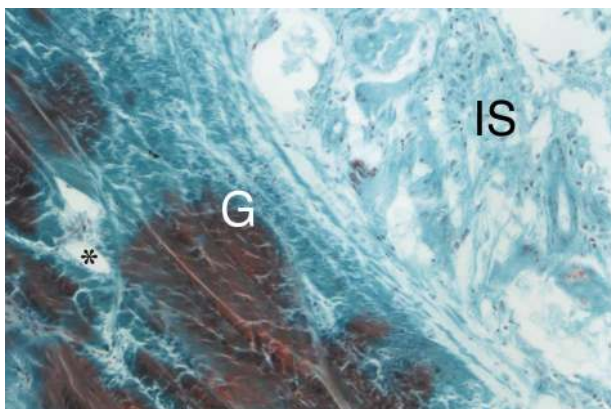


FIGURE 7. Photomicrograph of a transversal section from the tibial tunnel at 24 weeks showing the interface between the graft (G) and the former implant site (IS). The screw is degraded, leaving hypercellular connective tissue. The graft appears viable with vessels (*) and a normal number of cells (Masson Goldner's trichrome stain, original magnification $\times 200$).

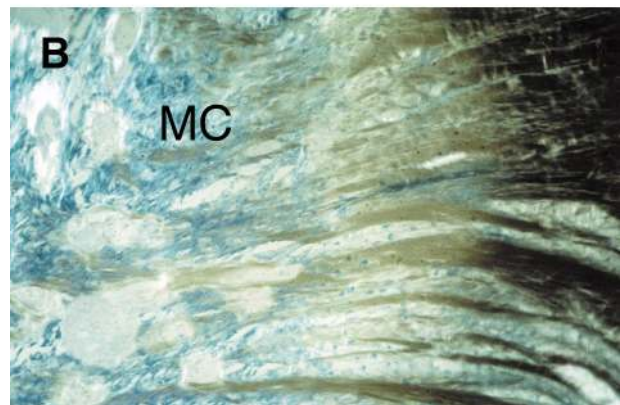
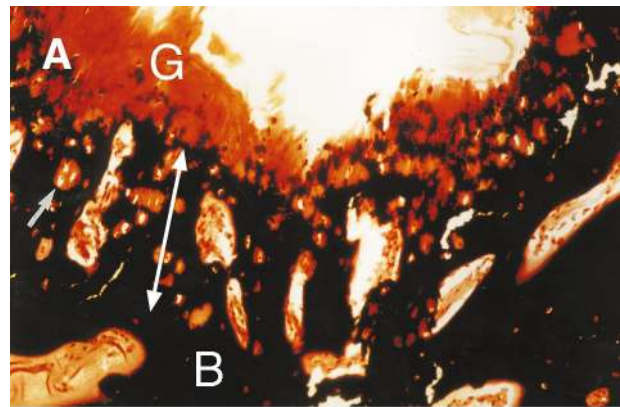


FIGURE 8. (A) Photomicrograph of the tibial graft insertion site at the joint surface at 24 weeks showing the former site of graft rupture (top). There is a broad transition zone consisting of mineralized cartilage (between arrows) and the fibrocartilaginous part of the graft (G). There are chondrocytes (arrow) embedded in the zone of mineralized cartilage (Safranin O–van Kossa stain, original magnification $\times 63$). (B) Higher magnification photomicrograph under polarized light showing the collagen fibers extending into the band of mineralized cartilage (MC) (Alcian blue stain, partially polarized light, original magnification $\times 126$).

with a wide regular ligamentous insertion. Serial section with Alcian blue and Safranin O–van Kossa staining showed a distinct transition zone consisting of mineralized cartilage blending with the woven bone and the fibrocartilaginous part of the graft (Fig 8).

