Papers

Differential detection of type II collagen N-terminal and C-terminal denaturation epitopes in degrading cartilage

L J Croucher, A P Hollander

Abstract

***Aims*—To investigate the relative stability of collagen metabolites in degrading cartilage.**

***Methods*—New antipeptide antibodies to denaturation epitopes located in the N-terminal and C-terminal regions of the type II collagen helix have been made and characterised. Type II collagen fragments in the conditioned medium from cultures of degrading bovine nasal cartilage were detected by immunoblotting with the new antisera as well as by N-terminal sequencing. The antibodies were also used in immunohistochemical studies of normal and osteoarthritic human cartilage.**

***Results*—Type II collagen fragments with an apparent molecular mass of approximately 30 kDa were detected in cartilage conditioned media using antibody AH12L3, which recognises N-terminal epitope AH12. The N-terminal sequence of one of these fragments matched exactly a sequence in the N-terminal region of type II collagen. Antibody AH9L2, which recognises C-terminal epitope AH9, did not bind to any protein bands in the immunoblotted culture medium. In immunohistochemical studies, antibody AH12L3 detected extensive regions of degraded collagen in osteoarthritic cartilage and a more restricted pattern of staining in non-arthritic cartilage. Far less immunostaining was apparent in all cartilage specimens with antibody AH9L2.**

***Conclusions*—These results indicate that the N-terminal region of type II collagen is more resistant to proteolysis than the C-terminal region, an observation that has important implications for the choice of epitopes that are likely to be good markers of damage to cartilage collagen in patients with arthritis.**

(*J Clin Pathol: Mol Pathol* 1999;**52**:323–331)

Keywords: type II collagen; denaturation; cartilage

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Accepted for publication
14 September 1999

Type II collagen and aggrecan, the two major components of articular cartilage, are degraded and lost from this tissue in arthritis and destruction of the collagen scaffold is thought to be irreversible, leading to loss of cartilage integrity and eventual joint failure.[1] The collagen network provides the tissue with tensile strength and restrains the swelling pressure exerted by hydrated aggrecan, the molecule responsible for compressive stiffness.[2] The pathological mechanisms leading to type II collagen degradation in arthritis are poorly understood. We and others have studied collagen degradation in cartilage explant culture systems,[3 4] as well as in human arthritic cartilage specimens.[5 6] However, there are very little data describing the relation between the biochemical processes of collagen degradation and the clinical, pathological events in osteoarthritis (OA)[1] or rheumatoid arthritis (RA).

There is now considerable evidence that degradation of type II collagen is a major feature both of OA and RA. Histological evidence of damage to collagen in OA cartilage was first described by Mankin *et al* in 1971,[7] and Venn and Maroudas[8] subsequently measured the collagen degradation in OA as a loss of hydroxyproline. Electron microscope studies of RA cartilage have demonstrated collagen degradation at the articular surface, especially at the cartilage–pannus junction.[9 10] More recently, antibodies specific to molecular forms of type II collagen have been used to study degradation of this molecule in more detail. When a fibrillar collagen molecule is cleaved in its triple helix by a collagenase, the resulting fragments denature, thereby losing their helical conformation.[11 12] The process of denaturation can be detected using antibodies to peptide sequences from the α-chain. Antibodies raised to cyanogen bromide (CNBr) fragmented peptides of type II collagen were shown to react with the denatured molecule, but not with the intact, native collagen.[13] Monoclonal antibody COL2-3/4m, raised to a synthetic peptide from a sequence in the CB11 region of the type II collagen α-chain, was used as the basis of an inhibition enzyme linked immunosorbent assay (ELISA) for measuring collagen degradation in OA cartilage,[5] as well as in chondrocyte or cartilage explant culture systems.[3 14] These denaturation antibodies have also been of use in immunohistochemical studies to localise regions of

cartilage containing denatured type II collagen.[6 13 15]

The matrix metalloproteinases (MMPs) are the enzymes thought most likely to be responsible for degradation of extracellular matrix proteins because they are generally active at neutral pH.[16–18] In particular, there are three known collagenases, namely interstitial collagenase (MMP-1; EC 3.4.24), neutrophil collagenase (MMP-8; EC 3.4.24.34), and collagenase 3 (MMP-13; EC 3.4.24.-; the full MMP-13 EC number has not yet been assigned), each of which could contribute to cartilage collagen degradation.[12 19 20] They all cleave type II collagen at a specific bond, Gly^{975}–Leu^{976}, in each of the α-chains, although secondary and tertiary cleavages might also occur.[20–22] The membrane bound metalloproteinase MT1-MMP (MMP-14) also cleaves at this site.[23] Neoepitope antibodies to the cleavage site termini have been described,[21 22] and used to demonstrate that cleavage of the Gly^{975}–Leu^{976} bond is responsible for at least some of the damage to type II collagen molecules in OA cartilage. Cleavage at sites closer to the N-terminus and C-terminus of the triple helix, or in the telopeptides, might also lead to depolymerisation of the type II collagen fibrillar network. The metalloproteinase stromelysin (MMP-3) has been shown in vitro to cleave the native type II collagen molecule at two sites within the N-telopeptide,[24] whereas the cysteine proteinase cathepsin K cleaves the helix of type II collagen, close to its N-telopeptide.[25] However, the in vivo importance of such cleavages has not been studied extensively.

The aim of our study was to determine the susceptibility of the terminal regions of the type II collagen triple helix to proteolysis in degrading cartilage, to elucidate the proteolytic mechanisms that contribute to joint failure in arthritis.

Materials and methods

HUMAN CARTILAGE

Human femoral condylar or femoral head articular cartilage was obtained from patients undergoing total hip or knee arthroplasty for OA, or from hip joints replaced after osteoporotic fracture of the femoral neck. A scalpel was used to cut full thickness slices from loaded regions of the femoral condyles of knee joints, and from all areas of the femoral heads. The cartilage was stored at −20°C.

