View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Osteoarthritis and Cartilage (2001) **9,** 308–315
© 2001 OsteoArthritis Research Society International
doi:10.1053/joca.2000.0390, available online at http://www.idealibrary.com on IDEAL®

1063–4584/01/040308+08 $35.00/0

Osteoarthritis and Cartilage

Journal of the OsteoArthritis Research Society International

Type II collagen degradation in articular cartilage fibrillation after anterior cruciate ligament transection in rats

R. Stoop*†**, P. Buma*, P. M. van der Kraan†, A. P. Hollander‡§, R. Clark Billinghurst‡‖, T. H. M. Meijers‡¶, A. R. Poole‡ and W. B. van den Berg†

**Orthopaedic Research Laboratory, †Department of Rheumatology, University Hospital Nijmegen, The Netherlands. ‡Joint Diseases Laboratory, Shriners Hospital for Children and Division of Surgical Research, Dept. of Surgery, McGill University, Montreal, Canada. §Present address: Department of Human Metabolism, University of Sheffield, U.K. ‖Present address: Department of Clinical Sciences, Colorado State University, U.S.A. ¶Present address: Mt Sinai Hospital, Toronto, Canada. **Present address: Department of Orthopedic Surgery, Rush-Presbytarian-St. Luke's Medical Center, Chicago, Illinois, U.S.A.*

Summary

Objective: To investigate the kinetics of early cartilage changes in mechanically induced osteoarthritis (OA) and the association of these changes with damage to the type II collagen network.

Methods: Experimental OA was induced by anterior cruciate ligament transsection in the rat knee joint (ACLT-OA). Animals were sacrificed after 2, 7, 14, 28 and 70 days. Knee joints were evaluated using routine histology and immunohistochemistry for denatured (unwound) type II collagen to detect collagen damage. An antibody recognizing the collagenase cleavage site in type II collagen was used to study the role of collagenase in this process.

Results: The first changes of the articular cartilage after anterior cruciate ligament transection occurred in the superficial zone. These changes included loss of superficial chondrocytes, swelling of the remaining chondrocytes and superficial fibrillation. The swelling of the chondrocytes did not result from a change towards the hypertrophic phenotype, since these cells did not stain for type X collagen. A marked increase in denatured type II collagen staining was present in the fibrillated areas. Staining of the collagenase cleavage site showed the same distribution as denatured collagen but was clearly less intense. Collagen damage could never be detected before fibrillation occurred and was not present in non-fibrillated areas.

Conclusions: These results indicate that in this model cartilage degeneration starts at the articular surface and that this degeneration is associated with a localized expression of type II collagen degradation products. © 2001 OsteoArthritis Research Society International

Key words: Type II collagen, Osteoarthritis, Anterior cruciate ligament transection, Rats, Immunohistochemistry, Cartilage degradation, Type X collagen.

Introduction

In osteoarthritis (OA), articular cartilage is damaged with a loss of function. Although little is known of the initiating events in cartilage degradation in OA, a number of studies suggest that changes of the superficial cartilage layer are among the earliest events[1–3]. In both human[4,5] and in canine experimental OA[6,7] the tensile stiffness of the superficial zone decreased by 40–60%, accompanied by an increase of the water content of the cartilage[7,8]. Biomechanical calculations suggest that damage of the surface zone leads to increased loading of the cartilage matrix[9], resulting in higher stresses on the underlying cartilage. This may provide a sequence of events in which the degeneration of the superficial zone develops into fibrillation of the cartilage and eventually results in erosions and ulcerations[10–11]. The loss of tensile strength and the increased water content suggest a significant change, i.e. disruption or disorganization, in the network of highly orientated type II collagen fibrils in the superficial cartilage[12].

Immunolocalization of type II collagen degradation products confirmed degeneration of the collagenous network of the superficial zone during late stage human OA[2,13]. Disruption of this network as an initiating event in experimental OA is suggested by Panula and co-workers[14]. In dogs with a tibial valgus osteotomy they found a large increase in collagen-induced optical path difference that occurred before surface fibrillation was histologically visible. This suggests a disruption of the superficial collagen network in the early phases of OA, preceding fibrillation and further degeneration of the cartilage. In human OA cartilage several studies show an elevated activity of matrix metalloproteinases, including an up-regulation of MMP-1

Received 6 July 2000; revision requested 13 October 2000; final revision received 7 November 2000; accepted 12 November 2000; published online 9 March 2001.

P. M. van der Kraan is funded by 'Het Nationaal Reumafonds', The Netherlands. A. R. Poole is funded by Shriners Hospitals for Children, Medical Research Council of Canada and National Institutes of Health, U.S.A.

Address correspondence to: Pieter Buma, Department of Orthopaedics, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Tel: +31 243614930; Fax: +31 243540230; E-mail: P.Buma@orthp.azn.nl

(collagenase-1)[15–19], suggesting that the changes in the collagenous network may be due to increased enzymatic activity of the chondrocytes.

