OsteoArthritis and Cartilage (2004) 12, 720–728

doi:10.1016/j.joca.2004.06.004

Osteoarthritis and Cartilage

International Cartilage Repair Society

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Detection of aggrecanase- and MMP-generated catabolic neoepitopes in the rat iodoacetate model of cartilage degeneration

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Summary

Objective: To characterize the time course of aggrecan and type II collagen degradation in the rat iodoacetate model of cartilage degeneration in relationship to the temporal sequence that has been described in human osteoarthritis (OA).

Design: Rats were injected intra-articularly in one knee joint with iodoacetate and damage to the tibial plateau was assessed from digitized images captured using an image analyzer. The articular cartilage from the tibial plateau was harvested, extracted and glycosaminoglycan (GAG) content was measured using the dimethylmethylene blue (DMMB) assay. Cartilage aggrecan neoepitopes were detected in cartilage extracts by Western blotting using antibodies recognizing the aggrecanase-generated C-terminal neoepitope NITEGE (BC-13) and the MMP-generated C-terminal neoepitope DIPEN (BC-4). A type II collagen collagenase-generated neoepitope was detected in cartilage extracts by ELISA using the Col2-3/4Cshort antibody; denatured collagen was detected using the Col2-3/4m antibody.

Results: Degenerative joint changes and proteoglycan (GAG) loss progressed with time after iodoacetate injection. Western blotting of cartilage extracts of iodoacetate treated rats demonstrated an increase in both aggrecanase- and MMP-generated epitopes with the NITEGE aggrecanase neoepitope being significantly elevated on days 7, 14 and 21 while DIPEN the MMP neoepitope was significantly elevated on days 7 and 14. The type II collagen neoepitope recognized by Col2-3/4Cshort was significantly increased in cartilage extracts of rats at days 14 and 21 after iodoacetate injection.

Conclusion: The proteoglycan fragments extracted from the knee cartilage of rats after the intra-articular injection of iodoacetate appeared to result from cleavage at both aggrecanase and MMP sites. Cleavage of type II collagen by collagenase was also detected after iodoacetate injection and occurred subsequent to the initiation of aggrecan loss. These observations serve to demonstrate similarities in the mechanisms of cartilage degeneration induced by iodoacetate to those seen in articular cartilage in OA.

Key words: Iodoacetate-induced arthritis, Neoepitopes, Matrix metalloproteinase, Aggrecanase.

Introduction

Osteoarthritis (OA) is a degenerative disease that occurs in a large proportion of the elderly and is characterized by cartilage matrix degradation. The degradation of cartilage that occurs in OA is a result of proteolytic cleavage of both of its structural components, aggrecan and type II collagen. The degradation of aggrecan occurs early in the process of cartilage degeneration[1] and is followed by the catabolism of collagen fibrils leading to the loss of cartilage structural integrity[2]. The loss of aggrecan from cartilage is believed to be due to the proteolytic cleavage within the interglobulin domain (IGD) between the G1 and G2 globular domains at two specific sites, Asn^{341}-Phe^{342} and Glu^{373}-Ala^{374}. A number of MMPs have been shown to cleave at the Asn^{341}-Phe^{342} (references 3–5) while the cleavage of the Glu^{373}-Ala^{374} is due to "aggrecanase" enzymatic activity[6–9]. ADAMTS-4 and ADAMTS-5 were the first aggrecanases to be identified and are members of the *A Di*sintegrin *A*nd *M*etalloproteinase with *T*hrombo*S*pondin motifs gene family[10,11]. More recently, a third ADAMTS (ADAMTS-1) has been shown to cleave aggrecan at the sites appropriate to classify it as an "aggrecanase"[12], while ADAMTS-9 has been shown to cleave one of the C-terminal aggrecanase sites albeit inefficiently[13].

Type II collagen is the major structural collagen in articular cartilage. A number of MMPs (MMPs 1, 8, 13, 14 and 18) cleave intact collagen between residues Gly^{775} and Leu^{776}. Neoepitope antibodies that recognize the carboxy-terminal neoepitope of this cleavage site have been developed[14] as well as antibodies to denatured collagen[15]. These antibodies have been used to demonstrate increased collagen cleavage and degradation in human and

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Received 20 October 2003; revision accepted 1 June 2004.

animal models of OA[14–17]. Likewise antibodies to the neoepitopes generated in aggrecan by the enzymatic activity of MMPs and aggrecanases have been developed and have been used in experimental systems of cartilage degradation[18–20].

