Monoclonal antibodies that specifically recognize neoepitope sequences generated by 'aggrecanase' and matrix metalloproteinase cleavage of aggrecan: application to catabolism *in situ* and *in vitro*

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Monoclonal antibodies have been prepared that react specifically with the neoepitopes present on proteoglycan degradation products generated from the proteolytic cleavage of aggrecan in the interglobular domain. Antibody BC-3 recognizes the new N-terminus (ARGSV...) on aggrecan degradation products produced by the action of the as yet uncharacterized proteolytic activity, 'aggrecanase', and antibody BC-4 recognizes the new C-terminus (...DIPEN) generated by the proteolytic action of matrix metalloproteinases. Specificity for these neoepitope sequences was determined in competitive e.l.i.s.a. using synthetic peptide antigens as inhibitors. Antibody BC-3 was used in the detection of aggrecan degradation products in the culture medium obtained from two different *in vitro* culture systems: bovine cartilage explants treated with either retinoic acid or interleukin-1, and secondly, rat chondrosarcoma cells treated with retinoic acid. Both interleukin-1 and retinoic acid treatment caused an increase in aggrecan catabolism resulting in an increased release to the medium of specific aggrecan degradation products containing the BC-3 neoepitope generated by the action of 'aggrecanase'. However, several additional aggrecan catabolites were present that were not immunoreactive with antibody BC-3. In addition, under control conditions, in the bovine cartilage cultures the BC-3 epitope was found on some of these aggrecan catabolites. In contrast, no immune-reactive material was found in the aggrecan degradation products present in control media of rat chondrosarcoma cells cultured in the absence of retinoic acid. Collectively, these results demonstrate that 'aggrecanase' activity is not a constitutive event in all cartilage culture systems and also suggest that proteolytic agents other than 'aggrecanase' are involved in aggrecan catabolism in normal turnover compared with pathological conditions. Antibody BC-4 was used to demonstrate the identity of the G1 domain of aggrecan following proteolytic cleavage of a purified G1-G2 preparation with collagenase, gelatinase A or stromelysin. The G2 product of this cleavage did not react with antibody BC-3, indicating that, under the experimental conditions used, none of these enzymes exhibited 'aggrecanase' activity. It is expected that both of these antibodies will play a pivotal role in detailed studies elucidating molecular mechanisms of aggrecan degradation and they will be particularly useful for the sensitive monitoring of aggrecan degradation products in tissue extracts and body fluids.

INTRODUCTION

The structure and biochemical composition of the large aggregating proteoglycan of articular cartilage (aggrecan) has been well documented and reviewed [1]. Briefly, it consists of an extended protein core which can be subdivided into several structural regions; at the N-terminus there are two globular domains, G1 and G2, which are then separated from a third globular domain, G3, at the C-terminus by a long extended central polypeptide segment to which over 100 glycosaminoglycan chains are attached [2]. The two N-terminal globular domains are in turn separated by a short polypeptide segment of approx. 150 residues termed the interglobular domain. The G1 domain of aggrecan interacts with hyaluronate and link protein to form large macromolecular aggregates, so facilitating its immobilization in the tissue [3–5]. The function of the G2 domain is still unclear; however, it shows considerable sequence homology with both the G1 domain and link protein. There is considerable heterogeneity in the individual aggrecan molecules present in proteoglycan aggregates, some of this heterogeneity being caused by post-translational proteolytic cleavage of the molecule in different domains.

Recent studies [6–12] indicate that the two major sites of proteolytic cleavage in human aggrecan (and aggrecan from other species) occur within the interglobular domain between amino acid residues Asn^{341}-Phe^{342} and Glu^{373}-Ala^{374} (human sequence enumeration) [13]. The latter cleavage site produces glycosaminoglycan-containing N-terminal fragments that are the major aggrecan degradation products isolated from synovial fluids of patients with arthritis [7,8] and are also found in media from cartilage explant cultures treated with retinoic acid or interleukin-1 [9–12]. The former cleavage site generates a C-terminal catabolic fragment containing the 50–60 kDa G1 domain that remains in the tissue complexed to hyaluronic acid [6]. Generation of these cleavage products by proteinases *in vitro* has only been achieved for the Asn^{341}-Phe^{342} site. The resultant G1 domain which accumulates in articular cartilage is generated by the action of several of the neutral metalloproteinases; MMP-1, -2, -3, -7, -8 and -9 [6,14,15]. The identity of the proteolytic activity responsible for the Glu^{373}-Ala^{374} aggrecan cleavage site is presently unknown, in spite of considerable experimentation characterizing the different catabolic products of aggrecan generated by pure proteinases and crude extracts from monolayer and explant culture systems [14–16]. However, the available evidence suggests that the proteolytic activity responsible for this cleavage in aggrecan has a specificity for Glu-Xaa peptide bonds, where Xaa is Ala, Gly or Leu [8,10–12]. This as yet uncharacterized proteolytic activity has been termed 'aggrecanase'.