Fifty-Two-Week Group: At 1 year, a normal direct ligament insertion with a transition zone consisting of mineralized cartilage and fibrocartilage was found in all specimens distally and proximally. This insertion was broader and more structured, with parallel fibers, compared with what was found at 24 weeks, and it had a morphologic structure comparable to a normal tendon insertion (Fig 9). The adjacent bone tissue had lamellar structure. The former implant site was still visible but there was a marginal ingrowth

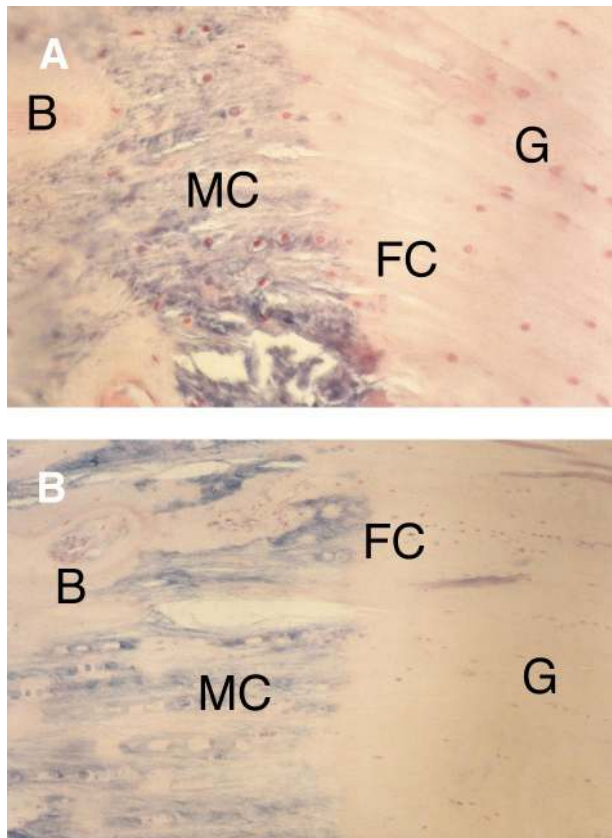


FIGURE 9. (A) Photomicrograph of a tibial graft insertion at 52 weeks, showing the 4 zones of a normal primary ligament insertion. There is a band of mineralized cartilage (MC) and fibrocartilage (FC) between the bone (B) and the graft (G) (Alcian blue stain, original magnification $\times 252$). (B) Photomicrograph of the femoral insertion of the extensor tibialis muscle for comparison, showing the same distinct transition zone consisting of mineralized cartilage (Alcian blue stain, original magnification $\times 126$).

of bone trabeculae. The intra-tunnel part of the graft was almost resorbed in some cases and in other cases it was thinned but well structured with a regular crimp.

Fluorescence Microscopy Findings

At 6 weeks, there was intensive fluorochrome activity at the implant-bone and the tendon-bone interface as well as more peripheral to the graft fixation site. In these specimens, the earliest bone growth started within the first week at the distal aspect of the graft fixation site, whereas later (3 weeks) there was also intensive fluorochrome activity closer to the articular tunnel aperture site (Fig 10).

In the 9-week specimens, fluorochrome activity at the tendon-bone and the implant-bone interface was

lower compared with the 6-week specimens, but it was still intense and showed the typical band-like appearance found during the remodeling of trabecular bone. There was a high xylenol orange activity at the articular tunnel aperture site indicating an intense new bone formation after 6 weeks (Fig 11A). At the tendon-bone interface, we found islets of high fluorochrome activity (Fig 11B) as a sign of the osseous replacement of the partially developed FIZ at the tendon-bone junction between 4 and 8 weeks.

At 12 weeks, an intense band-like remodeling activity at the tendon-bone interface was still present, whereas there was almost no fluorochrome activity at the implant-bone interface. The highest fluorochrome activity of all 3 fluorochromes was found at the articular tunnel aperture site, indicating continuous bone growth and remodeling between weeks 7 and 11 (Fig 12).

