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Early Degradation of Type IX and Type II Collagen with the Onset of Experimental Inflammatory Arthritis

Toshihisa Kojima,[1] Fackson Mwale,[1] Tadashi Yasuda,[1] Christiane Girard,[2] A. Robin Poole,[1] and Sheila Laverty[2]

Objective. **To determine whether following the onset of intraarticular inflammation, there is early damage to articular cartilage, specifically to types II and IX collagen, and the proteoglycan (PG) aggrecan, and whether measurement of the degradation products of these molecules in synovial fluid (SF) and serum may permit the detection of cartilage damage.**

Methods. **A rabbit model of rheumatoid arthritis, antigen (ovalbumin)–induced arthritis, was studied. Articular cartilage samples were analyzed by immunoassays for total type II collagen content, its denaturation and cleavage by collagenases, and for type IX collagen content. PG content was determined by colorimetric assay. In serum and SF, total PG content and collagenase-generated peptides of type II collagen were measured.**

Results. **After 6 days, both the PG content and the NC4 domain of type IX collagen were reduced in femoral and tibial cartilage, concomitant with the onset of arthritis. In only the tibial cartilage did this reduction in PG persist up to day 20. However, denatured type II collagen was increased in all cartilage samples, but only on day 20. In SF, the PG content was significantly reduced on day 20, and products of type II collagen cleavage by collagenase were significantly increased on both day 6 and day 20.**

Conclusion. **This study, which is the first of its kind examining changes in both types II and IX collagen and PG content, reveals early damage to both types of collagen as well as to PG in articular cartilage samples following induction of joint inflammation. SF analyses reveal this early damage and may be of value in the study and treatment of inflammatory arthritic diseases such as rheumatoid arthritis.**

Collagen fibrils of the cartilage matrix principally contain type II collagen, but types XI and IX collagen are present as well, the latter being found on the surface of the fibril (1). Aggrecan is the largest proteoglycan (PG) found in the interfibrillar matrix. The loss of PG and damage to type II collagen, because of its enhanced denaturation and cleavage by collagenase, are principle features of the articular cartilage matrix breakdown in both osteoarthritis (OA) and rheumatoid arthritis (RA) (1–5). Molecular abnormalities involving type IX collagen (6) and type II collagen (7) can each independently cause premature degeneration of human articular cartilage (e.g., in familial OA), demonstrating the importance of these collagens in the maintenance of the structure and function of the cartilage matrix.

It is important to examine both PG and these collagens in studies of early degradation of articular cartilage in arthritis. There is currently no information concerning the relative cleavage and degradation of types II and IX collagen in vitro or in vivo and how these features relate to PG degradation. It has been shown in culture that PG loss precedes cleavage and damage to type II collagen when cartilage degradation is induced by interleukin-1 (8–10). In studying the degradation of articular cartilage, it is important to use in vivo analyses, especially in the early stages of degradation, so that the relative attack on different matrix molecules can be more clearly understood.

We therefore selected a rabbit model of antigen-induced arthritis in which intraarticular inflammation can be induced in a single knee joint and compared with its contralateral control joint. Previous work has re-

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[1]Toshihisa Kojima, MD, PhD, Fackson Mwale, PhD, Tadashi Yasuda, MD, PhD, A. Robin Poole, PhD, DSc: McGill University, Montreal, Quebec, Canada; [2]Christiane Girard, DMV, Dipl ACVP, Sheila Laverty, MVB, Dipl ACVS: University of Montreal, St. Hyacinthe, Quebec, Canada.

Address correspondence and reprint requests to Sheila Laverty, MVB, Dipl ACVS, Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Montreal, 3200 rue Sicotte, St. Hyacinthe, Quebec J2S 7C6, Canada.

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vealed that there is early damage to PG in articular cartilage in this model (11) which, in many ways, resembles the intraarticular inflammation seen in RA. Information gained from such studies may therefore be of value in the management of early and established RA. We selected a larger animal since analytic work on inflammatory arthritis in rats and mice (such as antigen-induced arthritis or type II collagen–induced arthritis) is restricted to immunohistochemical analyses of joint cartilage samples. In the rabbit, quantitative immunoanalyses of cartilage changes are possible in view of the increased joint size and therefore increased mass of the articular cartilage that is available for study.

Using immunoanalyses that we recently developed, we demonstrated that following induction of experimental intraarticular inflammation in rabbits, there is early damage to PG (principally aggrecan) and to the collagen fibril in the extracellular matrix of cartilage. This damage involves early increases in collagenase activity, which can be detected by analyses of SF.

MATERIALS AND METHODS

Animals. Male New Zealand white rabbits 16–18 weeks old were used. The experimental protocol was approved by the Animal Care Committee of the Faculty of Veterinary Medicine, University of Montreal.

