OsteoArthritis and Cartilage (2005) **13,** 139–145

doi:10.1016/j.joca.2004.10.017

Osteoarthritis and Cartilage

International Cartilage Repair Society

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Increase in degraded collagen type II in synovial fluid early in the rabbit meniscectomy model of osteoarthritis[1]

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Summary

Objective: The objective of this study was to determine whether collagen type II breakdown products in synovial fluid (SF), detected by an enzyme-linked immunoassay, represent a useful marker for early events in osteoarthritis (OA) in the rabbit medial meniscectomy model.

Design: Complete medial meniscectomy was performed on the right knee joints of 32 rabbits. Balanced groups of rabbits were then sacrificed at 2, 4, 8, and 12 weeks post-surgery. An additional 8 unoperated and 11 sham-operated animals served as controls. SF lavages were performed on right and left knee joints of the same animals at sacrifice. The proteolytic epitope of type II collagen was monitored using an enzyme-linked immunoassay.

Results: Macroscopically visible surface fibrillation and focal erosions appeared as early as 2 weeks after meniscectomy in the femorotibial joint ($P < 0.01$). OA developed gradually during the later observation period, and then predominantly on the medial tibial plateau and medial femur. Significant histological alterations in cartilage, including a loss of proteoglycans, surface irregularities, and clefts, were detected at 2 weeks after meniscectomy ($P < 0.01$). Collagen type II epitope levels in SF lavage samples were elevated peaking at 2 weeks after meniscectomy ($P < 0.02$). Levels decreased at later time points, but they were still raised at 12 weeks ($P \leq 0.05$). Highly significant correlations were found between the SF collagen type II epitope levels and the macroscopic and microscopic scoring results (Spearman rho correlation coefficient, macroscopy—collagen type II epitope $r = 0.222$, $P = 0.025$; microscopy—collagen type II epitope $r = 0.436$, $P \leq 0.01$).

Conclusion: In this rabbit model of medial meniscectomy, levels of type II collagen fragments in SF appear to provide a useful marker of the early degenerative changes.

Key words: Articular cartilage, Osteoarthritis, Collagen type II, Medial meniscectomy, Rabbit.

Introduction

Meniscal damage is a frequent result of knee injury, in particular following traffic accidents and sports trauma[1,2]. It is well known that meniscectomy often leads to degenerative (posttraumatic) arthritis in patients and experimental animals[3–6].

Animal models are useful in osteoarthritis (OA) research because they provide insights on early cellular and molecular events. Complete medial meniscectomy in the rabbit leads to the development of OA[7–12]. This model has mostly been characterized at the macroscopic and microscopic level. An increase in collagen metabolism follows the meniscectomy early[9,11]. Medial meniscectomy is an animal model with a well defined starting point of the arthritic disease process and is highly relevant to the human patient as this procedure is performed in the human patient. In this regard, the medial meniscectomy model is different from other rabbit models, e.g., the Moskowitz model[13], the Hulth–Telhag model[14] or chemical models (e.g., Ref.[15]) which are artificial and not found in the human patient. Many details of this complete medial meniscectomy model of the rabbit remain to be studied.

After water, collagen type II is the principal component by weight of articular cartilage. It has been shown that damage to the collagen network is an early event in the development of OA, including in particular the rabbit model of medial meniscectomy[16–21]. There is a need for cartilage biomarkers that can provide an index of the course of the human disease, to monitor the effects of therapy and aid pharmacological studies. If biomarkers of early disease events can be found, they potentially would be useful in the diagnosis and monitoring of disease development.

[1]This study was supported by governmental grants (BMBF 01GG9851; NIH AR37318).
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Received 17 March 2004; revision accepted 22 October 2004.

Antibodies against specific degradation products of type II collagen have been developed (e.g., Atley[22]) for use in enzyme-linked immuno sorbent assays (ELISA) of different body fluids[23–25]. In this study, we have used the rabbit complete medial meniscectomy model to induce the early stages of OA. As this model has potential value in surgical and pharmacological studies, we then set out to determine whether collagen type II breakdown products in synovial fluid (SF) detected by an ELISA represent useful markers for early events in OA development.