BOVINE CARTILAGE EXPLANT CULTURES

Bovine nasal septum cartilage was obtained from freshly killed adult cows and cultured in DMEM (Gibco Life Technologies, Paisley, UK), with the following supplements: penicillin G (2000 IU/ml), streptomycin (0.1 mg/ml), Hepes buffer (10 mM), and L-glutamine (2 mM) (all from Gibco), as described previously.[3] The medium was changed on days 7, 14, and 21 of culture. Matrix degradation was stimulated by the addition of recombinant human interleukin 1α (IL-1α; a kind gift of Dr C Reynolds, National Cancer Institute, Frederick, Maryland, USA) to the medium at 50 ng/ml on days 0 and 7. Medium from the third week of culture was collected and stored at −20°C before analysis.

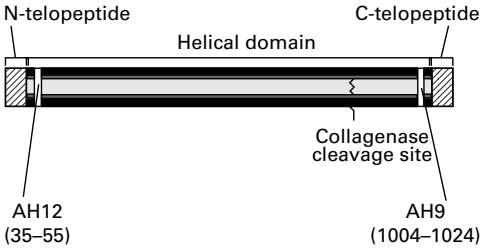

Figure 1 Location of peptide epitope sequences used to generate new anti-type II collagen antibodies. Residue numbers are shown in brackets, where the first amino acid of the N-telopeptide has been taken as residue 1.

ANTIPEPTIDE ANTIBODIES TO TYPE II COLLAGEN DENATURATION EPITOPES

Antibodies were raised in rabbits to the following peptides: AH9, G-P-P(OH)-G-P-R-G-R-S-G-E-T-G-P-A-G-P-P(OH)-G-N-P(OH); and AH12, G-A-P-G-P-Q-G-F-Q-G-N-P-G-E-P-G-E-P-G-V-S. AH9 is located in the C-terminal region of the human α1(II) chain, beginning at residue 1004 (where residue 1 is the N-terminal amino acid of the N-telopeptide), and AH12 is located in the N-terminal region of the human α1(II) chain, beginning at residue 35 (fig 1). They were chosen on the basis of favourable hydrophilicity profiles and on their minimal sequence identity with other collagen α-chains, as described previously.[5] The peptides were synthesised, using standard 9-fluorenylmethyloxycarbonyl (FMOC) chemistry, by Dr A Moir, Kreb's Institute, University of Sheffield, UK. They were each made with an additional N-terminal cysteine for conjugation to keyhole limpet haemocyanin (KLH; Calbiochem-Novabiochem Corporation, San Diego, California, USA). Each peptide was coupled to KLH through its N-terminal cysteine, using the coupling reagent bromoacetic acid N-hydroxysuccinimide ester (Sigma-Aldrich Chemical Company, Poole, Dorset, UK), as described previously.[26] New Zealand white or dwarf lop rabbits were immunised subcutaneously with KLH conjugated peptide, 200 mg/animal, dissolved in phosphate buffered saline (PBS), and emulsified with an equal volume of complete Freund's adjuvant (Sigma). Blood samples were taken after the fourth immunisation and tested concurrently with pre-immune sera from the same animals for reactivity with specific epitopes by direct binding ELISA, as described below.

DIRECT BINDING ELISA

Heat denatured type II collagen was prepared by heating a solution of bovine nasal septum type II collagen (Sigma), in 0.1 M sodium carbonate buffer (pH 9.2) at 80°C for 20 minutes. The solution of heat denatured type II collagen was diluted to 40 μg/ml in carbonate buffer, and 50 μl was added to each well of Immulon-2 ELISA plates. The heat denatured type II collagen was allowed to adsorb passively to the bottom of the wells at 4°C for 72–96 hours, before removal of unbound protein with three washes of PBS containing 0.1% vol/vol Tween

20 (PBS/Tween; Sigma). The wells were blocked with PBS containing 1% wt/vol bovine serum albumin (BSA; Sigma), at 50 μl/well for 30 minutes at room temperature, before a final wash in PBS/Tween. Other plates were coated in the same manner with immunising peptides, each at 2 μg/ml. Antibodies were added to coated plates at various dilutions in PBS. After incubation at 37°C for 90 minutes, plates were washed three times in PBS/Tween. Secondary antibody, alkaline phosphatase labelled goat antirabbit immunoglobulin (Southern Biotechnology Associates, Birmingham, Alabama., USA), diluted 1/1000 in PBS containing 1% wt/vol BSA and 0.1% vol/vol Tween 20, was added at 50 μl/well, and incubated for a further 90 minutes at 37°C. Plates were washed as above, with a final wash in distilled water, before the addition of alkaline phosphatase substrate, 0.5 mg/ml disodium *p*-nitrophenyl phosphate (Sigma), prepared in 8.9 mM diethanolamine, 0.25 mM $MgCl_2$ (pH 9.8), 50 μl/well. After incubation at ambient temperature for 10–20 minutes, absorbance was measured at 405 nm on an MRX microplate reader (Dynatech Labs, Billinghurst, West Sussex, UK).

PURIFICATION OF IgG FROM RABBIT ANTIPEPTIDE ANTIBODIES

Rabbit antisera were diluted 1/4 in 20 mM sodium acetate buffer (pH 5.0) and 15 ml of this solution was applied to a 5 ml protein G Sepharose 4 fast flow column (Pharmacia Biotech, St Albans, UK). The flow rate was maintained at 1 ml/minute throughout the procedure. A UV detector linked to the column outflow was used to monitor protein elution at 280 nm. When all unbound protein had been flushed through the column with sodium acetate buffer, bound IgG was eluted with 0.1 M glycine/HCl (pH 2.7), collected in 2 ml fractions. These were neutralised at the point of collection with 1–2 drops of 1 M Tris/HCl (pH 9.0). The absorbance of the fractions was determined at 280 nm on a spectrophotometer (Perkin-Elmer Ltd, Beaconsfield, Buckinghamshire, UK) and those containing the protein peak were pooled. The IgG was dialysed extensively against PBS and centrifuged to remove any insoluble IgG precipitate, followed by a final determination of IgG concentration by measurement of the absorbance at 280 nm. Aliquots were stored at −20°C.