To study the kinetics of degeneration of the articular cartilage during OA and the changes of the collagen network, we investigated damage of type II collagen during the early phases of experimental, mechanically-induced OA. We performed immunolocalization directed against denatured (unwound) type II collagen[13] to study damage to type II collagen. An antibody recognizing the collagenase cleavage site on the three-quarter fragment of type II collagen[20] was used to investigate collagenase cleavage in the destruction of this molecule. We show in this model that cartilage degeneration starts at the articular surface and that this degeneration is associated with a localized degradation of type II collagen.

Material and methods

ANIMALS

Male Wistar rats (220–240 g) were obtained from the central animal department breeding facility of the University of Nijmegen. They were kept in pairs in cages with a sawdust bedding and had free access to a standard diet and tap water.

ANTERIOR CRUCIATE LIGAMENT TRANSECTION

Rats were anesthetized by inhalation of 2% fluothane (Zeneca, Macclesfield, U.K.) in oxygen/nitrous oxide. After shaving the knee joint, the skin was disinfected with iodine and a parapatellar skin incision was made on the medial side of the joint. An incision on the medial side of the patellar tendon provided access to the joint space after which the patella was dislocated laterally with the leg in extension. The anterior cruciate ligament was transected using a custom-made retrograde-cutting hook. A positive anterior drawer test ensured complete transection of the ligament. After relocation of the patella, the wound was closed with vicryl 5/0 (polyglactin 910) braided absorbable suture (Ethicon, Edinburgh, U.K.). The skin was closed with two staples and Buprenorfinehydrochloride (80 μl, s.c., Temgesic®, Reckitt & Colman products, Kingston-upon-Hull, U.K.) was given as an analgesic. Care was taken to keep the operation area moist with saline and all operation procedures were performed using a surgical microscope. This procedure is modified from the protocol described by Williams *et al*.[21]

Sham operations were performed in the contralateral knee joint and consisted of opening of the joint space, subluxation of the patella and closing of the wound. All procedures were approved by the animal welfare committee.

HISTOLOGY

For routine histology, animals were killed by cervical dislocation after 2, 7 or 21 (N=24) days, or, in a second experiment, after 2, 4 and 10 weeks (N=24). Whole knee joints were dissected, fixed in 4% formalin buffered with 0.1 M phosphate buffer (pH 7.4) and decalcified with 5% formic acid. After embedding in paraffin, 7 μm sections were cut and stained with hematoxylin/eosin or with safranin O/fast green.

For immunohistochemistry, 24 animals were used and killed after 2, 4 and 10 weeks after ACL-transection. These knee joints were dissected and decalcified in 10% EDTA (Titriplex III, Merck, Darmstadt, Germany) and 7.5% polyvinylpyrrolidone (PVP, M_r 29,000, Serva, Brunswick, Amsterdam, The Netherlands) in 0.1 M Tris buffer (pH 7.4) for 4 weeks at 4°C[22]. After extensive rinsing with 7.5% PVP in 0.1 M Tris buffer, tissue blocks were rapidly frozen in liquid nitrogen and stored at −70°C. Coronal whole knee joint sections (7 μm) were cut at −18°C on a Bright 3050 cryostat and mounted on glass microscope slides precoated with 3-aminopropyltriethoxysilan (Sigma, St Louis, MO)[2]. Sections were air dried for 1 h and stored at −70°C until further use.

ANTIBODIES

To visualize type II collagen degradation, we used antibodies Col2-3/4C_{short} (20) and Col2-3/4m (13). Col2-3/4C_{short} is a rabbit polyclonal antibody directed against the COOH terminus of the three-quarter fragment generated by cleavage of native type II collagen by mammalian collagenases. Col2-3/4m is a mouse monoclonal IgG_1 that reacts with an epitope exposed in denatured, but not in native (helical), type II collagen. It recognizes a 13-residue amino-acid sequence which is located in the CB11 region of the three-quarter piece produced by collagenase cleavage of type II collagen.

Type X collagen was detected with a polyclonal rabbit antibody raised against a human NC1 synthetic peptide. The human and mouse sequences of this peptide have only a single amino acid difference. This antibody did not show cross-reactivity against collagen type I, II, IX and XI. In earlier studies it was shown that the collagen type X polyclonal antibody specifically stained collagen type X in murine joints[23].

IMMUNOHISTOCHEMISTRY

After thawing, the sections were fixed in freshly prepared 4% formaldehyde (5 min) and washed extensively in 0.1 M phosphate buffered saline (pH 7.4; PBS) for 15 min. To enhance the permeability of the extracellular matrix, glycosaminoglycans were removed by incubating the sections with 1% hyaluronidase (testicular, type I-s, EC 3.2.1.35, Sigma, St Louis, MO) in PBS, for 30 min at 37°C.

Endogenous peroxidase activity was blocked by incubation of the sections with freshly prepared 1% (vol/vol) H_2O_2 in absolute ethanol for 30 min. Non-specific staining was blocked by incubation of the sections with 10% normal goat serum (Col2-3/4C, anti-type X collagen) or with 10% normal horse (Col2-3/4m) serum in PBS with 1% bovine serum albumin (Sigma, St Louis, MO).

