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Chapter 6

Functional Changes in Articular Cartilage after Meniscal Allograft Transplantation. A Quantitative Histochemical Evaluation in Rabbits

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Submitted
Abstract

Purpose: To evaluate quantitatively functional changes in articular cartilage after meniscus transplantation in rabbits.

Type of study: Experimental study.

Methods: Thirty rabbits were divided into 5 groups. Group A and Group C were subjected to meniscectomy only. Group B and Group D underwent meniscal transplantation immediately after meniscectomy. Group E had delayed transplantation at 6 weeks after meniscectomy. Six nonoperated knees served as controls. Functional changes in articular cartilage were examined at 6 weeks (Groups A, B) and 1 year (Groups C, D, E, controls) after surgery by measuring lactate dehydrogenase (LDH) activity in chondrocytes as a measure of their vitality and proteoglycan content of the extracellular matrix as a measure of its quality.

Results: At 6 weeks and 1 year follow-up, no significant differences were found between the immediate transplant group and postmeniscectomy group. The delayed transplant group showed a significantly decreased proteoglycan content as compared with the postmeniscectomy group. No significant differences in cellular LDH activity were found between the immediate transplant group and postmeniscectomy group at 6 weeks and 1 year. However, the delayed transplant group showed a significant decrease in LDH activity as compared with the postmeniscectomy group.

Conclusions: Immediate meniscal transplantation in rabbits did not significantly reduce degenerative changes of articular cartilage in comparison with meniscectomy on a short-term and long-term basis, whereas delayed transplantation lead to more degenerative changes than meniscectomy.

Clinical relevance: Before meniscus transplantation can be considered as an alternative to meniscectomy in clinical practice, it has to be determined whether this procedure has any protective effect on articular cartilage on the long term.
Introduction

Experimental and clinical studies have revealed an increased incidence of degenerative changes of articular cartilage in the knee joint after meniscectomy.\textsuperscript{1-4} Meniscal allograft transplantation is used to replace the native meniscus in an effort to prevent these degenerative changes. Application of meniscal allografts in clinical practice has progressed to a point where relief of pain may be expected on a short-term basis, but long-term protective effects on articular cartilage after meniscal transplantation have not yet been demonstrated conclusively.\textsuperscript{5,6} In experimental histological studies, it has been shown clearly that knees receiving a meniscal transplant immediately after meniscectomy developed fewer degenerative changes than knees that underwent meniscectomy only.\textsuperscript{7-10} On the other hand, it has been reported that meniscal transplantation with an interval between meniscectomy and implantation may even lead to more degenerative changes than meniscectomy only.\textsuperscript{7-8} However, these studies evaluated the structural but not the functional changes in articular cartilage after meniscal transplantation.

The aim of the present study was to evaluate with quantitative histochemical means functional changes in the remaining articular cartilage in rabbit knees following meniscal transplantation on a long-term basis. Several studies have demonstrated that activity of chondrocytes and levels of proteoglycans in articular cartilage are decreased in osteoarthritic joints.\textsuperscript{11-14} In the present study, vitality of chondrocytes is determined quantitatively by measuring cellular lactate dehydrogenase (LDH) activity, which is considered to be an indicator of biosynthetic capacity in chondrocytes.\textsuperscript{11,15,16,17} The quality of the extracellular matrix of cartilage was determined by measuring quantitatively the proteoglycan content in cartilage on the basis of the intensity of Safranin O staining.\textsuperscript{13,18}