Although the etiology of OA is poorly understood, numerous animal models that mimic aspects of the disease have been developed to study the pathophysiology of the disease and to evaluate potential therapeutics. Kalbhen and Blum first described the iodoacetate-induced model of degenerative arthritis in chickens[21] and subsequently the model was performed in a number of other species[22–26]. The severity of the cartilage lesions, suppression of mobility and inhibition of proteoglycan synthesis were directly related to the dose of iodoacetate[27]. Recently, we have demonstrated the upregulation of MMPs in knee cartilage from iodoacetate-injected rats and inhibition of the degenerative changes by several MMP inhibitors[28]. In the present study we have investigated the mechanisms of cartilage degeneration in the rat iodoacetate model by exploring the temporal relationship between MMP- and aggrecanase-generated aggrecan metabolism as well as aggrecan and type II collagen cleavage in cartilage degradation using neoepitope specific antibodies.

Materials and methods

ANIMALS

Sprague–Dawley male rats weighing 220–230 g (Harlan, Indianapolis, IN) were housed singly in wire cages in sanitary ventilated animal rooms with controlled temperature, humidity and regular light cycles. Rodent chow (Ralston-Purina, Richmond, IN) and water were available *ad libitum*. Animals were acclimated for at least 1 week before use.

All animals were housed, fed, and handled in compliance with the standards set forth by the Animal Welfare Act as amended. Where standards are not indicated in the Animal Welfare Act the recommendations on HHS Publications (NIH) No. 85-23, "Guide for the Care and Use of Laboratory Animals", were followed.

INDUCTION OF IODOACETATE-INDUCED ARTHRITIS

Arthritis was induced by a single intra-articular injection of iodoacetate into the knee joint of rats anesthetized using (3:1) CO_2/O_2. A 10 mg/ml concentration of monosodium iodoacetate (MIA) (Aldrich Chemical, Milwaukee, WI) was prepared using injectable saline as the vehicle. After appropriate anesthesia each rat was positioned on its back and the left leg was flexed 90 degrees at the knee. The patellar ligament was palpated below the patella and the injection was made into this region. Each rat received 0.025 ml intra-articular injection into the left knee using a glass gas tight syringe with a 27 gauge $\frac{1}{2}$ inch needle. Care was taken not to advance the needle in too far into the cruciate ligaments.

Since only a small amount of cartilage is obtained from rat knees and different extraction protocols are required for aggrecan and collagen analysis, two separate experiments were performed. One experiment was used for processing knee cartilage for aggrecan analysis and a second experiment for collagen analysis as described below. Each experiment utilized groups of 10 rats sacrificed 0, 1, 3, 7, 14 and 21 days after iodoacetate injection for a total of 60 rats for each experiment. The left knees of the sacrificed animals were disarticulated and the tibial plateau imaged using an Optimas image analyzer. The tibial plateau was used for image analysis because it provided a relatively flat surface compared with the femoral condyles, allowing the image analysis camera to focus on the entire cartilage surface. The severity of damage in the magnified images was assessed by three independent observers in a blinded manner using a scale of increasing severity (0 = normal; 4 = maximum severity) as described previously[28].

ANALYSIS OF AGGRECAN METABOLISM

Articular cartilage was harvested from the tibial plateau of rats at various times after iodoacetate injection. The cartilage from 10 rats at each time point was randomly pooled into five groups containing cartilage from two rats to provide sufficient material for quantitation of proteoglycan and for Western blots. The cartilage was blotted dry, weighed and extracted with 4 M GuHCl in 0.05 M sodium acetate containing the proteinase inhibitors 0.01 M EDTA, 0.1 M 6-aminohexanoic acid, 0.005 M benzamidine HCl and 0.01 M *N*-ethylmaleimide for 48 h at 4°C. Following extraction the cartilage residue was digested with papain and the proteoglycan content of the cartilage extract and papain digest was measured as sulphated glycosaminoglycan (GAG) using a dimethylmethylene blue assay[29] with chondroitin sulphate-C from shark cartilage (Sigma Chemical, Inc., St Louis, MO) as standard.

Proteoglycan fragments in the guanidine extracts of cartilage from the GAG studies outlined above were analyzed as described previously[19]. There were five pools of cartilage at each time point containing cartilage from two rats. One set of samples was used to work out experimental conditions (antibody concentration loading amounts) and the other four samples were used to run four separate Western blots. Briefly, the guanidine extracts were dialyzed and deglycosylated with chondroitinase ABC (Sigma Inc.), keratanase (Seikagaku) and keratanase II (Seikagaku) at 37°C for 2–4 h. Samples were dialyzed against deionized water, lyophilized and separated under reducing conditions on 4–12% gradient Tris/glycine SDS-PAGE gels (Novex, Inc., Frankfurt, Germany). Samples were loaded such that the extract from an equal wet weight of cartilage was run in each lane. The proteins were electrophoretically transferred to nitrocellulose membranes. Immunoblotting was performed as previously described[30] using monoclonal antibodies recognizing the C-terminal aggrecan neoepitope NITEGE (BC-13)[18] and the MMP-generated C-terminal aggrecan neoepitope VDIPEN (BC-4)[18]. Blots were digitized and the density of immunostained bands was quantified using NIH image software. The band with the highest density on each Western blot was assigned a value of 100 density units and the densities of all other bands were compared to the 100 unit band. This allowed the comparison between different Western blots.