Sections were incubated overnight with the Col2-3/4C (1/800), the Col2-3/4m antibody (1/800) or the anti-type X collagen (1/4000) at 4°C in a humidified chamber. Biotin-labeled goat anti-rabbit and horse anti-mouse antibodies (DAKO A/S, Glostrup, Denmark, 1/400) were used as secondary antibodies (1 h, room temperature). A biotin–streptavidin detection system (Vectra elite kit, Vectra, Burlingham, CA) was used according to the manufacturer's recommendations. The peroxidase was detected using tablets containing 10 mg 3′,3′diaminobenzidine (Sigma, St Louis, MO) dissolved in 15 ml PBS with 12 μl H_2O_2 (30%) for 5 min. After rinsing, sections were dehydrated and mounted with DPX (BDH, Poole, U.K.).

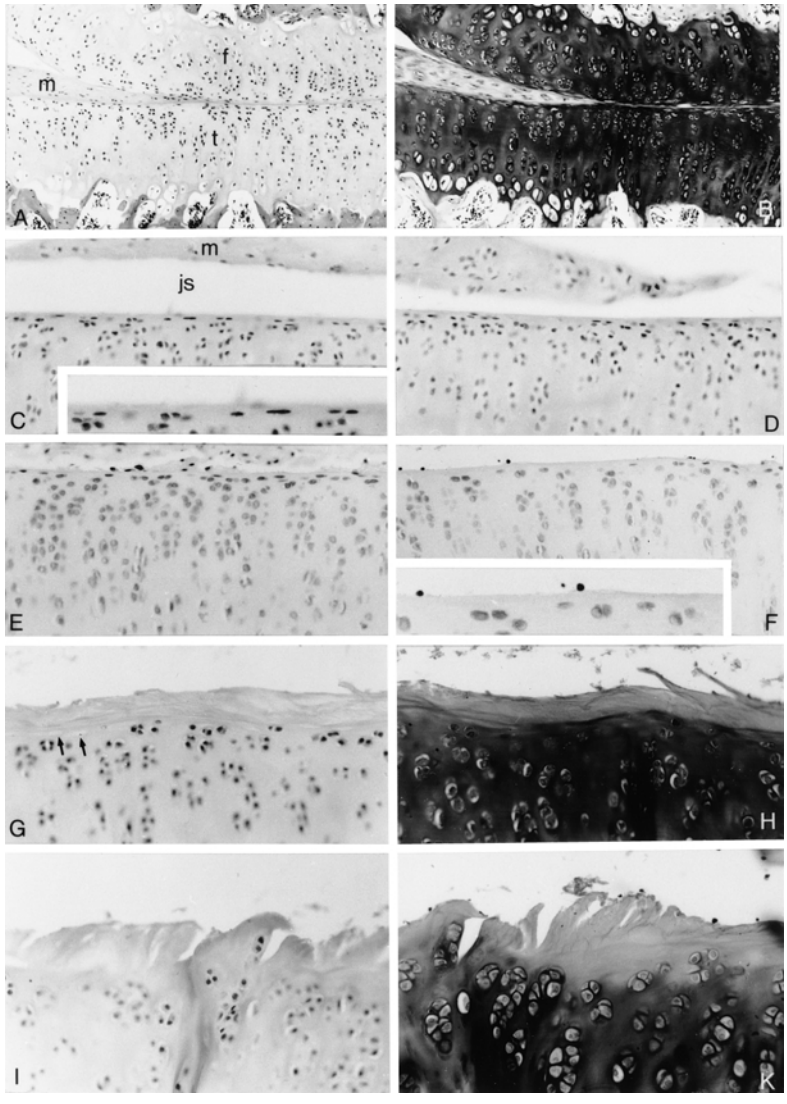

Fig. 1. Cartilage surface changes after ACL transection in the femoral–tibial compartment of the rat knee joint. (A), (C–G), (I) HE-stained sections. (B). H, K. Safranin O/fast green stained sections. (A–C). Control cartilage had a smooth cartilage surface with flat superficial chondrocytes orientated parallel to the cartilage surface [insert in (C)]. No proteoglycan depletion could be observed. In sham-operated animals (D) and in the 2-day group (E) no changes compared with control cartilage were observed. (F) After 7 days, slight loss of superficial chondrocytes was observed. The remaining chondrocytes had a swollen appearance [insert in (F)]. After 4 weeks (G), (H) surface fibrillation was found on almost the entire cartilage surface. A sharp transition between fibrillated and non-fibrillated cartilage was visible. Arrows in (G) indicated empty lacunae. In fibrillated areas, loss of safranin O staining was observed (H). The surface fibrillation had increased after 10 weeks (I), (K), again showing loss of safranin O staining in the superficial cartilage zone (K). Magnification: (A), (B) 100×; (C–K) 200×, inserts 400×.

Results

BODY WEIGHT

Two days after operation a small loss of body weight was observed in both ACL transected and sham-operated rats, but from 1 week after the operation until the end of the experiment the body weight of rats in both groups steadily increased. No statistical differences could be observed between ACL transected and sham-operated animals.