Abbreviations used: CPC, cetylpyridinium chloride; DMEM, Dulbecco's modified Eagle's medium; DMMB, Dimethylmethylene Blue.

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In a recent publication [17] we described a novel experimental approach of identifying proteolytic products by preparing monoclonal antibodies that are specific for the neoepitopes resulting from the action of proteases on extracellular matrix macromolecules. In the present paper we describe the production of two novel antibodies; one specific for the C-terminal neoepitope in the G1 domain that accumulates when aggrecan is cleaved by metalloproteinases, and the other specific for the N-terminal neoepitope generated by the action of 'aggrecanase' in the interglobular domain. These antibodies have been denoted BC-4 and BC-3 respectively, and have been used to characterize aggrecan catabolic products in a number of different experimental situations.

EXPERIMENTAL

Immunization and fusion

The antigens used for immunization were synthetic peptide–ovalbumin conjugates containing the peptide sequences ARGSVILTVKGGC or CGGFVDIPEN coupled to the carrier protein ovalbumin though the C- or N-terminal cysteine respectively (Figure 1). These sequences correspond to the new N- or C-terminal sequence on aggrecan catabolic products produced by 'aggrecanase' and MMP-1, -2, -3, -7, -8 and -9, respectively. The procedures used for peptide synthesis and conjugation were as described by Hughes et al. [17]. Procedures for immunization, cell fusion and hybridoma selection were as described by Caterson et al. [18,19]. After 10–14 days in culture, aliquots from wells containing putative hybridomas were tested in e.l.i.s.a. for the presence of antibodies with specificity directed against the original immunizing antigen but negative reactivity against ovalbumin–peptide conjugates containing an unrelated peptide sequence (either IQAENGGC or HLLVEAGGC). Hybridomas that only showed positive reactivity against the immunizing antigen were expanded in cell culture, cloned to limiting dilution and used for ascites production [17]. Antibody isotype was determined using direct e.l.i.s.a. and an isotype screening kit, supplied by Southern Biotechnology (Birmingham, AL, U.S.A.) with antigen-coated plates to capture the antibody in the hybridoma supernatants. The specificity of antibodies BC-3 and BC-4 was established by competitive inhibition e.l.i.s.a. with a variety of synthetic peptides using procedures described previously [17].

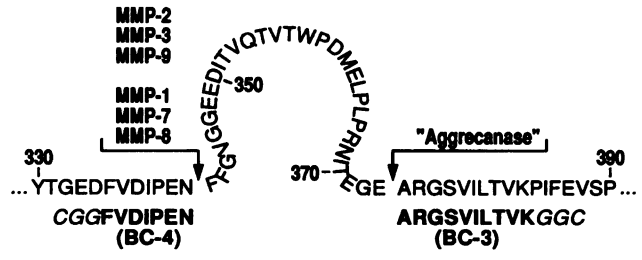

Figure 1 Schematic representation of the interglobular domain of human aggrecan

The amino acid sequence of the interglobular domain [13] is depicted, showing the sole cleavage site for MMP-2, MMP-3 and MMP-9, the additional N-terminal cleavage site for MMP-1, MMP-7 and MMP-8 [14], and the 'aggrecanase' cleavage site [10,11]. The sequences of the synthetic peptides used for monoclonal antibody BC-3 and BC-4 production are indicated below, with the authentic aggrecan sequence in bold and the linkage sequence used for conjugation to ovalbumin in italics.