Development of antigen-induced arthritis. Rabbits were earmarked and randomly assigned to experimental groups. The baseline animals (n = 5) were euthanized before immunization. All other rabbits (n = 12 per group) were sensitized with an emulsion of 4 mg of ovalbumin (OVA) (chicken egg albumin, grade VI; Sigma, St Louis, MO) in 0.5 ml of Freund's complete adjuvant (Sigma) and 0.5 ml of phosphate buffered saline. The emulsion was administered intradermally, in 100-μl volumes, at 5 intrascapular sites. A similar booster injection (OVA in Freund's incomplete adjuvant) was given 2 weeks later. Four days after the booster injection (day 0), knee joints were challenged with intraarticular (IA) injection of 1.5 mg of OVA in 0.5 ml of saline into the left knee and an equivalent volume of sterile saline alone into the right knee. In the control group, both joints were injected with 0.5 ml of sterile saline alone. Hypnorm, 0.2 ml/kg intramuscularly (fentanyl citrate; Janssen Animal Health, Janssen Pharmaceutica, Beerse, Belgium), was used for neuroleptanalgesia prior to IA injections.

The animals were examined daily for joint swelling. The diameter of the joint was measured at the same site (mediolateral measurement at the level of the proximal limit of the tibial crest) each day for 6 days using 15-cm dial calipers (General, Tool Canada, Pointe Claire, Quebec, Canada). Analgesia with buprenorphine HCl, 0.3 mg/kg subcutaneously (Temgesic; Reckitt & Coleman, Kingston-upon-Hull, UK), was provided for 6 days following IA injection. On day 6 and day 20 following IA injection of OVA, 6 rabbits from each group (OVA-treated and control) were euthanized.

Serum, SF, cartilage, and synovial membrane sampling. Blood samples were obtained on days −21, −11, −4, 3, 6, 10, 17, and 20 from the auricular artery of the ear. Lavage synoviocentesis was performed immediately after euthanasia, by injecting 1.5 ml of sterile saline into the joint space, followed by 10 flexions and extensions, and immediate synoviocentesis. This was also performed following neuroleptanalgesia with an intramuscular injection of Hypnorm 1 week before IA challenge. Both serum and SF samples were centrifuged at 3,000 revolutions per minute for 20 minutes, and the supernatants were frozen at −70°C until analyzed.

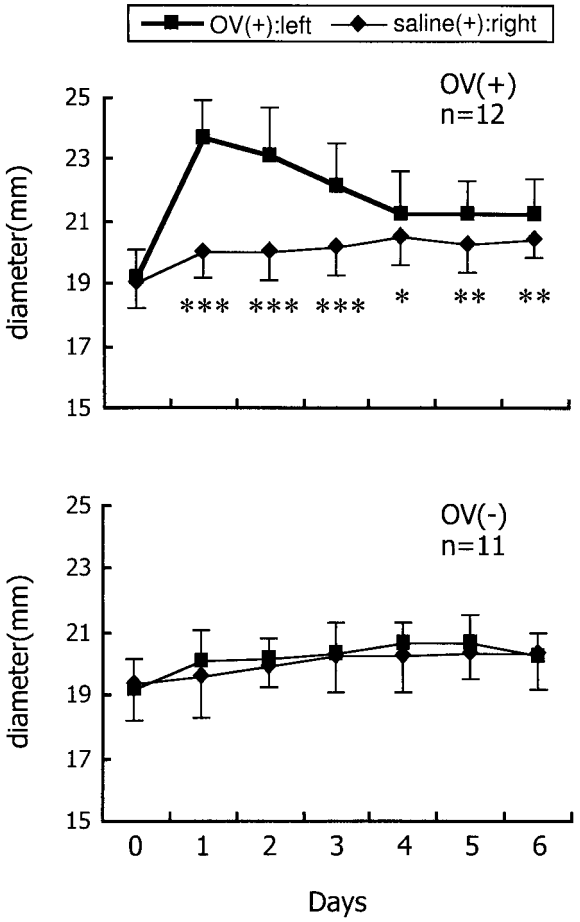

Figure 1. Joint swelling in ovalbumin (OV)–injected (+) and OV-uninjected (−) NZW rabbits. Values are the mean and SD. * = $P < 0.05$, ** = $P < 0.01$, and *** = $P \leq 0.0001$ for left knees versus right knees, by paired t-test.

The articular cartilage was harvested by shaving from the patellofemoral and femorotibial surfaces of the femoral condyles and the tibial plateau down to the subchondral bone. Samples were stored at −20°C until analyzed.