HISTOLOGICAL CHANGES AFTER ACL TRANSSECTION

The articular cartilage from the sham-operated knee joints was histologically normal, except that occasionally some dead cells were found in the central part of the medial tibia. In the unstable knee, degenerative changes were found in the tibial-femoral compartment, but in most animals patellar cartilage was unaffected even 10 weeks post-operatively.

SURFACE CHANGES

In ACL-transected knee joints clear changes of the surface of the articular cartilage were present, ranging from swelling of the superficial chondrocytes to complete loss of the superficial chondrocytes and fibrillation of the superficial zone. In control knee joints [Fig. 1(A), (C)], the articular cartilage had a smooth surface lined with flat

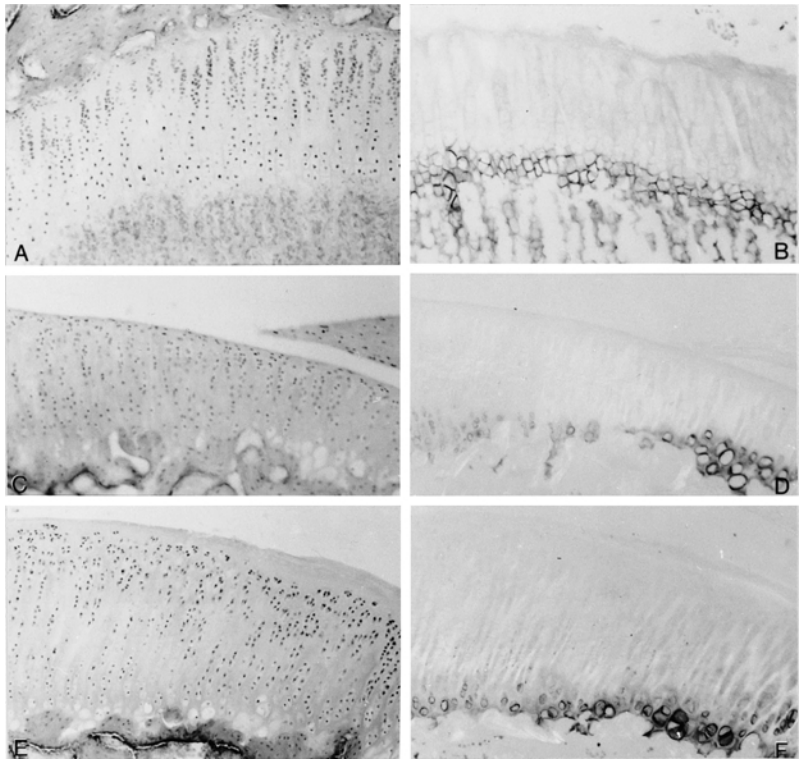

Fig. 2. Type X collagen expression in rat cartilage after anterior cruciate ligament transection. (A), (C), (D). HE-stained sections. (B), (D), (F). Type X collagen. Polarized light was used in (D) and (F) to show the contours of the cartilage. In control cartilage, type X collagen was present, as expected, surrounding hypertrophic chondrocytes in the epiphyseal growth plate (A), (B) and in the calcified zone of the articular cartilage (C), (D). (E), (F) No changes in type X collagen expression were found 10 weeks after ACL transection. Magnification: 100×.

superficial chondrocytes orientated parallel to the surface. Safranin O staining was present throughout the cartilage [Fig. 1(B)]. While the sham-operated [Fig. 1(D)] and 2 days ACL transected knee joints [Fig. 1(E)] closely resembled the control group, 7 days after ACL transection scattered chondrocyte loss was observed [Fig. 1(F)]. The remaining chondrocytes had a swollen appearance [insert in Fig. 1(F)]. Also slight fibrillation of the articular surface occurred. This process of fibrillation increased in severity throughout the duration of the experiment [4 weeks: Fig. 1(G), (H); 10 weeks: Fig. 1(I), (K)]. Especially in the 4-week group a sharp transition between the non-fibrillated and fibrillated areas was observed. In the fibrillated areas the flat superficial chondrocytes were not present or were no longer recognizable as such and empty lacunae were often observed [Fig. 1(G)]. Furthermore, loss of safranin O staining was always found in fibrillated cartilage [Fig. 1(H), (K)]. Cloning of chondrocytes was apparent in these damaged areas at 10 weeks [Fig. 1(K)].

TYPE X COLLAGEN EXPRESSION AFTER ACL-TRANSECTION

To investigate if the swelling of chondrocytes after ACL transection was caused by a change towards a hypertrophic phenotype, immunohistochemistry for type X collagen was performed. In control, non-operated cartilage, type X collagen staining was restricted to the hypertrophic zone of the epiphyseal growth plates [Fig. 2(A), (B)] and to the calcified zone of the cartilage [Fig. 2(C), (D)]. Staining was most intense around the hypertrophic cells, but type X collagen was also present in the interterritorial matrix. In OA knee joints [Fig. 2(E), (F)], type X collagen staining was not found around the swollen chondrocytes in the superficial cartilage zone or elsewhere in the non-calcified zone of the cartilage.

