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Research Article

A new immunoassay was developed to detect denaturation of type II collagen in osteoarthritis (OA). A peptide, alpha 1 (II)-CB11B, located in the CB11 peptide of type II collagen, was synthesized and used to produce a monoclonal antibody (COL2-3/4m) of the IgG1 (kappa) isotype. This reacts with a defined epitope in denatured but not native type II collagen and the alpha 3 chain of type XI collagen. The latter is present in very small amounts (about 1% wt/wt) in cartilage relative to the alpha 1 (II) chain. By using an enzyme-linked immunosorbent assay, type II collagen denaturation and total type II collagen content were determined. The epitope recognized by the antibody was resistant to cleavage by alpha-chymotrypsin and proteinase K which were used to extract alpha 1 (II)-CB11B from the denatured (alpha-chymotrypsin soluble) and residual native (proteinase K soluble) collagen alpha-chains, respectively, present in human femoral articular cartilage. Type II collagen content was significantly reduced from a mean (range) of 14% (9.2-20.8%) of wet weight in 8 normal cartilages to 10.3% (7.4-15.0%) in 16 OA cartilages. This decrease, which may result in part from an increased hydration, was accompanied by an increase in the percent denaturation of type II collagen in OA to 6.0% of total type II collagen compared with 1.1% in normal tissue. The percent denaturation was ordinarily [...]

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Increased Damage to Type II Collagen in Osteoarthritic Articular Cartilage Detected by a New Immunoassay

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Abstract

A new immunoassay was developed to detect denaturation of type II collagen in osteoarthritis (OA). A peptide, $\alpha 1(\text{II})$ -CB11B, located in the CB11 peptide of type II collagen, was synthesized and used to produce a monoclonal antibody (COL2-3/4m) of the IgG₁(κ) isotype. This reacts with a defined epitope in denatured but not native type II collagen and the $\alpha 3$ chain of type XI collagen. The latter is present in very small amounts (about 1% wt/wt) in cartilage relative to the $\alpha 1(\text{II})$ chain. By using an enzyme-linked immunosorbent assay, type II collagen denaturation and total type II collagen content were determined. The epitope recognized by the antibody was resistant to cleavage by α -chymotrypsin and proteinase K which were used to extract $\alpha 1(\text{II})$ -CB11B from the denatured (α -chymotrypsin soluble) and residual native (proteinase K soluble) collagen α -chains, respectively, present in human femoral articular cartilage. Type II collagen content was significantly reduced from a mean (range) of 14% (9.2–20.8%) of wet weight in 8 normal cartilages to 10.3% (7.4–15.0%) in 16 OA cartilages. This decrease, which may result in part from an increased hydration, was accompanied by an increase in the percent denaturation of type II collagen in OA to 6.0% of total type II collagen compared with 1.1% in normal tissue. The percent denaturation was ordinarily greater in the more superficial zone than in the deep zone of OA cartilage. (*J. Clin. Invest.* 1994; 93:1722–1732.) Key words: arthritis • cartilage • collagen II • collagenase • degradation

Introduction

Type II collagen and aggrecan (a large, aggregating proteoglycan) are the two major components of the extracellular matrix of cartilage. The collagen, which is present in a fibrillar form, provides tensile strength whereas the aggrecan is responsible for

compressive stiffness of cartilage (1–3). Degradation and loss from the matrix of either of these molecules could contribute significantly to destruction of articular cartilage and loss of its function in patients with arthritic diseases. The proteolytic degradation of proteoglycan has been widely studied (3–6). In contrast, there have been very few studies of type II collagen degradation, owing to a lack of adequate methodology with which to detect damage to specific collagen molecules and their release. Electron microscopic studies have revealed damage to collagen fibrils in articular cartilage in RA (7). Damage to collagen at the junction of cartilage with pannus (8) and at the articular surface (9) has also been noted in RA. In osteoarthritis (OA),¹ Venn and Maroudas (10) used estimations of hydroxyproline to demonstrate a decrease in the collagen content as a percentage of wet weight of femoral head cartilage compared with normal (postmortem) cartilage. Such analyses cannot distinguish different types of collagen in the cartilage.