Material and Methods

Thirty mature female New Zealand white rabbits were divided into 5 groups of 6 animals each. The right knees were operated on. Groups A and C were subjected to medial meniscectomy. Groups B and D underwent meniscal allograft transplantation immediately after medial meniscectomy. In group E, delayed meniscal allograft transplantation was performed at 6 weeks after meniscectomy. Six weeks after operation, groups A and B were sacrificed by intravenous injection of sodium pentothal. The other groups were sacrificed at 1 year after (the first) operation. The nonoperated left knee joints of 6 rabbits (3 animals of group D and 3 animals of group E) were selected at random before operation to serve as control group. Approval of this study was obtained from the local ethical committee for animal experiments.
Surgical Technique
All animals were premedicated with an intramuscular dose of ketamine (50 mg/kg) and xylazine hydrochloride (8 mg/kg). Rabbits were anaesthetized by inhalation of halothane, oxygen, and nitrous oxide via a mask. After disinfection of the skin, the right knee was approached by a medial parapatellar arthrotomy through the patellar fat pad and gentle lateral displacement of the extensor mechanism using a sterile technique. Medial menisci, including the meniscal wall, were resected sharply along the periphery, dividing the coronary ligament, and were detached from its anterior and posterior tibial bone attachments at the junction of the ligamentous attachment and the meniscal fibrocartilage. Care was taken to avoid injuring collateral and cruciate ligaments. Harvested meniscal grafts were immersed in sterile saline. Acute transplantation was performed immediately after meniscectomy by suturing an appropriately sized fresh allograft, selected from previously removed menisci, in the recipient bed using 3-6-0 polypropylene sutures. The anterior and posterior horns of the graft were reattached anatomically to the appropriate ligamentous structures without using bone plugs; the midportion was sutured to the medial collateral ligament. Allograft position and mobility were controlled in knee flexion and extension, and under valgus and varus stress. The capsule, periarticular tissues, and skin were closed with interrupted 3-0 polyglactin sutures. Delayed transplantation was performed by a 2-step procedure with an interval of 6 weeks between meniscectomy and transplantation using an appropriately sized fresh allograft immersed in sterile saline. The surgical procedure was as described above. All allografts were obtained from different animals used in this study and were re-implanted within 2 hours after harvesting. After operation, animals received subcutaneous analgesic (buprenorfine, 0.05 mg/kg) during 24 hours and were able to move and exercise freely. Antibiotic prophylaxis was given for 72 hours (enrofloxacin 5%, 5 mg/kg), perioperatively.

Preparation of Cryostat Sections
After dissection of the knee joints, skin and superficial muscle layers were removed and the joints were immediately embedded in an aqueous solution of 8% (weight per volume) gelatin white (Sigma, St. Louis, MO, USA) and frozen slowly in liquid nitrogen as previously described. Sections were cut on a motor-driven cryostat fitted with a retraction microtome (Bright, Huntingdon, UK) and a tungsten carbide-tipped knife (Spikker, Zevenaar, The Netherlands) at a cabinet temperature of -25°C. The angle between knife and surface of the tissue block was 8°. After the block was trimmed to the desired level, transparent tape (Scotch tape 800; 3M, St. Paul, MN, USA) was fastened with a stiff brush onto the section surface of the block. The microtome knife then cut underneath the tape at a low but constant speed and 10-μm thick sections attached to tape were obtained without loss of tissue integrity. Pieces of tape with adherent sections were fixed on glass slides with ordinary tape. Coronal serial sections of the intact knee joints were prepared including menisci,
femur, and tibia. Sections were made in this plane to allow for comparative observations in medial and lateral compartments of the knees in each section.

**Histochemical Staining**

From each knee joint, 1 section was stained with Safranin O (Merck, Darmstadt, Germany) (Figure 1). To ensure that sections were taken from the same position of the knee in all animals, they were selected on the basis of the presence of sutures attaching the transplanted meniscus to the collateral ligament. These sections were air-dried for 60 minutes at room temperature and subsequently fixed in 4% paraformaldehyde (Merck) and 1% glutaraldehyde (Merck) in cacodylate buffer (0.1 M, pH 7.4) for 30 minutes at room temperature. After rinsing in distilled water, sections were stained in a solution of 300 mg Safranin O in 100 ml aqueous 0.125 N HCl solution, pH 1.0, for 30 minutes at room temperature. Then, sections were rinsed thoroughly in distilled water, air-dried, and embedded in euparal.

LDH activity was stained in another randomly chosen section from each knee joint that was adjacent to the section used for Safranin O staining (Figure 1). Sections were air-dried (10 minutes, 37° C) and then incubated at 37° C for 5 minutes using preheated incubation medium according to the method described by Van Noorden and Vogels and Van Noorden and Frederiks. The medium consisted of 100 mM phosphate buffer, pH 7.45, containing 18% polyvinyl alcohol (Sigma), 5 mM azide, and 5 mM tetranitro blue tetrazolium chloride (tetranitro BT; Serva, Heidelberg, Germany). Tetranitro BT was dissolved in equal amounts of dimethylformamide and ethanol before addition to the incubation medium giving a final concentration of each solvent of 2%. LDH activity was demonstrated with the addition of 150 mM sodium L-lactate (Serva), 3 mM NAD\(^+\) (Boehringer, Mannheim, Germany), and 0.32 mM 1-methoxyPMS (Serva). The reaction was stopped immediately by washing sections in tap water of 60° C to remove the viscous incubation medium. Sections were mounted in glycerol-jelly. For control incubations, lactate was omitted from the incubation medium as described by Van Noorden and Vogels. All control incubations resulted in completely unstained sections.

