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Graft remodeling during growth following anterior cruciate ligament reconstruction in skeletally immature sheep

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Abstract
Introduction Ruptures of the anterior cruciate ligament are being diagnosed with increasing frequency in skeletally immature individuals. It was our aim to investigate the graft remodelling process following an autologous, transphyseal reconstruction of the anterior cruciate ligament (ACL) in skeletally immature sheep. We hypothesized that the ligamentisation process in immature sheep is quicker and more complete when compared to adult sheep.
Materials and methods Skeletally immature sheep with an age of 4 months underwent a fully transphyseal ACL reconstruction using an autologous tendon. The animals were subsequently sacrificed at 3, 6, 12 and 24 weeks following surgery. Each group was characterised histomorphometrically, by immunostaining (VEGF, SMA), by transmission electron microscopy (TEM) and biomechanically (UFS Roboter).
Results The histomorphometric analysis and presence of VEGF and SMA positive cells demonstrated a rapid return to a ligament like structure. The biomechanical analysis revealed an anteroposterior translation that was still increased even 6 months following surgery.
Conclusion As in adult sheep models, the remodeling of a soft tissue graft used for ACL reconstruction results in a biomechanically inferior substitute. However, the immature tissue seems to remodel faster and more complete when compared to adults.

Keywords Anterior cruciate ligament · ACL · Knee · Biomechanics · Sheep · Graft remodeling · Histomorphology · Immunohistochemistry · Electron microscopy · Skeletally immature

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Introduction

Intraligamentous ruptures of the anterior cruciate ligament (ACL) are being diagnosed with increasing frequency [5, 6, 19, 26]. Discussion is still ongoing on how and when to best treat this injury in patients with open growth plates. Several problems have been reported with this injury in this younger patient subpopulation. As a consequence, the rate of revision surgery is reported to be higher than in adult ACL reconstructive surgery [10, 16].

It is known from previous animal studies that different ligaments are unique in their growth, development and aging [31]. It has further been shown that the rate of skeletal maturation causes significant changes to ligaments whereby, for example the stiffness increases during growth

[32]. Recently, several authors have emphasized the need for more basic research in that field to better understand this injury and to optimize treatment options for this young patient subpopulation [4, 14, 23].

It was the aim of the present study to describe the graft remodeling process in a model of skeletally immature sheep following an autologous ACL reconstruction, both histologically and biomechanically. We hypothesized that the ligamentisation process in immature sheep is quicker and more complete when compared to adult sheep.

Materials and methods

Study design

All procedures were performed with permission of the local governmental animal rights protection authorities (Ref. No. 05/933) and in accordance with the National Institute of Health guidelines for the use of laboratory animals. Thirty-two black headed sheep of 4 months of age underwent a fully transphyseal reconstruction of their right ACL. The left knee served as a control. The amount of longitudinal growth corresponded grossly to the amount of remaining growth in girls with a skeletal age between 9 and 12 years [24]. Four groups of eight animals each were sacrificed at 3, 6, 12 and 24 weeks following surgery respectively. Two animals of each group were used for the histological workup, and six animals for the biomechanical analysis.

Operative technique

The right knee joint was exposed through an anteromedial incision with release of the medial parapatellar retinaculum. The patella was displaced laterally, the anterior fat pad was sharply separated, and the plica synovialis and the ACL exposed and removed. A split of the ipsilateral superficial flexor digitorum tendon and gastrocnemius tendon was harvested and used as a soft tissue graft. The tibial and femoral tunnels were created using a fully transphyseal technique. Graft fixation was achieved using the Endobutton proximally and the Suture Washer device distally (both: Smith and Nephew Endoscopy, Andover, MA, USA). A 5 mg/kg dose of procain-benzylpenicillin (long-acting penicillin and dihydrostreptomycin) was given post-operatively as an antibiotic. Buprenorphin was injected immediately as well as 6 and 24 h postoperativeley. Carprofen was given in a dose of 2 mg/kg for the first three postoperative days. The weight of the animals was followed, and postoperative mobilisation was recorded on a daily basis according to a specific grading system as previously suggested [12].

Gross inspection and tissue sampling

Immediately following surgery, the knee joints of the animals dedicated for histological workup were exposed through an anteromedial arthrotomy. The grafts were macroscopically analyzed to determine the integrity. The ACL graft was then sharply dissected and fixed in an aldehyde solution. The hind limbs foreseen for biomechanical analysis were exarticulated in the hip joint and frozen in full extension at −20°C. Twelve hours prior to biomechanical testing, they were thawed at room temperature. Following kinematic testing with the capsuloligamentous structures in place, the capsule was sharply dissected and the graft was checked for its integrity.