TYPE II COLLAGEN DEGRADATION DURING SURFACE FIBRILLATION

In cartilage from unoperated knee joints, type II collagen degradation could only be observed in the hypertrophic zone of the calcified cartilage zone. Both denatured type II collagen [Fig. 3(A)] and the collagenase-cleavage site in type II collagen [Fig. 3(B)] were found around hypertrophic chondrocytes. Furthermore, an intense line of type II collagen cleavage was present at the calcified cartilage-bone interface [Fig. 3(B)].

In contrast, a marked increase in type II collagen denaturation was found in fibrillated areas of ACLT-OA knee joints [Fig. 3(C)]. A sharp transition in the staining pattern was found between the fibrillated and non-fibrillated areas. In non-fibrillated cartilage, an increase in denatured collagen was never found. Interestingly, almost no collagenase cleavage site [Fig. 3(D)] could be observed in areas showing collagen denaturation.

In areas that were heavily eroded denatured type II collagen was abundantly present [Fig. 3(E), (F)], while moderate collagenase cleavage site immunoreactivity could be detected [Fig. 3(G)]. In the 10 weeks' group a similar staining pattern was found: denatured type II collagen stained intensely in fibrillated areas [Fig. 3(H)], while comparatively little collagenase cleavage site was present [Fig. 3(I)].

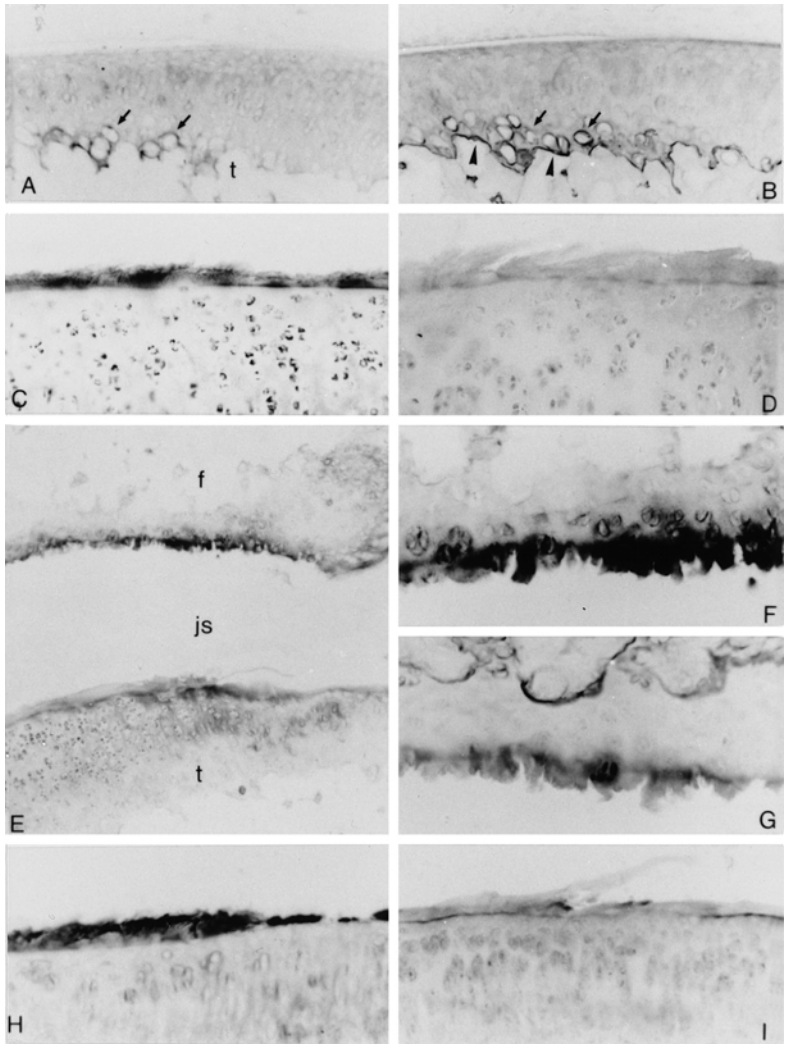

Fig. 3. Type II collagen degradation in the superficial zone of rat articular cartilage after ACL transection. (A), (B). In control cartilage, collagen type II denaturation (A) and the collagenase cleavage site (B) could only be observed around hypertrophic chondrocytes (arrows). Furthermore, the collagenase-cleavage site was present as an intense band on the cartilage–bone interface [arrowheads in (B)]. In surface fibrillation, denatured type II collagen was clearly present (C), but relatively little collagenase cleavage site was found (D). (E) In heavily eroded areas denatured type II collagen was abundantly present, but only moderate cleavage site was found (G). (F) Enlargement of (E). (H), (I). Cartilage surface, 10 weeks after ACL transection. (H), denatured type II collagen, (I), collagenase cleavage site in type II collagen. Magnification: (A), (B), (E) 100×; (C), (D), (F–I) 200×.