Specific studies of damage to type II collagen resulting in denaturation (unwinding) of the triple helix were first made by Dodge and Poole (11) and Dodge et al. (12), who showed that a polyclonal rabbit antiserum raised against cyanogen bromide peptides of type II collagen will react specifically with denatured, but not native (triple helical), type II collagen α chains or fragments thereof containing the recognized epitopes. The antiserum was used to demonstrate, by immunolocalization and immunochemical analysis of cartilage extracts, type II collagen cleavage in situ in OA and RA and type II collagen denaturation induced when cartilage is cultured with interleukin-1. These studies clearly demonstrated that arthritic cartilages exhibit increased staining for type II collagen denaturation. Moreover, whereas in aging and OA damage was first observed at and close to the articular surface, in RA early damage was usually most pronounced around chondrocytes in the deep zone, adjacent to subchondral bone (11, 12). This revealed that there are differences in the sites of cleavage of type II collagen that may be due to differences in the pathogenesis of these diseases (see also reference 3 for a discussion). The fact that early damage to type II collagen is predominantly pericellular/territorial suggests that in the majority of cases collagen damage was mediated by the chondrocyte. Subsequently we showed, with a specific, nontoxic hydroxamate inhibitor, that cleavage of type II collagen in human articular cartilage is mediated by metalloproteinases released from chondrocytes (13). Other immunochemical studies with the antiserum revealed that the metalloproteinase collagenase may in part be responsi-

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1. Abbreviations used in this paper: HDC, heat-denatured type II collagen; OA, osteoarthritis; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

ble for this denaturation. This is the only mammalian proteinase that can cleave the triple helix of this molecule (4).

At that time it was not possible to quantitate collagen denaturation by immunoassay. Moreover, because we showed that denatured collagen is retained in the fibril and not released (11, 12), it was necessary to develop a means of selectively extracting denatured type II collagen so that it could be measured. In order to assay collagen fragments and conduct other specific immunological studies, it is preferable to determine the content of just one epitope, of known location within the collagen molecule, by using a single monoclonal antibody in an immunoassay. Under these circumstances any one fragment will react either fully or not at all in the assay and so the quantity of denatured collagen and identity of such fragments containing the defined epitope can be more accurately determined. Furthermore, a protease with gelatinase-like activity that cleaves neither the chosen epitope nor native (helical) collagen could theoretically be used to extract those denatured collagen α -chains that are retained in the fibril.

To study damage to type II collagen in articular cartilage in arthritis, we developed an immunoassay based on our earlier immunochemical work (11, 12). Here we describe and use this assay to provide the first quantitative evidence for increased damage to type II collagen in situ and decreased content of total type II collagen in OA cartilage.

Methods

Tissue. Human articular cartilages were removed from the anterior (loaded) region of the femoral condyles of adult knee joints. OA cartilages were obtained at surgery from patients undergoing total joint arthroplasty. Site-matched nonarthritic articular cartilages of normal appearance were obtained at autopsy within 15 h postmortem from individuals with no known history or signs of arthritic/joint abnormalities. Patient details and Mankin grades of the normal and OA cartilage specimens are shown in Table I. Previously, cartilage from these same sites has been analyzed for the proteoglycan aggrecan (6).

Preparation of cartilage plugs. In that cutting cartilage can produce denaturation of type II collagen, care was taken to prepare defined plugs of cartilage using a specially made, standardized, stainless steel punch. This avoided variable results and the increased denaturation obtained by dicing the cartilage with a scalpel or by preparing frozen sections (data not shown). Wherever possible, a full depth slice of cartilage was taken from each femoral condyle (medial and lateral) and two full-depth cylindrical plugs, each ~ 4 mm in diameter by 2 mm deep, were prepared from each slice using the steel punch. One of the plugs was used for the histological assessment of Mankin grade, as previously described (6, 14). This did not include analysis of the calcified cartilage, hence the maximum grade in the group of OA cartilages was only 13. The other plug was used for the extraction and assay of type II collagen. In some experiments the full-depth plugs were approximately divided into an upper and a lower half by using a scalpel. The

upper 1 mm included the articular surface and upper-mid zone; the lower 1 mm consisted of lower-mid and deep zone cartilage. It was essential to standardize the tissue sample preparation in this way because cutting the cartilage causes denaturation of type II collagen.