Bovine cartilage explant cultures

Bovine nasal cartilage was diced into 2 mm^3 cubes and maintained in organ culture in Dulbecco's modified Eagle's medium (DMEM) without Phenol Red, containing 0.2 mg/ml BSA, 20 mM Hepes, 100 units/ml penicillin and 100 μg/ml streptomycin. Each culture, containing 1 g of cartilage in 10 ml of medium, was maintained for 1 day without additives, then continued in culture with no additives or in the presence of either retinoic acid (3×10^{-7} M) or interleukin-1β (20 units/ml). Medium was changed on days 2 and 3 then every two days thereafter. Analysis of the medium using the Dimethylmethylene Blue (DMMB) assay [20,21] showed stimulation of glycosaminoglycan release by retinoic acid and interleukin-1 when compared with control (untreated) cultures.

Macromolecules in culture media from days 4 and 5 were dialysed exhaustively against deionized water, freeze-dried, then dissolved at 2 mg/ml in 50 mM sodium acetate, pH 5.5. Cetylpyridinium chloride (CPC) in the same buffer was added to give a final concentration of 1% and the precipitated proteoglycans and proteoglycan fragments collected by centrifugation. The pellet was washed with 1% (w/v) CPC then dissolved in n-propanol/water (3:2, v/v). Two volumes of ethanol saturated with potassium acetate was added at 4 °C and the proteoglycan samples (now as their potassium salts) were collected by centrifugation. The pellet was washed twice with ethanol, then with ether and air dried [22].

Proteoglycan samples were dissolved at 5 mg/ml in 0.1 M Tris/acetate, pH 7.0, containing 10 mM EDTA, 10 mM iodoacetamide, 5 mM phenylmethanesulphonyl fluoride, 0.36 mM pepstatin A, 0.12 unit/ml keratanase (endo β-galactosidase, Sigma) and 0.06 unit/ml chondroitinase ABC and incubated at 37 °C overnight. Digestion was terminated by addition of SDS/PAGE sample buffer and incubation for 3 min in a boiling-water bath. Samples, representing 25 μg of proteoglycan, were analysed on 4–10% SDS/PAGE gradient gels followed by electroblotting to polyvinylidene difluoride membranes (Immobilon P, Millipore), which were probed for the BC-3 epitope using immunostaining techniques described previously [17]. Duplicate blots were stained with Coomassie Brilliant Blue R250 and the corresponding immunoreactive band was excised for N-terminal sequence analysis using an Applied Biosystems 473A peptide sequencer.

Swarm rat chondrosarcoma chondrocyte cultures

The cloned cell line Rx derived from the Swarm rat chondrosarcoma (kindly provided by Dr. James Kimura, Henry Ford Hospital, Detroit, U.S.A.) was cultured at a density of 1.6×10^7 cells per 100-mm-diam. dish in DMEM containing 5% (v/v) fetal-calf serum, gentamicin (50 μg/ml) and L-ascorbate (25 μg/ml added daily). On day 3 of culture, medium was removed and plates separated into two treatment groups; control (untreated) cultures and those to which 1×10^{-6} M retinoic acid was added. Control cultures received 3 μl of absolute ethanol, the vehicle used to dissolve the retinoic acid. Both groups were maintained in culture for a further 96 h, at which time the medium was removed and dialysed exhaustively against 0.05 M sodium acetate, pH 6.8, containing the following enzyme inhibitors; 10 mM EDTA, 5 mM benzamidine hydrochloride and 0.1 M 6-aminohexanoic acid. After dialysis the glycosaminoglycan content was determined using a modification of the DMMB assay [20,21]. The media from both groups were then pooled and the proteoglycan metabolites isolated by direct associative CsCl density-gradient centrifugation (starting density

1.5 g/ml). After centrifugation the tubes were divided into four fractions (A1–A4, bottom to top), which were then dialysed and assayed for their glycosaminoglycan content. In both groups 90% of the total glycosaminoglycan was recovered in their respective A1 fractions.

Samples (A1 fractions) containing proteoglycans were digested with chondroitinase ABC (0.1 unit/100 μg of glycosaminoglycan), dialysed, freeze-dried, redissolved in SDS/PAGE buffer at 1 μg of original proteoglycan/μl before electrophoresis on 4–15% SDS/PAGE gradient gels [23]. After electrophoresis the fractionated aggrecan degradation products were transferred to nitrocellulose, incubated in blocking solution [5% (w/v) BSA in 0.15 M NaCl, 0.01 M Tris/HCl, 0.02% NaN_3; Tris/saline/azide buffer] followed by a 1:1000 dilution of monoclonal antibody 2-B-6 (which recognizes chondroitin-4-sulphate stubs generated by chondroitinase pretreatment of proteoglycans) [18] or BC-3 in 1% (w/v) BSA in Tris/saline/azide for 1 h at room temperature. After three washes in Tris/saline/azide buffer, the nitrocellulose sheets were incubated with alkaline-phosphatase-conjugated rabbit anti-(mouse Ig) second antibody (Promega) and immunopositive bands visualized [17]. In general, the immunoblots were incubated for 10–30 min at room temperature to achieve optimum colour development.

