# Aggrecanase versus matrix metalloproteinases in the catabolism of the interglobular domain of aggrecan *in vitro*

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The importance of aggrecanase versus matrix metalloproteinase (MMP) enzymic activities in the degradation of aggrecan in normal and osteoarthritic (OA) articular cartilage *in vitro* was studied in order to further our understanding of the potential role of these two enzyme activities in aggrecan catabolism during the pathogenesis of cartilage degeneration. Porcine and bovine articular cartilage was maintained in explant culture for up to 20 days in the presence or absence of the catabolic stimuli retinoic acid, interleukin-1 or tumour necrosis factor-$\alpha$. Release of proteoglycan from cartilage was measured as glycosaminoglycan (GAG) release using a colorimetric assay. Analysis of proteoglycan degradation products, both released into culture media and retained within the cartilage matrix, was performed by Western blotting using antibodies specific for the N- and C-terminal neoepitopes generated by aggrecanase- and MMP-related catabolism of the interglobular domain of the aggrecan core protein (IGD). In addition, studies determining the mRNA expression for MMP-3 and MMP-13 in these same cultures were undertaken. These analyses indicated that all three catabolic agents stimulated the release of $> 80\,\%$ of the GAG from the articular cartilage over 4 days. The degree of GAG release corresponded to an increase in aggrecanase-generated aggrecan catabolites released into the media and retained within the cartilage. Importantly, there was no evidence for the release of MMP-generated aggrecan metabolites into the medium, nor the accumulation of MMP-generated catabolites within the tissue in these same cultures. Expression of the mRNAs for two MMPs known to be capable of degrading the aggrecan IGD, MMP-3 and MMP-13, was detected. However, increased expression of these MMPs was not correlated with aggrecan degradation. Analyses using porcine cartilage, cultured with or without catabolic stimulation for 12 h to 20 days, indicated that primary cleavage of the IGD by aggrecanase was responsible for release of aggrecan metabolites at both the early and late time points of culture. Cultures of late-stage OA human articular cartilage samples indicated that aggrecanase activity was upregulated in the absence of catabolic stimulation when compared with normal porcine or bovine cartilage. In addition, even in this late-stage degenerate cartilage, aggrecanase and not MMP activity was responsible for the release of the majority of aggrecan from the cartilage. This study demonstrates that the release of aggrecan from both normal and OA cartilage in response to catabolic stimulation *in vitro* involves a primary cleavage by aggrecanase and not MMPs.

Key words: articular cartilage, explant culture, extracellular matrix, proteoglycan degradation, proteolysis.

## INTRODUCTION

Articular cartilage contains high concentrations of the large aggregating proteoglycan aggrecan, a multi-domain molecule which is essential for the demanding functions of this highly specialized tissue. The high negative charge density of the glycosaminoglycan (GAG) chains present on aggrecan monomers in cartilage proteoglycan aggregates contributes significantly to the ability of articular cartilage to withstand compressive deformation during joint articulation. The loss of GAG-bearing aggrecan fragments from articular cartilage, and their release into the synovial fluid, is one of the central pathophysiological events in joint diseases such as osteoarthritis and rheumatoid arthritis. This depletion of aggrecan from the cartilage matrix compromises the weight-bearing properties of the tissue and contributes to further mechanical disruption of the cartilage. In order to develop optimal therapeutic and prophylactic pharmacological strategies for the management of the various arthropathies it is vital to determine the enzyme(s) primarily responsible for the release of GAG-bearing aggrecan metabolites from articular cartilage.

Amino acid sequence analysis of the aggrecan fragments released from the cartilage matrix into the synovial fluid of arthritic patients has revealed that the major catabolite has an N-terminus ARGSV…, resulting from cleavage between $Glu^{373}$ and $Ala^{374}$ (human sequence enumeration) [1,2]. The enzyme activity responsible for this cleavage is referred to as 'aggrecanase'. However, G1-bearing aggrecan fragments terminating with the amino acid sequence … DIPEN have been isolated from human articular cartilage [3], and degradation products initiating at $F^{341}$FGVG… have been detected in synovial fluid of arthritic patients [4,5] (G1 is globular domain 1

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; OA, osteoarthritic; RA, retinoic acid; IL-1, interleukin-1, TNF, tumour necrosis factor-$\alpha$; GAG, glycosaminoglycan; IGD, interglobular domain of the aggrecan core protein; DMMB, Dimethylmethylene Blue; MAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; TIMP, tissue inhibitor of metalloproteinase.

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of aggrecan, which contains the hyaluronan-binding region). Such degradation products can be generated *in vitro* by the action of a number of the matrix metalloproteinases (MMPs), with cleavage of the interglobular domain of the aggrecan core protein (IGD) occurring between $Asn^{341}$ and $Phe^{342}$ (human sequence enumeration), 32 amino acids N-terminal to the aggrecanase cleavage site [3,6–9]. The occurrence of these amino acid sequences on aggrecan catabolites suggests that cleavage of aggrecan by both aggrecanase and MMPs occurs *in vivo*.

The study of aggrecan IGD catabolism by aggrecanase and MMPs has been greatly facilitated by the development of antibodies which specifically recognize the new N- and C-terminal amino acid sequences ('catabolic neoepitopes') generated by cleavage at $Glu^{373}$-$Ala^{374}$ by aggrecanase (anti-ARGSV and anti-ITEGE) or at $Asn^{341}$-$Phe^{342}$ by MMPs (anti-FFGVG and anti-DIPEN) [5,10–13]. Studies utilizing antibodies to the C-terminal neoepitopes on aggrecan catabolites retained within the cartilage matrix have confirmed the presence of G1-bearing fragments resulting from cleavage at both the aggrecanase and MMP sites [14–19]. The presence of these different aggrecan catabolites in the synovial fluid and cartilage matrix may be explained by a number of possible catabolic pathways, as suggested by Lark et al. [16]; i.e. (i) primary cleavage at the aggrecanase site followed by secondary MMP catabolism of the G1-bearing fragment; (ii) primary cleavage at the MMP-site followed by secondary cleavage of the released GAG-bearing catabolite by aggrecanase; or (iii) independent cleavage of separate aggrecan molecules by the two enzyme activities.