Identification and synthesis of a type II collagen B-cell epitope. Sites containing putative B cell epitopes within the type II collagen α chains were predicted by the identification of hydrophilic domains in the amino acid sequence, according to the method of Hopp and Woods (15). Only the cyanogen bromide peptide 11 of the $\alpha 1$ (II) chain was analyzed for hydrophobicity/hydrophilicity, because this 279-amino acid peptide resides within the triple helix and has already been shown to be immunogenic in rabbits (11). Additional criteria for selection of a suitable peptide sequence were as follows: (a) The epitope should not include any hydroxylysine residues (these could be involved in cross-link formation and may be substituted with carbohydrate chains that could block antibody-binding). (b) The epitope should be well conserved between species (to permit species cross-reactivity). (c) The epitope should have minimal homology with sequences from other proteins and collagen α chains. (d) The epitope should be resistant to hydrolysis by proteinase K and α -chymotrypsin, which were used to extract from cartilage native and denatured collagen respectively.

For the purposes of analysis, it was assumed that all proline and lysine residues in the "Y" position of the Gly-X-Y repeat sequence were hydroxylated. However, the hydroxyl group did not appear to contribute significantly to the hydrophobicity of cyanogen bromide peptide 11, in that the hydrophobicity/hydrophilicity profile was almost identical when hydroxyproline and hydroxylysine hydrophathy values were replaced with those of proline and lysine respectively.

Peptides up to 23 amino acids long containing a centrally located hydrophilic domain, with an amino-terminal cysteine for conjugation to ovalbumin and a carboxy terminal tyrosine (for iodination if required), were synthesized at a 0.25-mmol scale, using standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry, on an model 431A solid-phase peptide synthesizer (Applied Biosystems, Inc., Foster City, CA). The crude peptides were purified by reverse-phase chromatography (Prep-10 Aquapore C8 column, Applied Biosystems Inc.) using an acetonitrile gradient in 0.1% trifluoroacetic acid.

Preparation of a monoclonal antibody. The chosen peptide, $\alpha 1$ (II)-CB11B, was conjugated to ovalbumin through its amino-terminal cysteine, using the coupling reagent bromoacetic acid-*N*-hydroxysuccinimide ester (Sigma Chemical Co., St. Louis, MO), as previously described (16). BALB/c mice were immunized five times at 2-wk intervals by i.p. injection with 100 μ g of the conjugated peptide emulsified with complete (first immunization) or incomplete (subsequent immunizations) Freund's adjuvant. Serum samples from the mice were then tested in an ELISA (see below) for reactivity with both peptide and heat-denatured type II collagen (HDC). Splenocytes from the mouse showing the best serum antibody response were fused to SP2/0 myeloma cells and monoclonal antibodies were prepared using conventional hybridoma technology (17, 18). Cloned hybridoma cells that produced antibodies reacting with both HDC and the immunogenic peptide were expanded in vivo as an ascites (18). The antibody isotype was determined using a commercial isotype screening kit (Southern Biotechnology Inc., Birmingham, AL).

Purification of collagens and preparation for analysis. Bovine and human type II collagen and bovine types I and III collagens were prepared by differential salt precipitation, as previously described (11, 19). Culture medium from bovine growth plate hypertrophic chondrocytes, enriched for type X collagen by differential salt precipitation, was kindly donated by Dr. M. Alini (Joint Diseases Laboratory, Shriners Hospital, Montreal). Bovine type XI collagen was a generous gift from Dr. J. J. Wu and Dr. D. Eyre, Department of Orthopedics, University of Washington, Seattle. HDC was prepared by heating a 1-mg/ml solution of type II collagen in PBS for 20 min at 80°C. Cyanogen bromide peptides of bovine type II collagen were prepared as described previously (11).