Synovial membrane histopathologic evaluation. A specimen of the synovial membrane was collected from each femorotibial joint, lateral to the patella, fixed in 10% buffered formalin, and embedded in paraffin. Six-micrometer–thick sections were stained with hematoxylin–phloxine–saffron. The presence of synoviocyte hyperplasia, synovial membrane erosion, fibrin, fibrosis, inflammatory cell infiltrates (polymorphonuclear cells, macrophages, lymphocytes, or plasma cells), and angiogen-

Table 1. Histopathologic scoring of synovial membranes in OVA-injected and control groups*

Evaluation point, study group	Synovial hyperplasia	Synovial erosions	Fibrin	Polymorphonuclear leukocytes	Macrophages	Lymphocytes	Plasma cells	Joint score
Day 6								
OVA-injected animals (n = 6)	1.83 ± 0.41†	1.17 ± 0.75	1.33 ± 0.52	1.33 ± 0.52	0	1.33 ± 0.52†	1.5 ± 0.83	8.5 ± 2.7†
Saline-injected controls (n = 6)	0	0	0	0	0	0	0	0
Day 20								
OVA-injected animals (n = 6)	1.0 ± 0.63	0	0	0.83 ± 0.75	0.33 ± 0.52	0.33 ± 0.52	0.83 ± 0.75	3.3 ± 2.7
Saline-injected controls (n = 5)	0	0	0	0	0	0	0	0

* Values are the mean ± SD. See Materials and Methods for explanation of scoring system.
† $P < 0.05$ versus ovalbumin (OVA)–injected group on day 20.

esis was evaluated and scored semiquantitatively on a scale of 0–3, where 0 = absent, 1 = mild, 2 = moderate, and 3 = severe. A total joint score was obtained by adding the individual scores for each parameter studied (maximum score 18).

Extraction and assay of cartilage for collagenase-cleaved, denatured, and total type II collagen content. The method used to extract Col2-3/4m (4) and Col2-3/4C_{short} (2) epitopes from cartilage was described previously (2,3). The α-chymotrypsin extracts were analyzed for both the collagenase-generated Col2-3/4C_{short} neoepitope and the intrachain hidden Col2-3/4m epitope. The undigested cartilage was incubated overnight at 56°C with proteinase K. The total collagen content in the cartilage was determined from the content of Col2-3/4m in both the α-chymotrypsin and proteinase K digests.

Determination of PG content. The hyaluronidase-digested SF and both the α-chymotrypsin and proteinase K cartilage digests were assayed for glycosaminoglycan, which represents primarily a measure of PG content, using a modification of the colorimetric 1,9-dimethylmethylene blue dye assay (12).

Determination of the NC4 domain of type IX collagen content in the cartilage. The NC4 domain of type IX collagen α1(IX) chain in the cartilage was measured from both the α-chymotrypsin and proteinase K digests using a new immunoassay recently developed in this laboratory (13).

Determination of DNA content in the cartilage. The DNA content of the articular cartilage was measured in the proteinase K digest as described previously (2,14). The contents of Col2-3/4C_{short}, Col2-3/4m, and NC4 epitopes were expressed as moles of peptide per microgram of cartilage DNA. PG content was expressed as micrograms per microgram of cartilage DNA.

Assays of SF and serum for collagen degradation products. SF was digested overnight at 37°C with 0.4 turbidity-reducing units/ml of *Streptomyces* hyaluronidase (Sigma) in 10 m*M* sodium acetate, pH 5.0, with proteinase inhibitors (1 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride, 1 m*M* iodoacetamide, and 5 μg/ml of pepstatin A) to decrease the viscosity of the fluid. This digest and serum were assayed for collagenase-generated neoepitope of type II collagen, Col2-3/4$C_{long\ mono}$ (C2C), using the immunoassay developed in this laboratory (15). The C2C antibody does not cross-react with collagenase-generated neoepitopes of type I collagen, which are also recognized by Col2-3/4C_{short} antibody. We could not use the C2C antibody to determine the collagenase-generated neoepitope of type II collagen in the cartilage because this epitope is digested by α-chymotrypsin. The epitope content recognized by Col2-3/4$C_{long\ mono}$ antibody was expressed as picomoles of peptide per milliliter of SF or serum.

Statistical analysis. The effect of OVA injection was analyzed by comparisons between saline-injected right knee joints and OVA-injected left knee joints by use of paired *t*-tests for analytic data. We also performed paired *t*-tests between the right and left joints in the baseline and control groups to detect possible differences. In serum samples, two-way repeated-measures analysis of variance was fitted with the factors of treatment and time. For histologic analyses, the Mann-Whitney U test was applied.

RESULTS

Findings of clinical evaluation of antigen-induced arthritis. Joint swelling resulted from IA injection of OVA and was clearly detectable up to day 6 and peaking on day 1, compared with saline-injected contralateral joints (Figure 1). No significant between-joint differences were detected in control rabbits. One rabbit in the control group was eliminated during the study because of acquired trauma to the knee during the experimental period.