EXTRACTION OF RAT ARTICULAR CARTILAGE FOR QUANTITATION OF COLLAGENASE CLEAVAGE NEOEPITOPE AND DENATURED TYPE II COLLAGEN

Articular cartilage was harvested from the tibial plateau rats at various times after iodoacetate injection, blotted dry, weighed and extracted overnight at 37°C with α-chymotrypsin (~0.3 mg/15 mg of cartilage) in 50 mM Tris–HCl, pH 7.6, containing 1 mM EDTA, 1 mM iodoacetamide and

10 μg/ml of pepstatin A (Sigma Chemical Co.) as previously described[14,15]. The α-chymotrypsin activity was inhibited by incubating for 20 min at 37°C with *N*-tosyl-L-phenylalanine-chloromethyl ketone (TPCK; Sigma Chemical Co.) at a final concentration of 160 μg/ml. The samples were then centrifuged and the supernatants were assayed using an ELISA assay for the Col2-3/4Cshort neoepitope[14] and for denatured collagen using the mouse monoclonal antibody (Col2-3/4m) to the interchain epitope CB11B[15]. The remaining insoluble portion of the cartilage was digested overnight at 56°C with 1 mg/ml proteinase K in 50 mM Tris–HCl containing the proteinase inhibitors described above. Proteinase K was inactivated by boiling for 20 min. The percent collagen cleaved was calculated from the amount of Col2-3/4Cshort neoepitope assayed in the chymotrypsin extract expressed as a percentage of the total collagen content (total Col2-3/4m epitope in chymotrypsin and proteinase K extracts).

STATISTICAL ANALYSIS

Quantitative data from the GAG and aggrecan neoepitope studies were analyzed using ANOVA and data from the collagen degradation studies were evaluated using a non-homogeneous analysis of variance model.

Results

CARTILAGE AND MATRIX DEGRADATION INCREASE WITH TIME AFTER IODOACETATE INJECTION

Tibial degeneration

The injection of 0.25 mg of iodoacetate into the left knee of rats resulted in a time related increase in the severity of cartilage degeneration as revealed by image analyses of the tibial plateau. There was virtually no macroscopic cartilage degeneration until 7 days after iodoacetate injection and then cartilage damage progressed from days 7 to 21 (Fig. 1).

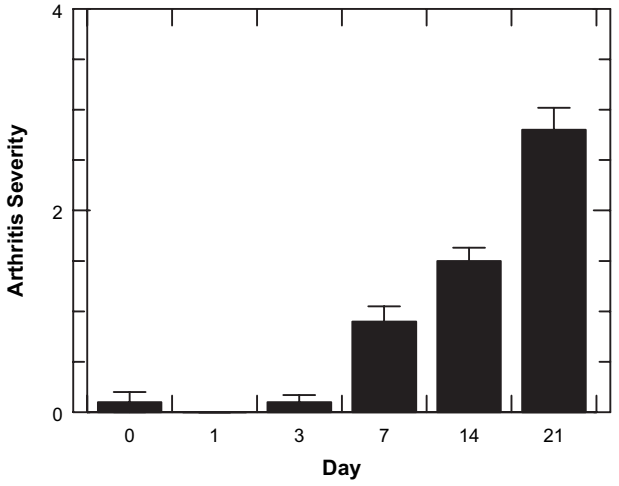

Fig. 1. Time-dependent effect of the injection of iodoacetate on the severity of tibial plateau degeneration in rat knees. The severity of knee joint degeneration in rats injected with 0.25 mg of iodoacetate was evaluated at varying times after injection and the cartilage was collected for biochemical evaluation of aggrecan degradation. The severity of damage to the tibial plateau was assessed using magnified images captured with an image analyzer using a scale of increasing severity (0 = normal; 4 = maximum severity) as described in Materials and Methods. The data are presented as mean ± S.E.M. from 10 rats per group.

Aggrecan loss and degeneration in tibial plateau

There was a decrease in extractable GAG in the cartilage from the iodoacetate-injected knees with time (Fig. 2). There was no significant change in GAG content on days 1 and 3 after iodoacetate injection compared to the day 0 animals; however, there was a significant decrease in GAG content on days 7, 14 and 21 ($P < 0.001$) (Fig. 2). The GAG content on day 21 was significantly lower than that on all other days ($P < 0.002$). The change in GAG content was consistent with the increase in the cartilage damage scores.