Hydroxyproline assay. Some samples were assayed for hydroxyproline using a colorimetric method (20).

Table I. Patient Details

Disease group	n	Mean age yr	Age range yr	Male/female %	Median Mankin grade	Range of Mankin grades
Normal	8	56	28–81	50:50	2	0–6
OA	16	69	57–84	57:43	5	2–10

Detection of mouse antibodies to synthetic $\alpha 1(II)$ -CB11B peptide and HDC. The wells of Immulon-2 flat bottomed ELISA plates (Dynatech Laboratories, Inc., Chantilly, VA) were each coated with 2 μ g of peptide or HDC, in 50 μ l of 0.1 M carbonate buffer, pH 9.2, by passive adsorption for 24–48 h at 4°C. Nonspecific binding wells were incubated with 50 μ l of buffer. The plates were washed three times with PBS containing 0.1% vol/vol Tween-20. Uncoated binding sites were blocked by incubation with 50 μ l/well of 1% wt/vol BSA in PBS (PBS-BSA) for 30 min at room temperature. The plates were washed once with PBS-Tween and 50 μ l of diluted mouse serum or hybridoma culture supernatant was added to individual wells. After incubation for 90 min at 37°C the plates were washed three times with PBS-Tween and then alkaline phosphatase-conjugated goat anti-mouse IgG, IgM, and IgA (Zymed Laboratories, San Francisco, CA) diluted 1:500 in PBS-BSA-Tween was added at 50 μ l/well. The plates were incubated for 90 min at 37°C and then washed three times with PBS-Tween and once with distilled water. Alkaline phosphatase substrate, disodium *p*-nitrophenyl phosphate (Sigma Chemical Co.), was prepared fresh at 0.5 mg/ml in 8.9 mM diethanolamine, 0.25 mM MgCl₂, pH 9.8, and 50 μ l added to each well for 20–30 min at 37°C. The absorbance was measured at 405 nm on a Multiskan plus MKII plate reader (ICN/Flow, Mississauga, ON).

Inhibition ELISA for denatured type II collagen. Linbro 96-well round-bottom microtiter plates (ICN/Flow) were used for preincubations. They were precoated with 100 μ l/well of PBS-BSA for 30 min at room temperature and washed once with PBS-Tween. The outermost wells were not used, to minimize the effects of evaporation. There were six nonspecific binding wells on each plate, which contained 100 μ l each of 50 mM Tris-HCl, pH 7.6 (Tris). 50 μ l/well of monoclonal ascitic fluid, diluted appropriately from (1 in 5,000 to 1 in 10,000) with Tris to provide a detectable but inhibitable level of binding, was added to each of 54 wells of the preincubation plates. The antibody in 6 of the 54 wells on each plate was mixed with 50 μ l/well of Tris, to indicate maximum binding in the absence of inhibitory epitope. The antibody in all 48 test wells was mixed with 50 μ l/well of standard $\alpha 1(II)$ -CB11B peptide, HDC, or cartilage extracts containing denatured collagen, diluted appropriately with Tris. All standards and samples were tested in duplicate wells. The plates were sealed with parafilm and incubated overnight at 37°C in a humidified incubator. A multichannel pipette was used to transfer 50 μ l of each preincubated sample to the equivalent well of an Immulon-2 ELISA plate, coated with 2 μ g/well HDC and blocked with PBS-BSA as described above. It was essential to transfer all the samples from any one plate within a short space of time (~45 s) in order to maximize accuracy. The ELISA plates were each incubated for exactly 30 min at room temperature and then washed three times with PBS-Tween. Second antibody and alkaline phosphatase substrate were prepared and added as described above, except that the plates were incubated with the second antibody for 2 h. The mean absorbance from the six nonspecific binding wells was subtracted from the absorbance value of all other wells on the same plate. The percentage inhibition of binding by samples or standards was calculated relative to the mean absorbance from the six maximum binding wells on the same plate, which represented 0% inhibition (100% binding).