In attempts to elucidate the actual sequence of catabolic events, researchers have studied *in vitro* models of normal and accelerated aggrecan catabolism using either cartilage explants or chondrocyte monolayer cultures exposed to catabolic agents such as retinoic acid (RA), interleukin-1 (IL-1) or tumour necrosis factor-$\alpha$ (TNF) [12,20–25]. However, no studies have simultaneously evaluated the generation of the N- and C-terminal neoepitopes which result from the action of both aggrecanase and MMPs *in vitro*. In the present study we have utilized articular cartilage explant cultures as models for studying mechanisms of normal and accelerated aggrecan catabolism. Neoepitope-specific antibodies were used to detect aggrecan fragments, both retained within the cartilage matrix and released into culture media, that were produced by either aggrecanase or MMP cleavage. The generation of the four different catabolic products was evaluated both with time in culture and in the presence or absence of three different catabolic stimuli (RA, IL-1 and TNF).

## MATERIALS AND METHODS

### Preparation and culture of cartilage explants

Full-depth articular cartilage was dissected from bovine (2 week old immature and 18–24 month old mature) or porcine (3–6 month old) metacarpophalangeal joints of six different animals. Explants (10–20 mg wet weight) were cultured for 72 h at 37 °C in a humidified atmosphere of 5% $CO_2$/95% air in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Paisley, U.K.) containing 50 $\mu$g/ml gentamicin and 10% (v/v) fetal bovine serum. Explants were then washed ($3 \times 5$ min) in serum-free DMEM and cultured in triplicate in individual wells of 24-well tissue culture plates containing 1 ml of serum-free DMEM with or without $10^{-6}$ M all-*trans*-RA (Sigma-Aldrich, Poole, Dorset, U.K.), 10 ng/ml recombinant human IL-1 (Sigma) or 100 ng/ml recombinant human TNF-$\alpha$ (Sigma). Most of the cartilage explants were maintained in serum-free culture for 4 days; however, in some experiments porcine articular cartilage was maintained in culture for a variety of time points between 12 h and 20 days. The culture medium was changed every 4 days with fresh RA-, IL-1- or TNF-supplemented medium added at each time. Human articular cartilage was obtained from knee joints harvested at the time of total joint replacement for osteoarthritis from three individuals aged 65–70 years. Cartilage explants were cultured for 4 days as described above, except that IL-1$\beta$ rather than IL-1$\alpha$ was used. At the termination of all cultures, the cartilage explants were blotted dry, weighed and extracted as described below.

### Extraction and quantification of proteoglycan

Proteoglycans retained within the cartilage matrix were extracted for 48 h at 4 °C using 10 vol. of 4 M guanidinium hydrochloride/0.05 M sodium acetate, pH 6.8, containing 0.01 M EDTA, 0.1 M 6-aminohexanoic acid, 0.005 M benzamidine HCl and 0.01 M *N*-ethylmaleimide as proteinase inhibitors. Proteoglycan content in the medium and cartilage extracts was measured as sulphated GAG by colorimetric assay [26] using Dimethylmethylene Blue (DMMB) and chondroitin sulphate-C from shark cartilage (Sigma) as a standard. The effect of treatment on the release of GAG (expressed as a percentage of GAG release relative to control cultures from the same species) was analysed using a one-sample Student's *t*-test. Differences in the release of GAG associated with species and treatment were analysed using a two-factor analysis of variance. All data were analysed using the Stat View package for Macintosh (Acura, Berkeley, CA, U.S.A.), with $P$ values $\leqslant 0.05$ being considered statistically significant.

### SDS/PAGE and Western blotting

Proteoglycan fragments in both conditioned medium and cartilage extracts were precipitated overnight at 4 °C by the addition of 3 vol. of absolute ethanol, deglycosylated, subjected to SDS/PAGE and transferred to nitrocellulose membranes as described previously [25].

To standardize the loading of samples on to the gels, different methods were employed for media and extracts, based on the particular aggrecan catabolites present in these two samples. In culture media, predominantly GAG-bearing catabolites are released, and thus in order to compare the relative abundance of the different N-terminal neoepitope-bearing catabolites, these samples were loaded on an equal GAG basis. Control cultures release significantly less GAG compared with catabolically stimulated cultures, thus loading on an equal GAG basis removes the potential bias of analysing significantly less aggrecan fragments in control lanes. In contrast, the G1-bearing aggrecan molecules retained within the cartilage matrix would be a mixture of intact monomers containing the GAG-attachment domains, and the free G1 domains that result from cleavage within the IGD. In control cultures, the cartilage extract contains a significantly higher GAG content than cartilage from catabolically stimulated cultures, therefore these samples were loaded on the basis of an equivalent wet weight of tissue (i.e. extract from 10 mg of wet tissue).

Immunoblotting of membranes and incubations with primary and secondary antibodies was performed as previously described [27] using monoclonal antibodies (MAbs) or polyclonal antiserum as follows: MAb BC-13 (1:250) and MAb BC-3 (1:2000; [11]) recognizing the aggrecanase generated C- and N-terminal IGD neoepitopes ...ITEGE and ARGSV... respectively; polyclonal anti-DIPEN (1:250; [13,19]) and MAb BC-14 (1:1000; [11,28]) recognizing the MMP-generated C- and N-terminal IGD neoepitopes ... DIPEN and FFGVG... respectively; and MAb

2-B-6 (1:10000) recognizing a chondroitinase-generated chondroitin-4-sulphate disaccharide epitope. All Western blots were scanned and differences in staining intensity of digitized bands were assessed using NIH Image (version 1.55) software.