Tibial cartilage extracted from the knee joint of rats at various times after iodoacetate injection was analyzed by Western blotting using antibodies to neoepitopes generated at the aggrecan cleavage sites produced by aggrecanase (NITEGE) or MMPs (DIPEN). A 70 kDa doublet and a single 50 kDa protein were the two predominant NITEGE terminating G1 fragments [Fig. 3(A)]. The 70 kDa doublet was consistent with that observed in other species[19]. While we have not previously observed a 50 kDa terminating catabolite, it is consistent in size with a minor band detected in extracts of retinoic acid treated human articular cartilage[31]. The full range of proteins present in a pool of cartilage extracts from all time points is shown in Fig. 3(B), lane 1. Western blot analysis of the cartilage extract excluding the primary antibody demonstrated that this band was specific [Fig. 3(B), lane 2] and both the 50 and 70 kDa bands were observed when a duplicate filter was probed with a polyclonal anti-NITEGE serum (generously provided by Dr John Mort, Shriners Hospital, Quebec, Canada) [Fig. 3(B), lane 3]. The 50 kDa NITEGE terminating fragment was present in the day 0 samples and must have resulted from earlier catabolism and accumulation during the rats' lifetime. A single DIPEN terminating aggrecan

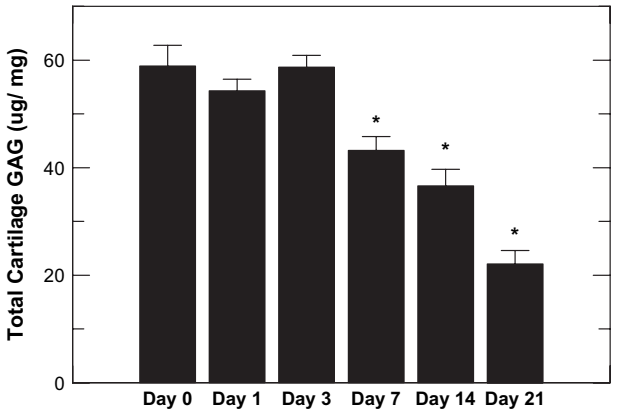

Fig. 2. Time-dependent loss of glycosaminoglycan (s-GAG) after intra-articular injection of iodoacetate into the knee of rats. The s-GAG content of knee cartilage from rats at various times after iodoacetate injection (shown in Fig. 1) was measured using the dimethylmethylene blue method. The cartilage from 10 rats at each time point was randomly pooled into five groups containing cartilage from two rats and the data are presented as the mean ± S.E.M. ($N = 5$) amount of GAG/mg of wet weight of cartilage. *Denotes significance ($P < 0.05$) compared to day 0.