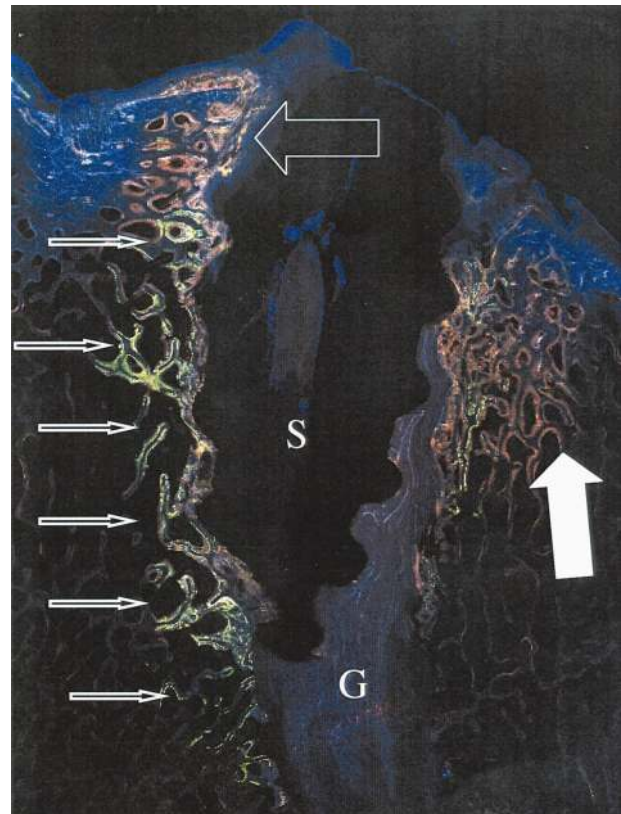


FIGURE 10. Fluorescence microscopy of a longitudinal tibial section at 6 weeks presenting all 3 given fluorochromes. There is an intense bone growth after 1 week close to the graft fixation site as indicated by the calcein green fluorescence (small arrows). At 3 weeks, there is intense bone growth closer to the articular tunnel aperture (transparent arrow) and more peripheral to the graft fixation site (white arrow). (S, screw site; G, graft tissue.)