### Preparation of MMP-digested aggrecan fragments

MMP-digested aggrecan fragments were used as positive controls on Western blots probed with anti-DIPEN and BC-14 antibodies. Porcine articular cartilage, which had been freeze–thawed three times, was digested at 37 °C for 16 h in 100 mM Tris, pH 7.4, containing 150 mM NaCl, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ with 1 mM *p*-aminophenylmercuric acetate and 50 $\mu$g/ml recombinant human pro-MMP-1 (a gift from Dr. Peter Mitchell, Pfizer, Groton, CT, U.S.A.). Proteoglycan fragments released from the tissue were precipitated with 3 vol. of absolute ethanol as described for culture media samples. The MMP-1-digested cartilage matrix was washed five times with PBS before extraction for 48 h at 4 °C as described earlier. The MMP-1-generated aggrecan fragments were deglycosylated and subjected to electrophoresis and Western blotting as described earlier.

### RNA extraction and reverse transcription-PCR (RT-PCR)

Replicate day-4 explants, cultured identically with those processed for Western blotting, were utilized for total RNA extraction. Total RNA was extracted from cartilage explants and analysed by RT-PCR using primers corresponding to cDNA sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), MMP-3 and MMP-13, as described [29]. Dye-terminator sequencing was performed on each cDNA obtained using an ABI Prism 377 sequencer (Perkin-Elmer Biosystems, Foster City, CA, U.S.A.) to confirm the integrity of the PCR products. Experiments were replicated using cartilage from three separate tissue samples from each species.

## RESULTS AND DISCUSSION

### Proteoglycan catabolism in 4-day cultures of bovine and porcine cartilage

In 4-day cultures of bovine (both immature and mature) and porcine cartilage, exposure to RA, IL-1 and TNF resulted in a significant increase ($P < 0.005$ for all analyses) in GAG release from cartilage explants over that seen in control cultures (Table 1). The response of mature bovine cartilage to IL-1 was significantly less ($P < 0.03$) than immature bovine cartilage or porcine cartilage, consistent with previous reports demonstrating a decrease in the responsiveness of articular cartilage to IL-1 with increasing age [30]. The proteoglycan metabolites released into the culture media were evaluated by Western blotting with MAbs 2-B-6, BC-3 and BC-14 (Figure 1). No differences were observed between immature and mature bovine cartilage explants with respect to the results of Western blot analysis (results not shown). Western blots throughout the paper therefore present data from only immature bovine samples, but the results are representative of mature tissue. Aggrecan metabolites reactive with MAb 2-B-6 (Figure 1A) ranged in molecular mass from > 250 kDa to approx. 90 kDa, as has been previously described [21]. In stimulated cultures there was a decrease in the high-molecular-mass 2-B-6-positive bands (Figure 1A); however, the banding pattern was not markedly different between the three catabolic agents, suggesting that similar cleavages of the core protein were occurring regardless of the stimuli used.

Immunoblot analysis with MAb BC-3 of the proteoglycan metabolites released into the culture media is shown in Figure 1(B). In control cultures little or no BC-3-immunoreactive material was detectable. The medium from cultures stimulated with RA, IL-1 and TNF contained BC-3-reactive bands ranging in mass from approx. 250–40 kDa (Figure 1B). Thus, in all cultures in which an increase in the release of aggrecan was detected using the DMMB assay (Table 1), there was an increase in BC-3-reactive metabolites. These results confirm previous

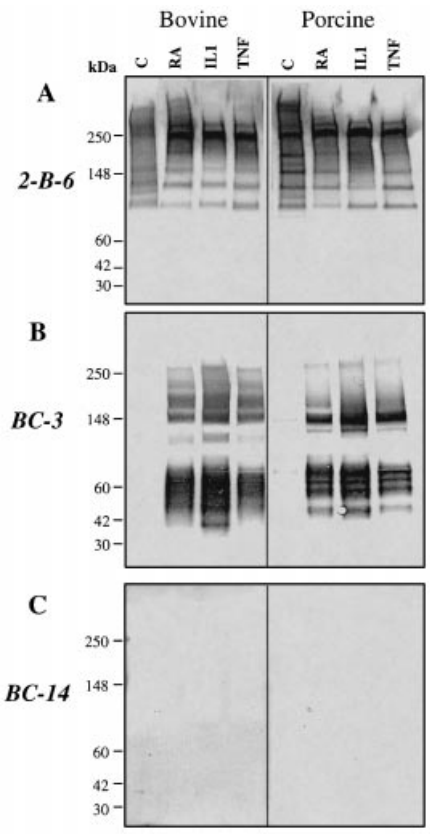


**Figure 1 Western blot analysis of proteoglycan fragments released into culture media**

Bovine and porcine articular cartilage was cultured as explants for 4 days in the absence (C) or presence of the catabolic agents RA, IL-1 and TNF. Western blots were probed with: (**A**) MAb 2-B-6 recognizing chondroitinase-generated chondroitin-4-sulphate stubs; (**B**) MAb BC-3 recognizing the aggrecanase-generated neoepitope ARGSV ...; and (**C**) MAb BC-14 recognizing the MMP-generated neoepitope FFGVG ... The migration positions of pre-stained globular protein standards are shown on the left.

**Table 1 Release of proteoglycan from cultures stimulated for 4 days with RA, IL-1 and TNF as a percentage of control cultures (means ± S.E.M.; $n = 6$ animals)**

| | Release of proteoglycan (% of control) | | |
|---|---|---|---|
| | Immature bovine | Mature bovine | Porcine |
| RA | 450 ± 50 | 533 ± 49 | 502 ± 28 |
| IL-1 | 639 ± 82 | 413 ± 34* | 615 ± 62 |
| TNF | 377 ± 50 | 462 ± 28 | 378 ± 50 |

* Significantly different from IL-1 value of immature bovine and porcine proteoglycan.