Histology and immunohistochemistry

The tissues were fixed in 3.7–4% buffered formalin for 18–24 h. All samples underwent an automated dehydration process in graded alcohols and were embedded in paraffin wax. Sections were cut at 2–5 μm. Sections were stained with hematoxylin–eosin. For immunohistochemistry, endogenous peroxidase was quenched (ChemMate™ DAKO Envision™ Detection Kit, Dako, Germany) and non-specific sites were blocked with the use of ChemMate™ Antibody Diluent (Dako, Germany). The sections were then incubated with a monoclonal antibody to Alpha Smooth Muscle Actin (SMA, clone 1A1, Sigma-Aldrich Chemie GmbH, Germany) at a dilution of 1:1,000 for 30 min at room temperature as previously described [17]. SMA is known to play a role in the remodeling process of transplanted tendon grafts [17]. A mouse-anti-human antibody of Vascular Endothelial Growth Faktor (VEGF, clone C-1, Santa Cruz Biotechnology, Germany) was used to asses the neovascularisation process as previously described [20]. Negative controls were done for both antibodies. All of the sections were then incubated with an anti-mouse secondary antibody of the Envision+™ System (Dako, Germany). Counterstaining was done with hematoxylin.

Histomorphometric analysis

The volumetric densities of cells, blood vessels and extracellular matrix were calculated using a morphometric method previously described by Weibel [27]. Three sections were cut in 100 μm intervals, stained with hematoxylin–eosin and six test areas were examined using systematic random sampling at 100× magnification. A regular 50 point lattice was used. The volumetric density (V_V) of a defined structure (V_{Vs}) corresponds to the ratio of

points lying on the structure (P_S) to the total number of test points in a reference area (P_{REF}). For all calculations the following figure was used: $V_{Vs} = \frac{P_S}{P_{REF}} = \frac{V_S}{V_{REF}}$.

Transmission electron microscopy (TEM)

The harvested tissue was immediately fixed in 2.5% glutaraldehyde (Polysciences, Warrington, PA, USA) in 0.1 M sodium cacodylate, pH 7.3 (Schuchardt, Hohenbrunn, Germany) and postfixed in 2% osmium tetroxide (Polysciences, Warrington, PA, USA) in the same buffer. Specimens were dehydrated in ethanol and embedded in epoxy resin (Serva, Heidelberg, Germany). Thin sections stained with aqueous uranyl acetate and lead citrate were examined with a Philips EM 301 operating at 80 KV.

Biomechanical evaluation

The ovine knee was mounted in a sensor-guided robot (KUKA KR 15/1, KUKA Robotics, Augsburg, Germany) in a specially designed testing fixture. The tibia was mounted to the force-moment sensor (IpeA, Berlin, Germany) and the femur to a fixed mounting block. A tibial anatomical coordinate system was kinematically established for each specimen. The geometric center of rotation was calculated from preliminary flexion–extension and internal–external rotation motions about the 90° flexion position of the specimen. The internal–external rotation axis was located parallel to the axis of the tibial diaphysis, and the flexion–extension axis orthogonal to the plane of passive flexion–extension. Laxity of the specimens was tested by applying anteroposterior displacement at 90° of flexion. During this anterior–posterior motion, the tibia was allowed to freely translate about the remaining two axes, and rotate in the varus–valgus and internal–external directions, thus allowing coupled secondary motions to occur. Anteroposterior displacement was applied until a force of 50 N in both directions respectively was detected, and the direction of motion was reversed. Loading was applied over three complete anterior–posterior motion cycles at a displacement rate of 0.1 mm/s. The third hysteresis curve was taken for the calculation of the anteroposterior translation. Stiffness was calculated from the ends of the loading portion of the hysteresis curve of the drawer testing as a measure of the rate of load take-up of the reconstruction. The cross sectional area of each specimen was measured three times using a laser micrometer (Takikawa Engineering, Tokyo, Japan). The means of the three measurements of each specimen were used for statistical analysis.

Data analysis

All data were analyzed by a professional statistician using the software SPSS version 14.0 for windows (Chicago, IL, USA). The biomechanical data were analyzed with a two way ANOVA (the first dependent variable being the time of evaluation following surgery, the second being the operated or intact knee). This test was again followed by a Students *t* test for independent variables. The differences were considered to be significant at a probability level of $p \leq 0.05$.

Results

Postoperative course

Two animals died post-operatively of pneumonia and had to be excluded from the study leaving 30 animals for the final evaluation. All animals demonstrated a quick return to full mobilization within 6 weeks as measured by the mobility score. On two animals, an effusion of the right knee joint was aspirated for diagnostic reasons and a bacterial infection ruled out.

Gross inspection

The gross inspection of the ACL reconstructed knees after sacrifice demonstrated intact knee joints with no meniscal lesion and no major signs of osteoarthritis. All grafts were in place and covered by a synovial sheath as early as 3 weeks following surgery (Fig. 1).

HE staining

The hematoxylin–eosin staining at 3 weeks following surgery revealed an inhomogeneous picture with predominantly acellular areas. However, other regions of the graft already demonstrated a cell-repopulation (Fig. 2a). After 6 weeks, the graft was highly vascularized and contained a high number of myofibroblasts between the collagen fibers (Fig. 2b). During the following 6 weeks of the remodeling process, the collagen fibers became more organized and the vasculature and cell density decreased (Fig. 2c). Six months following surgery, collagen fibers were densely packed and parallel aligned. The fibroblasts and some remaining capillaries were arranged between these collagen fibers (Fig. 2d).