Discussion

The first degenerative changes in rat articular cartilage after transection of anterior cruciate ligament (ACL) were found in the matrix and chondrocytes of the superficial zone of the articular cartilage, as in human[1,2,10] and experimental OA in other animal models[7,16,24]. These changes consisted of loss of superficial chondrocytes, resulting in empty lacunae, while the remaining chondrocytes had a more enlarged appearance, resembling chondrocytes from deeper zones. These results are in accordance with observations in ACL models in beagles[24] and American fox hounds[25]. In these dog models, loss of superficial chondrocytes and enlargement of the remaining chondrocytes of the superficial zone were the first changes found. However, in these animal models this only occurred 1–2 months after operation, compared to 14 days in the rat model.

Enlargement of superficial chondrocytes may have several underlying mechanistic causes. First, it may be caused by the decreasing integrity and support of the pericellular matrix. Second, enlargement of chondrocytes may indicate swelling prior to hypertrophy or third, may occur as a sign of increased metabolic activity due to cell division or increased synthesis of matrix components. In an attempt to discriminate between these possibilities we looked at type X collagen expression. It has been described that type X collagen is expressed in the superficial layer of normal articular cartilage[26,27]. If the enlargement of the remaining chondrocytes represented a change from normal articular chondrocytes into hypertrophic chondrocytes, and by this an increase in the normal type X expression, immune-localization of type X collagen could be expected. Type X collagen was clearly present in areas known to contain hypertrophic chondrocytes like the growth plate[28–31] and the calcified zone of articular cartilage[32,33]. No changes in the staining pattern for type X collagen were found in either sham-operated or ACL-transected rats. This indicates that the enlargement of chondrocytes did not

result from a change into the hypertrophic phenotype. Although a number of studies showed an increase in type X collagen expression in OA cartilage, this probably correlates with relatively late events in the OA process. In most studies increased type X collagen staining could only be detected in chondrocyte clusters[33–35] or in fibrillated areas[35,36]. Instead of a change into the hypertrophic phenotype, the increase in size of the chondrocytes may reflect an increased ability for synthesis of extracellular matrix molecules or proteases as a response to the changed loading pattern. Such increase in synthetic capacity was also previously found in dog cartilage after ACL-transection[24,25], as indicated by an increase of the endoplasmic reticulum content of the superficial chondrocytes. Finally, it may also indicate that the cells are changing into chondroblasts with the capacity of cells division or that the swelling is made possible simply by the lesser constraint exerted by the damaged pericellular matrix.

The degeneration of the surface zone progressed as a very localized process, as represented by the differences in proteoglycan staining between the fibrillated (safranin O staining absent or reduced) and non-fibrillated areas. That the changes at the articular cartilage surface also involved damage to the collagenous network was demonstrated with immunostaining for type II collagen degradation products. Increased staining for denatured type II collagen was found only in fibrillated areas, never in the underlying cartilage. This indicates that the degeneration of the collagenous network is a very focal process, which starts at the articular surface. In recent studies of our laboratory, in which we studied collagen degradation in two mice stains that showed spontaneous OA, we used the same antibodies as in this study[37]. The pathology observed in these mice strains is comparable to other spontaneous OA models in small laboratory animals, such as the guinea-pig, but is different from the ACLT-OA in the rat. Localized defects were found, but the surface fibrillation as found in rats was not present. If we compare the neoepitopes, we found that also in these mice strains the collagenase cleavage site also showed very little increase in the degenerated sites. The COL-2-3/4m antibody was localized to a very small rim at the outer edge of the degenerated area.

An explanation for the fibrillation in the early OA in the rat might be an especially thick tangential collagen layer in rats. If we compared the thickness of the tangential collagen layer in rats and dogs in picrosirius red stained sections, we found that the layer in the rat is relatively thick compared to that in the dog. This indicates that the fibrillation we observed might be a phenomenon that becomes particularly clear in early OA in the rat. In human OA damage to type II collagen also was found to start at the articular surface where proteoglycan is lost[2].

The importance of collagenases in the induction of the type II collagen denaturation in the superficial cartilage zone is not completely clear. In comparison with denatured collagen, the collagenase cleavage site was always less intense and in a number of sections only staining for denatured collagen was found. This might reflect direct mechanical damage to the collagenous network due to the changed loading pattern, which will expose the denatured collagen epitope but not the collagenase cleavage site.

On the other hand, a number of studies have indicated the presence of collagenases (most prominently MMP-1) in the articular surface zone of experimental and human OA cartilage, both on the mRNA[18,19] and on the protein level[17,38]. This implies a role for the cleavage of type II collagen by collagenases during the OA process irrespective of whether this process is spontaneous or induced mechanically, as in our study. The low immunoreactivity might therefore be explained by a difference in processing after the initial cleavage of type II collagen. The denatured type II collagen epitope lies relatively far removed from the cleavage site and is probably more resistant to further degradation, for example by gelatinases, than the collagenase cleavage site where the removal of only one amino acid leads to loss of immunoreactivity[20]. Moreover, Kafienah *et al*.[39] recently showed that cathepsin K is able to cleave native alpha1(II) chains in the N-terminal region of the helical domain rather than at the well-defined collagenase cleavage site which leads to exposure of the COL2-3/4m epitope. However, whether this destructive pathway plays a role in the osteoarthritic process remains to be established.