Digestion of purified aggrecan G1-G2 domain with matrix metalloproteinases

Aggrecan G1-G2 domain from pig laryngeal cartilage was prepared and purified using published procedures [24] and dissolved in 10 mM $CaCl_2$, 100 mM NaCl, 50 mM Tris/HCl, pH 7.5, at 1.0 mg/ml. Purified rabbit bone stromelysin (MMP-3) and gelatinase (MMP-2) were gifts from Dr. Gillian Murphy, Strangeways Research Laboratory, Cambridge, U.K., and fibroblast collagenase (MMP-1) catalytic fragment [25] was provided by Dr. Tim Cawston, Addenbrooks Hospital, Cambridge, U.K. Purified aggrecan G1-G2 domain was digested for 24 h at 37 °C with 100 μg/ml MMP-1, 2.5 μg/ml MMP-2 or 2.5 μg/ml MMP-3. MMP-2 and MMP-3 were activated with aminophenyl mercuric acetate prior to incubation with the purified G1-G2 preparation. After digestion, samples were incubated at 37 °C for 18 h with keratanase (0.025 unit per 30 μl sample, Seikagaku) in 50 mM Tris/acetate, pH 7.5, containing proteinase inhibitors (10 mM EDTA, 100 μg/ml cystatin, 1 mM phenylmethanesulphonyl fluoride, 0.1 M 6-aminohexanoic acid, 5 mM benzamidine hydrochloride). After keratanase treatment the samples were subjected to SDS/PAGE in 5% gels [26] and either silver stained or electrophoretically transferred to an immobilon P membrane before immunolocation analysis with monoclonal antibody BC-4. The 85–90 kDa digestion product generated by all of these enzymes (MMP-1, -2 and -3) was subjected to N-terminal sequence analysis to verify that they all had the N-terminal sequence FFG... which is characteristic of the MMP cleavage products reported previously [12].

RESULTS

Characterization of monoclonal antibody BC-3 specificity

A single mouse was immunized with an ovalbumin conjugate substituted with a synthetic peptide (ARGSVILTVKGGC) containing the neoepitope representing the N-terminal sequence of the 'aggrecanase' cleavage site (Figure 1). One resulting hybridoma clone, denoted BC-3, showed strong reactivity with the immunizing antigen and with a conjugate containing the shorter aggrecan-related peptide ARGSVIGGC, but no reactivity with unrelated peptide conjugates nor with the carrier protein. The BC-3 hybridoma cell line was cloned by limiting dilution, and its antibody isotype determined as being an IgG1 heavy chain and κ light chain. Competitive e.l.i.s.a., using peptide antigens ARGSVILTVKGGC and EARGSVILGGC in free solution, demonstrated that internalization of the N-terminal alanine residue resulted in complete loss of inhibition (Figure 2a). This result establishes that the free N-terminal sequence ARGSVI... is an essential part of the BC-3 neoepitope.