Histomorphometric analysis

The histomorphometric analysis reflects these morphological results. Cell density and the amount of vessels inside

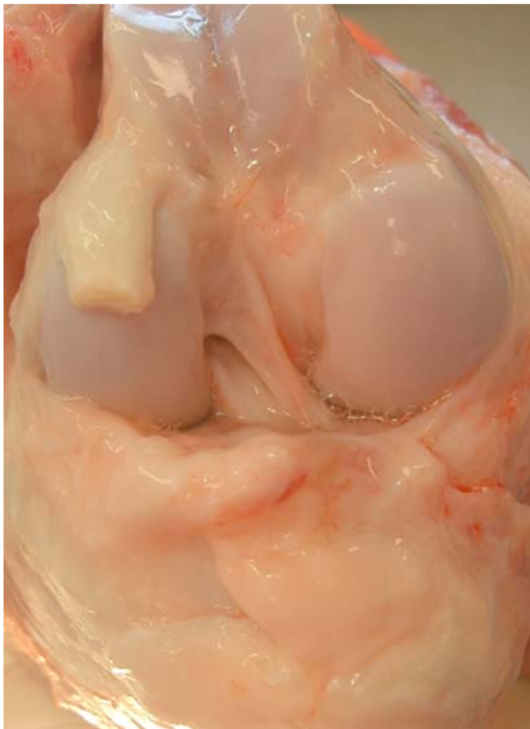

Fig. 1 Macroscopic appearance of an ACL reconstructed knee at time of sacrifice 6-month following surgery. Note the synovial sheath surrounding the graft. There was no evidence of meniscal lesions or major articular cartilage lesions in all specimens

the graft increased during the initial 6 weeks. Thereafter, the remodeling process advanced by formation of extracellular matrix (Fig. 3).

VEGF staining

In the early stages of the graft remodeling process, VEGF could be demonstrated in the ingrowing vessels and connective tissue cells. This staining was weak at 3 weeks (Fig. 4a). At 6 weeks, only scattered cells within the extracellular matrix stained positive for VEGF (Fig. 4b). The strongest staining in nearly all cells could be observed 12 weeks following surgery (Fig. 4c). Finally, at 24 weeks, only some pericytes of the vessels remained positive for VEGF (Fig. 4d).

SMA staining

The repopulating cells of the graft at 3 weeks following surgery contained SMA (Fig. 5a). Therefore, these cells are myofibroblasts by definition. During the next 6 weeks, the myofibroblasts and the pericytes of proliferated vessels demonstrated an increased staining for SMA (Fig. 5b). The following period of the graft remodeling process demonstrated a decreased intensity of SMA staining (Fig. 5c). Similar to VEGF, 6 months following surgery only pericytes and smooth muscle cells of the vessels remained stained (Fig. 5d).

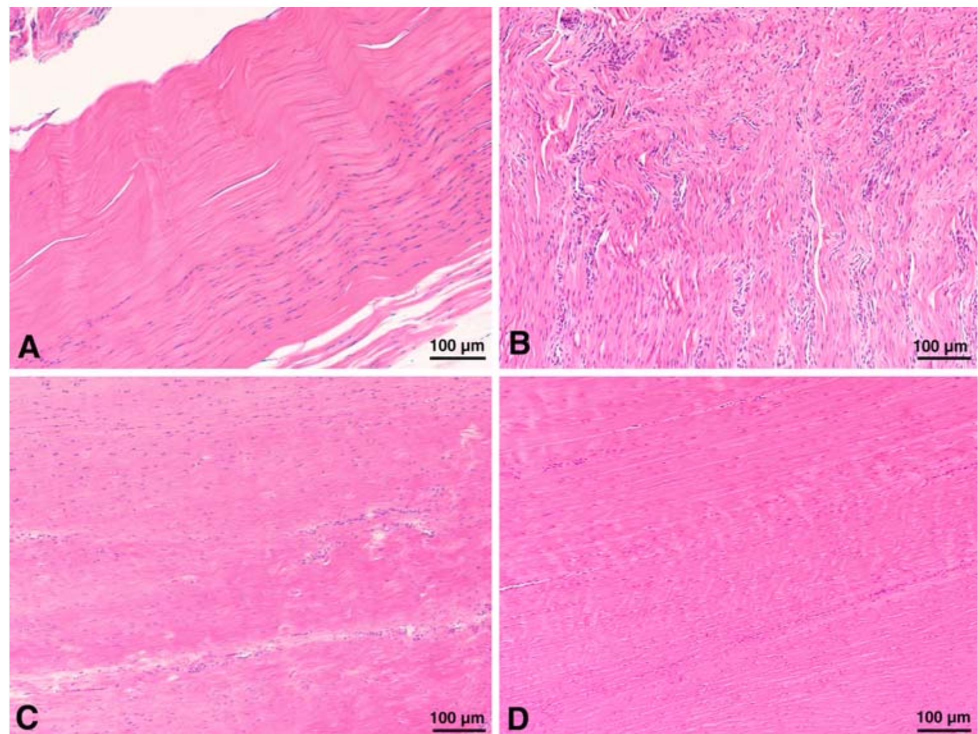

Fig. 2 Morphology of the transplant healing after 3 (**a**), 6 (**b**), 12 (**c**) and 24 (**d**) weeks. After 3 weeks the graft becomes repopulated partly (**a**). After 6 weeks the highest density of proliferating micro vessels and connective tissue cells can be seen (**b**). The reorganization of the collagen fibers increases after 12 weeks (**c**) and is finished by a regular parallel arrangement after 24 weeks (**d**). Paraffin, H&E staining