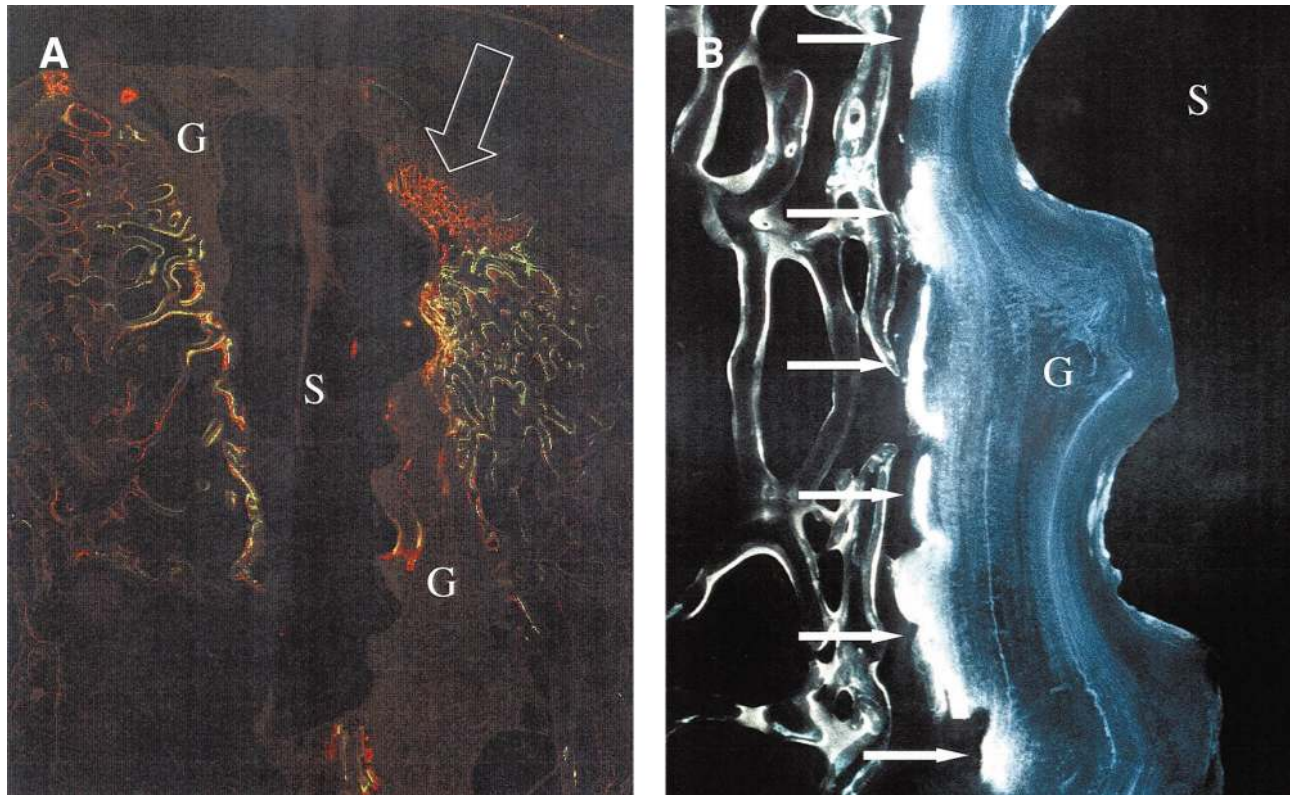


FIGURE 11. (A) Fluorescence microscopy of a longitudinal tibial section at 9 weeks. There is still intense bone growth close to the graft fixation site as indicated by all 3 fluorochromes (4 to 8 weeks). Note the intense xylenol orange fluorescence at the articular site (arrow), indicating a narrowing of the tunnel aperture at 6 weeks. (S, screw site; G, graft tissue.) (B) Higher magnification of a longitudinal tibial section at 9 weeks presenting calcein green and tetracycline fluorescence. The intense islet-like fluorescence (arrows) at the tendon-bone interface indicates an osseous bridging of the partially developed FIZ between 4 and 8 weeks.

In the 24-week specimens, almost no fluorochrome activity could be detected at the tendon-bone interface. At that time, there was still intense fluorescence of all 3 fluorochromes at the articular tunnel aperture site. Fluorochrome distribution indicated a continuous appositional narrowing of the aperture site between weeks 13 and 23 (Fig 13).

At 52 weeks, we found a further narrowing of the tunnel aperture site as well as deeper within the former graft tunnel. At that time, the former implant site was partially filled with dense lamellar bone. The former tendon-implant interface was replaced by dense bone showing a continuous band-like bone apposition as indicated by the homogeneous fluorescent bands (Fig 14).

DISCUSSION

The bony incorporation of a soft-tissue graft in the bone tunnel is the basic requirement for long-time

survival of the graft tissue. Several studies have investigated the tendon-to-bone healing of soft-tissue grafts histologically in laboratory animals as well as in humans during second-look or revision procedures.^{11-16,20-28} Different intra-articular and extra-articular animal models have been studied and, thus, different time frames of a proper tendon-to-bone healing have been described. A major limitation in all these studies, as well as in the present one, is the early strength loss of the graft during the remodeling process, leading to midsubstance failure at a certain time.¹⁷ Therefore, a clear correlation between specific histologic findings of graft incorporation and biomechanical parameters does not exist.

Previous studies describe the tendon-to-bone healing progress via a zone of vascular, highly cellular fibrous tissue, which we defined as the FIZ, which undergoes a maturation process until its matrix consists of orientated collagen fibers and the FIZ-graft interface becomes indistinct.^{11,13} The development of

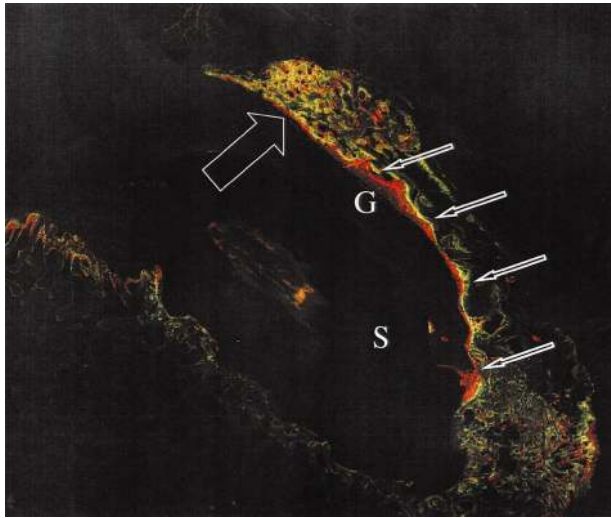


FIGURE 12. Fluorescence microscopy of a longitudinal femoral section at 12 weeks showing all 3 fluorochromes. There is almost no osseous activity at the implant-bone interface, whereas there is still marked remodeling activity at the tendon-bone interface (small arrows). Note the intense fluorescence of all 3 fluorochromes at the articular tunnel aperture site (arrow), indicating a continuous bone growth and remodeling at the surface over the whole labeling period (7 to 11 weeks). (S, screw site; G, graft tissue.)