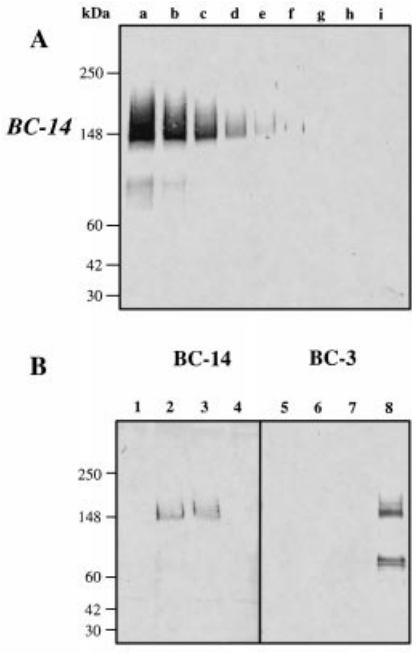


**Figure 2 Detection and susceptibility to secondary cleavage of IGD aggrecan fragments initiating with the neoepitope sequence FFGVG**

(**A**) Serial dilutions of aggrecan catabolites released from the cartilage were immunoblotted with MAb BC-14 recognizing the MMP-generated neoepitope FFGVG … Two-fold serial dilutions of deglycosylated aggrecan fragments were loaded that contained: lane a, 20 $\mu$g of GAG; b, 10 $\mu$g of GAG; c, 5 $\mu$g of GAG; d, 2.5 $\mu$g of GAG; e, 1.2 $\mu$g of GAG; f, 0.6 $\mu$g of GAG; g, 0.3 $\mu$g of GAG; h, 0.15 $\mu$g of GAG; and i, 0.08 $\mu$g of GAG. (**B**) Immunoblot analysis (BC-14 lanes 1–4 and BC-3 lanes 5–8) of: conditioned medium with no endogenous BC-14- or BC-3-reactive metabolites (lanes 1 and 5); FFGVG … initiating metabolites generated by MMP-1 digestion of porcine articular cartilage (lanes 2 and 6); digestion of the FFGVG … substrate with conditioned medium (lanes 3 and 7); and digestion of intact aggrecan monomers in conditioned medium (lanes 4 and 8). The migration positions of pre-stained globular protein standards are indicated on the left.

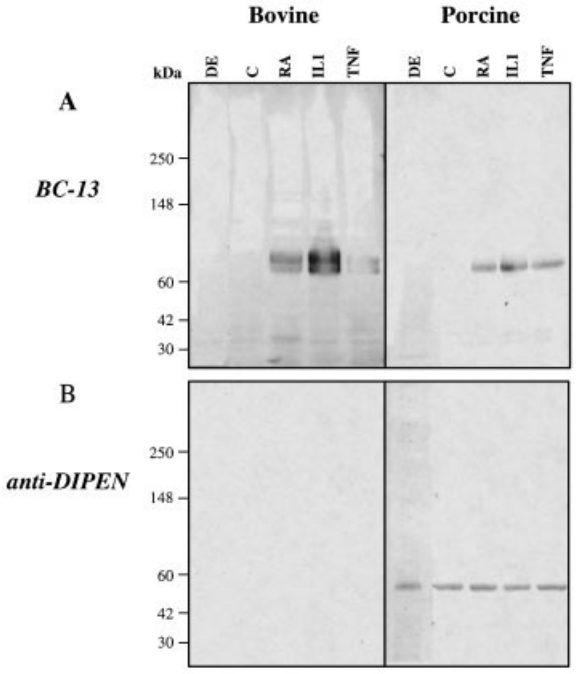


**Figure 3 Western blot analysis of proteoglycan fragments retained within the cartilage matrix**

Aggrecan fragments were extracted from the cartilage of 4-day cultures of normal bovine and porcine articular cartilage and immunoblotted with: (**A**) MAb BC-13 recognizing the aggrecanase-generated neoepitope … ITEGE; and (**B**) anti-DIPEN antisera recognizing the MMP-generated neoepitope … DIPEN. Extracts analysed were from a direct extract of fresh uncultured cartilage (DE) and cartilage cultured as explants in the absence (C) or presence of RA, IL-1 and TNF. The migration positions of pre-stained globular protein standards are indicated on the left.

findings demonstrating that catabolic stimulation of cartilage explants results in an increase in aggrecanase-generated aggrecan metabolites in the culture media [12,20–25]. No BC-3-positive bands were observed above 250 kDa, indicating that the high-molecular-mass bands observed with MAb 2-B-6 had not been cleaved within the IGD. The lower-molecular-mass (40–80 kDa) aggrecanase-generated BC-3-positive bands did not co-localize with 2-B-6 reactivity, thus indicating cleavage of these molecules at their C-terminus to remove the chondroitin sulphate-rich region. No differences in the BC-3 banding pattern were observed between the three catabolic agents, indicating that the catabolic processes occurring in the C-terminus of the metabolites were the same. Immunoblotting of identical media samples with MAb BC-14 failed to detect any immunoreactive proteoglycan degradation products in any cultures of normal bovine or porcine cartilage ($n = 6$; Figure 1C).

To determine whether the absence of any MMP-generated catabolites in the culture media was due to the sensitivity of the assay used, 2-fold serial dilutions of deglycosylated MMP-1-generated aggrecan catabolites were Western blotted with MAb BC-14 (Figure 2A). A linear decrease in the intensity of the BC-14-immunoreactive bands was observed between 20 and 0.6 $\mu$g of GAG. The sensitivity of the BC-14 Western blotting technique was calculated based on the assumption that all of the GAG released from freeze–thawed porcine cartilage in response to MMP-1 digestion initiated with the N-terminus $F^{342}$FGVG … . Culture media samples (Figure 1C) were all loaded at 20 $\mu$g of GAG per lane. Therefore the presence of BC-14-reactive aggrecan metabolites would have been detected in the culture media if as little as 0.6 $\mu$g of the GAG, or 3.0% of the loaded sample, initiated with the N-terminus FFGVG. It was also considered that secondary cleavage of MMP-generated aggrecan metabolites by aggrecanase could mask the detection of any such catabolites in culture media. To test whether such a cleavage could occur, MMP-1-generated metabolites initiating with FFGVG … were incubated with soluble aggrecanase generated in conditioned medium from IL-1-stimulated porcine chondrocytes as previously reported [27]. Western blot analyses of this conditioned medium with MAbs BC-14 and BC-3 showed the lack of endogenous aggrecan catabolites in this preparation (Figure 2B, lanes 1 and 5). The MMP-1-generated metabolites were shown by Western blot to contain BC-14 (FFGVG …) but not BC-3 (ARGSV …) reactive fragments (Figure 2B, lanes 2 and 6). Incubation of the FFGVG … fragments in the medium containing soluble aggrecanase failed to reduce the BC-14 reactivity and did not generate BC-3 reactivity (Figure 2B, lanes 3 and 7). It was confirmed that the conditioned medium contained aggrecanase but not MMP activity through the generation of BC-3- but not BC-14-reactive catabolites when intact aggrecan was digested (Figure 2B, lanes 4 and 8). These results indicate that MMP-generated aggrecan metabolites are not secondarily cleaved by soluble aggrecanase.