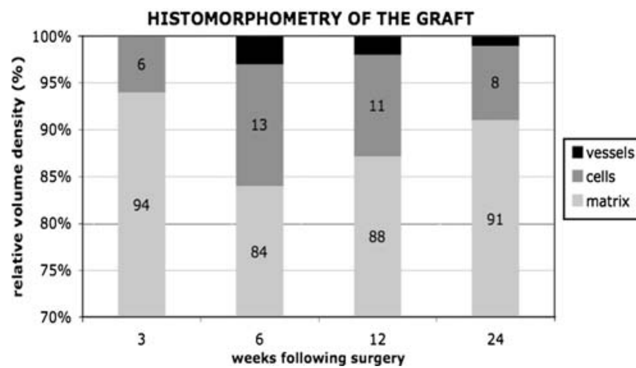

Fig. 3 Relative volume density of blood vessels, cells and extracellular matrix in the graft throughout the remodeling process. The density of cells and microvessels increases until 6 weeks and normalizes towards 24 weeks

TEM

After 3 weeks, only some apoptotic cells were present in the extracellular matrix containing bundles of about 100 nm thick collagen fibers and remnants of proteoglycans (Fig. 6a). Six weeks following surgery, a high density of myofibroblasts was demonstrated. The presence of nucleoli and the predominance of the rough endoplasmic reticulum reflected their activity of the remodeling process, which is a combination of degradation and biosynthesis (Fig. 6b). Thick collagen fibers are degraded whereas small diameter fibrils of about 30 nm are simultaneously synthesized. The actin network inside the myofibroblasts is diminished during the remodeling process (Fig. 6c). The extracellular matrix contained a very high density of parallel-arranged collagen fibers that are interspersed by single fibroblasts with long processes (Fig. 6d). In the cytoplasm, only few remaining actin filaments and few cell organelles were visible, demonstrating that the remodeling process was finished.

Biomechanics

Anteroposterior translation

The intact contralateral knees demonstrated a mean AP drawer displacement of 3.25 ± 0.38 mm. At 3 weeks following surgery, the average AP translation of the reconstructed right knees increased to 8.52 ± 1.04 mm (162.15%, $p < 0.005$). Measurements at 6 and 12 weeks still indicated significantly more translation than the intact knees, but demonstrated reduced amounts of AP translation compared to 3 weeks. Finally, at 24 weeks, the ACL reconstruction gained further stability with a remaining difference of 3.47 ± 1.8 mm when compared to the intact contralateral knees (increase of 106%, $p < 0.05$), (Fig. 7).

Drawer-stiffness

The drawer stiffness of the intact left knees was 33.38 ± 4.7 N/mm. At 3 weeks, the stiffness of the operated knees was significantly lower when compared to the