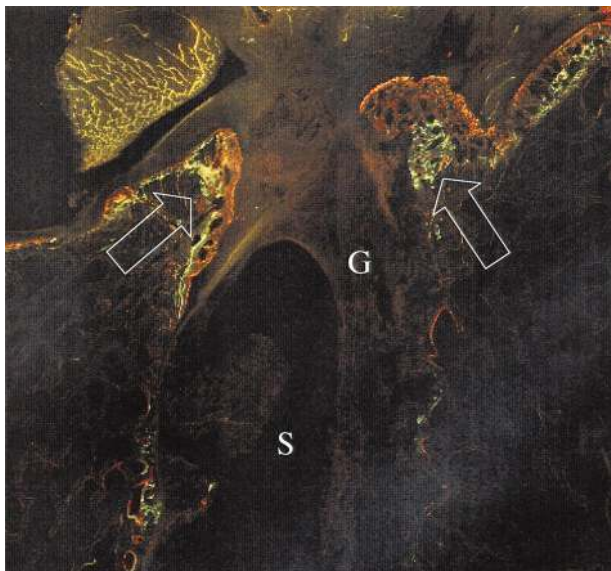


FIGURE 13. Fluorescence microscopy of a longitudinal tibial section at 24 weeks showing all 3 fluorochromes. Note that there is almost no osseous activity either at the implant-bone interface or the tendon-bone interface between 13 and 23 weeks, whereas there is still intense bone growth and remodeling at the articular tunnel aperture site (arrows). (S, screw site; G, graft tissue.)

Sharpey-like collagen fibers bridging the bone and the graft has been described and is viewed as the earliest sign of osseous integration.^{11,13,14} Rodeo et al.¹¹ described a broad FIZ which was still visible after 26 weeks. They found the first Sharpey-like fibers developed at 4 weeks. Blickenstaff et al.¹² and Grana et al.¹³ investigated semitendinosus tendon healing in an intra-articular model in rabbits. They observed an obliteration of the FIZ-graft interface at 12 weeks and found the first Sharpey-like fibers at 3 weeks. In another study Liu et al.¹⁴ investigated the matrix composition during tendon-to-bone healing by immunohistochemical analysis in an extra-articular model in rabbits. At 6 weeks, there was a broad and disorganized FIZ and Sharpey-like fibers were found, displaying collagen type II expression.

Our first research question asked about the progression of tendon-to-bone healing histologically when using anatomic interference fit fixation. Although different animal models were used, a distinct difference between the previous studies and the present study could be found. In the present study, the development of a FIZ was only partial or already osseously replaced at 6 weeks as shown by fluorescence microscopy. The main difference between the studies is that in the present one the tendon graft was fixed by a press-fit mechanism. Although the main weakness of our study

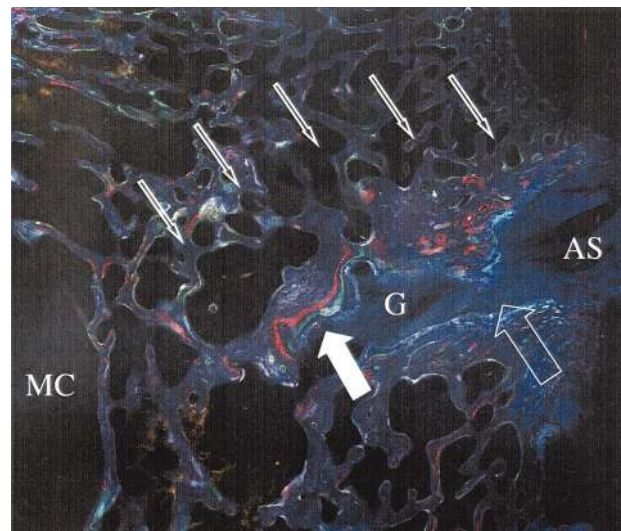


FIGURE 14. Fluorescence microscopy of a longitudinal tibial section at 52 weeks showing all 3 fluorochromes. The former screw site (indicated approximately by the small arrow) is partially replaced by dense lamellar bone. There is a narrowing of the graft tunnel (transparent arrow) by continuous bone ingrowth as indicated by the homogeneous and broad fluorochrome bands (white arrow). There is normal remodeling activity in the periphery. (G, graft tissue; MC, medullary cavity; AS, articular tunnel aperture site.)

is the lack of a control group with nonanatomic fixation, it is reasonable to hypothesize that the tendon-to-bone healing may at least in part progress by direct contact healing without the development of a FIZ. Furthermore, when a FIZ was partially present, its maturation process was completed between 9 and 12 weeks.