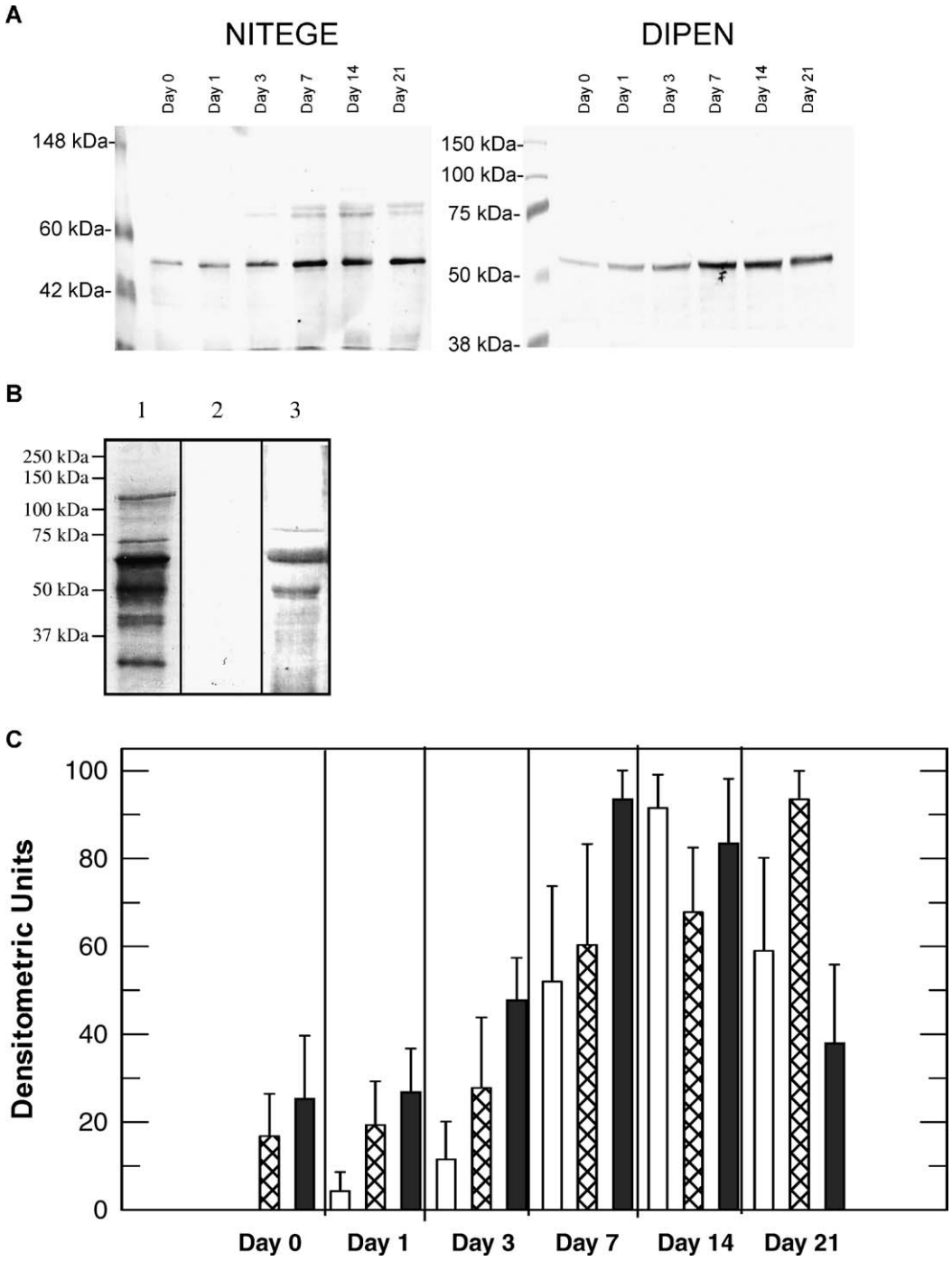

Fig. 3. Western blot analysis of cartilage extracts from rats at various times after iodoacetate injection (shown in Fig. 1). Cartilage was collected at various times after iodoacetate injection and immunoblotted with antibodies recognizing the aggrecanase-generated NITEGE and MMP-generated DIPEN terminating aggrecan neoepitopes. The results from a single Western blot representative of four individual Western blots are shown (A). As a control for the specificity of the primary antibody (BC-13) a Western blot of pooled joint extracts was developed without BC-13 (B, lane 2) or with a polyclonal anti-NITEGE (generously provided by Dr John Mort) (B, lane 3). (B, lane 1) shows a coomassie stain of the full range of proteins present in the cartilage extract. The relative amount of each epitope was measured by densitometry (C). The data are presented as mean $\pm$ S.E.M. from the four Western blots. The 70 kDa doublet C-terminal NITEGE protein is shown as an open bar, the 50 kDa C-terminal NITEGE protein as a hatched bar and the C-terminal DIPEN terminating 55 kDa protein as a solid black bar.

metabolite ($\sim$55 kDa) was observed in the rat cartilage and was similar in size to that seen in other species [Fig. 3(A)][19].

The intensity of the NITEGE bands changed significantly with time as determined by densitometry. The density of both the 70 and 50 kDa NITEGE bands was significantly ($P < 0.01$) increased compared with day 0 on days 7, 14 and 21 [Fig. 3(C)]. The staining intensity of the DIPEN band also changed significantly with time. The increased staining observed on day 3 did not reach statistical significance, whereas the staining observed at days 7 and 14 was

significantly greater than that observed at day 0 [Fig. 3(C)]. Interestingly, the staining intensity of the DIPEN band decreased at day 21 and was not significantly different from day 0.

COLLAGEN CLEAVAGE INCREASES WITH TIME AFTER IODOACETATE INJECTION

Since only a small amount of cartilage is obtained from rat knees and a different extraction protocol was required for assessment of collagen cleavage a separate rat iodoacetate experiment was performed. Rats were sacrificed at various times after a single intra-articular injection of 0.25 mg of iodoacetate into the left knee; the severity of arthritis was determined and the tibial cartilage was extracted for quantitation of collagen neoepitopes. Although there was a time related increase in the severity of cartilage degeneration as assessed by gross pathology using image analysis (Fig. 4), the amount of Col2-3/4C neoepitope present in cartilage did not significantly increase during the first week after iodoacetate injection but was significantly elevated on days 14 and 21 ($P < 0.01$) compared to day 0 controls (Fig. 5).

The percentage of denatured collagen as measured with the Col2-3/4m antibody was significantly higher than the Col2-3/4Cshort epitope in both control rats and iodoacetate-injected animals (Fig. 5). However, the Col2-3/4m epitope was not significantly higher on days 14 and 21 compared to day 0 controls (Fig. 5).