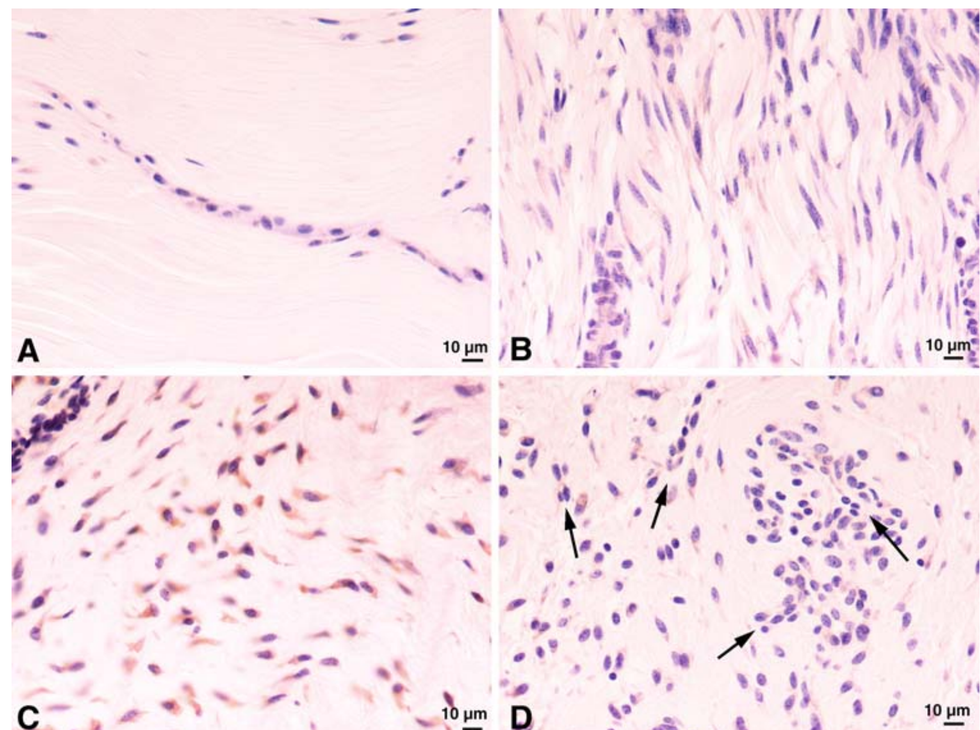

Fig. 4 Immunohistochemistry of VEGF in the transplant during the graft remodeling for 3 (**a**), 6 (**b**), 12 (**c**) and 24 (**d**) weeks. The ingrowing connective tissue cells become positive (**a**, **b**) and synthesize the highest concentrations of VEGF after 12 weeks (**c**). The pericytes around the vessels (lined by *arrows* in **d**) contain the product even after 24 weeks. Paraffin, H&E staining

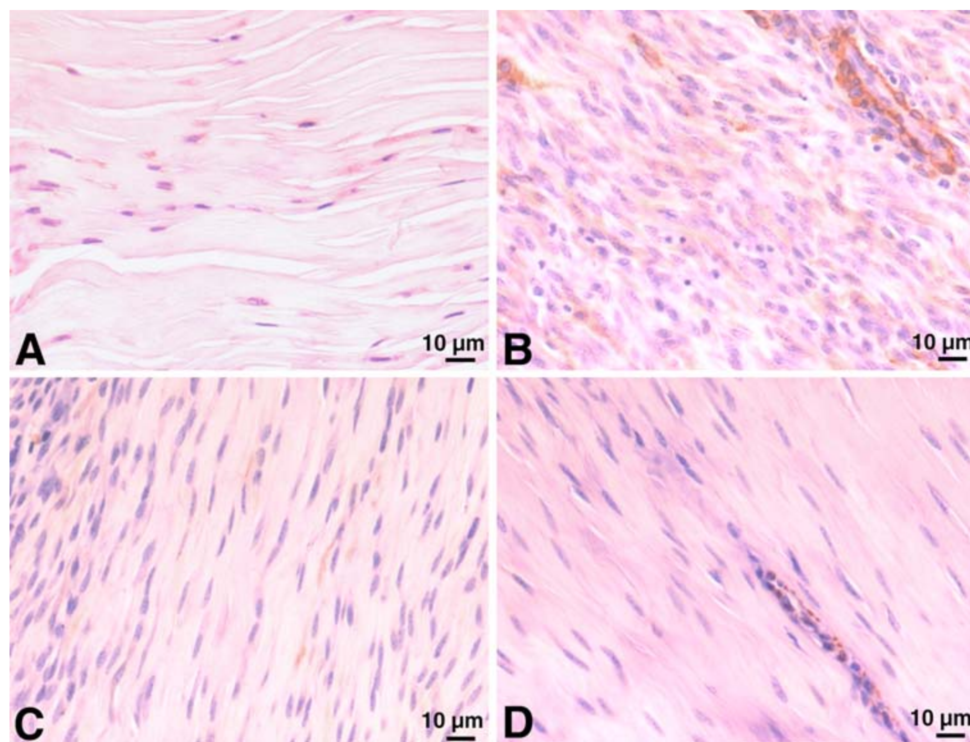

Fig. 5 Comparison of the SMA-positive cells in the graft after 3 (**a**), 6 (**b**), 12 (**c**) and 24 (**d**) weeks. SMA-positive myofibroblasts are present during their repopulation after 3 weeks (**a**) and are proliferating intensely after 6 weeks (**b**). During the remodeling of the extracellular matrix the amount of SMA is diminished after 12 weeks (**c**) and remained only positive in the smooth muscle cells of the vessels after 24 weeks (**d**). Paraffin, H&E staining

intact ACL (21.41 ± 4.29 N/mm, $p < 0.05$). During the remaining course of the remodeling process, the stiffness of the graft increased significantly to 30.87 ± 1.46 N/mm at 24 weeks ($p < 0.005$). At 24 weeks, the remaining side-to-side difference was not significant and the stiffness of the operated knees equaled that of the intact knees. (Figure 8).

Cross sectional area

The mean cross sectional area of the intact ACLs was 23.39 ± 3.51 mm^2. There was a significant increase in the cross sectional area of the grafts throughout the remodeling process from 25.11 ± 7.25 mm^2 at 3 weeks to 51.07 ± 14.9 mm^2 at 24 weeks ($p < 0.005$). Table 1.