We next examined the proteoglycan fragments extracted from the articular cartilage both before ('direct extract') and after culture. Western blot analyses of cartilage extracts with MAbs BC-3 and BC-14 revealed little or no immunoreactive bands respectively, indicating that the majority of such aggrecan fragments were released from the cartilage matrix (results not shown). Thus the lack of BC-14 in culture media (Figure 1C) was not due to the retention of these fragments within the tissue. No BC-13-reactive aggrecan fragments were detected in extracts of cartilage taken before culture or in control cultures (Figure 3A). However, in extracts of cartilage stimulated with RA, IL-1 and TNF, BC-13-reactive bands were observed. The increase in BC-13 in stimulated cultures was co-ordinate with the increase in both GAG- and BC-3-reactive metabolites in the media, indicating that a primary cleavage of the IGD by aggrecanase was occurring. A single BC-13-positive band of approx. 70 kDa was detected in stimulated porcine cartilage, whereas the same 70 kDa band and a second larger band (approx. 80 kDa) were observed in bovine cartilage extracts. The detection of two bands terminating with … NITEGE has previously been reported [12] and is believed to be associated with differences in N-linked glycosylation [13,31]. The finding of a single band in porcine tissue suggests that in this species such glycosylation variants are not present, although three sites for potential N-glycosylation in the porcine G1 have been described [32]. Immunoblot analysis of cartilage extracts with anti-DIPEN antiserum revealed no bands in any bovine cartilage extracts (Figure 3B). This finding was not unexpected, since the bovine IGD contains a Ser rather than an Asn at amino acid 341 [33] and as such the anti-DIPEN antiserum used in the present study recognizes the bovine sequence of … DIPES with sensitivity an order of magnitude lower (P. J. Roughley and J. S. Mort, unpublished work). In porcine cartilage, however, a single anti-DIPEN immunoreactive band of approx. 60 kDa was observed in the direct extract and cartilage extracts from all culture conditions, including controls. Importantly, the intensity of … DIPEN staining did not change with any culture when compared with the direct extract of the cartilage before culture (Figure 3B). Immunoblotting of media samples with MAb BC13 and anti-DIPEN revealed no reactivity (results not shown), indicating that the majority of G1-containing aggrecan metabolites were retained within the cartilage matrix rather than being released into the culture media (i.e. they were still bound in hyaluronan–link protein complex). Thus, the anti-DIPEN immunoblot results were consistent with the lack of BC-14-positive aggrecan metabolites in the culture medium and strongly suggested that no primary cleavage of the IGD by MMPs was occurring during the culture period in either control or stimulated cultures.

The detection of … DIPEN-terminating aggrecan metabolites in the direct extract of porcine cartilage is consistent with reports in other species and indicates an accumulation of this neoepitope in the cartilage over the life of the animals [14–19]. However, it is not clear whether these … DIPEN metabolites detected in articular cartilage are generated by the action of MMPs on intact aggrecan or the aggrecanase-generated G1 metabolite. We therefore investigated the potential of MMPs to generate the … DIPEN epitope by secondarily cleaving aggrecanase-generated G1 fragments. The G1 fragments in day-4 IL-1-stimulated porcine cartilage (predominantly NITEGE-terminating, see Figure 3A) were separated from intact glycosylated aggrecan using anion-exchange chromatography. The isolated G1 fragments were incubated with or without MMP-1 for 18 h and the resultant metabolites were analysed by Western blot analysis using MAb BC-13 and anti-DIPEN antiserum. MMP-1 digestion reduced the BC-13 reactivity and increased the anti-DIPEN reactivity

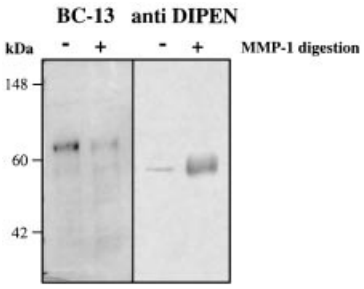


**Figure 4 Western blot analysis of aggrecan fragments generated by secondary digestion of aggrecanase-generated metabolites by MMPs**

Aggrecan metabolites with the C-terminal sequence … NITEGE were incubated with and without MMP-1 and the resultant products were immunoblotted with MAb BC-13 recognizing the aggrecanase-generated neoepitope … NITEGE and anti-DIPEN antisera recognizing the MMP-generated neoepitope … DIPEN. The migration positions of pre-stained globular protein standards are indicated on the left. (+) and (−) indicate incubation with or without MMP-1 respectively.

(Figure 4), indicating that secondary cleavage of aggrecanase-generated G1 metabolites by MMPs can occur. Similar results have recently been reported using immunohistological analysis following MMP-3 digestion of murine articular cartilage [34]. Taken together, these results raise the possibility that detection of the … DIPEN neoepitope in articular cartilage may not be solely due to primary MMP-generated aggrecan release from articular cartilage, but could also result from secondary cleavage of aggrecanase-generated metabolites. In addition, it has recently been demonstrated that the … DIPEN neoepitope can be generated *in vitro* by cathepsin B, in addition to the MMPs [35]. The generation of … DIPEN by cathepsin B was observed principally at acidic pH ($\leqslant 5.5$); however, sufficient exopeptidase activity of this enzyme remained at more neutral pH to eventually generate this neoepitope [35]. Cathepsin B has been detected in normal articular cartilage and levels of this enzyme are increased in osteoarthritic cartilage [36]. Furthermore, prolonged exposure to IL-1 has been shown to induce matrix depletion of cartilage explants, with a subsequent increase in the release of cathepsin B when compared with intact explants [37]. The possibility therefore exists that cathepsin B may also play a role in the generation of anti-DIPEN-reactive fragments.