CLEAVAGE OF NATIVE TYPE II COLLAGEN BY MMP-13

To help demonstrate the specificity of the antibodies used in our study, recombinant human MMP-13 (a kind gift from Dr P Mitchell, Pfizer Central Research, Groton, Connecticut, USA) was used to cleave bovine type II collagen into its characteristic 1/4 and 3/4 fragments. The enzyme was activated by incubation with 1 mM *p*-aminophenyl-mercuric acetate (Sigma) for one hour at 37°C. Bovine type II collagen, dissolved in 0.1 M Tris/HCl (pH 7.6), containing 10 mM $CaCl_2$, was incubated with the activated enzyme at a molar ratio of 50 : 1 for 40 hours at 30°C. The enzyme was inhibited by the addition of 20 mM EDTA. Aliquots of the cleaved collagen were stored at −20°C.

WESTERN IMMUNOBLOTTING

Samples of conditioned culture media, purified collagens, or collagenase cleaved collagens were separated under reducing conditions on 10%, 1 mm thick, 7 × 8 cm mini-protean sodium dodecyl sulphate polyacrylamide gels (SDS–PAGE; Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), as described previously.[3] Human and bovine type I collagen, bovine type II collagen, bovine type III collagen, and human type IV collagen were all purchased from Sigma. Human type II collagen was a kind gift from Dr AR Poole, Shriner's Hospital, Montreal, Canada. The electrophoresed samples were then transferred to a nitrocellulose membrane (Bio-Rad Laboratories) under standard conditions. Transferred proteins were then either stained with amido black (Bio-Rad; 0.1% wt/vol in 10% vol/vol acetic acid) or immunoblotted as follows. The membranes were blocked overnight at ambient temperature with 3% wt/vol BSA in PBS. After a brief wash in PBS/Tween, membranes were incubated with a purified IgG preparation of the anticollagen antibody, diluted appropriately in 3% BSA/PBS, for two hours at ambient temperature. The membranes were washed three times in PBS/Tween before incubation with alkaline phosphatase labelled goat antirabbit immunoglobulin (Southern Biotechnology), diluted 1/1000 in 3% BSA/PBS, for 30 minutes at ambient temperature. After three further washes in PBS/Tween and a final rinse in distilled water, the membranes were incubated with an alkaline phosphatase substrate kit, containing a solution of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad). After optimal colour development, the reaction was stopped by rinsing the membrane in distilled water.

N-TERMINAL SEQUENCING

Media samples from cartilage cultures were electrophoresed, after a pre-run of the gel with dithiothreitol and glutathione (both from Sigma) as free radical scavengers, according to the method of Dunbar and Wilson.[27] Electrophoretic transfer to a polyvinyledine difluoride (PVDF) membrane (Bio-Rad) was carried out at 4°C in 50 mM 3-[cyclohexylamino]-1-propane sulphonic acid (CAPS) buffer (Sigma) with 10% methanol, for two hours at 200 mA. N-terminal sequencing of the excised bands was carried out by Dr B Dunbar, protein sequencing unit, University of Aberdeen, Scotland.

IMMUNOHISTOCHEMISTRY

Cartilage pieces were frozen in OCT embedding medium (BDH Laboratory Supplies, Poole, Dorset, UK). Sections (7 μm) were cut using a cryostat (Bright, Huntingdon, Cambridgeshire, UK) and attached to slides precoated with 3-aminopropyl triethoxysilane (ICN Biomedicals Ltd, Thame, UK), as

described previously,[28] to ensure maximum adherence of the section for uniform staining. Sections were either stained immediately or stored at −20°C. They were fixed in 4% vol/vol paraformaldehyde (Sigma) and immunostained essentially as outlined previously,[6] with some amendments. Aldehyde groups were blocked with normal goat serum, diluted 1/10 in PBS, and the permeability of the extracellular matrix was maximised with chondroitinase ABC lyase (ICN Biomedicals) containing proteinase inhibitors, as described previously.[6] Sections were probed with IgG purified from anticollagen antibodies. The optimum dilution for each antibody was determined by matching the IgG concentration of the antibody with that of non-immune rabbit IgG (Vector Laboratories Burlingame, California, USA), which produced negligible background staining of the same tissue when used under the same conditions. More specific controls were performed by pre-absorption of AH12L3 and AH9L2 with their respective peptides at 100 µg peptide/ml of appropriately diluted antibody, for one hour at 37°C, before incubation with the sections. A biotin–avidin detection system was used. Sections were incubated with biotin labelled goat antirabbit secondary antibody (Southern Biotechnology), diluted 1/100. Bound antibody was detected using avidin–biotin–peroxidase in the ABC Elite Biotin-Streptavidin Kit (Vector Laboratories), according to the manufacturer's directions. Peroxidase was reacted with the substrate 3,3'-diamonobenzidine (Vector Laboratories), with the addition of nickel, to form a grey black stain. Sections were dehydrated through graded alcohols before mounting permanently in DPX (BDH).

IMMUNOHISTOCHEMICAL SCORING

All immunostained sections were scored for intensity of staining by one observer (APH) under blinded conditions as follows. A value of 0–3 was assigned for the surface, mid, and deep zones of cartilage (top 20%, middle 40%, and lower 40%, respectively, from the articular surface). Scores were as follows: 0, no apparent staining; 1, weak/diffuse staining; 2, moderate staining; 3, intense staining. A final score was then calculated as the sum of the values for each of the three zones. Differences in scores between patient groups or antibodies were calculated using the two tailed Mann-Whitney U test; $p < 0.05$ was taken as significant.

Results

GENERATION OF ANTIBODIES TO N-TERMINAL AND C-TERMINAL EPITOPES IN TYPE II COLLAGEN

Antiserum AH9L2 was obtained from a rabbit immunised with AH9. IgG was purified from this antiserum using a protein G column and then characterised for reactivity with different antigens by direct binding ELISA. It bound well to peptide AH9 (fig 2A) and to heat denatured type II collagen (figure 2C). Similarly, antiserum AH12L3 was obtained from a rabbit immunised with peptide AH12. Purified AH12L3 reacted well with peptide AH12 in a direct binding ELISA (fig 2B), and also bound to heat denatured type II collagen (fig 2C). Antibody AH9L2 clearly had negligible affinity for AH12, as did AH12L3 for AH9 (compare fig 2A and 2B). There was some difference between the two antibodies in their recognition of heat denatured type II collagen (fig 2C).