Discussion

The aim of the present study was to investigate the graft remodeling process following an ACL reconstruction during growth, both histologically and biomechanically. The process of graft remodeling following the transplantation of an autologous, non-vascularized graft has been described in several previous adult animal studies [2, 3, 8, 22, 30]. Today it is widely accepted, that even a fully remodeled graft displays inferior histological and biomechanical properties and does not undergo a process of full “ligamentisation” [2, 15]. Previous studies have subdivided the biological responses of the graft following an ACL reconstruction into the phases of avascular necrosis, revascularization, cellular proliferation, and matrix remodeling [1, 8]. In our study of skeletally immature sheep, the qualitative analysis of the H&E stained sections displayed similar stages throughout the healing process at 3, 6, 12 and 24 weeks. This is reflected by the quantitative histomorphometric analysis according to the system of Weibel [27].

While the stages of graft remodeling in our study were similar to those of previous adult studies, the timeframe seems to be different in the present study of immature sheep. Weiler in his study on graft remodeling found only few newly formed vessels at 6 weeks following surgery and its highest density at 12 weeks [30]. In contrast, our grafts demonstrated the highest vascularity at 6 weeks. The revascularization of the graft is a crucial step in the remodeling process as it enables cell infiltration from the blood system, which is thought to be essential for tissue healing [11]. However, tissue degradation caused by vascular ingrowth may be an important factor contributing to the decrease of tensile strength of the graft during the remodeling phase [20, 33]. It has been shown in a model of the healing rabbit medial collateral ligament, that such histological “flaws” are associated with reduced biomechanical properties of the ligament [25]. Recently, it has also been shown in an ovine model of ACL reconstruction, that the local administration of VEGF to the graft increases its vascularity, but results in a significantly larger antero-posterior translation when compared to untreated grafts [33]. It might therefore be beneficial, if the highest