Taken together, the results of immunoblotting with BC-3, BC-13, BC-14 and anti-DIPEN indicate that the aggrecanase-generated metabolites detected in the media of day-4 cultures were the direct result of primary cleavage of the IGD by this enzyme. In addition, the lack of detectable MMP-generated metabolites within the media, together with the fact that no change in the intensity of staining for … DIPEN-terminating fragments could be detected, indicated that no primary cleavage by MMPs was occurring during the course of the 4-day cultures.

### Aggrecan catabolism associated with different times of culture

To establish that MMP-generated aggrecan catabolism was not occurring earlier than 4 days in culture, porcine articular cartilage from three animals was cultured for between 12 h and 4 days (Table 2). A significant increase in GAG release when compared with control cultures was induced after 12 h of culture in the presence of IL-1 ($P = 0.02$) but not TNF or RA. After 24 h of culture in the presence of all three catabolic agents there was a significant increase in GAG release when compared with control cultures ($P < 0.03$ for RA, IL-1 and TNF). The response to IL-1

**Table 2 Mean (±S.E.M.) ($n = 3$) cumulative proteoglycan release ($\mu$g of GAG/mg wet weight) over time in culture from porcine articular cartilage explants**

| | Cumulative proteoglycan release ($\mu$g of GAG/mg wet weight) | | |
|---|---|---|---|
| | 12 h | 24 h | 4 days |
| Control | 1.14 ± 0.04 | 1.88 ± 0.07 | 5.88 ± 0.20 |
| RA | 1.53 ± 0.14 | 5.16 ± 0.20* | 30.64 ± 0.72* |
| IL-1 | 6.17 ± 1.67* | 12.14 ± 1.01* | 42.40 ± 0.50* |
| TNF | 1.70 ± 0.06 | 5.71 ± 0.64* | 31.07 ± 1.67* |

* Significantly different from control value at the same time point.

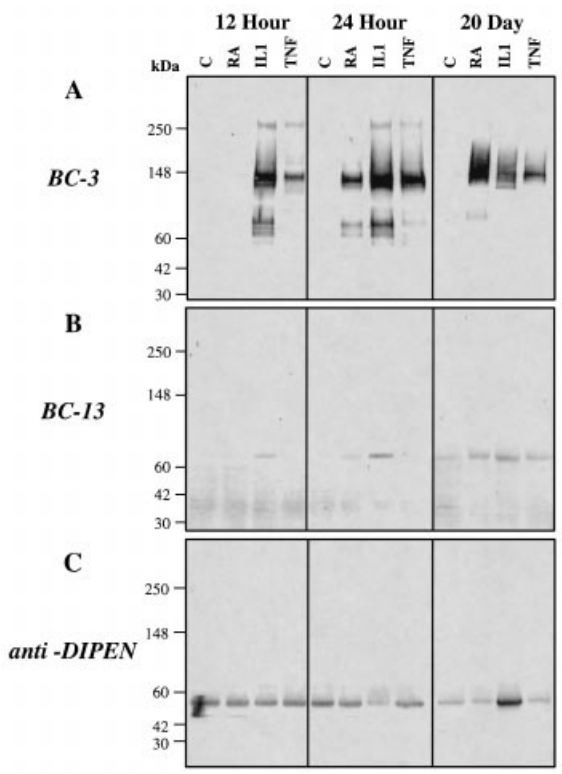


**Figure 5 Western blot analysis of proteoglycan degradation products generated over time in culture**

Western blot of aggrecan metabolites detected in explants of porcine articular cartilage cultured for 12 h, 24 h or 20 days in the absence (C) or presence of RA, IL-1 and TNF. (**A**) Aggrecan catabolites released into the culture media over the first 12 and 24 h or days 16–20 of culture were immunoblotted with MAb BC-3 recognizing the aggrecanase-generated neoepitope ARGS ...; (**B**) aggrecan catabolites extracted from the cartilage matrix of 12 and 24 h or 20-day cultures were immunoblotted with MAb BC-13 recognizing the aggrecanase-generated neoepitope ... ITEGE; (**C**) aggrecan catabolites extracted from the cartilage matrix of 12 and 24 h or 20-day cultures were immunoblotted with anti-DIPEN antisera recognizing the MMP-generated neoepitope ... DIPEN. The migration positions of pre-stained globular protein standards with their apparent molecular masses are indicated on the left.

was significantly greater than with RA or TNF ($P < 0.001$ for both agents). Western blotting of aggrecan catabolites released into the media or retained in the matrix after 12 and 24 h of culture with MAbs BC-3, BC-14, BC-13 and polyclonal anti-DIPEN antiserum was performed (Figure 5). At all time points where increased GAG release into the media was observed compared with control cultures (Table 2), a co-ordinate increase in BC-3-positive aggrecan fragments was also evident (Figure 5A). The size-distribution of the BC-3-positive metabolites was very similar to that observed in the day-4 culture medium. Interestingly, at 12 h the increase in GAG release associated with TNF treatment was not statistically significant, but an increase in BC-3-reactive metabolites was observed (Table 2 and Figure 5A). This is most likely due to the greater sensitivity of the Western blotting technique compared with the DMMB assay. MMP-generated BC-14-reactive fragments were not observed at any time point in the presence or absence of catabolic stimulation (results not shown). Consistent with these results, increased BC-13-reactive metabolites were observed in cartilage extracts from 12 h IL-1 and 24 h RA, IL-1 and TNF cultures, whereas no difference in anti-DIPEN staining intensity was detectable between samples (Figure 5B). Thus, the earliest release of GAG in response to catabolic stimulation was again associated with primary cleavage of the IGD by aggrecanase and not by MMPs.