**Quantitative Analysis**

Safranin O-stained sections and LDH activity-stained sections were analyzed quantitatively using the QWinPro image analysis software (Leica, Cambridge, UK) in the interactive mode. Illumination of the sections was performed with white light. Articular cartilage of femur and tibia were delineated by hand and the cartilage area was taken as 100%. Then areas of the articular cartilage that were considered to be positive for Safranin O staining were delineated manually after a threshold was selected that discriminated areas negative and positive for Safranin O staining (Figure 1). Safranin O-positive areas were calculated as percentages of total areas of cartilage matrix.
Chondrocytes positive for LDH activity were delineated automatically using a constant threshold. This constant threshold could be used because of the large differences of absorbance between LDH-positive chondrocytes and extracellular matrix or dead LDH-negative chondrocytes (Figure 1).

**Statistical Analysis**
Data were statistically analysed using the nonparametric Kruskal-Wallis test in multiple comparisons between different groups, followed by post-hoc comparisons with the Nemenyi test. Statistical significance was set at $p \leq 0.05$.

**Figures 1A-D.** Photomicrographs of articular cartilage of the medial tibial plateau in rabbit knees of a control knee (A,B) and a knee showing degenerative changes (C,D). (A) Safranin O staining in control; (B) Lactate dehydrogenase activity in control; (C) Safranin O staining at 1 year after meniscectomy; (D) Lactate dehydrogenase activity at 1 year after meniscectomy. (c, cartilage; js, joint space; b, bone; *, areas showing extracellular matrix with decreased content of proteoglycans; **, areas showing chondrocytes virtually without LDH activity; arrows indicating surface articular cartilage. Original magnification x40).
Results

Macroscopical Assessment of Menisci
None of the menisci showed extrusion. Capsular ingrowth was observed in all menisci that were transplanted immediately after meniscectomy. Six weeks after transplantation, 2 of 6 meniscal allografts in group B showed degenerative changes. One year after immediate transplantation (group D), degenerative changes were found in 3 of 6 transplants. After delayed transplantation (group E), 2 of 6 menisci were completely degenerated after 1 year follow-up. All other transplants in group E healed to the periphery, but all showed degenerative changes and shrinkage. One of these transplants developed a calcification in the anterior horn. Evidence of meniscal regeneration was observed in 5 of 6 rabbits at 6 weeks after meniscectomy without transplantation (group A) and in 3 of 6 rabbits at 1 year after meniscectomy only (group C). These observations were not correlated with the subsequent analysis of the articular cartilage.

Changes in Articular Cartilage
Figure 1 shows Safranin O and LDH activity staining in control knees and knees at 1 year follow-up after meniscectomy. It is clear that the operation resulted in reduced amounts of cartilage. The following quantitative analysis has been performed to analyse the quality of the remaining cartilage. The mean decrease in Safranin O-staining intensity and LDH activity in tibial and femoral articular cartilage in each group are presented in the Figures 2 and 3. In general, changes in quality of extracellular matrix and vitality of chondrocytes due to treatment were similar in cartilage of the tibial plateau and femoral condyle. Control joints showed hardly any areas of cartilage with decreased Safranin O staining, whereas joints of animals in groups A-D showed 5-15% of the remaining cartilage with decreased amounts of proteoglycans. Delayed transplantation resulted in over 30% of the area of the remaining cartilage to be affected. Nine percent of the cartilage in the control group showed LDH activity. The delayed transplant group showed LDH activity in 4% of the cartilage. The differences between groups A-D and the control group were less distinct.

Statistical Analysis
All groups of animals that had been operated on demonstrated a significant decrease in the area of the cartilage with healthy amounts of proteoglycans as compared with the control group (p < 0.01 for both the medial tibial plateau and femoral condyle in groups A-E). Knee joints examined at 1 year after meniscectomy and 1 year after immediate transplantation did not show a significant further decrease in proteoglycan content when compared with knee joints examined at 6 weeks after meniscectomy only and 6 weeks after immediate transplantation, respectively. No significant differences in proteoglycan content were found between the immediate transplant
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Figure 2. Mean percentage area ± standard deviation of articular cartilage showing decreased Safranin O staining of the medial tibial plateau (MTP) and the medial femoral condyle (MFC) for the nonoperated group (control), group A (6 weeks after meniscectomy), group B (6 weeks after meniscal transplantation), group C (1 year after meniscectomy), group D (1 year after immediate meniscal transplantation), and group E (1 year after meniscectomy followed by delayed meniscal transplantation at 6 weeks afterwards).