Proteolytic cleavage of native type II collagen by recombinant interstitial collagenase. Bovine native type II collagen was initially dissolved in 0.5 M acetic acid and then diluted to a final concentration of 0.5 mg/ml in 0.1 M Tris-HCl, pH 7.6, containing 10 mM CaCl₂ and recombinant human interstitial collagenase (MMP-1; kindly donated by Dr. Michael Lark, Merck, Sharpe & Dohme, Rahway, NJ) which had been activated by incubation with 0.25 mM aminophenyl mercuriacetate in the same buffer for 10 min at 37°C. The final molar ratio of human collagenase to collagen was 1:5. The control contained type II collagen in buffer with no collagenase. The samples were incubated for 20 h at 30°C then the collagenase was inhibited by the addition of 10 mM EDTA (final concentration). The samples were tested for immunoreactivity by immunoblotting and immunoassay.

Proteolytic cleavage of HDC and $\alpha 1(II)$ -CB11B peptide by clostridial collagenase. HDC and $\alpha 1(II)$ -CB11B were dissolved in Tris con-

taining 5 mM CaCl₂ and 14 U/ml chromatographically purified collagenase form III (bacterial collagenase) from *Clostridium histolyticum* (Advance Biofactures Corp., Lynbrook, NY). Controls contained HDC or $\alpha 1(II)$ -CB11B in Tris with 5 mM CaCl₂ but no collagenase, or collagenase in Tris with 5 mM CaCl₂ but no HDC, nor $\alpha 1(II)$ -CB11B peptide. All the tubes were incubated overnight at 37°C and the collagenase was then inhibited by the addition of EDTA to a final concentration of 0.1 M. The samples were each tested for reactivity in the inhibition ELISA for denatured type II collagen.

Proteolytic cleavages of HDC by α -chymotrypsin and proteinase K. Aliquots of HDC at concentrations of 0.25 mg/ml or less were prepared in Tris containing 0.5 mg/ml of type VII α -chymotrypsin or proteinase K (both from Sigma Chemical Co.). Control tubes contained only HDC, or 0.5 mg/ml α -chymotrypsin or 0.5 mg/ml proteinase K. The samples were all incubated overnight at 37°C (α -chymotrypsin) or 56°C (proteinase K). The α -chymotrypsin was inactivated by addition of 115 μ g/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma Chemical Co.) in Tris, predissolved in ethanol (4% vol/vol, final concentration). Proteinase K was inactivated by boiling for 10 min. Degradation of the HDC by each proteinase was confirmed by SDS-PAGE (see below). The degraded collagens and control samples were each tested for reactivity in the inhibition ELISA for denatured type II collagen.

Extraction and assay of native and denatured collagen from cartilage plugs. Because α -chymotrypsin, like all mammalian proteinases apart from interstitial collagenase, cannot cleave intact, triple helical type II collagen, this enzyme can be used to selectively degrade any collagen in cartilage which has already been denatured in situ. Conversely, cartilage can be fully solubilized by incubation with proteinase K at 56°C (its optimal temperature). Complete denaturation of the extracted collagen can then be ensured by heating. Therefore cartilage plugs were routinely extracted, first with α -chymotrypsin and then proteinase K. The plugs were distributed individually into Eppendorf tubes. To each tube was added 500 μ l of 1 mg/ml α -chymotrypsin in Tris containing 1 mM iodoacetamide, 1 mM EDTA, and 10 μ g/ml pepstatin-A (all from Sigma Chemical Co.). The samples were incubated overnight at 37°C. The α -chymotrypsin was inhibited by addition of 200 μ l per tube of 0.4 mg/ml TPCK in Tris containing 4% vol/vol ethanol. The α -chymotrypsin extract was separated from the residue. Each residue was then digested with 500 μ l of 1 mg/ml proteinase K in Tris containing the same protease inhibitors as described above. After digestion overnight at 56°C, no residue remained. The proteinase K was inactivated and the extracted collagen fully denatured by boiling the samples for 10 min. In order to measure any native collagen extracted by α -chymotrypsin, a 300 μ l aliquot of each α -chymotrypsin extract was mixed with 100 μ l of the 1 mg/ml proteinase K solution and incubated and then boiled as described for cartilage residues. The TPCK-inhibited α -chymotrypsin and boiled proteinase K had no inhibitory effect themselves in the inhibition ELISA.