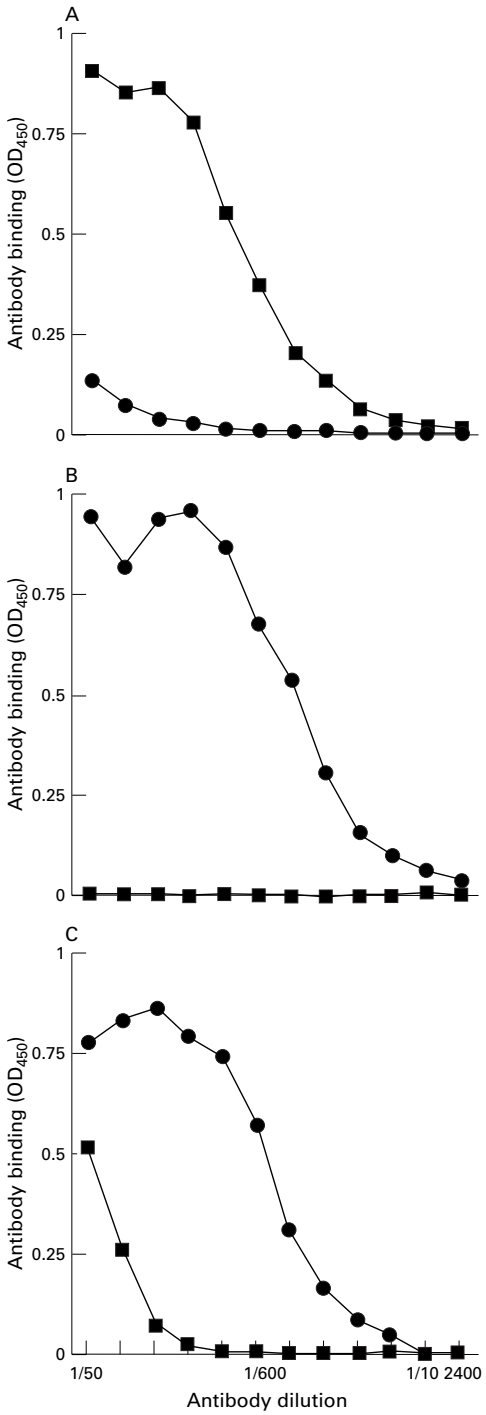

Figure 2 Immunoreactivity of IgG preparations from anticollagen antibodies with immunising peptides and heat denatured type II collagen, determined by direct binding enzyme linked immunosorbent assay (ELISA). ELISA plates were coated with peptide or heat denatured type II collagen and incubated with serial dilutions of IgG, as described in the methods section. Results are shown for wells coated with (A) peptide AH9, (b) peptide AH12, or (C) heat denatured type II collagen. Antibody AH9L2, closed square; antibody AH12L3, closed circle.

This was most likely because the standard bovine type II collagen preparation used in our study contained large fragments recognised by AH12L3 (figs 3C and 4C). These fragments were not detected with AH9L2 (figs 3B and 4B), presumably because they had been generated by C-terminal cleavage of the α-chains. Neither of these antisera was reactive with native type II collagen in a direct binding ELISA (data not shown). These results indicate that AH9L2 will specifically detect the C-terminal epitope to which it was raised, in denatured but not intact type II collagen, and AH12L3 will react specifically with its N-terminal epitope in denatured type II collagen.

SPECIFICITY OF REACTION WITH TYPE II COLLAGEN

The new antibodies were tested for reactivity with various bovine and human collagens. In western immunoblotting experiments both antibodies were used at their optimal dilutions of 1/1000 (AH9L2) and 1/10 000 (AH12L3). They were found to react specifically with the α1(II) chain of bovine as well as human type II collagen (fig 3). In addition, AH12L3 detected the crosslinked β component and some lower molecular mass collagen fragments naturally present in the purified collagen preparations. Neither antibody detected any of the components of bovine and human type I collagen, bovine type III collagen, or human type IV collagen (fig 3B and C). The reactivity of both antibodies with type II collagen was abolished by preincubating them with their immunising peptides at 100 μg/ml for one hour at 37°C before use (data not shown), indicating that the antibodies bind to the type II collagen through their specific epitopes.

DETECTION OF COLLAGENASE GENERATED FRAGMENTS OF TYPE II COLLAGEN

Cleavage of bovine type II collagen by MMP-13 generated the expected 3/4 and 1/4 fragments (figure 4A). Antibody AH9L2 detected its epitope in the 1/4 fragment and intact α1(II) chain, but not in the 3/4 fragment (fig 4B). The β component of type II collagen is also visible in fig 4B, but is not detectable with AH9L2 in fig 3B; different preparations of type II collagen were used for these two experiments. Presumably, the higher molecular mass fragment also detected with this antibody is a crosslinked dimer of the 1/4 fragment, as has been noted previously.[21 22] In contrast, antibody AH12L3 detected its epitope in the 3/4 fragment and in the intact α1(II) chain, but not in the 1/4 fragment (fig 4C). These findings confirm the specificity of AH9L12 for a C-terminal epitope and AH12L3 for an N-terminal epitope of the α1(II) chain.