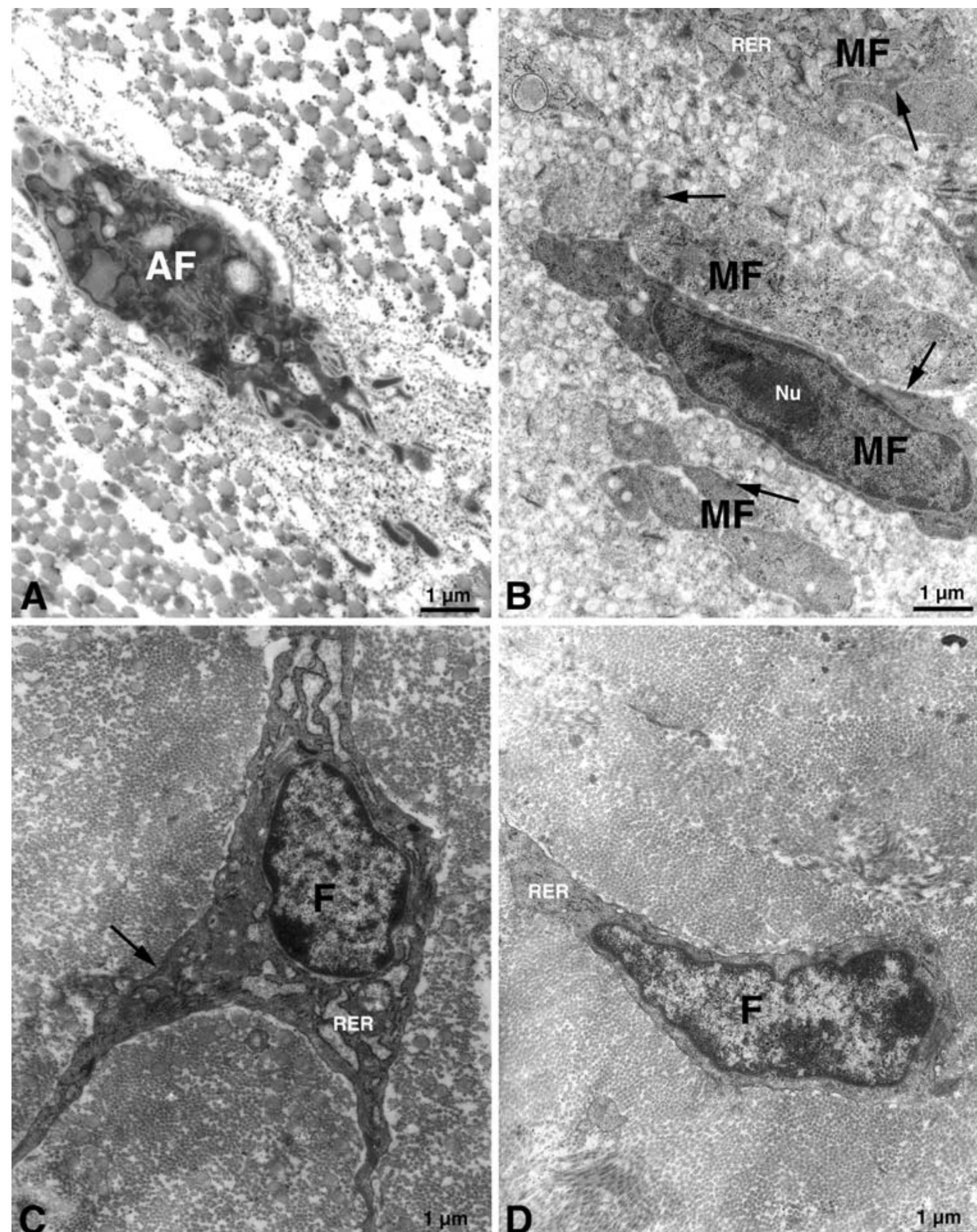

Fig. 6 Ultrastructure of the extracellular matrix of the graft during the healing process after 3 (**a**), 6 (**b**), 12 (**c**) and 24 (**d**) weeks. The graft after 3 week consists of apoptotic cells (*AF*) embedded between thick collagen fibrils and dots of aggregated proteoglycans. The proliferating myofibroblasts (*MF*) are characterized after 6 weeks (**b**) by nucleoli (*Nu*) inside their nuclei and enormous rough endoplasmic reticulum (*RER*). Both are responsible for the systematic remodeling of the extracellular matrix. Their extensive actin network (*arrows* in **b**) demonstrates their ability to actively contract and to withstand the longitudinal stress along the graft. After 12 weeks (**c**) the fibroblasts are intermingled with long processes between the newly formed thin collagen fibrils, whereas the thick collagen fibrils are degraded continuously. The passive mechanic strength is responsible for diminishing the actin cortex (*arrow* in **c**). After 24 weeks the graft consists of parallel arranged and densely packed thin collagen fibrils. Single fibroblasts with sparse biosynthetic activity lie in-between. Epon, staining with uranyl acetate and lead citrate, transmission electron microscopy

vascularity is reached early following surgery and decreases soon, within the period of protected rehabilitation.

In the present study, fibroblasts stained strongly positive for VEGF within the first 12 weeks. Immunostaining for VEGF has been used in a previous adult sheep study on the graft remodeling process following soft tissue ACL reconstruction [20]. Similar to our study, Peterson et al. found the strongest VEGF immunostaining within the first 12 weeks after surgery and a decrease thereafter. They concluded, that VEGF is involved in the complex process of graft remodeling following ACL reconstruction [20]. However, the presence of VEGF positive fibroblasts at 12 weeks exceeds the period of highest vascularity at 6 weeks. Thus, other angiogenic factors, such as a reduced oxygen tension, have to be considered to be even more potent in this initially necrotic tissue.

The presence of SMA containing myofibroblasts in the early stages of graft remodeling has been documented in a previous study [18]. These myofibroblasts are thought to be involved in the earliest stages of graft remodeling and are also present in the native adult ACL and tendon [18, 30]. In the present study, 6 months following surgery SMA positive cells were only present adjacent to vessels within the graft. This is again in contrast to a previous study, where myofibroblasts could be found even 104 weeks following surgery [30]. It remains unclear whether this finding demonstrates a superior remodeling process in skeletally immature sheep. The electron microscopic results

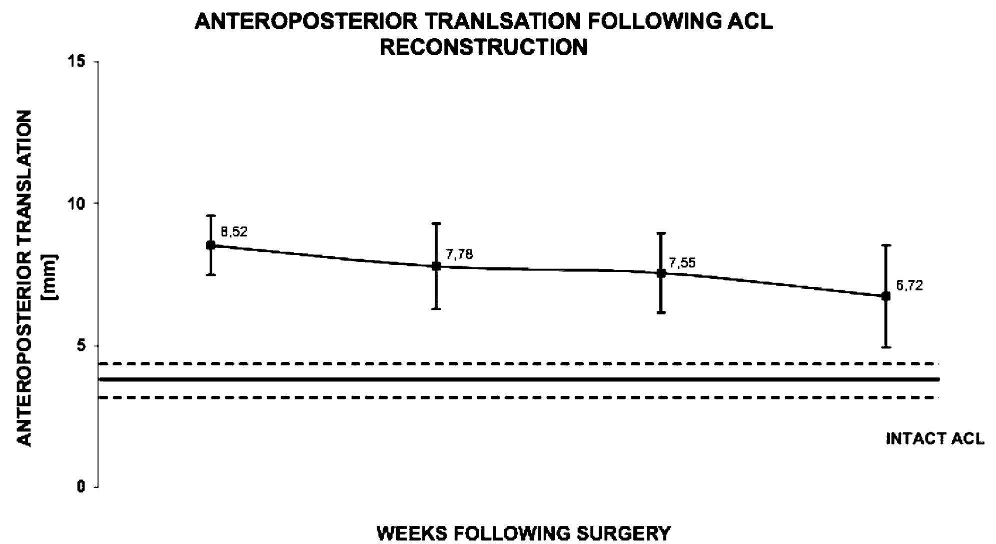

Fig. 7 Biomechanical evaluation of the anteroposterior translation of the ACL reconstructed knee. The horizontal line demonstrates the mean value for AP displacement of the intact contralateral knees and the dotted lines the corresponding standard deviation