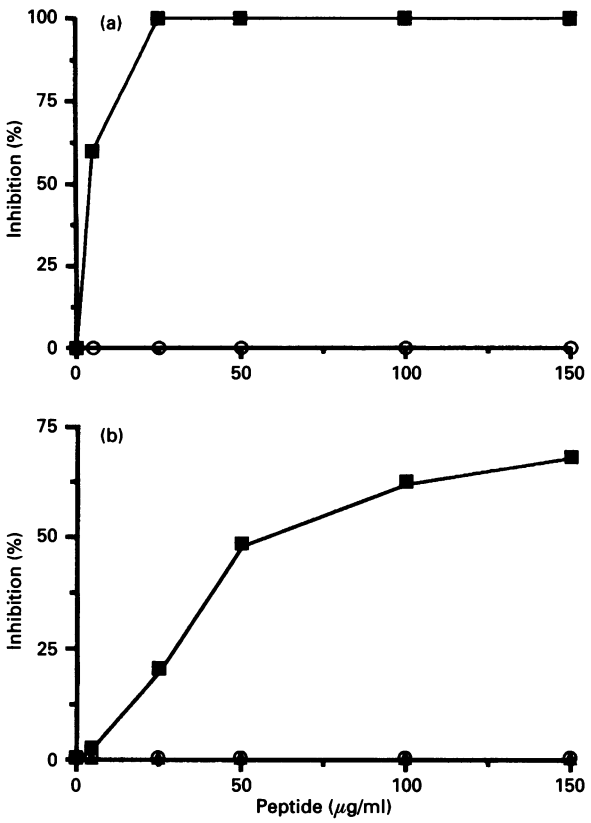

Figure 2 Competitive inhibition e.l.i.s.a. using monoclonal antibodies BC-3 and BC-4

Plates were coated with the ovalbumin–peptide conjugates that were used as immunizing antigens at 3 μg/ml. Inhibition analyses were performed using synthetic peptides as competing antigens. (**a**) For BC-3 analyses the peptides were ARGSVILGGC (■) and EARGSVIG (○). (**b**) For BC-4 analyses the peptides were GFVDIPEN (■), GFVDIPE (○) and GFVDIPENF (▲).

Application of BC-3 for the elucidation of 'aggrecanase' action

Several laboratories [9–11] have demonstrated that 'aggrecanase' products are generated from interleukin-1-treated bovine cartilage explant culture systems. Ilic et al. [12] have described work indicating that similar degradation products are produced after retinoic acid treatment of bovine cartilage maintained in explant culture. Therefore we investigated the immunoreactivity of monoclonal antibody BC-3 with cartilage degradation products generated by interleukin-1- or retinoic acid-treated bovine articular cartilage explant cultures *in situ*. In addition, Swarm rat chondrosarcoma cells maintained in monolayer culture in the presence of retinoic acid were analysed to investigate whether 'aggrecanase'-generated products were also characteristic of *in vitro* culture systems.

Immunolocation analysis of aggrecan degradation products released into the culture medium of the bovine cartilage explant

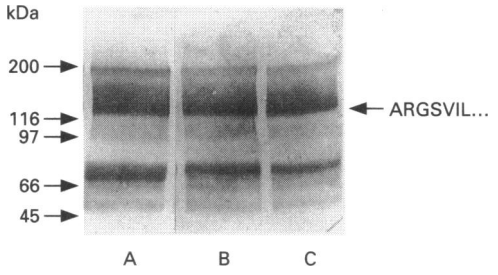

Figure 3 Analysis of bovine cartilage explant culture media using BC-3

Localization of BC-3 neoepitope was performed on proteoglycan fragments isolated from the medium of bovine articular cartilage cultured in the presence of (lane A) 3×10^{-7} M retinoic acid, (lane B) 20 units/ml interleukin-1β, and (lane C) no additives. The N-terminal sequence of the fragment in the major band obtained from the equivalent location on a companion blot is indicated. Equal quantities of medium proteoglycan (assessed as DMMB-reactive material) were applied to each well.

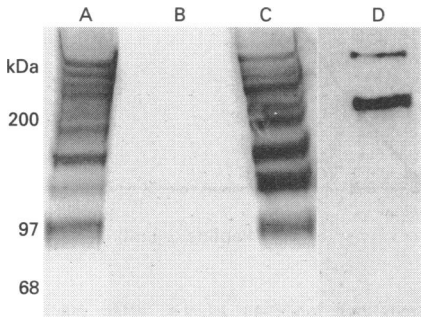

Figure 4 Analysis of rat chondrosarcoma cell culture media using BC-3

Localization of BC-3 neoepitope was performed on proteoglycan fragments from the media of control (untreated, lanes A and B) and retinoic acid-treated (1×10^{-6} M, lanes C and D) chondrocyte cultures. Immunolocation was performed with antibody 2-B-6 (anti-chondroitin 4-sulphate, lanes A and C) or with antibody BC-3 (lanes B and D).

cultures treated with either retinoic acid or interleukin-1 identified BC-3 immunopositive bands (Figure 3). Sequencing of the major BC-3 band at 120 kDa gave the N-terminus ARGSVIL..., consistent with the cleavage site reported for the action of 'aggrecanase' [10]. BC-3-positive immunoreactivity was also seen with additional aggrecan degradation products, but there was insufficient material present to determine their N-terminal sequences. Both interleukin-1 and retinoic acid stimulated proteoglycan fragment release into the culture medium above that seen in control cultures. However, when equal weights of proteoglycan fragments were analysed, similar staining patterns were seen for the BC-3 epitope for all three culture conditions. This indicates that the same degradative mechanism is acting in both stimulated and unstimulated tissue in this experimental system.