Figure 3. Mean percentage area ± standard deviation of healthy chondrocytes on the basis of their LDH activity in articular cartilage of the medial tibial plateau (MTP) and the medial femoral condyle (MFC) for the nonoperated group (control), group A (6 weeks after meniscectomy), group B (6 weeks after meniscal transplantation), group C (1 year after meniscectomy), group D (1 year after immediate meniscal transplantation), and group E (1 year after meniscectomy followed by delayed meniscal transplantation at 6 weeks afterwards).
group and the postmeniscectomy group at 6 weeks and 1 year follow-up. However, the delayed transplant group showed significantly more areas with decreased Safranin O staining than the postmeniscectomy group at 1 year follow-up (p < 0.01 for both the medial tibial plateau and femoral condyle).

Compared to the nonoperated control group, no significant differences in vitality of chondrocytes as determined by LDH-activity staining were found in the postmeniscectomy group and the immediate transplant group at 6 weeks and 1 year follow-up. However, delayed transplantation caused diminished vitality of chondrocytes (p < 0.05). No significant differences in LDH activity were found between the immediate transplant group and the postmeniscectomy group at 6 weeks and 1 year follow-up. The delayed transplant group showed a significant decrease in LDH activity as compared with the postmeniscectomy group at 1 year follow-up (p < 0.05 for both the medial tibial plateau and femoral condyle).

Discussion

The outcome of meniscal allograft transplantation must be examined on the basis of prevention of osteoarthritic changes in knees that underwent meniscectomy. To evaluate changes in articular cartilage, several scoring and grading systems have been used. However, these semiquantitative methods often suffer from bias. Quantification of changes in articular cartilage using image analysis allows a more objective comparison between specimens. Early degenerative changes of articular cartilage are characterized by decreased amounts of proteoglycans in the extracellular matrix and increased rates of proliferation of chondrocytes in clones. In later stages of the disease, hypocellularity is observed as well. However, the number of chondrocytes in osteoarthritic cartilage is not correlated directly with the metabolic activity of these cells. Therefore, numbers of chondrocytes are less relevant as a parameter for the quality of articular cartilage than metabolic activity of these cells. Indications of metabolic activity of chondrocytes can be obtained by determination of activities of key enzymes of particular metabolic pathways. Because of their low oxygen uptake, chondrocytes depend largely on anaerobic glycolysis for the reoxidation of NADH to NAD, which is required for biosynthesis of proteoglycans. LDH can be considered as a key enzyme for this anaerobic metabolism in chondrocytes. In mice that spontaneously develop osteoarthritis and closely resembles osteoarthritis in humans, Altman demonstrated a focal loss of LDH activity in chondrocytes in precisely those regions of cartilage where the disease starts to manifest. This was not due to death of chondrocytes because the cells were viable. This finding suggests that chondrocytes in osteoarthritic knees already show decreased enzyme activity before cell death occurs.

In the present study, quantitative cytochemistry has been used to evaluate the anaerobic metabolism of chondrocytes. Cryostat sections were used giving the
opportunity to investigate the metabolism in cartilage without loss of enzyme activity by fixation, demineralisation, or embedding of the tissue. In general, osteoarthritic cartilage shows a mild to moderate decrease in proteoglycan content proportional to the severity of the disease.\textsuperscript{24} Measuring differences in intensity of Safranin O staining using image analysis can be used as a simple quantitative histochemical method to follow changes in proteoglycan content of the articular cartilage.\textsuperscript{19} To determine whether meniscal allografts are effective in reducing degenerative changes of the articular cartilage as observed after meniscectomy, a comparison with meniscectomized knees at the same postoperative time is required.