DETECTION OF TYPE II COLLAGEN FRAGMENTS IN CARTILAGE EXPLANT CULTURES

To determine the relative stabilities of the AH9 and AH12 epitopes in a catabolic environment, a bovine nasal cartilage explant culture system was used. This model has been described previously in detail.[3] When the nasal cartilage explants are cultured with IL-1α the type II collagen remains intact throughout the first two weeks. There is then a large increase in MMP activity, which coincides with the release of type II collagen into the tissue culture medium, which can be detected by immunoassay.[3] Therefore, we collected the conditioned medium from the third week of culture with IL-1α, in experiments using bovine nasal cartilage from four individual animals. In each case we confirmed, by the above immunoassay, that most of the type II collagen

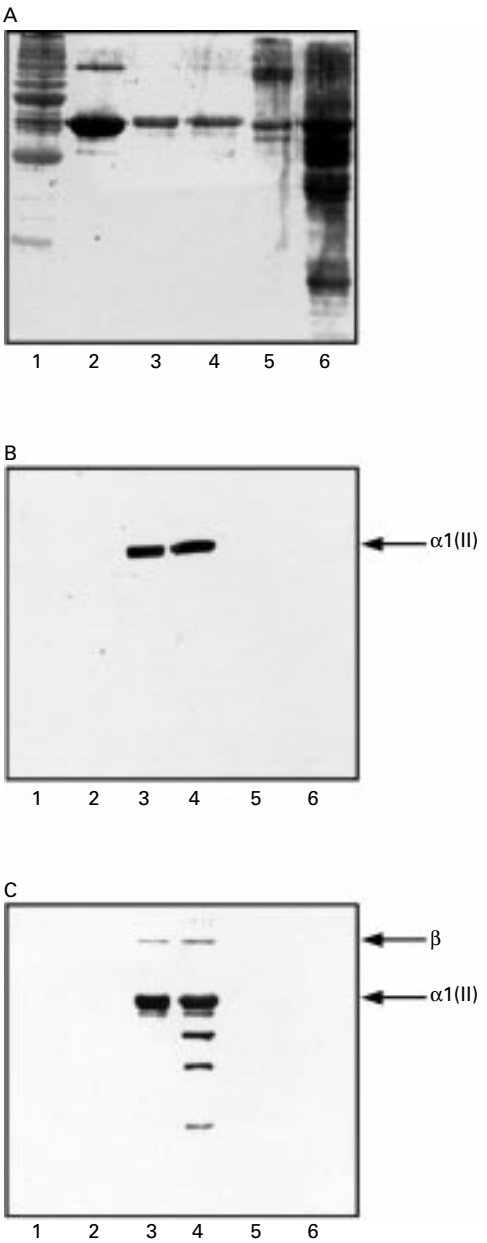

Figure 3 Specificity of N-terminal and C-terminal antibodies for type II collagen. Purified collagens were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (10% gels) and transferred on to a nitrocellulose membrane, as described in the methods section. (A) Amido black staining of all the transferred proteins; (B) immunoblotted with antibody AH9L2; (C) immunoblotted with antibody AH12L3. Lane 1, human type IV collagen; lane 2, bovine type III collagen; lane 3, human type II collagen; lane 4, bovine type II collagen; lane 5, bovine type I collagen; lane 6, human type I collagen. The migration position of type II collagen α and β components is indicated.

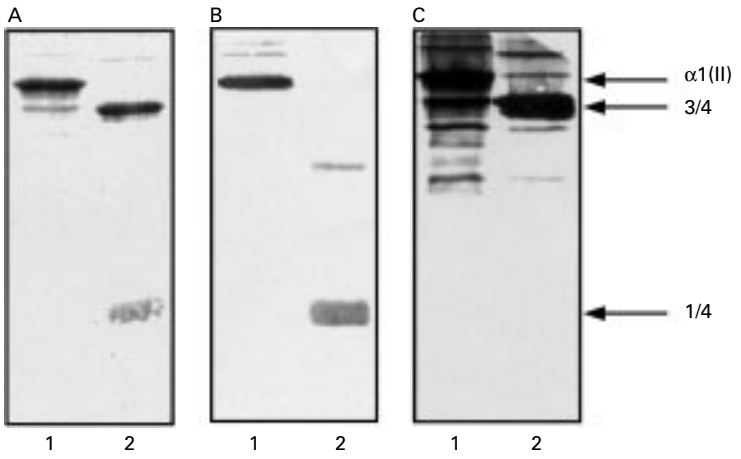

Figure 4 Detection of specific gelatinolytic fragments of type II collagen. Bovine type II collagen was cleaved by recombinant human matrix metalloproteinase 13 (MMP-13), the fragments separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (10% gels) and transferred on to a nitrocellulose membrane, as described in the methods section. (A) Amido black staining of all the transferred proteins; (B) immunoblotted with AH9L2; (C) immunoblotted with antibody AH12L3. Lane 1, uncleaved type II collagen; lane 2, type II collagen cleaved by MMP-13. The migration positions of the intact α1(II) chain as well as the 1/4 and 3/4 fragments are indicated.

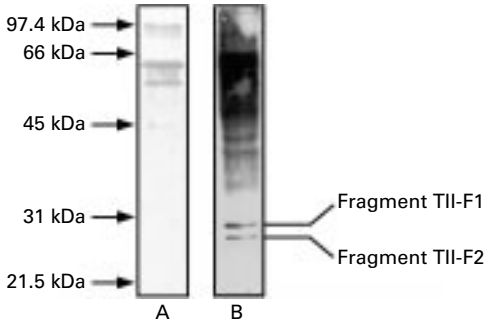

Figure 5 Detection of type II collagen fragments in culture medium conditioned by degrading cartilage. Bovine nasal cartilage was cultured with recombinant human interleukin 1α (IL-1α) for three weeks and the culture medium was changed every seven days. Third week medium was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (10% gels) and transferred on to a nitrocellulose membrane, as described in the methods section. (A) Immunoblotted with AH9L2; (B) immunoblotted with AH12L3. Migration positions of standard molecular mass markers are indicated by arrows. The type II collagen fragments consistently detected in four separate experiments are also shown.