Methods

SURGERY AND BODY FLUIDS COLLECTION

The study was performed with 51 male New Zealand White rabbits aged 10 months. X-rays were taken to ensure their skeletal maturity at the time of surgery. The rabbits were housed in individual cages and received standard food *ad libitum*. Anesthesia was induced with 80–100 mg/kg intra-muscular (i.m.) Ketavet (Pfizer GmbH, Karlsruhe, Germany) and 4–6 mg/kg i.m. Rompun 2% (Bayer AG, Leverkusen, Germany). For intra- and postoperative analgesia, 0.02–0.05 mg/kg Temgesic (Essex Pharma GmbH, Munich, Germany) was given i.m. before surgery, after surgery and 8 h later again.

In all operated animals, a medial parapatellar arthrotomy was performed on the right knee joint under sterile conditions. Thirty-two rabbits had an open meniscectomy with complete excision of the medial meniscus from the medial joint capsule and complete detachment from their ligamentous insertions. Eleven rabbits underwent a sham procedure in which the knee joint (including the fat pad) was fully opened and the meniscus was exposed as was done in the meniscectomized rabbits. Eight control rabbits were left unoperated.

Animals were sacrificed as follows: at 2 weeks (meniscectomy $n = 8$, sham $n = 7$, control $n = 2$), at 4 weeks (meniscectomy $n = 8$, sham $n = 2$, control $n = 2$), at 8 weeks (meniscectomy $n = 8$, sham $n = 2$, control $n = 2$), and at 12 weeks (meniscectomy $n = 8$, control $n = 2$) after surgery.

The study was approved by the Institutional Review Board and the local German government (Regierungspräsidium Darmstadt).

MACROSCOPY AND HISTOLOGY

Macroscopic grading was performed on all right and left knee joints. The nature of the lesions was classified as: 0 = normal, 1 = focal surface roughness, 2 = widespread surface irregularity, 3 = beginning surface fibrillation, 4 = severe surface fibrillation, 5 = beginning erosion, 6 = severe erosion, 7 = slight ulceration, and 8 = severe ulceration. In addition, the location of the lesions in the joint was recorded by a specific nine-area grid of each medial and lateral tibia, medial and lateral femur, and patella surface. This grid follows the proposed classification of the International Cartilage Repair Society[26] and was adapted to the rabbit knee. Always, the most severe lesion in an area of the grid accounted for the score. The macroscopic grading was performed in a blinded manner in consensus by two observers at the time of dissection.

Four micrometer sections of the central medial and lateral tibial plateaus were stained with hematoxylin & eosine and safranin O (SO). Histological grading was performed with a dedicated grading system accounting for the pathologic alterations of proteoglycan content, matrix structure, cellularity, tidemark duplication, and osteophyte formation (modified Mankin score[27], see Table I).

SF SAMPLING

At weeks 2, 4, 8 and 12 after surgery, SF lavages were performed on right and left knee joints. 1.5 ml of sterile Ringer's solution was injected and the knees moved 10 times through a full range of motions. The aspirated lavage volumes were recorded. SFs were centrifuged at 3000 rpm in a microcentrifuge prior to freezing. Samples were kept frozen at −70°C until analysis.

IMMUNOASSAY OF THE CROSS-LINKED C-TELOPEPTIDE DOMAIN OF TYPE II COLLAGEN (COL2CTX) IN SFs

Collagen type II was measured in SF lavage samples using an ELISA. The ELISA uses the antibody 2B4 raised against the "EKGPDP" amino acid sequence, which recognizes a proteolytic epitope in the C-telopeptide cross-linking domain of type II collagen. This assay has been previously reported[22–24,28]. Sixty microliters of sample was used per measurement. Samples were assayed in duplicate. Briefly, microtiter plates (Nunc Maxisorp) were coated with the synthetic peptide, EKGPDP, linked to bovine serum albumin (BSA) by glutaraldehyde. SF lavage samples were diluted 1:4 with assay buffer (50 mM Tris, 0.2% (v/v) Tween 20, 0.2% (w/v) BSA, pH 7.8). The synthetic peptide, EKGPDP, after treatment in solution with glutaraldehyde to derivatize the α- and ε-amino groups was used as an assay calibrator. Samples or calibrators were incubated with mAb 2B4 conjugated to horseradish peroxidase for 16 h at 4°C. Color was developed using the peroxidase substrate 3,3′,5,5′-tetramethyl benzidine and the peroxidase reaction was stopped with 1.0 M sulfuric acid and the optical density measured at 450 nm. Results were expressed as ng of peptide equivalent per ml of joint fluid. The intra-assay coefficient of variation was $<10\%$ and the inter-assay coefficient of variation was $<15\%$ in these rabbit samples.