In contrast, the constitutive action of 'aggrecanase' was not observed in cultured rat chondrosarcoma cells (Figure 4). However, evidence of 'aggrecanase' action was observed when the rat chondrosarcoma cells were stimulated with retinoic acid. The pattern of BC-3 immunoreactivity was quite distinct from that seen in the bovine cartilage explant system. This lack of 'aggrecanase' action in control cultures was not due to the absence of proteolytic degradation as such, as immunolocation with monoclonal antibody 2-B-6 revealed at least nine distinct

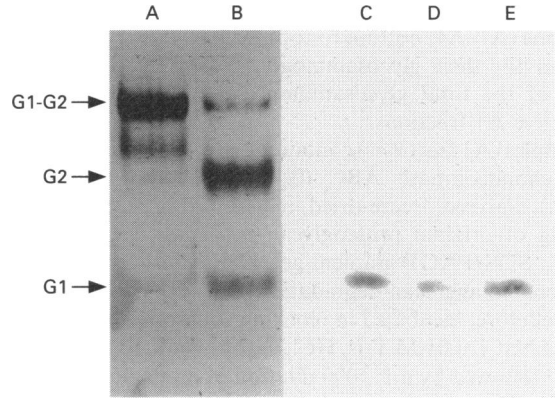

Figure 5 Analysis of metalloproteinase cleavage of purified pig G1-G2 domains using BC-4

Lane A, silver stain of undigested G1-G2 fragment; lane B, silver stain of G1-G2 fragment digested with MMP-3 (stromelysin); lanes C, D and E, BC-4 immunoreactivity of degradation product produced by MMP-3 (stromelysin), MMP-2 (gelatinase) and MMP-1 (collagenase) respectively. Silver staining patterns of G1-G2 digestion products were similar for all three enzymes.

core protein fragments under both conditions. Not all fragments stained with equal intensity, and several of the prominent bands were not BC-3 reactive. This would imply that proteinases other than 'aggrecanase' must be acting in this system.

Characterization of monoclonal antibody BC-4 specificity

A single mouse was immunized with an ovalbumin conjugate substituted with a synthetic peptide (CGGFVDIPEN) containing the neoepitope representing the C-terminal sequence present on the G1 domain product of metalloproteinase action (Figure 1). One of the resulting hybridoma clones, denoted BC-4, showed strong reactivity with the immunizing antigen, but no reactivity with unrelated peptide conjugates nor with the carrier protein. The BC-4 hybridoma cell line was cloned by limiting dilution and its antibody isotype determined as being IgG2a heavy chain and κ light chain. Competitive e.l.i.s.a. of monoclonal antibody BC-4 against a series of unconjugated peptides showed that removal of the C-terminal asparagine residue or the addition of a phenylalanine residue at the C-terminus resulted in a complete loss of inhibition (Figure 2b). These results demonstrate the importance of the free C-terminal sequence ... DIPEN as an essential component of the BC-4 epitope.

Application of BC-4 to study metalloproteinase action

A purified preparation of pig aggrecan G1-G2 domains, as a substrate for MMP-1, -2 and -3, was used to study the action of these enzymes in the aggrecan interglobular domain. A typical fragment pattern following digestion with MMP-3 reveals two products by silver staining; an 85–90 kDa G2 fragment and a 50–56 kDa G1 fragment (Figure 5, lane B). Amino acid sequence analysis of the 85–90 kDa G2 fragment revealed the N-terminus FFGVG... Assuming a single cleavage occurs [27], this result implies that the second fragment should have the C-terminal sequence ... DIPEN (Figure 1). Immunoanalysis with monoclonal antibody BC-4 confirmed this assumption. Immunolocation of the G1 and G2 fragments resulting from digestion with stromelysin (MMP-3, Figure 5, lane C), gelatinase A (MMP-2, Figure 5, lane D) and fibroblast collagenase (MMP-1, Figure

5, lane E) gave an immunopositive band at 50–56 kDa, but no reactivity with the 85–90 kDa digestion product or the 150 kDa undigested G1-G2 starting material. In addition, the 85–90 kDa G2 product of this cleavage did not react with antibody BC-3, indicating that under the experimental conditions used none of these enzymes exhibited 'aggrecanase' activity [28].