Findings of histopathologic evaluation of antigen-induced arthritis. The changes observed in OVA-treated animals were characterized by mild-to-moderate synovial hyperplasia with mild-to-moderate inflammation. Polymorphonuclear and plasma cells predominated and were associated with lymphocytes and some macrophages. Mild-to-moderate erosions with exudation of fibrin were observed in 5 animals, 4 of which were noted on day 6. There was no evidence of fibrosis and neoangiogenesis.

The synovial membrane from the saline-injected

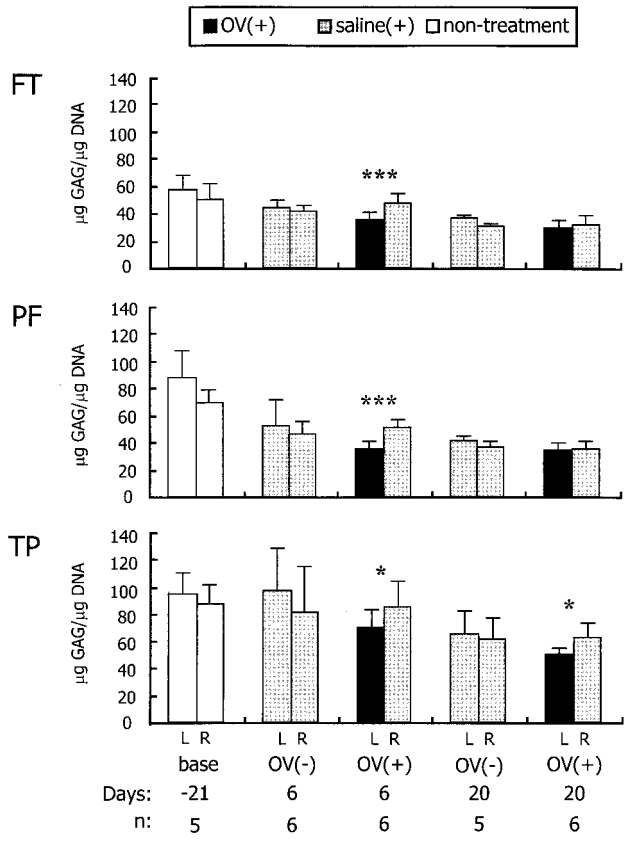

Figure 2. Proteoglycan (PG) content in cartilage from ovalbumin (OV)–injected (+) and OV-uninjected (−; non-treatment) NZW rabbits. PG content in α-chymotrypsin and proteinase K digests of cartilage were determined by dimethylmethylene blue assay. Values are the mean and SD. $* = P < 0.05$ and $*** = P < 0.001$ for left knees versus right knees, by paired t-test. FT = femorotibial surface of femoral condyles; PF = patellofemoral surface of femoral condyle; TP = tibial plateau; GAG = glycosaminoglycan; base = baseline group (see Materials and Methods for details); n = number of animals.

right knee joint was normal in all OVA-treated animals. No pathology was observed in the synovia from the left and right joints of the control rabbits (data not shown). The total joint scores in OVA-treated rabbits are shown in Table 1. Scores were higher on day 6 (acute phase) than on day 20 (chronic phase) for each histologic parameter except macrophages.

Findings of cartilage and SF analyses. Results of cartilage analyses were expressed in terms of DNA content since analyses of the DNA contents per wet weight (~1 μg/mg) revealed that there were no significant differences ($P \leq 0.05$) between the left and right knees in any experimental group. P values ranged from 0.11 to 0.82 in the femorotibial cartilage, from 0.08 to 0.53 in the patellofemoral cartilage from the femoral condyles, and from 0.11 to 0.49 in the tibial plateau cartilage. Dry weights could not be determined since the drying process causes denaturation of type II collagen.

Findings of PG degradation analyses. Since DNA contents were ~1 μg/mg wet weight, the PG content in control joints was similar to published results for bovine nasal cartilage (10). In uninjected baseline animals (−21 days), we detected no significant differences in cartilage PG content between joints. In OVA-treated rabbits, a reduction in cartilage PG content was detected on day 6 in all IA sites and on day 20 in the tibial plateau of only the OVA-injected joints. Control animals, which did not receive IA injections of OVA, showed no significant differences in cartilage PG content (Figure 2). The progressive decline in PG content with time is a reflection of growth-related changes, since these rabbits had not finished growing.

The release of PG into SF did not reflect the reduction in tissue content. The PG content in SF was significantly decreased in OVA-injected joints on day 20 compared with contralateral saline-injected joints, and although not significantly different, the PG content was also reduced on day 6. In baseline and control rabbits, there was no significant difference between right and left knee joints in terms of PG release into SF (Figure 3).