STATISTICAL ANALYSIS

The macroscopic data were analyzed statistically by comparing specific areas of the knee joint and area sums across all joint sites (Wilcoxon and Mann–Whitney *U* test).

Data from the SF measurements were compared intra-individually using the Wilcoxon test. Inter-individual comparisons (using the Mann–Whitney *U* test) were only performed based on the difference between operated and non-operated contralateral knees because assays of control, sham, and meniscectomy groups were not performed on the same days. Data were calculated in absolute amounts (ng per ml of lavaged fluid and ng per total knee joint). Macroscopic, microscopic and SF data were compared by nonparametric methods using the Spearman rho correlation coefficient. The SPSS® (version 11, Chicago, IL) package was used for statistical calculations.

Results

GENERAL

None of the operated, sham-operated or unoperated animals ($n = 51$) showed signs of infection during the

Table I
Characteristics of the histologic score as used for evaluation (modified Mankin score[27])

Superficial zone	SO-staining	0/1/2/3	Normal/focal loss of staining/widespread loss of staining/none
	Cartilage structure	0/1/2/3/4/5	Normal/focal surface irregularities/widespread irregularities/beginning fibrillation/progressive fibrillation/tissue loss
	Clefts	#	Number
	Cells	0/1/2	Normal/slightly decreased/decreased
Transitional zone	SO-staining	0/1/2/3	Normal/focal loss of staining/widespread loss of staining/none
	Clefts	#	Number
	Cells	0/1/2	Normal/slightly decreased/decreased
	Cartilage loss	0/5	No/yes
Upper radial zone	SO-staining	0/1/2/3	Normal/focal loss of staining/widespread loss of staining/none
	Clefts	#	Number
	Cells	0/1/2	Normal/slightly decreased/decreased
	Cartilage loss	0/5	No/yes
Deep radial zone	SO-staining	0/1/2/3	Normal/focal loss of staining/widespread loss of staining/none
	Clefts	#	Number
	Cells	0/1/2	Normal/slightly decreased/decreased
	Cartilage loss	0/5	No/yes
Cloning		0/1	No/yes
Osteophytes		0/1/2/3	None/early/progressive/prominent

study, later confirmed by histopathological evaluation (see Wachsmuth *et al.*[29]). Apart from knee-joint effusions (see Fig. 1) and periarticular soft tissue edema, the postoperative course was uneventful for all animals.

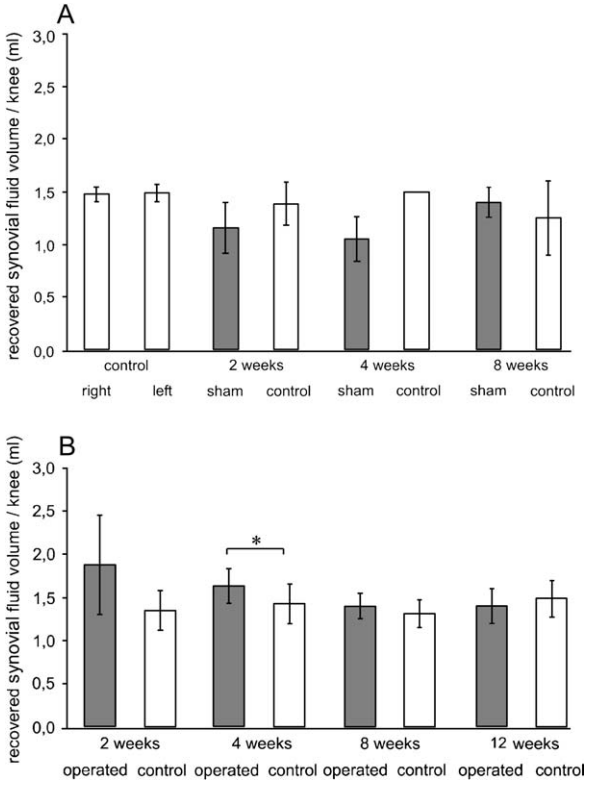

Fig. 1. Mean volumes (±SD) of SF lavaged (by injecting 1.5 ml Ringer's solution) from right (operated) and left (unoperated) rabbit knee joints. (A) Unoperated and sham-operated control groups and (B) meniscectomy groups during the study period (2, 4, 8, and 12 weeks). The return volumes of left knees (control) did not change within the study period. Due to synovial effusion, lavage volumes were significantly higher at 4 weeks after meniscectomy when compared to the control knee (paired Student's *t* test* = $P < 0.01$).