Sharpey and Ellis²⁹ described perforating fibers that extend across the bone anchoring the soft tissue to the bone. The presence of these fibers should be considered to represent an indirect tendon or ligament insertion, as can be found for the periosteal attachment of tendons.^{13,30} The periosteal attachment, however, should be viewed as a dynamic anchorage of soft tissue to bone allowing certain shear movements. It has been hypothesized that, in grafts fixed nonanatomically, shear in the tunnel may lead to the development of such an indirect insertion and allow minor longitudinal movements between the graft tissue and the surrounding bone.³¹ In fact, in the present study, Sharpey-like fibers were maximally pronounced at areas of expected high stresses, such as at the articular tunnel aperture site, or in the intra-tunnel part where the graft was not directly in contact with the surrounding bone.

Previous studies described the development of a zone of fibrocartilage with chondroid cells aligning between Sharpey-like fibers^{15,26,32} as we found at the intra-tunnel part of the tendon-bone interface. Furthermore, we found no mineralized cartilage tissue adjacent to the fibrocartilage in these areas; thus, the presence of fibrocartilage does not necessarily imply that a direct ligament insertion has been developed. The presence of fibrocartilage with its chondroid cell transformation may account for continuously acting tensile and compressive forces. In contrast, a direct type of ligament or tendon insertion consists of 4 distinct transition zones of ligament, fibrocartilage, mineralized cartilage, and bone.^{30,33} This type of ligament insertion serves well to transmit tensile forces while the mineralized cartilage tidemark allows a gradual force transmission by reducing the stiffness gradient between the bone and the zone of fibrocartilage.^{33,34} Previous animal studies using a bone–patellar tendon–bone graft have shown that the implanted natural direct insertion of the patellar tendon maintains its structure during the remodeling.^{16,35,36} To our knowledge, no previous study has found a direct ligament insertion at the joint surface with these specific transition zones after soft-tissue graft or tendon implantation into a bone tunnel. The present study

clearly shows such a direct ligament insertion to be developed at 24 weeks.

Based on our findings, we believe that 2 different mechanisms of tendon-to-bone healing could be found in soft-tissue graft ACL reconstruction—intra-tunnel healing and surface healing. The development of Sharpey-like fibers, the maturation of the FIZ, and the subsequent development of a fibrocartilaginous insertion have been described in several reports and were found in the intra-tunnel part of the tendon-to-bone healing process in the present study. The force distribution at such an intra-tunnel insertion is almost parallel to the bone surface and may thus explain the development of an indirect type of insertion with a zone of fibrocartilage. In contrast, at the joint surface, the forces act mainly perpendicular as is found with the regular ACL insertion. In the present study, such a direct type of ligament insertion was found, which may have its origin at the mineralized cartilage of the articular cartilage blending with the graft tissue. We believe, however, that to allow for the development of a direct insertion at the articular tunnel aperture site where high shear forces may act on the tendon-bone interface, certain graft-tunnel motions have to be neutralized. By using an interference fit fixation technique, these graft-tunnel motions may be neutralized and thus promote the development of a direct type of ligament insertion at the joint surface.

Our second research question asked about the progression of bone growth and remodeling at the graft fixation site. We found high osseous activity at the bone-implant interface up to 6 weeks, indicating that bone growth and remodeling induced by drilling, dilation, and screw insertion may be terminated at that time. In contrast, bone growth at the tendon-bone interface as a part of the intra-tunnel tendon healing process was terminated at 12 weeks as indicated by the fluorescence microscopy findings in the 24-week specimens. This observation clearly shows that the intra-tunnel tendon-bone healing and remodeling may be terminated around the 12th week if interference fit fixation is used. At the articular tunnel aperture site, a high osseous activity was first found at 3 weeks, but osseous narrowing of the aperture site was maximally pronounced at 6 weeks as indicated by fluorescence microscopy. Remodeling activity at the osseous tunnel aperture site could be found throughout the whole experimental period, but intense bone growth could only be found before the 24th week. This observation indicates that the calcified part of the direct ligament insertion at the articular tunnel aperture site forms between 6 and 24 weeks in the present animal model.