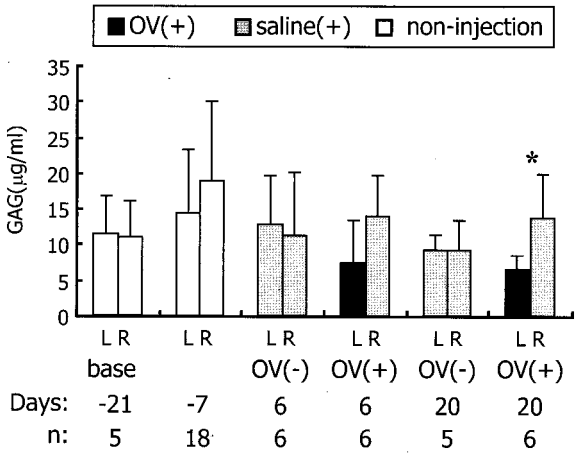

Figure 3. Proteoglycan (PG) content in synovial fluid from ovalbumin (OV)–injected (+) and OV-uninjected (−; non-injection) NZW rabbits. PG content in hyaluronidase digests was determined by dimethylmethylene blue assay. Values are the mean and SD. $* = P < 0.05$ for left knees versus right knees, by paired t-test. GAG = glycosaminoglycan; base = baseline group (see Materials and Methods for details); n = number of animals.

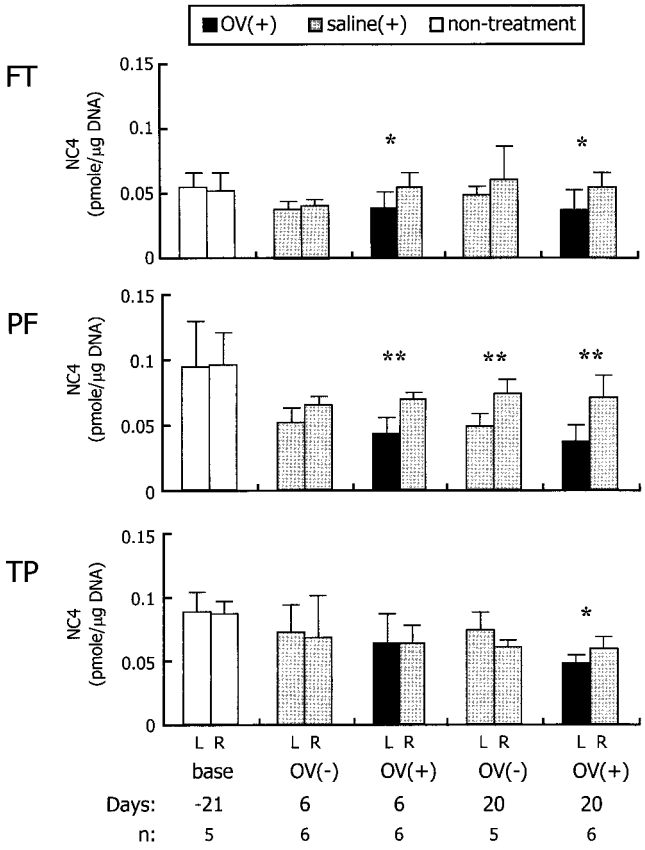

Figure 4. Content of the NC4 domain of type IX collagen in cartilage from ovalbumin (OV)–injected (+) and OV-uninjected (−; non-treatment) NZW rabbits. NC4 domain content in α-chymotrypsin and proteinase K digests was determined by immunoassay. Values are the mean and SD. * = $P < 0.05$ and ** = $P < 0.01$ for left knees versus right knees, by paired *t*-test. FT = femorotibial surface of femoral condyles; PF = patellofemoral surface of femoral condyle; TP = tibial plateau; base = baseline group (see Materials and Methods for details); n = number of animals.

Content and degradation of type IX collagen. In OVA-treated animals, the cartilage NC4 domain content was significantly decreased in the femorotibial and patellofemoral cartilage of OVA-injected joints compared with the contralateral joints on day 6 and in all sites within the OVA-injected joints on day 20. In baseline and control rabbits, no significant differences in the NC4 content were detected between right and left knees, except in the patellofemoral cartilage on day 20 (Figure 4).

Collagenase-cleaved, denatured, and total type II collagen content. No significant differences in the total type II collagen content of articular cartilage were observed between joints in the baseline, control, and OVA-treated groups during the experimental period (Figure 5). Cleavage of type II collagen by collagenase(s) in the cartilage was also not significantly different in either the baseline, control, or OVA-treated rabbits when comparing right and left knees (Figure 6). However, on day 20, an increase in denatured type II collagen was clearly detectable in all compartments of the OVA-treated injected joints compared with the contralateral saline-injected joints. No such differences were observed at any other time in the baseline, control, and OVA-treated rabbits (Figure 7).