MACROSCOPIC EVALUATION

Macroscopically, alterations in the medial tibial plateaus and medial femoral condyles were more frequent after meniscectomy compared with contralateral knees of each operated animal and control (non-operated) animal knees. Macroscopically visible surface fibrillation and focal erosions were observed as early as 2 weeks after meniscectomy in the femorotibial joint (Fig. 2), but not on the patella surface. These characteristics of OA progressed gradually

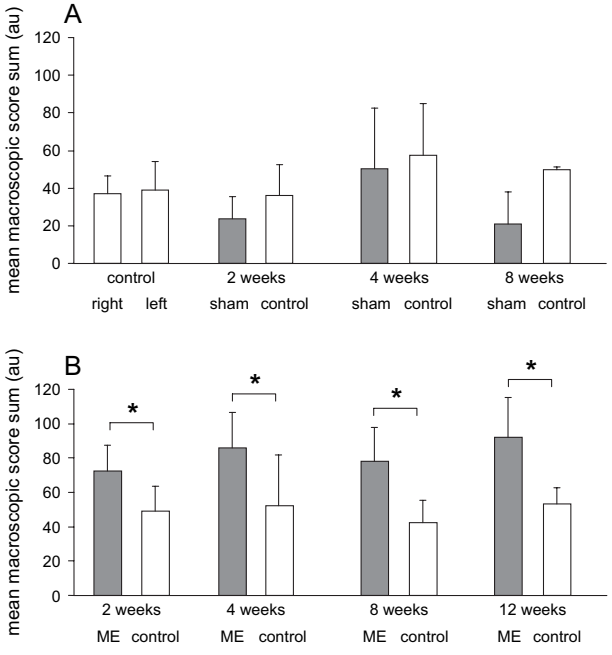

Fig. 2. Combined macroscopic score results (means ± SD) for all knee joint surfaces after complete medial meniscectomy. (A) Unoperated (=control) and sham-operated control groups and (B) meniscectomy (ME) groups during the study period (2, 4, 8, and 12 weeks). In this mild, slowly progressing model, cartilage surface changes are already evident at 2 weeks after surgery (Wilcoxon, * = $P < 0.01$ at all time points).

Table II
Number of areas per nine-area grid with macroscopically detected lesions at different time points after meniscectomy. We superimposed a nine-area grid on each joint surface[28]. No lesions were observed on patellar cartilage. Eight animals were evaluated at each time point

	Tibia medial	Tibia lateral	Femur medial	Femur lateral	Total
2 weeks	3	—	3	—	6
4 weeks	6	—	3	—	9
8 weeks	6	1	2	1	10
12 weeks	9	1	2	1	13

at later time points, predominantly in the vicinity of the medial tibial plateau and the medial femur (Table II). They were obvious when the operated knees were compared with their contralateral control knees.

The macroscopic scores of the pathological changes in the meniscectomized knees were statistically significant when compared to the contralateral knees of the same animals and to non-operated knees of control animals (Fig. 2). The scores for meniscectomized knees were already elevated 2 weeks after surgery and remained so throughout the 12 weeks of study. Summed scores for right (operated) knees were higher than those for left (unoperated) knees.

Table II summarizes the number of areas within the nine-area grid covering each joint site showing macroscopic lesions. The surface area of the macroscopic lesions increased with time. Sham-operated and unoperated rabbits did not develop OA-like lesions (data not shown). OA-like changes gradually progressed over 12 weeks, predominantly on the medial tibial plateau and medial femur.

HISTOPATHOLOGICAL EVALUATION

Significant histological changes were evident at both medial and lateral central tibial plateaus at 2 weeks after meniscectomy (see Fig. 3). Loss of SO-staining (indicating a loss of proteoglycans), surface irregularities, and clefts were observed (see Fig. 4). These characteristic signs of early OA were seen in animals only after meniscectomy. Higher histological scores were seen at medial surfaces when compared with the lateral joint surfaces at 2, 4, 8, and 12 weeks post-meniscectomy (see Fig. 3).

Microscopic and macroscopic score results of the central tibial plateaus demonstrated a significant correlation (Spearman rho; $r = 0.375$; $P < 0.001$; $n = 102$).