was released into the culture medium at that time point (not shown). The media were each immunoblotted using AH9L2 and AH12L3. The results shown in fig 5 are from one representative experiment. Antibody AH12L3 detected a series of bands in the week three medium (fig 5B). However, only two of them were distinct in all four experiments. These two bands, labelled as fragments TII-F1 and TII-F2, had an apparent molecular mass of approximately 30 kDa. Their detection was inhibited by preincubation of the antibody with 100 μg/ml of AH12 (not shown), indicating that the antibody was binding through its epitope in the N-terminal region of the α1(II) chain. To confirm these observations, week three medium was separated electrophoretically and transferred to a PVDF membrane, as described in the methods section. The two bands corresponding to fragments TII-F1 and TII-F2 were excised and their N-terminal

Table 1 N-terminal sequencing of fragment TII-F1

Cycle	*PTH amino acid*	*Yield (pmol)*	*Human α1(II) sequence (residues 42–56)*
1	F	17.2	F
2	Q	13.2	Q
3	G	11.0	G
4	N	10.0	N
5	P	7.0	P
6	G	8.5	G
7	E	6.2	E
8	P(OH)	ND	P(OH)
9	G	7.1	G
10	E	4.7	E
11	P(OH)	ND	P(OH)
12	G	6.0	G
13	V	6.0	V
14	S	3.0	S
15	G	4.9	G

The band corresponding to fragment TII-F1 (fig 5) in week 3 medium from interleukin 1α stimulated cultures of bovine nasal cartilage explants was excised from a PVDF membrane and its N-terminal sequence determined, as described in the methods section.
ND, not determined; PTH, phenylthiohydantoin.

sequences determined. The results (table 1) show that the first 15 residues of fragment TII-F1 correspond exactly to the human type II collagen sequence, starting eight residues from the N-terminus of AH12. We were unable to obtain a sequence from band TII-F2.

In contrast, no specific bands were detected with antibody AH9L2 (fig 5A), indicating that the C-terminal region of the collagen molecule had been degraded more extensively or the epitope itself had been cleaved by a chondrocyte derived proteinase. The diffuse, high molecular mass bands weakly detected using antibody AH9L2 were still apparent when the antibody had been preincubated with 100 μg/ml of AH9 (not shown), indicating that they were non-specific.

DETECTION OF TYPE II COLLAGEN FRAGMENTS IN HUMAN ARTICULAR CARTILAGE BY IMMUNOHISTOCHEMISTRY

Having demonstrated in an in vitro model of cartilage degradation that the N-terminus of the type II collagen α-chain persists longer than the C-terminus, it was important to determine whether this apparent differential suseptibility to degradation might be important in human disease. We have shown previously that peptide epitopes in denatured type II collagen can be detected by immunohistochemical staining of human articular cartilage, presumably because fragments of the denatured α1(II) chains are retained in the extracellular matrix by crosslinks. We used the same approach to determine whether epitopes AH9 and AH12 could be detected in degrading cartilage. Antibody dilutions were optimised as described in the methods section. AH12L3 was used at a dilution of 1/2000 and AH9L2 at 1/800.

Samples from 16 different patients with OA and eight non-arthritic patients were analysed. Table 2 shows the results. The numerical scores for AH12L3 staining were significantly higher than those for AH9L2 staining, both in OA and non-arthritic cartilage, with the overall scores for OA cartilage being higher than those for non-arthritic cartilage. Figure 6 shows the immunohistochemical localisation of dena-

Table 2 Immunohistochemical scores for human cartilage sections stained with N-terminal and C-terminal type II collagen denaturation antibodies

OA cartilage			*Non-arthritic cartilage*		
Sample	*AH12L3*	*AH9L2*	*Sample*	*AH12L3*	*AH9L2*
1	3	1	1	2	0
2	4	2	2	0	0
3	6	3	3	1	0
4	3	1	4	2	0
5	9	4	5	0	0
6	9	4	6	0	0
7	7	1	7	1	0
8	9	4	8	2	0
9	9	2	Median	1*	0
10	9	2			
11	4	1			
12	9	2			
13	9	4			
14	6	2			
15	9	4			
16	9	4			
Median	9**	2			

Cartilage sections were immunostained for N-terminal and C-terminal epitopes (AH12L3 and AH9L2, respectively). Staining intensities of the superficial layer, mid, and deep zones of extracellular matrix were scored as described in the methods section. The sum of these three scores is shown for each specimen. Statistical comparisons were performed using the Mann-Whitney U test.
*$p < 0.02$; **$p < 0.0001$ (*v* AH9L2).

tured type II collagen epitopes in a typical example of OA femoral head cartilage from one of the patients. The results with antibody AH12L3 were essentially as described previously for antibody COL2-3/4m.[6] Moderate interterritorial staining was seen throughout the depth of the OA cartilage, with a thin band of intense staining at the articular surface (fig 6A). Although there was some non-specific staining of the articular surface in the control (fig 6E), this was less than that observed with specific antibody. Deep zone chondrocytes showed pronounced pericellular staining with AH12L3 (fig 6C). Sections were also stained with antibody COL2-3/4m. The pattern of staining with this antibody was similar to that seen with AH12L3, although the intensity of staining was greater with COL2-3/4m (not shown). This was consistent with results described previously for COL2-3/4m.[6] In contrast, staining with antibody AH9L2 was generally weaker in all zones (fig 6B and D), and this difference between the antibodies was significant (table 2). The intensity of staining with AH9L2 was not much greater than with the non-immune control serum (fig 6E). We cannot rule out the possibility that epitope AH9 is exposed by denaturation and then masked by another cartilage matrix molecule. However, a more likely explanation is that after cleavage of the type II collagen triple helix by a collagenase in OA cartilage, the C-terminal epitope, AH9, is degraded and cleared from the extracellular matrix more rapidly than the N-terminal epitope, AH12.