The denatured collagen content represented ~2.5% of the total collagen in control cartilage samples. The content of denatured and type II collagen (4) and collagen cleavage products (2,10) in control cartilage was similar to published values for adult human and

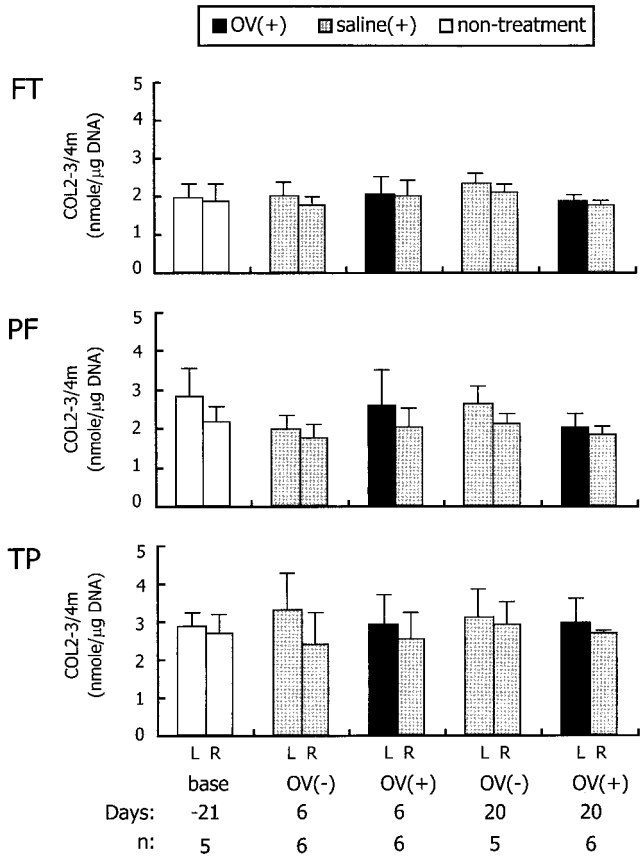

Figure 5. Total type II collagen content in cartilage from ovalbumin (OV)–injected (+) and OV-uninjected (−; non-treatment) NZW rabbits. The combined COL2-3/4m epitope content in α-chymotrypsin and proteinase K digests was determined by immunoassay. Values are the mean and SD. There were no significant differences between left and right knees in any group, by paired *t*-test. FT = femorotibial surface of femoral condyles; PF = patellofemoral surface of femoral condyle; TP = tibial plateau; base = baseline group (see Materials and Methods for details); n = number of animals.

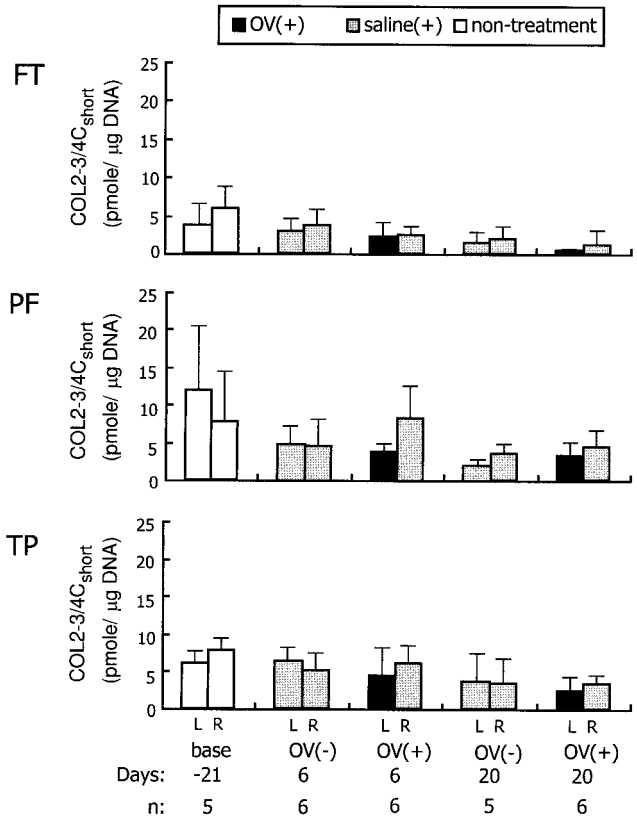

Figure 6. Content of cleaved type II collagen in cartilage from ovalbumin (OV)–injected (+) and OV-uninjected (−; non-treatment) NZW rabbits. The COL2-3/4C$_{short}$ epitope content in α-chymotrypsin digests was determined by immunoassay. Values are the mean and SD. There were no significant differences between left and right knees in any group, by paired *t*-test. FT = femorotibial surface of femoral condyles; PF = patellofemoral surface of femoral condyle; TP = tibial plateau; base = baseline group (see Materials and Methods for details); n = number of animals.

bovine articular cartilage (4,10). In normal cartilage, the collagenase-cleaved collagen represented ~15% of the denatured collagen. This often decreased severalfold with cartilage damage, reflecting a selective loss of the cleavage neoepitope.