ANALYSIS OF COLLAGEN TYPE II FRAGMENTS IN SF

Recovered volumes of SF lavaged from right and left knee joints, sorted by type of surgery during the study period (2, 4, 8, and 12 weeks), are shown in Fig. 1. Volumes from left (non-operated) knees were relatively constant during the study period. Due to synovial effusion, volumes from the operated right knees were higher than those from left knees at the early time points (2 weeks ($P = 0.073$) and 4 weeks ($P = 0.008$)). Epitope levels in SF lavage samples were significantly higher and peaked at 2 weeks after meniscectomy on the operated side compared with control side (Fig. 5). At 2 weeks, the joint effusion also was evident both quantitatively as clinical observation and by recovered volume of fluid (Fig. 1). Generally, col2CTx epitope levels decreased at later time points, but they were still higher at 12

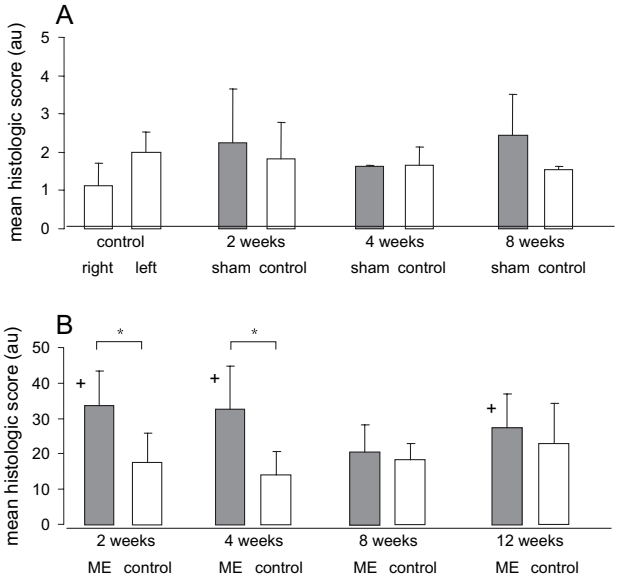

Fig. 3. Microscopic score results (means ± SD) of the tibial plateaus. (A) Unoperated and sham-operated control groups and (B) meniscectomy (ME) groups during the study period (2, 4, 8, and 12 weeks). (A) Differences between unoperated and sham-operated knee joints were not found. In this mild model, statistically significant cartilage changes (*) are detected between right and left tibial plateaus at 2 and 4 weeks after surgery (Wilcoxon, $P < 0.05$). Also, meniscectomized right knees were statistically significantly different (+) from unoperated knees at 2, 4, and 12 weeks (Mann–Whitney U, $P < 0.01$).

weeks compared to values from control knees [Figs. 5, 6(A)]. For the statistical analysis of col2CTx levels in SF, measured levels were compared uncorrected (ng/ml assayed lavage volume) and corrected (ng/knee joint) for differences in the return volumes. The correction was performed by multiplying the measured amount of epitope (ELISA result: ng col2CTx per ml assayed SF lavage) with the obtained return volume of each individual joint (ml SF lavage return). Differences between the uncorrected right and left knee joint SF lavage epitope levels (intra-individual differences) were significant at 2, 4, 8 (Wilcoxon test: $P < 0.02$), and 12 weeks (Wilcoxon test: $P = 0.025$). As shown in Fig. 6(A), differences between the corrected right and left knee joint lavage epitope levels (intra-individual differences) were also elevated at 2, 4, 8 ($P < 0.02$) and 12 weeks post-meniscectomy ($P = 0.050$). In the unoperated animals, there were no differences between the right and left knees in uncorrected and corrected [Fig. 6(B)] epitope levels.

CORRELATION OF SF LEVELS OF COLLAGEN TYPE II FRAGMENTS IN SF WITH MACROSCOPIC AND MICROSCOPIC SCORE RESULTS

The macroscopic score sums of all joint sites were correlated with the corrected and uncorrected SF levels. An overall correlation of $r = 0.193$ ($P = 0.052$) was calculated for uncorrected SF levels (ng/ml assayed lavage volume) and an overall correlation of $r = 0.222$ ($P = 0.025$) for corrected SF levels (ng/knee joint).

When the col2CTx levels were compared with the microscopic score results of tibial plateaus, again, a highly significant correlation ($P \leq 0.001$) was demonstrated for uncorrected ($r = 0.434$) and corrected ($r = 0.436$) SF levels.