DISCUSSION

The results presented in this paper using BC-3 illustrate two important points. First, 'aggrecanase' activity is not a constitutive event in all cartilage culture systems, and secondly, proteolytic agents other than 'aggrecanase' are clearly involved in aggrecan degradation in some systems. In our work, constitutive 'aggrecanase' action was observed in a bovine cartilage explant culture system but not in a rat chondrocyte culture system. This difference could be due to variation in several parameters, e.g. site where tissue was harvested, duration of culture, age and species of animal, or pathological status. Other results (C. E. Hughes, unpublished work) indicate that alternative culture conditions can result in non-constitutive expression of 'aggrecanase' activity for explants of bovine cartilage (both bovine nasal and bovine articular cartilage), suggesting that culture conditions *per se* are important. Such differences may be a consequence of variation in the synthesis of autocrine factors such as interleukin-1. This explanation might also account for the action of proteolytic activities other than 'aggrecanase' that occur under some conditions. One cannot therefore assume that a single agent, such as 'aggrecanase', is responsible for all aggrecan degradation *in vivo*.

The use of BC-4 provides a rapid and sensitive means for definitively identifying the C-terminus of the aggrecan G1 fragment that accumulates following the action of matrix metalloproteinases. It still remains to be established whether this is the only C-terminus produced or whether there are additional sites of proteolytic action that modify the initial cleavage product. This question could be resolved by use of BC-4 in quantitative analyses (e.g. immunoprecipitation). It would also be of interest to prepare antibodies directed against the C-terminal neoepitope generated by the action of 'aggrecanase' in the interglobular domain to determine whether the G1 domain product accumulates in the cartilage matrix without further modification. Indeed the availability of paired reagents recognizing both the C-terminal and N-terminal neoepitopes generated by a single proteolytic reagent will be extremely useful in the study of cartilage turnover, as they enable both the retained and released products to be analysed.

In this paper, we have used newly developed neoepitope antibody technology [17] to clearly identify two different aggrecan cleavage products produced by matrix metalloproteinases and the as yet unidentified cartilage proteinase activity 'aggrecanase'. Studies using monoclonal antibody BC-3 have demonstrated that products generated from 'aggrecanase' activity can be detected in the media of interleukin-1- or retinoic acid-treated cartilage explant or chondrocyte monolayer cultures. Use of antibody BC-3 to detect these 'aggrecanase'-generated degradation products is much more sensitive than the N-terminal sequence analysis that has previously been used [6–12,14]. As little as 10 μg of glycosaminoglycan-containing aggrecan catabolites (representing approx. 1 μg of protein distributed between 5–10 bands) can be easily detected by Western blot analysis of culture media. This sensitivity should allow for the development of quantitative immunoassays for the analysis of aggrecan catabolites in body fluids. Such analyses will facilitate monitoring of the progression of arthritic diseases and the efficacy of drug intervention.

Additional practical applications can readily be envisaged using these two immunological reagents. The use of BC-3 should facilitate the purification and subsequent biochemical characterization of 'aggrecanase', as it provides a specific method for monitoring this proteolytic activity. Furthermore, the use of BC-4 will facilitate the identification of metalloproteinase-generated aggrecan G1 domains that may accumulate in cartilage. With aging, G1 domains are known to accumulate [29]; but whether a single site of cleavage is responsible for their generation is as yet unknown. It can also be envisaged that these reagents will have a ready application to studies of the proteolytic mechanism of aggrecan degradation, particularly in arthritic diseases.

Note added in proof (received 21 November 1994)

The authors wish to acknowledge a recent Research Communication [30] that utilizes antibody BC-3 to provide evidence that neutrophil collagenase (MMP-8) can exhibit 'aggrecanase' activity *in vitro*.