In SF, this loss from cartilage was reflected by a significant increase in the collagenase-generated neoepitope in OVA-injected joints compared with contralateral saline-injected joints on both day 6 and day 20 (Figure 8). There was no significant difference in the SF content of the neoepitope in the baseline and control groups. These changes were not reflected in serum.

DISCUSSION

Little is known about whether there is damage to collagen as well as to PGs in articular cartilage following the onset of inflammation such as that which occurs in inflammatory joint diseases such as RA. Recent analyses of human articular cartilage, however, have revealed that type II collagen is degraded within months after rupture of the anterior cruciate ligament, which can frequently result in the development of a posttraumatic OA (16). Early changes in articular cartilage can be conveniently studied in animal models.

Previous work has focused mainly on analyses of changes involving the PG aggrecan (11,17). In the present study, we determined how this inflammation affects not only this PG, but also types II and IX collagen of the collagen fibril. We elected to analyze changes on days 6 and 20 since those would likely reflect early damage to cartilage at the end of the initial acute phase of inflammation (days 0–4) and during the early chronic phase (days 4–20). In other studies of joint inflammation in

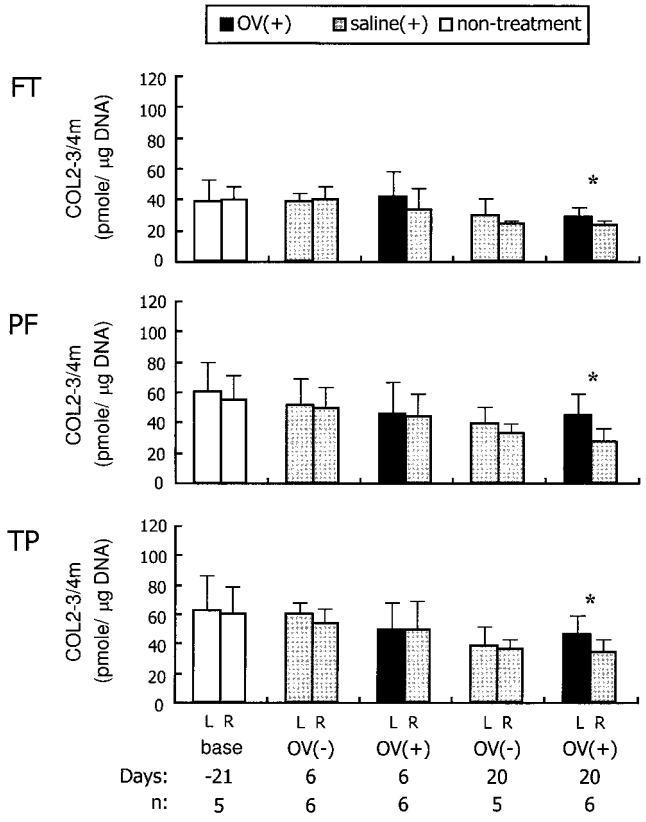

Figure 7. Content of denatured type II collagen in cartilage from ovalbumin (OV)–injected (+) and OV-uninjected (−; non-treatment) NZW rabbits. The COL2-3/4m epitope content in α-chymotrypsin digests was determined by immunoassay. Values are the mean and SD. * = $P < 0.05$ for left knees versus right knees, by paired *t*-test. FT = femorotibial surface of femoral condyles; PF = patellofemoral surface of femoral condyle; TP = tibial plateau; base = baseline group (see Materials and Methods for details); n = number of animals.

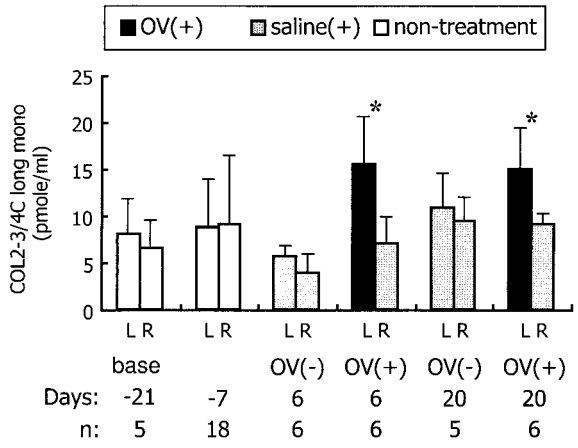

Figure 8. Collagenase-generated peptides of type II collagen in synovial fluid from ovalbumin (OV)–injected (+) and OV-uninjected (−; non-treatment) NZW rabbits. The COL2-3/4C$_{long\ mono}$ epitope in synovial fluid after hyaluronidase digestion was determined by immunoassay. Values are the mean and SD. $* = P < 0.05$ for left knees versus right knees, by paired *t*-test. base = baseline group (see Materials and Methods for details); n = number of animals.