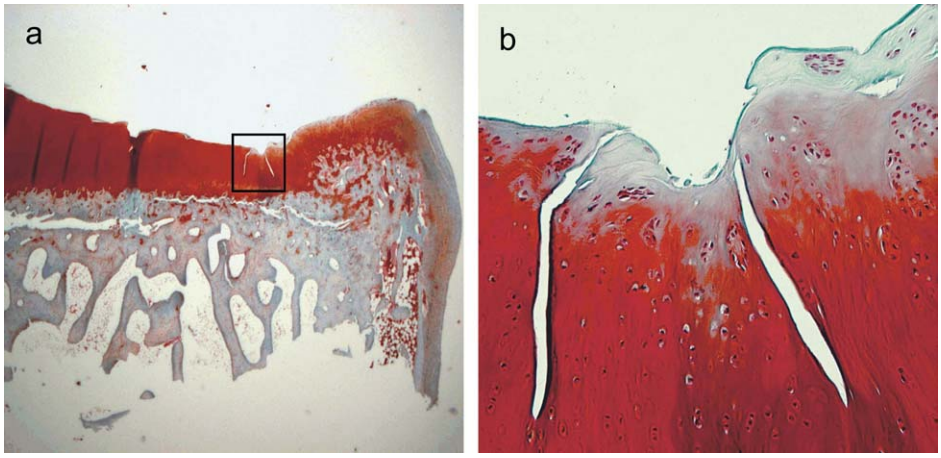

Fig. 4. SO-staining shows loss of proteoglycan, formation of osteophytes, clefts and chondrocyte cloning at 12 weeks after medial meniscectomy.

Discussion

There is a need for reliable cartilage biomarkers to detect the early phases of OA and for monitoring disease progression. Ideally, they should be non-invasive and responsive to beneficial effects of surgical and pharmacological interventions on osteoarthritic joints. At present, the beginning of OA can only be clearly defined in patients after injury or when animal models for (posttraumatic) OA are employed.

Meniscectomy has been described as a model of OA in several species[4,9,10,30]. Previous biomechanical studies have shown drastic changes in the loading pattern of articular cartilage after meniscectomy[31–33]. In the dog, sharp postoperative increases followed by later decreases in levels of proteoglycan markers have been described[30]. In this canine model, macroscopic OA-like lesions were evident within 12 weeks of surgery. In a recent human study, Lohmander *et al.*[24] found that collagen type II proteolytic epitope (col2CTx) levels in SF increased within hours after traumatic joint injury and remained elevated over many years.

In this rabbit meniscectomy study, macroscopic changes were already observed at 2 weeks after complete meniscectomy, i.e., the earliest observation point. The OA-like lesions gradually progressed over the 12-week observation period. The area of affected cartilage surface increased and osteophytes grew. The macroscopic and histological observations are consistent with early events in (posttraumatic) OA.

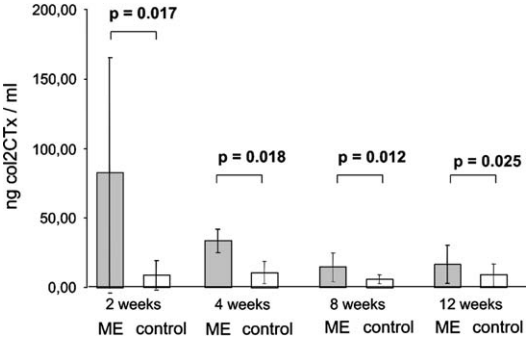

Fig. 5. col2CTx epitope levels (uncorrected = ng/ml assayed SF volume) from both knee joints after meniscectomy at 2, 4, 8, and 12 weeks. Right knees were meniscectomized (ME), left knees unoperated contralateral control knees (control).