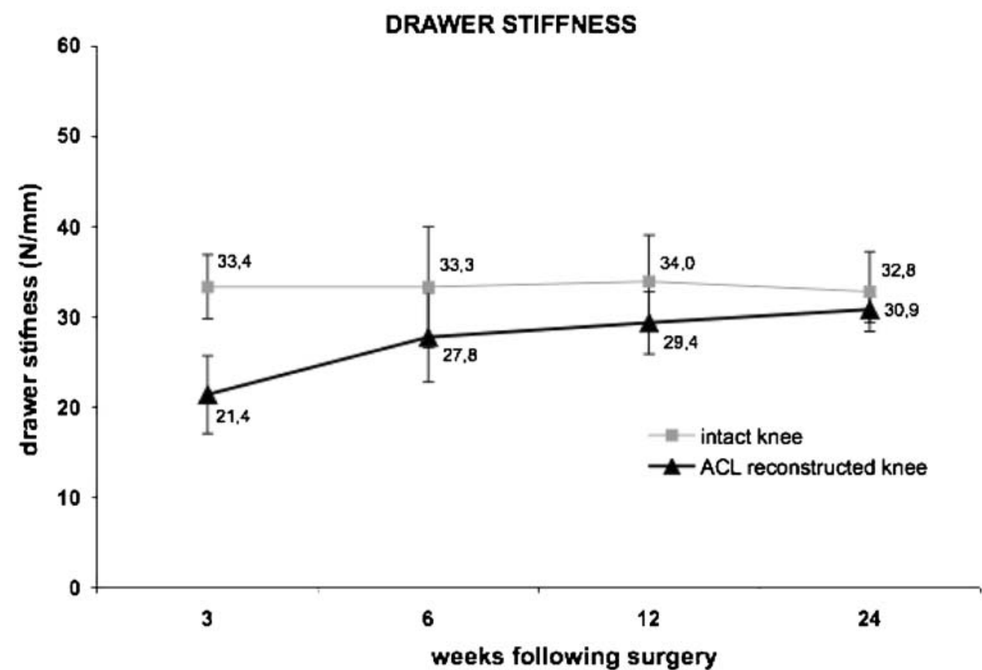

Fig. 8 Biomechanical evaluation of the stiffness (N/mm) of the native ACL and the ACL reconstructed knee and their corresponding standard deviations

Table 1 Results of biomechanical testing, mean, SD and significances

Weeks following surgery	AP translation (mm)	Stiffness (mm/N)	Cross sectional area (mm^2)
3	8.52 ± 1.04[a]	21.41 ± 4.29[a]	25.11 ± 7.25
6	7.78 ± 1.50[a]	27.78 ± 4.94	36.64 ± 14.84
12	7.55 ± 1.40[a]	29.37 ± 3.46	39.53 ± 13.42[a]
24	6.72 ± 1.80[a]	30.87 ± 1.46	51.07 ± 12.71[a]
Intact ACL	3.25 ± 0.38	33.37 ± 3.57	23.45 ± 3.39

[a] Significantly different from the intact ACL

demonstrated a shift toward smaller fibrils, which is in line with previous reports [7, 28]. Thus, there does not seem to be a beneficial effect of skeletal immaturity to the type of collagen produced during graft remodeling.

Finally, the biomechanical evaluation is again in agreement with a number of previous studies on ACL reconstruction in adult ovine and caprine models (Table 2). Full restoration of AP translational stability following ACL

Table 2 Previous studies on ACL reconstruction with biomechanical evaluation of knee stability in sheep

Author	Animal	Graft	Fixation	AP translation at 6 months (mm)
Cummings [9]	Goat	BPTB	Interference screw, Steinmann pin	8 (isolated anterior translation)
Weiler [29]	Sheep	BPTB	Interference screw	3.81
Schindelheim [21]	Sheep	BPTB	Staple	4.0
Present study	Sheep	Soft tissue graft	Endobutton, Suture Washer	3.47

Comparison of AP translations 6 months following surgery

reconstruction is difficult to achieve in these large animal models [9]. Jackson et al. [13] even reported AP translations at 6 weeks following surgery that were more similar to joints that had the ACL transected, but not reconstructed, than to ACL intact knees. A comparison of our results to similar studies is difficult, as the grafts used, the fixation techniques and the testing methods may be different. However, Table 2 provides an overview of previous studies and allows comparing AP translations at six months following surgery. Compensative hypertrophy of the graft may interfere with the anteroposterior translation and is again in line with previous studies as are the stiffness data in our study [28, 29].

Cummings et al. [9] in their studies could show that an ACL reconstruction in ovine and caprine models always results in inferior biomechanical results when compared to human reconstructions. This has to be kept in mind as a limitation of the present study when trying to transfer animal studies to clinical practice. The strengths of the present work are that it is the first study on graft remodeling following an ACL reconstruction during growth. Multiple methods have been combined to understand the interaction between morphology and functionality.

The management of injuries of the ACL during growth still poses a challenge to the orthopaedic surgeon. The data of the present animal study may allow for the statement, that the biology of the graft remodeling process is superior to that of adult sheep studies. The resulting biomechanical properties may have some clinical relevance. However, they do not allow to advocate as a more accelerated rehabilitation protocol following ACL reconstruction in these young patients.

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