rats, we have observed evidence of early damage to articular cartilage in the acute phase but more pronounced subsequent damage during the development of joint destruction (15). Moreover, recent studies of mice have revealed the importance of conducting longitudinal analyses to identify the progressive involvement of proteinases in the development of articular cartilage damage (17–19). Articular cartilage was analyzed to determine whether there are compartmental differences in the knee in response to IA inflammation, as has been observed by other investigators in murine studies (18,19). In addition, we examined body fluids to determine whether early changes are also reflected in these sites.

Only those joints injected intraarticularly with OVA showed inflammation and damage to articular cartilage. Animals that did not receive IA injections of OVA showed no differences between joints, except in the case of type IX collagen, where a reduced content of the NC4 domain was observed in the left knee joints of control animals on day 20. We have no explanation for this difference.

The NC4 domain of type IX collagen protrudes from the surface of the type II collagen fibril. It has been suggested that this collagen may provide a molecular link between the fibrils and other interfibrillar matrix components such as PG (20). The molar ratio of the NC4 domain to that of type II collagen is ~1:30–40 $\times$ 10^3. This very low level contrasts with a much higher content of this collagen as purified from nonarticular human hyaline cartilage samples, where a content of ~5% type IX and 90% type II collagen has been reported (21). This would translate into a molar ratio of type IX:type II of ~1:15. However, using the same methodology as we used here, we have determined that in cultured bovine growth plate cartilage, the minimal molar ratio of type IX collagen bearing the NC4 domain to type II collagen is ~1:50 (13), indicating that the content of the type IX collagen NC4 domain is much lower in rabbit articular cartilage than in other cartilages examined to date.

Our studies reveal that loss of the NC4 domain is an early event that accompanies PG loss in most compartments and precedes detectable damage to type II collagen within the cartilage. However, our SF analyses revealed that type II collagen cleavage by collagenase was indeed an early event, in that it occurred when type IX collagen and PG were first degraded and lost from the cartilage. However, this increased cleavage was never detectable in articular cartilage until day 20, when we observed increased denaturation, which results from collagen cleavage. The lack of detection of the cleavage product in cartilage likely results from rapid proteolysis of the denatured α chains of the three-quarter and one-quarter fragments, which are then susceptible to cleavage by proteinases such as matrix metalloproteinase 1 (MMP-1), MMP-2, MMP-3, MMP-9, and MMP-13.

In recent studies of the intraarticular degeneration of articular cartilage in the mouse, the induction of increased denaturation (18) and collagenase cleavage (18,19) of type II collagen were observed immunohistochemically as early events that accompanied cleavage of aggrecan by the metalloproteinase and aggrecanase(s) (19). These changes can vary according to the joint compartment (18), as was the case in our studies. The nature of the collagenase(s) involved in the cleavage of type II collagen in the present studies remains to be established. Recently, we have found evidence to indicate that chondrocytes utilize primarily collagenase 3 (MMP-13) in collagen cleavage of bovine (induced by interleukin-1α) (10) and human (22) OA articular cartilage. In rabbit inflammatory arthritis, collagenase 1 (MMP-1) and MMP-13 may also be contributed by activated synovial cells (23).

How type IX collagen is degraded is not known. But MMP-3, which is involved in PG degradation (19), has been reported to be capable of cleaving type IX collagen within its NC2 domain (24). A cleavage site produced by an unknown proteinase has also been identified close to or within the NC4 domain (13).

SF analyses of PG did not reflect the reduction

in PG content in articular cartilage. Only on day 20 were significant differences observed, and then, in contrast to earlier reports, PG was reduced. This could be explained if the inflammation resulted in the release of PG as a product of degradation of newly synthesized molecules. Inflammation has been shown to inhibit PG aggrecan synthesis via induction of interleukin-1 (17). Thus, it would reduce the substrate available for proteolysis. Similar observations of a reduction of PG aggrecan have been made in SF from patients with OA, where the PG content was inversely related to joint inflammation (25).

In conclusion, there is rapid and extensive extracellular matrix degradation involving both PG and collagens in articular cartilage that is caused by joint inflammation in the early phase of antigen-induced arthritis in rabbits. Degradation of both collagen fibrils and PG are also reflected in the SF, but collagen cleavage by collagenase is detected much earlier. Analyses of SF of humans with arthritis may provide an opportunity to study early damage to articular cartilage in diseases such as in RA.

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