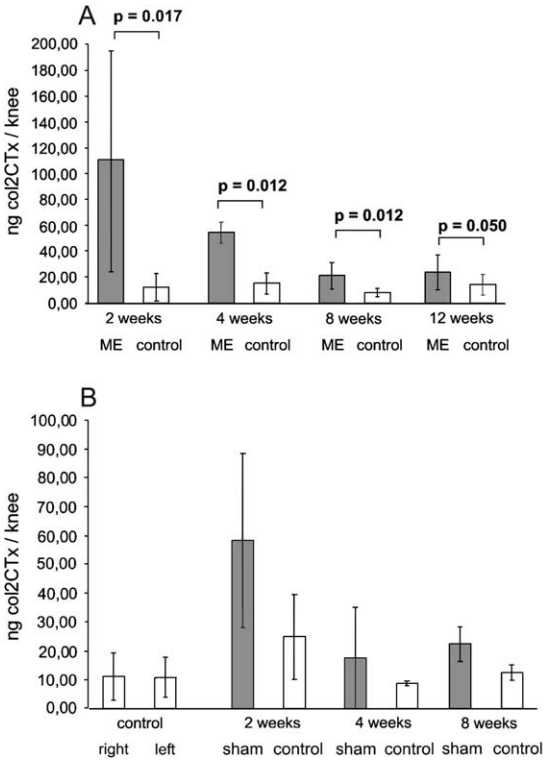

Fig. 6. (A) col2CTx epitope levels (corrected = ng/knee) from both knee joints after meniscectomy at 2, 4, 8, and 12 weeks. At 12 weeks, the difference was at the level of significance. Right knees were meniscectomized (ME), left knees unoperated intra-individual control knees (control). (B) col2CTx epitope levels (corrected = ng/knee) from both knee joints in unoperated animals (right and left) and at 2, 4, and 8 weeks after sham surgery (right sham-operated and left contralateral control). Levels at all time points were not significantly different (including the sham surgery group at 2 weeks; $p = 0.063$).

Direct collection of neat SF from the normal rabbit knee joints is unreliable, using either open or puncture methods. Given that a normal rabbit knee joint contains as little as 50 μl of SF, as determined by magnetic resonance imaging [34], this is not surprising. For this reason, we chose to use a lavage technique described by others even though it has the potential for unreliable estimates of the dilution factor[35]. To provide some assurance of reproducibility we calculated the results two ways, per ml lavage of SF and per whole knee joint, with no difference in general findings.

Although in posttraumatic animal models of OA the process of cartilage degradation may proceed through different mechanisms from natural OA, these models offer the advantage of a controlled starting point of the disease process. Damage to the collagen network is believed to be an early event in OA[10,16–21]. Collagen type II degradation epitopes are therefore expected to be useful indicators of early events (e.g., 2B4[22], Col2-3/4[36], Col 234CEQ[37], 9A4[38], F2603[39], 5109[40]). This should be especially true after meniscectomy alone[38]. As articular cartilage is the predominant source of collagen type II in the knee joint, collagen type II epitopes present in SF should stem from articular cartilage, perhaps preferentially from the surface zone. This suggestion is consistent with data from immunohistochemical studies in different animal models (e.g., Stoop *et al.*[20]).

In this study, col2CTx (cross-linked C-telopeptide domain of type II collagen) in SF increased 2 weeks postoperatively then fell. This observation is consistent with other reported studies of posttraumatic OA, e.g., Refs.[19,20,24,30]. Indeed, two recent studies of anterior cruciate ligament transection in the dog found higher collagen type II proteolytic epitope levels soon after injury[28,41].

The later fall in collagen type II in SF could reflect a slowing of a transient cartilage response to the trauma. Previous observations suggested an acute phase of cartilage matrix remodeling after meniscectomy, with early proteoglycan loss and collagen network swelling[11,31]. A cross-sectional human study with the same assay found that levels of the C-telopeptide epitope and the collagen type II C-propeptide in SF increased within a week of knee injury and gave a similar temporal pattern[24].

Thus, the presented results are in good agreement with the human results. This supports rabbit meniscectomy as a useful model of OA, and confirms that in the rabbit the epitope can be detected and behaves similarly.

Interestingly, sham-operated knee joints also produced higher levels of collagen type II epitope at 2 weeks [see Fig. 6(B)]. Increases in collagen type II degradation have been described by Rogart *et al.*[42] after sham surgery on the contralateral knee joint in the rabbit Hulth–Telhag model. One possible cause of the transient sham-operated joint response[9] is stimulation of the articular chondrocytes by signaling molecules (e.g., pro-inflammatory cytokines) released from the injured synovium and joint capsule or as a result of the hemarthrosis. Thus, synovitis is linked to an increase in collagen type II levels in human SF[24,43]. Joint bleeding, which often follows surgery, can result in damaged articular cartilage, e.g., Refs.[44,45]. A less likely cause is altered mechanical loading patterns that accelerate biomarker release from the cartilage[46].

In summary, the present findings support the concept that collagen type II degradation markers show promise in monitoring early events in the process of OA development in animal models as well as in human disease.

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