In general, staining of the non-arthritic cartilage tissue was far less intense for specimens from each of the seven patients examined. Figure 7 shows a typical example of non-arthritic femoral head cartilage from one patient. The interterritorial staining seen with antibody AH12L3 was of a low intensity, apart from a thin band of staining at the articular surface (fig 7A). The extent and intensity of staining around chondrocytes was also much reduced compared with OA cartilage. This pattern is similar to that described for immunostaining of non-arthritic femoral condylar cartilage from older individuals, using antibody COL2-3/4m.[6] Again, there was virtually no staining with antibody AH9L2 (fig 7B), in most cases it was at the same level as the non-immune control (fig 7C), providing further evidence that the C-terminal region of the α1(II) chain is degraded more rapidly than the N-terminal region. This observation was confirmed by the immunostain scoring data, which showed significantly greater staining with AH12L3 than with AH9L2. Specific controls, using AH12L3 and AH9L2 preabsorbed with their respective peptides, were also performed on some OA and non-arthritic sections. The intensity of staining with these controls was found to be similar to that of the non-immune controls shown in fig 7. Conversely, when preabsorbed with the non-immunising peptides, staining with each antibody was not reduced, indicating that AH12L3 and AH9L2 do indeed bind specifically to their respective epitopes (not shown).

To ensure that the increased staining seen with AH12L3 in OA cartilage was not the result of unmasking of the AH12 epitope, as a consequence of proteoglycan loss during the disease process, some non-arthritic cartilage sections were treated with hyaluronidase in-

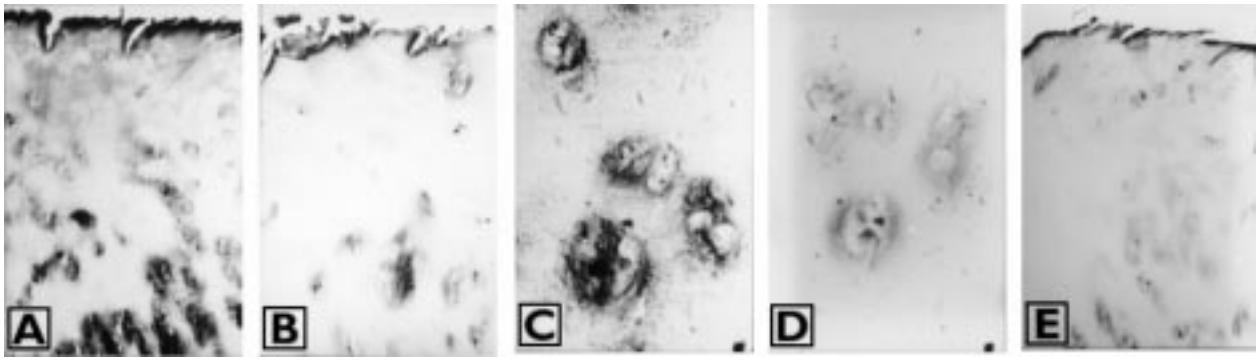

Figure 6 Detection of denatured type II collagen epitopes in human osteoarthritic cartilage. Femoral head cartilage from a patient with osteoarthritis was obtained at arthroplasty. Frozen sections were prepared and immunostained as described in the methods section. (A) And (C) immunostained with antibody AH12L3; (B) and (D) immunostained with antibody AH9L2; (E) background staining with IgG from non-immune rabbit serum. The exposure time was identical for each photograph. Original magnifications were ×35 (A, B, and E) or ×140 (C and D). In (A), (B), and (E), the articular surface is located towards the top of each photograph.

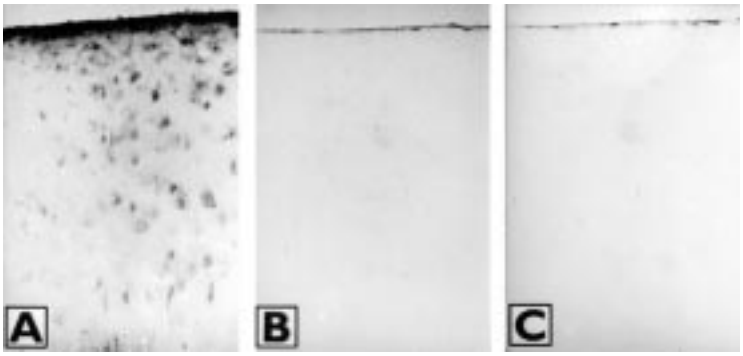

Figure 7 Detection of denatured type II collagen epitopes in non-arthritic human cartilage. Femoral head cartilage from a patient with a femoral neck fracture was obtained at arthroplasty. Frozen sections were prepared and immunostained as described in the methods section. (A) Immunostained with antibody AH12L3; (B) immunostained with antibody AH9L2; (C) background staining with IgG from non-immune rabbit serum. The exposure time was identical for each photograph. Original magnification, ×35. The articular surface is shown towards the top of each photograph.

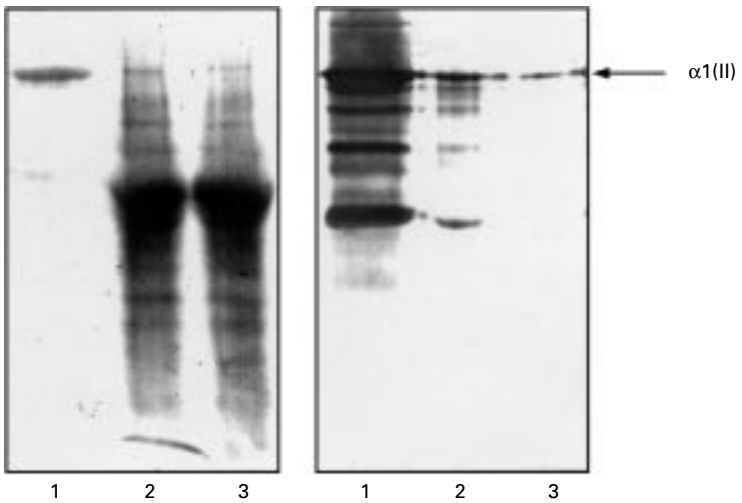

Figure 8 Detection of epitopes in human cartilage extract. Human cartilage was extracted with 4 M guanidinium HCl and immunoblotted with antibody AH12L3 after separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (10% gels). (A) Amido black staining of all the transferred proteins; (B) immunostaining with antibody AH12L3. Lane 1, standard type II collagen; lane 2, cartilage extract spiked with standard type II collagen; lane 3, cartilage extract only. The migration position of the intact α1(II) chain is indicated.