Porcine cartilage was also cultured for 20 days in the presence or absence of catabolic stimuli to determine whether MMP-generated catabolism could be detected during late-stage aggrecan release. In control cultures, 60 ± 5 % of the GAG content was released cumulatively into the culture media. All three catabolic agents significantly increased the release of GAG ($P < 0.05$ for all agents) to > 90 %, with > 80 % being released in the first week of culture. BC-3-positive aggrecan catabolites were detected in the media of day-4 to day-20 RA, IL-1 and TNF (but not control) cultures (Figure 5A), whereas BC-14-reactive catabolites were not detectable at any time point in control or stimulated cultures. The BC-3-positive metabolites in the day-20 culture medium (Figure 5B) were restricted in size to the higher-molecular-mass species (approx. 148 kDa), indicating that these metabolites had been minimally processed at their C-terminus. BC-13-positive catabolites were detected in cartilage extracts of catabolically stimulated, but not in control cultures, up to day 16, and in both control and stimulated cultures at day 20 (Figure 5B). The BC-13 immunoreactivity in control cultures was weaker than that observed in stimulated cultures, consistent with the reduced release of aggrecan in the former. A single major catabolite immunoreactive with the anti-DIPEN antiserum was observed in all samples, with no difference in staining intensity detectable between samples up to day 16. In day-20 cartilage extracts, however, an increase in the anti-DIPEN immunoreactivity was evident in extracts of cartilage cultured in the presence of IL-1 (Figure 5C). Given that no BC-14-reactive catabolites could be detected in the media of these day-20 IL-1 cultures, it seems likely that the increase in DIPEN was associated with secondary catabolism of aggrecanase-generated G1 fragments by MMPs or cathepsin B [35]. Confirmation of such a secondary cleavage was not possible using the present methodologies, since proteolysis of aggrecanase-generated G1 fragments by MMPs or cathepsin B would result in the release of a small 32 amino acid ... ITEGE-terminating fragment. Such a small peptide would potentially: (i) not be precipitated by ethanol; (ii) be lost on dialysis; (iii) run at the buffer front in SDS/PAGE; and/or (iv) not be quantitatively transferred to nitrocellulose.

### Expression of MMP-3 and MMP-13 mRNA in cartilage samples

The results of RT-PCR using primers specific for GAPDH, MMP-3 and MMP-13 are shown in Figure 6. Little or no mRNA for MMP-3 was observed in control cartilage. The expression of MMP-3 mRNA was increased in response to both IL-1 and TNF stimulation for 4 days in both species and in response to RA in

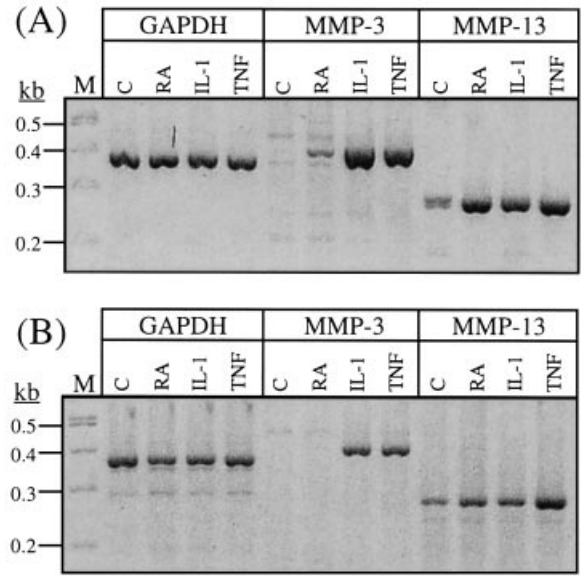


**Figure 6 RT-PCR analysis of MMP mRNAs in cartilage explants**

RT-PCR of mRNA extracted from 4-day explant cultures of normal bovine (**A**) and porcine (**B**) articular cartilage was performed using GAPDH-, MMP-3- and MMP-13-specific primers. Explants were cultured in the absence (C) or presence of RA, IL-1 and TNF. Migration of DNA size markers is shown in lane M with their sizes indicated on the left.

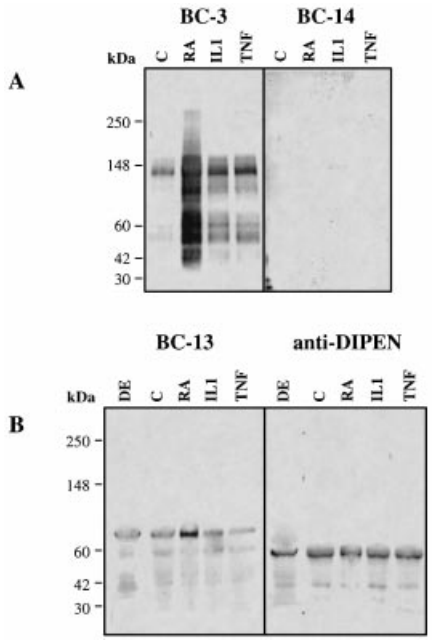


**Figure 7 Western blot of aggrecan metabolites detected in explants of OA human articular cartilage**

Extracts analysed were from a direct extract of fresh uncultured OA human cartilage (DE) and cartilage cultured for 4 days as explants in the absence (C) or presence of RA, IL-1 and TNF. (**A**) Aggrecan catabolites released into the culture media were detected with MAb BC-3 recognizing the aggrecanase-generated neoepitope ARGS ... and MAb BC-14 recognizing the MMP-generated neoepitope FFGV ...; (**B**) aggrecan catabolites extracted from the cartilage matrix were immunoblotted with MAb BC-13 recognizing the aggrecanase-generated neoepitope ... ITEGE and anti-DIPEN antisera recognizing the MMP-generated neoepitope ... DIPEN. The migration positions of pre-stained globular protein standards with their apparent molecular masses are indicated on the left.

bovine cartilage. Expression of MMP-13 mRNA was apparent in control cartilage. In bovine and, to a lesser extent, porcine cartilage, this expression appeared to be upregulated by exposure to all three catabolic agents. It is noteworthy that mRNAs for two MMPs (MMP-3 and MMP-13) capable of degrading the aggrecan IGD were expressed in these cartilage explants and yet no MMP-related cleavage of the IGD could be demonstrated. MMPs are secreted in an inactive form and once activated may be readily inhibited by the tissue inhibitors of metalloproteinases (TIMPs). The lack of catabolism of aggrecan by MMPs observed in the present studies may be associated with limited MMP secretion, a lack of activation of secreted MMPs and/or the presence of TIMP levels in excess of MMPs.