In the present study, no differences in LDH activity in chondrocytes between the groups were observed at 6 weeks and 1 year follow-up, which can be considered as short term and long term, respectively. The results at 6 weeks suggest that after meniscectomy as well as after meniscal transplantation chondrocytes are as vital as in control cartilage. At 1 year follow-up, the metabolic activity of the chondrocytes after meniscectomy and after immediate transplantation is still similar as in the control cartilage, whereas the LDH activity of chondrocytes in the articular cartilage in the delayed transplant group is clearly decreased. The proteoglycan content was significantly decreased in all experimental groups as compared with the control group. At 6 weeks and 1 year follow-up, differences between the meniscectomy group and the group that underwent immediate transplantation were not observed. Approximately 10\% of the remainder of the cartilage showed decreased amounts of proteoglycans. However, levels of proteoglycans in the articular cartilage in the delayed transplant group were significantly decreased as compared to both the meniscectomy group and the immediate transplant group at 1 year follow-up.

Combining the changes in activity of the chondrocytes with the changes in proteoglycan content in the present study, it can be concluded that chondrocytes remain similarly active after meniscectomy and after immediate meniscal transplantation as in control cartilage. Nevertheless, at 6 weeks follow-up there is a net decrease in total proteoglycan content after meniscectomy whether or not followed by immediate transplantation. Probably, this is the result of release of proteoglycans from the cartilage. No further decrease in proteoglycan content was observed at 1 year in the meniscectomy group and in the group subjected to immediate transplantation. In the delayed transplant group, the reduced vitality of chondrocytes is accompanied by a depletion of proteoglycans in the remainder of the cartilage. It should be pointed out that the data provide insight in histochemical changes in the part of articular cartilage that is not lost by the osteoarthritic process. The amounts of destroyed articular cartilage were not included in the present study.

In a previous study by Rijk et al.,\textsuperscript{8}, no significant differences in structural changes were demonstrated between the meniscectomy group and the transplant group at 6 weeks follow-up. The results at 1 year after surgery showed clearly that knees that received a meniscal transplant immediately after meniscectomy developed fewer degenerative changes than knees that were subjected to meniscectomy only. In the current study, it is demonstrated that the remainder of the cartilage in meniscectomized
knees showed the same proteoglycan content and LDH activity as the articular cartilage in knees subjected to immediate transplantation at 1 year. Apparently, the rate of metabolic activity and the proteoglycan content in the remaining cartilage in meniscectomized knees did not decrease as compared with the immediate transplant group despite the increased structural damage. Rabbits that underwent delayed transplantation showed significantly more degenerative structural changes than rabbits in the postmeniscectomy group and a decrease in proteoglycan content and LDH activity in the remaining cartilage as well. Thus delayed transplantation not only results in more structural damage to the articular cartilage than meniscectomy, but the remaining cartilage is also of less quality.

Our findings are in agreement with those of Mankin et al., who reported that with increasing severity of degenerative changes, synthetic mechanisms in chondrocytes fail and cartilage is destroyed. Aagaard et al. stated that the altered shape of the condyles attributable to ridge formation after meniscectomy may cause a mechanical conflict between the transplant and the condyles, which then could be responsible for the increased degenerative changes in the delayed transplant group. In addition, the twofold insult to knees subjected to delayed meniscal transplantation where the cartilage was exposed to the effects of drying for a longer period, could be at least partly responsible for the poor outcome in this group. Cummins et al. reported that immediate and delayed meniscal allograft transplantation in rabbits at 3 months follow-up offer protection to the articular cartilage of knees after meniscectomy and that delayed transplantation may reverse initial degenerative changes. The current results suggest that when initial degenerative changes attributable to meniscectomy are reversed, it is only temporary. The findings of the present study strongly suggest that delayed transplantation is not a good clinical alternative to treat patients with symptomatic degenerative joint disease after meniscectomy.

The results from the present study cannot simply be extrapolated to the situation in humans. Whether human meniscal allograft transplantation can either prevent or postpone the onset of osteoarthritis remains to be established. Nevertheless, rabbit models have proven to be one of the better models for the human situation because of similarities in histological and biochemical aspects of rabbit and human articular cartilage. On the other hand, the weight-bearing profile in rabbit knees is different from that in human knees. Furthermore, surgical procedures in small animals such as the rabbit are rather difficult and this may introduce artefacts that are hard to interpret when comparing data obtained in small animals with those obtained in larger animals.

It can be concluded that immediate meniscal allograft transplantation in rabbit knees did not significantly reduce degenerative changes of articular cartilage when compared with meniscectomy only. Moreover, after delayed meniscal transplantation, the articular cartilage showed even more degenerative changes than after meniscectomy. Therefore, clinical application of delayed meniscus transplantation has to be considered with care.
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