In conclusion, this study shows that after transection of the anterior cruciate ligament in rats the first changes in the cartilage occurred in the superficial zone. The development of fibrillation was highly correlated in time and space with an increase in denatured type II collagen staining. Collagen damage could never be detected before fibrillation occurred and was not present in non-fibrillated areas. This indicates that in this model initial cartilage mechanical overloading of the surface of the cartilage mainly induces degeneration. The absence of Col2-3/4C_{short} suggests that collagenases play a minor role in the generation of degeneration, which is associated with a localized destruction of the type II collagen network.

Acknowledgments

We would like to thank Diny Versleyen and Elly Vitters for their histological support and Gerry Grutters, Henny Eickholt and Geert Poelen for their biotechnical support.

Abbreviations

ACL	Anterior cruciate ligament
MMP	Matrix metallo proteinase
OA	Osteoarthritis
PVP	Polyvinylpyrrolidone
PBS	Phosphate-buffered saline

References

1. Mitchell N, Lee ER, Shepard N. The clones of osteoarthritic cartilage. J Bone Joint Surg Br 1992;74: 33–8.
2. Hollander AP, Pidoux I, Reiner A, Rorabeck C, Bourne R, Poole AR. Damage to type II collagen in aging and osteoarthritis starts at the articular surface, originates around chondrocytes, and extends into the cartilage with progressive degeneration. J Clin Invest 1995; 96:2859–69.
3. Vignon E, Arlot M, Hartmann D, Moyen B, Ville G. Hypertrophic repair of articular cartilage in experimental osteoarthrosis. Ann Rheum Diseases 1983; 42:82–8.
4. Akizuki S, Mow VC, Müller F, Pita JC, Howell DS, Manicourt DH. Tensile properties of human knee joint cartilage: I. Influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus. J Orthop Res 1986;4:379–92.