stead of chondroitinase before incubation with AH12L3, to remove the proteoglycan molecules in their entirety. No difference in staining pattern or intensity was seen between hyaluronidase and chondroitinase treated sections, indicating that the AH12 epitope is available to AH12L3, despite the presence of large proteoglycans (not shown). As an additional control, we determined whether the more extensive staining of cartilage sections with AH12L3 compared with AH9L2 might be the result of binding of the AH12L3 antibody to a cartilage component other than type II collagen. Non-covalently bound cartilage matrix proteins were extracted from human cartilage in 4 M guanadinium chloride, dialysed into 50 mM Tris/HCl, separated by 10% SDS-PAGE, and immunoblotted with antibody AH12L3. The same extracts were also spiked with standard bovine type II collagen for comparison. The results indicate that the cartilage extract contains some type II collagen, but no other bands were detected with the antibody (fig 8).

Discussion

Type II collagen, like other fibrillar collagen molecules, consists of a 1000 residue helical region flanked by short telopeptides.[29 30] Native collagen is considered to be very resistant to proteolysis, except by specific collagenases.[11 12 16] Conversely, denatured collagen (gelatin) is thought to be highly susceptible to proteolytic attack by specific gelatinases as well as many other enzymes.[16] Our original hypothesis was that the terminal regions of type II collagen might be particularly resistant to proteolysis after denaturation. Our findings have confirmed this hypothesis for the N-terminal helix, but not for the C-terminus.

We and others have shown previously that the degradation of collagen in human arthritic cartilage or in cultured cartilage explants is the result of initial cleavage by a collagenase, followed by gelatinolytic processing of the cleaved, denatured α-chains.[3 5 21 22] OA articular cartilage obtained from patients with end stage disease at the time of joint replacement was shown both by immunohistochemistry and immunoassay to contain denatured type II collagen.[5 6 13 15] The use of cleavage site neoepitope antibodies has shown that at least some of the denaturation of cartilage collagen must be a result of cleavage of the triple helix at the Gly^{975}–Leu^{976} bond by a collagenase.[21 22] Our immunohistochemical findings show that the denatured type II collagen in OA cartilage contains an N-terminal epitope (AH12), but very little C-terminal epitope (AH9). This suggests that the C-terminal region of the type II collagen α-chain is cleared from OA cartilage relatively quickly after collagenolysis and denaturation. The pattern of staining for AH12 was very similar to the immunostaining that we described previously for epitope CB11B, detected by monoclonal antibody COL2-3/4m.[5] CB11B is located 357 residues C-terminal to AH12. However, both epitopes are within the 3/4 fragment generated by collagenolytic cleavage. We have also demonstrated previously that type II collagen collagenase cleavage site neoepitopes can be detected in OA cartilage by immunohistochemistry.[22] The intensity and distribution of immunostaining for these neoepitopes was generally less than for AH12, but greater than for epitope AH9 (M Vankemmelbeke *et al*, 1997, unpublished observations). Epitope AH9 is located 209 residues C-terminal to the collagenase cleavage site. It might be that the C-terminal end of the collagenase generated 1/4 fragment is particularly susceptible to proteolysis.

Our conclusion that the C-terminal region of type II collagen is relatively unstable in a catabolic environment is supported by the observation that epitope AH9 cannot be detected in culture medium conditioned by degrading cartilage, whereas the N-terminal epitope AH12 can be detected, as confirmed by N-terminal sequencing of one of the collagen fragments.

Our results suggest that the helical region of type II collagen should not be considered as uniform in terms of its susceptibility to proteolysis in degrading cartilage. The Gly–Leu bond that is cleaved by collagenases,

located 239 amino acid residues away from the C-terminal telopeptide, is in a region of the triple helix that is rich in imino acids and therefore relatively lax, as has been shown for collagen types I and III.[31–33] The 1/4 fragment generated by cleavage of this bond is slightly less thermally stable than the 3/4 fragment, rendering it more susceptible to gelatinolytic activity.[34 35] Conversely, there is some evidence to suggest that the region of the α-chain of fibrillar collagens where the N-telopeptide is linked to the triple helix is relatively rigid, as a result of a network of interchain hydrogen bonds.[36] A 5' splice mutation in the COL1A1 gene has been identified in a patient with type IV osteogenesis imperfecta.[37] The mutation resulted in deletion of a short peptide from a region close to the N-terminus of the type I collagen triple helix, and this abnormal collagen was found to have a reduced melting temperature, indicative of its relative instability. Taken together, the available data suggest that the N-terminal region of the triple helix, close to its telopeptide, might be more resistant to proteolysis than the C-terminal region.

We have shown recently that cathepsin K can cleave native type II collagen close to its N-terminus.[25] The cleavage site is C-terminal to epitope AH12. Therefore, we cannot rule out the possibility that an accumulation of this epitope in human OA cartilage is a result of cathepsin K cleavage.

In our study, we chose to focus on epitopes close to the ends of the α-chains. Previous studies have used antibodies directed to epitopes located centrally within the 3/4 fragment of collagenase cleaved collagen or to the collagenase cleavage site itself.[5 21 22] We cannot rule out the possibility that other regions of type II collagen might be even more stable than the N-terminus, and a detailed comparison of a range of epitopes is warranted, because this might further our understanding of how cartilage collagen is degraded in arthritis. Such studies might also aid in the selection of epitopes for use as serum markers of cartilage collagen degradation.

We thank Dr P Mitchell (Pfizer Central Research, Groton, Connecticut, USA) for providing us with recombinant human MMP-13, Mr B Dunbar (protein sequencing unit, University of Aberdeen, Scotland) for protein sequencing, Dr A Moir (Kreb's Institute, University of Sheffield, UK) for peptide synthesis, and Dr S Smith and Mr A Bassett (Sheffield Hybridoma, University of Sheffield, UK) for help with the purification of IgG from antisera. This project was supported in part by a grant (to APH) from the Nuffield Foundation Oliver Bird Fund.

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