Discussion

We have shown that progressive degeneration of articular cartilage develops in the knees of rats following intra-articular injection of iodoacetate. In association with this degeneration, the cartilage contains an increased content of aggrecan epitopes reflecting an increase in both aggrecanase- and MMP-generated cleavage. The loss of aggrecan as detected by GAG content of the extracted cartilage increased with time in the rat iodoacetate model and the appearance of both aggrecanase and MMP neoepitopes was temporally related to the aggrecan loss with both epitopes significantly elevated at day 7. We also found that type II collagen undergoes increased cleavage by collagenase as reflected by the increase in the amount of neoepitope detected by the Col2-3/4Cshort antibody in iodoacetate-injected rats. However, this increase was clearly observed subsequent to the increase in proteoglycan loss and cleavage as has been shown in culture[32]. The data suggest that both aggrecanase and MMPs play a role in the cartilage degradation that occurs in the iodoacetate model.

In the present study we have utilized detection of the neoepitope sequences NITEGE and DIPEN to indicate aggrecanase or MMP cleavage of the aggrecan interglobular domain, respectively. Although MMP-8 can cleave at the aggrecanase site to generate NITEGE neoepitope, this only occurs after prolonged cleavage and is therefore considered unlikely to account for generation of this neoepitope *in vivo*[33]. Similarly, although cathepsin B can

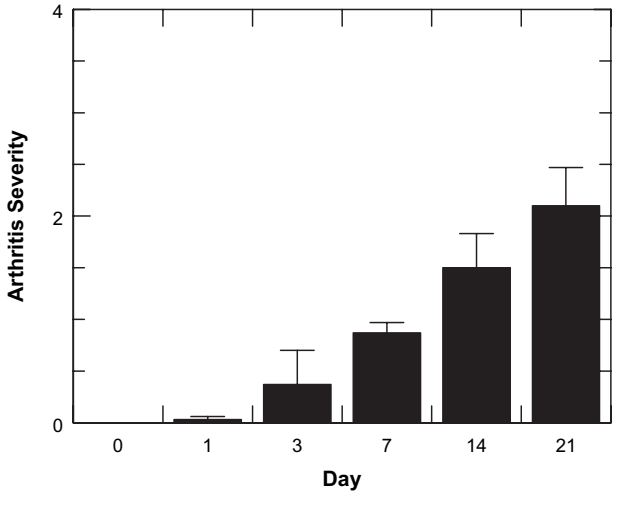

Fig. 4. Time-dependent effect of the injection of iodoacetate on the severity of joint degeneration in rat knees. The severity of tibial articular cartilage degeneration in rats injected with 0.25 mg of iodoacetate was evaluated at varying times after injection and the cartilage was collected for biochemical evaluation of collagen degradation. The data are presented as mean ± S.E.M. from 10 rats per group.

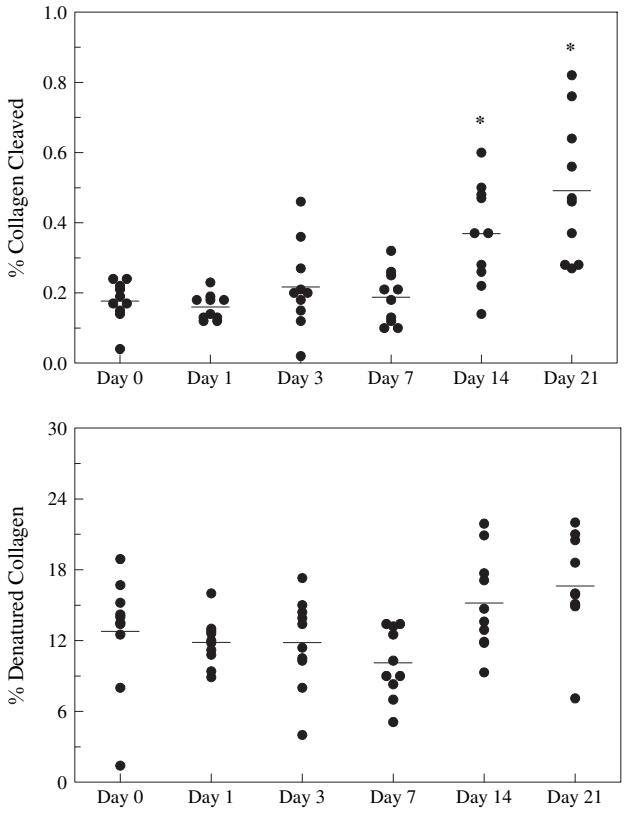

Fig. 5. Time course of type II collagen cleavage in the rat iodoacetate-induced arthritis model. Articular cartilages from rat knees harvested at various times after iodoacetate injection (shown in Fig. 4) were extracted with α-chymotrypsin and assayed for Col2-3/4Cshort and Col2-3/4m epitopes as described in Materials and Methods. Data are expressed as % cleavage of type II collagen (Col2-3/4Cshort; A) or as % collagen denaturation (Col2-3/4m; B) in chymotrypsin extracts of cartilage as a percent of total collagen content and the mean is shown as a horizontal line. Total collagen content was determined by the sum of the Col2-3/4m epitope from the chymotrypsin and proteinase K extracts. *Denotes statistical significance at $P > 0.05$.