### Aggrecan catabolism in osteoarthritic (OA) human articular cartilage

Whereas it appears from the present studies that aggrecanase is the primary proteolytic activity responsible for aggrecan cleavage in cartilage, MMP-generated aggrecan metabolites have been shown to occur in arthritic human knee-joint synovial fluids [5,38]. Since no evidence for MMP-generated aggrecan catabolism could be found in the present study using normal articular cartilage of bovine or porcine origin, cartilage derived from late-stage human OA knees ($n = 3$) was evaluated. Increased GAG release was observed with RA- (368 % of control), IL-1- (157 % of control) and TNF- (166 % of control) stimulation of human OA cartilage. Immunoblot analysis of aggrecan metabolites released into the culture medium and extracted from the matrix of OA human cartilage is shown in Figure 7. In contrast with the normal bovine and porcine tissue (Figure 1), media from control cultures of OA human cartilage contained BC-3-positive aggrecan metabolites (Figure 7A). Catabolic stimulation increased the BC-3-reactive metabolites in proportion to the increase in GAG release observed. In control, RA- and TNF-stimulated OA human cartilage, no BC-14-reactive material was detectable. In one of the three individuals tested, however, a single weakly staining BC-14-positive band (145–150 kDa) was observed in medium from the IL-1-treated culture (Figure 7A, lane IL-1). Detection of this MMP-generated metabolite in only one of the three samples tested correlates with the detection of a similarly sized aggrecan catabolite, initiating with the sequence FFG ..., in approx. 50 % of arthritic synovial fluid samples [38]. In contrast with normal cartilage, IL-1 stimulation in OA human cartilage has been shown to significantly enhance the secretion of MMPs and decrease the synthesis of both TIMP-1 and TIMP-2 [39]. In addition, MMPs may act preferentially on aggrecan molecules that have been extensively processed from their C-terminus and have accumulated in tissue over time [38]. The aggrecanase-generated catabolites released from human cartilage were smaller than those seen in bovine and porcine cultures (compare Figures 1B and 7A), indicating that the aggrecan monomers in the human tissue had been more extensively processed at their C-terminus. In contrast with normal bovine and porcine cartilage (Figure 2A), extracts of both uncultured and control human OA cartilage revealed BC-13-reactive catabolites (Figure 7B, lane C). This is likely to be associated with an accumulation of this aggrecanase-generated catabolite in the diseased cartilage, and is consistent with the increased aggrecanase activity found in this OA human tissue (Figure 7A, control) compared with normal bovine or porcine cartilage. An increase in BC-13-reactive material was only observed in response to RA stimulation, consistent with the markedly increased GAG release observed with this catabolic agent. The fact that an increase in BC-13 was not detected in association with IL-1 and

TNF stimulation, while a mild increase in BC-3 was observed, is likely to be associated with differing sensitivities of the two antibodies. The predominant anti-DIPEN immunoreactive aggrecan metabolite in the direct extract of OA human cartilage (Figure 7B) was of similar size to that in the porcine cartilage, and the intensity of staining did not vary with any of the culture conditions. An anti-DIPEN-reactive band of smaller (approx. 40 kDa) molecular mass was also observed in human OA cartilage, but again the staining intensity did not alter with the experimental culture conditions. Taken together, these results suggest that in late-stage OA cartilage, high concentrations of inflammatory cytokines such as IL-1 may result in some low levels (approx. 3% of the released GAG) of primary MMP-generated catabolism of aggrecan, as indicated by the release of an FFGVG… initiating catabolite. However, aggrecanase was still the major activity present in all samples examined, and an increase in aggrecan catabolism by this enzyme, and not MMPs, was correlated with the increase in GAG release associated with catabolic stimulation.

In conclusion, the release of aggrecan metabolites into culture medium from normal bovine and porcine articular cartilage stimulated with RA, IL-1 and TNF results from an increase in aggrecanase activity in these cultures. Importantly, we have demonstrated that in the culture media of either control or catabolically stimulated cultures of normal articular cartilage, the levels of aggrecan fragments initiating with FFGVG… (resulting from primary MMP-generated cleavage of aggrecan) were $< 3\%$ of the released GAG-bearing aggrecan fragments. Furthermore, by simultaneously evaluating the generation of the C-terminal fragments retained within the cartilage matrix, we have demonstrated for the first time that the BC-3-positive aggrecan fragments detected in the culture media of catabolically stimulated articular cartilage resulted from primary cleavage of the IGD by aggrecanase, rather than a secondary cleavage by this enzyme after an initial MMP-related cleavage of the aggrecan IGD. It should be noted that in the present study, only the contributions of chondrocyte-derived enzyme activities in aggrecan IGD cleavage were evaluated. Within the joint, additional enzymes involved in cartilage degradation may also be derived from the synovium, white blood cells or serum. Additionally, proteoglycan fragments contained within synovial fluid may originate from other intrasynovial structures which contain aggrecan, such as the meniscus [40]. It is apparent from the results of the present experiments that primary cleavage at the major MMP site in the aggrecan IGD, between amino acids $Asn^{341}$ and $Phe^{342}$, is of limited significance under conditions of control or stimulated aggrecan turnover in articular cartilage *in vitro*.

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