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REFERENCES

1 Hardingham, T. E., Fosang, A. J. and Dudhia, J. (1992) in Articular Cartilage and Osteoarthritis: Aggrecan, The Chondroitin Sulfate/Keratan Sulfate Proteoglycan from Cartilage (Kuettner, K. E., Schleyerbach, R., Peyron, J. G. and Hascall, V. C., eds.), pp. 5–20, Raven Press, New York
2 Paulson, M., Morgelin, M., Wiedemann, H., Beardmore-Gray, M., Dunham, D., Hardingham, T. E., Heinegård, D., Timpl, R. and Engel, J. (1987) Biochem. J. **245**, 763–772
3 Hardingham, T. E. and Muir, H. (1972) Biochim. Biophys. Acta **279**, 401–405
4 Heinegård, D. and Hascall, V. C. (1974) J. Biol. Chem. **249**, 4250–4256
5 Hardingham, T. E. (1979) Biochem. J. **177**, 237–247
6 Flannery, C. R., Lark, M. W. and Sandy, J. D. (1992) J. Biol. Chem. **267**, 1008–1014
7 Sandy, J. D., Flannery, C. R., Neame, P. and Lohmander, L. S. (1992) J. Clin. Invest. **89**, 1512–1516
8 Lohmander, L. S., Neame, P. J. and Sandy, J. D. (1993) Arthritis Rheum. **36**, 1214–1222
9 Sandy, J. D., Boynton, P. E. and Flannery, C. R. (1991) J. Biol. Chem. **266**, 8198–8205
10 Sandy, J. D., Neame, P. J., Boynton, R. E. and Flannery, C. J. (1991) J. Biol. Chem. **266**, 8683–8685
11 Loulakis, P., Shrikhande, A. V., Davis, G. and Maniglia, C. A. (1992) Biochem. J. **284**, 589–593
12 Ilic, M. Z., Handley, C. J., Robinson, H. C. and Mok, M. T. (1992) Arch. Biochem. Biophys. **294**, 115–122
13 Doege, K. J., Sasaki, M., Kimura, T. and Yamada, Y. (1991) J. Biol. Chem. **266**, 894–902
14 Fosang, A. J., Neame, P. J., Last, K., Hardingham, T. E., Murphy, G. and Hamilton, J. A. (1992) J. Biol. Chem. **267**, 19470–19474
15 Fosang, A. J., Last, K., Knauper, V., Neame, P. J., Murphy, G., Hardingham, T. E., Tschesche, H. and Hamilton, J. A. (1993) Biochem. J. **295**, 273–276
16 Flannery, C. R. and Sandy, J. D. (1993) Trans. Ortho. Res. Soc. **39**, 190
17 Hughes, C. E., Caterson, B., White, R. J., Roughley, P. J. and Mort, J. S. (1992) J. Biol. Chem. **267**, 16011–16014
18 Caterson, B., Christner, J. E. and Baker, J. R. (1983) J. Biol. Chem. **258**, 8848–8854
19 Caterson, B., Calabro, T. and Hampton, A. (1987) in Biology of the Extracellular Matrix: A Series. Biology of Proteoglycans (Wight, T. and Mecham, R., eds.), pp. 1–26, Academic Press, New York
20 Farndale, R. W., Sayers, C. A. and Barrett, A. J. (1982) Connect. Tissue Res. **9**, 247–248
21 Ratcliffe, A., Doherty, M., Maini, R. N. and Hardingham, T. E. (1988) Ann. Rheum. Dis. Biol. **47**, 826–832

22 Roughley, P. J. and Barrett, A. J. (1977) Biochem. J. **167**, 629–637
23 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
24 Fosang, A. J. and Hardingham, T. E. (1989) Biochem. J. **261**, 801–809
25 Schnierer, S., Kleine, T., Gote, T., Hillemann, A., Knauper, V. and Tschesche, H. (1990) Biochem. Biophys. Res. Commun. **191**, 319–326
26 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry **10**, 2606–2616
27 Fosang, A. J., Neame, P. J., Hardingham, T. E., Murphy, G. and Hamilton, J. A. (1991) J. Biol. Chem. **266**, 15579–15582
28 Fosang, A. J., Last, K., Neame, P. J., Hughes, C. E., Caterson, B., Hardingham, T. E., Knauper, V., Murphy, G. and Tschesche, H. (1994) Trans. Ortho. Res. Soc. **40**, 48
29 Roughley, P. J., White, R. J. and Poole, A. R. (1985) Biochem. J. **231**, 129–138
30 Fosang, A. J., Last, K., Neame, P. J., Murphy, G., Knauper, V., Tschesche, H., Hughes, C. E., Caterson, B. and Hardingham, T. E. (1994) Biochem. J. **304**, 347–351

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