generate the DIPEN epitope *in vitro* through its exopeptidase activity, the acidic pH (<6.0) necessary for this reaction to proceed efficiently suggests that it may not be physiologically relevant[34]. Most recently, recombinant ADAMTS-4 was shown to generate the DIPEN epitope in solution phase digests of purified aggrecan *in vitro*[35]. However, using a different recombinant ADAMTS-4 or recombinant ADAMTS-1 or ADAMTS-5, cleavage at this classical MMP site was not found by other workers[36]. Numerous studies have also failed to demonstrate increased generation of DIPEN when cartilage explants are stimulated with a variety of catabolic agents *in vitro*, despite marked increases in aggrecanase activity and depletion of cartilage aggrecan[19,37,38]. These results suggest that generation of the DIPEN neoepitope by ADAMTS-4 may not be relevant in cartilage tissue *in vivo*. We therefore consider it reasonable to continue to ascribe the generation of the NITEGE and DIPEN epitopes in cartilage to the action of aggrecanases and MMPs, respectively.

In our previous study we demonstrated that administration of MMP inhibitors for the first 7 days following iodoacetate injection was able to significantly ameliorate the cartilage damage[28]. It may seem difficult to reconcile with the present data demonstrating that collagenase cleavage of collagen is not maximal until days 14–21 [Fig. 5(A)]. One possible explanation is that MMP activity in the early phases of iodoacetate-induced disease is responsible for the cleaving matrix components that bind to type II collagen and normally act to protect the fibril from catabolism. Using *in vitro* models of progressive cartilage breakdown, it has been shown that COMP and fibromodulin are cleaved by metalloproteinases and released from cartilage prior to subsequent collagenolysis[39,40]. Inhibiting the catabolism of these or other fibril-associated molecules such as decorin, biglycan or collagen type IX during the first week of iodoacetate-induced arthritis could stop or delay subsequent collagen proteolysis. Additionally, the early administration of MMP inhibitors in this disease model may prevent the initial activation of proteinases such as MMP-3 that are required for collagenase activation. In our previous study, analysis of cartilage collagenase and gelatinase was performed in such a way that we did not distinguish between active and proenzyme[28]. Abrogation of early MMP activity may also stop the catabolism of other matrix molecules such as fibronectin, fragments which could then stimulate aggrecanase and collagenase synthesis and activity[38,41].

Our results demonstrating the presence of MMP- and aggrecanase-generated cleavage fragments in the rat iodoacetate model using biochemical methodology are similar to those recently reported for several rodent models of OA using immunohistochemistry. In mice containing a transgene overexpressing active MMP-13 in their hyaline cartilage significant upregulation of pericellular and intracellular staining of chondrocytes for the Col2-3/4Cshort epitope was observed[17]. Collagen cleavage detected using the antibody Col2-3/4Cshort was also associated with histopathologic lesions in the STR/ORT model of OA in mice[42] and in fibrillated areas after anterior cruciate transection in the rat[43]. Similarly, the aggrecan degradation epitopes generated by MMPs (VDIPEN) and aggrecanase (NITEGE) colocalized in both the pericellular and matrix adjacent to OA lesions in the mouse STR/ORT model[44]. The presence of both MMP- and aggrecanase-generated aggrecan fragments has been detected in human articular cartilage from normal and OA patients[45]. In extracts of human osteoarthritic articular cartilage significantly more Col2-3/4Cshort epitope was detected than in nonarthritic human cartilage around chondrocytes[16]. Although both aggrecan and collagen degradation neoepitopes generated in the rat iodoacetate model were also observed in spontaneous and surgically induced rodent models (ACL) the distribution and amount of these epitopes may vary. Matrix degradation occurs rapidly and is widespread in the rat iodoacetate model, whereas damage is more focal, generally less severe and develops more slowly in the STR/ORT and rat ACL models.