5. Akizuki S, Mow VC, Müller F, Pita JC, Howell DS. Tensile properties of human knee joint cartilage. II. Correlations between weight bearing and tissue pathology and the kinetics of swelling. J Orthop Res 1987;5:173–86.
6. Setton LA, Mow VC, Müller FJ, Pita JC, Howell DS. Mechanical properties of canine articular cartilage are significantly altered following transection of the anterior cruciate ligament. J Orthop Res 1994; 12:451–63.
7. Guilak F, Ratcliffe A, Lane N, Rosenwasser MP, Mow VC. Mechanical and biochemical changes in the superficial zone of articular cartilage in canine experimental osteoarthritis. J Orthop Res 1994;12:474–84.
8. McDevitt C, Gilbertson E, Muir H. An experimental model of osteoarthritis; early morphological and biochemical changes. J Bone Joint Surg Br 1977;59: 24–35.
9. Setton LA, Zhu W, Mow VC. The biphasic poroviscoelastic behavior of articular cartilage: role of the surface zone in governing the compressive behavior [see comments]. J Biomech 1993;26:581–92.
10. Byers PD, Maroudase A, Oztop F, Stockwell RA, Venn MF. Histological and biochemical studies on cartilage from osteoarthrotic femoral heads with special reference to surface characteristics. Connect Tissue Res 1977;5:41–9.
11. Venn M, Maroudas A. Chemical composition and swelling of normal and osteoarthrotic femoral head cartilage. I. Chemical composition. Ann Rheum Dis 1977; 36:121–9.
12. Kempson GE, Muir H, Pollard C, Tuke M. The tensile properties of the cartilage of human femoral condyles related to the content of collagen and glycosaminoglycans. Biochim Biophys Acta 1973;297: 456–72.
13. Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, *et al.* Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. J Clin Invest 1994;93:1722–32.
14. Panula HE, Hyttinen MM, Arokoski JPA, Langsjö TK, Pelttari A, Kiviranta I, *et al.* Articular cartilage superficial zone collagen birefringence reduced and cartilage thickness increased before surface fibrillation in experimental osteoarthritis. Ann Rheum Dis 1998;57: 237–45.
15. Dean DD, Martel-Pelletier J, Pelletier JP, Howell DS, Woessner JFJ. Evidence for metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. J Clin Invest 1989;84:678–85.
16. Pelletier JP, Martel-Pelletier J, Altman RD, Ghandur-Mnaymneh L, Howell DS, Woessner JF Jr. Collagenolytic activity and collagen matrix breakdown of the articular cartilage in the Pond-Nuki dog model of osteoarthritis. Arthritis Rheum 1983;26:866–74.
17. Fernandes JC, Martel-Pelletier J, Lascau-Coman V, Moldovan F, Jovanovic D, Raynauld JP, *et al.* Collagenase-1 and collagenase-3 synthesis in normal and early experimental osteoarthritic canine cartilage: an immunohistochemical study. J Rheumatol 1998;25:1585–94.
18. Shlopov BV, Lie WR, Mainardi CL, Cole AA, Chubinskaya S, Hasty KA. Osteoarthritic lesions: involvement of three different collagenases. Arthritis Rheum 1997;40:2065–74.
19. Freemont AJ, Hampson V, Tilman R, Goupille P, Taiwo Y, Hoyland JA. Gene expression of matrix metalloproteinases 1, 3, and 9 by chondrocytes in osteoarthritic human knee articular cartilage is zone and grade specific. Ann Rheum Dis 1997;56(9):542–9.
20. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, *et al.* Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. J Clin Invest 1997;99:1534–45.
21. Williams JM, Felten DL, Peterson RG, O'Connor BL. Effects of surgically induced instability on rat knee articular cartilage. J Anat 1982;134:103–9.
22. Tonnaer EL, Kuijpers W, Peters TA, Ramaekers FC. Effect of EDTA on cytokeratin detection in the inner ear. J Histochem Cytochem 1990;38:1223–7.
23. van der Kraan PM, Vitters EL, Meijers THM, Poole AR, Van den Berg WB. Collagen type I antisense and collagen type IIa messenger RNA is expressed in adult murine articular cartilage. Osteoarthritis Cart 1998;6:417–26.
24. Orford CR, Gardner DL, O'Connor P. Ultrastructural changes in dog femoral condylar cartilage following anterior cruciate ligament section. J Anat 1983; 137(Pt 4):653–63.
25. Rucklidge GJ, Milne G, Robins SP. Collagen type X. A component of the surface of normal human, pig, and rat articular cartilage. Biochem Biophys Res Com 1996;224:297–302.
26. Eerola I, Salminen H, Lammi P, Lammi M, von der Mark K, Vurio E, *et al.* Type X collagen, a natural component of mouse articular cartilage. Arthritis Rheum 1998;41:1287–95.
27. Pidd JG, Gardner DL, Adams ME. Ultrastructural changes in the femoral condylar cartilage of mature American foxhounds following transection of the anterior cruciate ligament. J Rheumatol 1988;15: 663–9.
28. Alini M, Matsui Y, Dodge GR, Poole AR. The extracellular matrix of cartilage in the growth plate before and during calcification: changes in composition and degradation of type II collagen. Calcif Tissue Int 1992;50:327–35.
29. Schmid TM, Linsenmayer TF. Type X collagen. In: Mayne R, Burgeson RE, Eds. Structure and function of collagen types. New York: Academic Press 1987: 223–59.
30. Reichenberger E, Aigner T, von der Mark K, Stöss H, Bertling W. In situ hybridization on the expression of type X collagen in fetal human cartilage. Dev Biol 1991;148:562–72.
31. Remington MC, Bashey RI, Brighton CT, Jimenez SA. Biosynthesis of a disulfide bonded short chain collagen by calf growth plate cartilage. Biochem J 1983; 224:227–33.
32. Gannon JM, Walker G, Fischer M, Carpenter R, Thompson RC Jr, Oegema TRJ. Localization of type X collagen in canine growth plate and adult canine articular cartilage. J Orthop Res 1991;9:485–94.
33. Walker GD, Fischer M, Gannon J, Thompson RC Jr, Oegema TRJ. Expression of type-X collagen in osteoarthritis. J Orthop Res 1995;13:4–12.
34. von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Glückert K, *et al.* Type X collagen synthesis in human osteoarthritic cartilage. Indication of chondrocyte hypertrophy. Arthritis Rheum 1992;35:806–11.
35. Aigner T, Reichenberger E, Bertling W, Kirsch T, Stoss H, von der Mark K. Type X collagen expression in

osteoarthritic and rheumatoid articular cartilage. Virchows Arch B Cell Pathol 1993;63:205–11.
36. Eerola I, Salminen H, Lammi P, Lammi M, von der Mark K, Vuorio E, *et al*. Type X collagen, a natural component of mouse articular cartilage: association with growth, aging, and osteoarthritis. Arthritis Rheum 1998;41:1287–95.
37. Stoop R, van der Kraan PM, Buma P, Hollander AP, Billinghurst RC, Poole AR, *et al*. Type II collagen degradation in spontaneous osteoarthritis in C57Bl/6 and BALB/c mice. Arthritis Rheum 1999;42:2381–9.
38. Moldovan F, Pelletier JP, Hambor J, Cloutier JM, Martel-Pelletier J. Collagenase-3 (matrix metalloprotease 13) is preferentially localized in the deep layer of human arthritic cartilage in situ: in vitro mimicking effect by transforming growth factor beta. Arthritis Rheum 1997;40:1653–61.
39. Kafienah W, Brömme D, Buttle DJ, Croucher LJ, Hollander AP. Human cathepsin-k cleaves native type I and II collagens at the n-terminal end of the triple helix. Biochem J 1998;331(Pt 3): 727–32.