Tunnel enlargement has been described to be of concern in hamstring tendon graft ACL reconstruction, although no correlation between its occurrence and clinical findings has been shown so far.^{2,37,38} The maximum enlargement has been reported to occur within the first month after surgery, but the underlying mechanism is still unclear.² It has been hypothesized that biomechanical as well as biologic factors such as synovial access may be responsible for this phenomenon.² However, it should be clear that any kind of an osseous tunnel enlargement results in a broadened FIZ between the bone and graft tissue, leading thus to an impaired or prolonged maturation and narrowing of the FIZ. In the present study, a tunnel enlargement could not be found at all. Moreover, we found a continuous bone ingrowth and subsequent narrowing of the graft tunnel over the whole observation period. This finding is in line with a recent clinical study comparing different hamstring tendon graft fixation techniques in which only the anatomic joint line interference screw fixation did not lead to any tunnel enlargement.³⁸ Therefore, it is reasonable to assume that anatomic interference fit fixation may prevent tunnel enlargement, thus promoting tendon-to-bone incorporation. However, it is still unclear whether this is the result of the neutralization of graft-tunnel motions or the prevention of synovial access to the graft tunnel.

The third research question asked if screw degradation compromises osseous graft incorporation or intratunnel graft viability. We found that the implanted poly-(D,L-lactide) interference screw showed maximum degradation at 24 weeks as indicated by the high amount of foreign-body giant cells and macrophages. We observed that, after 6 to 9 weeks, the screw was separated from the intra-articular environment by connective tissue overgrowth that later blended with the graft insertion site at the joint surface. Therefore, we believe that if a certain osseous graft anchorage has been developed at the joint surface, the screw and the intra-tunnel tendon-bone interface is of less importance for maintaining graft fixation. Commonly used biodegradable interference screws consist of high-molecular weight poly-L-lactide, which is known to show no degradation within an appropriate time.³⁹⁻⁴³ Therefore, no clinical or experimental data have been reported so far with follow-up clearly showing that biodegradable material degradation has no adverse effect on soft-tissue graft incorporation. To judge the influence of material degradation on graft healing or graft viability, a material is required that shows maximum degradation within the study period. The chosen

material poly-(D,L-lactide) showed its maximum degradation at 24 weeks. Foreign-body reactions to biodegradable implants are associated with their final implant degradation^{39,44-46} and, in fact, we found foreign-body giant cells and macrophages at the former implant site phagocytosing implant remnants combined with a moderate inflammatory response. However, at that time the important graft anchorage was already located at the articular surface. Other studies have suggested that a high amount of low-molecular weight by-products being released during final implant degradation could be toxic or may lower the pH at the previous implant site.⁴⁷⁻⁴⁹ Thus, there is a risk that these by-products may induce adverse cellular effects that may compromise graft viability. We found that the graft tissue adjacent to the former implant site appeared normal at 24 weeks without any cellular pathologies and normal vascularity, indicating that degradation by-products may not directly influence the viability of the graft tissue within the tunnel. Furthermore, after screw degradation we found a partial osseous restitution of the former implant site, from which there was a further osseous narrowing of the graft tunnel.

In summary, we can conclude that anatomic interference fit fixation of a soft-tissue graft promotes tendon-to-bone incorporation, possibly because of the neutralization of graft-tunnel motions. Furthermore, the typical tunnel enlargement associated with non-anatomic soft-tissue graft fixation can possibly be avoided by interference screw fixation directly at the joint line. In contrast to previous reports, it is obvious that 2 different mechanisms of tendon-to-bone healing exist; intratunnel and surface healing. When using interference screw fixation, the intratunnel healing may only be important during the early healing stages, but after a solid surface healing has occurred the intratunnel part of the graft tissue may be stress shielded, resulting in a partial resorption and continuous tunnel narrowing over time. This process led to the development of a direct type of ligament insertion at the articular tunnel aperture site, resulting in a regular biologic anchorage, as is found in the native ACL.

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