Due to the difficulty in obtaining significant amounts of synovial fluid from rat knees in the iodoacetate model, the present study relied on extraction of rat cartilage and only detected fragments that remained in the tissue after being cleaved by MMPs or aggrecanase. Therefore, any aggrecan G1 fragment that diffused into the synovial fluid remained undetected. The presence of DIPEN C-terminal G1 epitopes in the rat cartilage could have resulted from the primary cleavage of aggrecan by MMPs or MMP truncation of G1 fragments originally cleaved at the aggrecanase NITEGE site. However, the presence of DIPEN epitope is consistent with the previously reported[28] presence of MMP activity in cartilage extracts after the injection of iodoacetate. The simultaneous presence of both NITEGE and DIPEN epitopes in the rat iodoacetate model differs from that observed in several animal models of rheumatoid arthritis where NITEGE was the prominent epitope early during aggrecan depletion, whereas DIPEN dominated during the collagen degradation phase[46]. However, both DIPEN and NITEGE epitopes colocalized in human OA lesions[45] and in spontaneous OA in mice[44]. These studies may suggest that the temporal relationship between aggrecanase and MMP activity in models of inflammatory arthritis in mice and naturally occurring OA in mice and man may differ.

The amount of collagen cleavage fragment detected in rat cartilage extracts by the Col2-3/4Cshort antibody increased almost threefold from day 0 to day 21 after iodoacetate injection. This increase was similar to that observed in articular cartilage extracts from OA patients compared to normals[14]. The percent of collagen cleavage epitope detected on Western blot using Col2-3/4Cshort at day 21 was 0.49% (Fig. 5) which is comparable to approximately 1.5% of this epitope detected in OA patients[14]. The amount of denatured collagen observed in the rat iodoacetate model using the Col2-3/4m antibody increased about 2–3% from day 0 to day 21. The relatively low quantity of Col2-3/4Cshort epitope detected in the arthritic animals in the present study as well as previous human studies[14] could be an underestimation due to enzymatic cleavage of the collagen by proteinases such that the Col2-3/4Cshort antibody neoepitope is degraded and/or released, whereas the Col2-3/4m epitope is relatively distant from the collagenase cleavage site and may be less susceptible to secondary cleavage and more likely to be retained in the matrix.

Aggrecanase cleavage as detected using an antibody to the NITEGE epitope in cartilage from iodoacetate-injected rats resulted in a 70 kDa doublet on Western blot that was consistent with observations in other species[19]. The 50 kDa band observed must have resulted from further proteolysis in the N-terminus of the G1 molecule without affecting the hyaluronic acid binding as it is retained in the cartilage. It is possible that there is a cleavage in one of the three disulphide bonded N-terminal loops that does not affect hyaluronic acid binding. The small N-terminal peptide that is presumably removed to generate this 50 kDa NITEGE

catabolite is most likely disulphide bonded and part of an intact G1. However, as all the gels are run under reducing conditions, this small peptide is released. The 50 kDa NITEGE terminating fragment was present in the day 0 samples and must have resulted from earlier catabolism and accumulation during the rats' lifetime. While this fragment has been generated by aggrecanase cleavage of the IGD, the enzyme responsible for the further N-terminal cleavage is unknown.

The decrease in DIPEN in cartilage extracts at day 21 in the present study appears to contradict the increase in collagenase-generated Col2-3/4Cshort epitope. However, a similar loss of DIPEN immunostaining was recently reported in the most degenerate cartilage in a mouse model of OA[47]. This late stage decrease in DIPEN may be explained by the release of G1 from the cartilage as the collagen network becomes more severely disrupted, accounting for the coordinate loss of NITEGE immunostaining in the most degenerate mouse cartilage[47] and the decrease in the 70 kDa NITEGE bands in the present study (Fig. 3). In contrast, there was no decrease in the 50 kDa NITEGE band in the cartilage extracts at day 21. This smaller NITEGE catabolite may only be generated through the action of aggrecanase and a second cell-associated proteinase. Therefore, as the cartilage degeneration progresses and the interterritorial matrix is lost, only pericellular newly synthesized aggrecan remains and generation of the 50 kDa but not 70 kDa NITEGE continues.

These results suggest that degradation of aggrecan in the rat iodoacetate model involves the enzymatic activities of both aggrecanases and MMPs. Furthermore, type II collagen cleavage as measured using the Col2-3/4Cshort antibody was also increased in the knee cartilage of iodoacetate-injected rats suggesting a role for MMPs of the collagenase subtype. The demonstration of an increase in cleavage neoepitopes generated by aggrecanase(s) and MMPs in articular cartilage in the rat iodoacetate model associated with cartilage degeneration serves to reveal the similarities in the pathological mechanisms observed in this model to that seen in articular cartilage in human OA. The cleavage of type II collagen appears temporally after the degradation of aggrecan, similar to observations made in cultured bovine articular cartilage degradation induced by interleukin-1α[32].

Acknowledgements

The authors would like to thank Dr Lin Fei for performing statistical analysis on the data for this manuscript.

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