Digestion of extracted collagen peptides with clostridial collagenase to confirm susceptibility of the extracted CB11B epitope to cleavage by this enzyme. Collagens were extracted from plugs of OA cartilage with α -chymotrypsin and proteinase K and the proteinases inhibited as described above. The extracts were each divided into two aliquots. To one of these was added 5 mM CaCl₂ and 14 U/ml clostridial collagenase and to the other, 5 mM CaCl₂ only. A control tube contained collagenase in Tris with 5 mM CaCl₂ but no cartilage extract. All tubes were incubated overnight at 37°C and the collagenase activity was inhibited with EDTA as described above. The samples were all tested for reactivity in the inhibition ELISA for denatured type II collagen.

Extraction of cartilage plugs with guanidinium chloride to determine the content of non-cross-linked collagen. In one experiment, two adjacent full-depth plugs were taken from each of five OA cartilages. One of the plugs from each specimen was extracted with α -chymotrypsin as described above. The other plug from each specimen was extracted for 72 h at 4°C with gentle rocking in 4 mM guanidinium chloride, 0.1 M Tris-HCl, pH 7.3 containing 1 mM iodoacetamide, 1 mM EDTA, and 10 μ g/ml pepstatin-A. The guanidinium chloride ex-

tracts were dialyzed exhaustively against Tris using a membrane with a molecular weight cutoff of 3,500. A 300- μ l aliquot of each of the α -chymotrypsin and guanidinium chloride extract was mixed with 100 μ l of 1 mg/ml proteinase K, incubated overnight at 56°C, and then boiled to inhibit the proteinase. All the extracts were tested for reactivity in the inhibition ELISA for denatured type II collagen.

Electrophoresis and immunoblotting. SDS-PAGE of purified collagens was performed using 7.5% and 10%, 1-mm-thick, 7-cm \times 8-cm mini-Protean gels, stained with either Coomassie Brilliant Blue or silver stain (Bio-Rad Laboratories, Mississauga, ON), as described by Dodge and Poole (11). The electrophoresed samples were transferred to a nitrocellulose membrane which was then blocked overnight at 4°C with PBS containing 3% wt/vol BSA (PBS-3% BSA) (11). The membrane was incubated for 1 h at room temperature with the monoclonal antibody or control mouse ascitic fluid diluted in PBS-3% BSA. After three washes with PBS-Tween, the membrane was incubated at 30 min at room temperature with the alkaline phosphatase conjugated goat anti-mouse second-step antibody described above, diluted 1 in 100 with PBS-3% BSA. The membrane was washed well with PBS-Tween and once with distilled water. Alkaline phosphatase substrate solution was prepared from a commercial kit (Bio-Rad) employing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. It was added and incubated with the membrane at room temperature until optimal color had developed. Further reaction was stopped by washing off the substrate solution with distilled water.

Determination of type II collagen content based on epitope analysis. The molecular weights of the human type II collagen α 1-chain and of peptide α 1(II)-CB11B were each calculated from their amino acid sequences using version 1.00 of Prosis software (Hitachi Software Engineering Co. Ltd., Brisbane, CA). It was assumed that there are 99 hydroxyproline residues and 20 hydroxylysine residues for every 1,000 total residues of the α 1(II) chain (21). On this basis, the molecular weights of the α 1(II) chain and α 1(II)-CB11B peptide were calculated as 98,291 and 2,231 respectively. Therefore, the microgram per milligram concentration of type II collagen extracted from cartilage was calculated by multiplying the concentration of extracted α 1(II)-CB11B by a factor of 44.

Statistical analysis. Significance of differences in total and denatured collagen was tested using the Mann-Whitney U-test for comparison between groups and the paired *t* test for comparison of upper 1-mm with lower 1-mm zones prepared from the same cartilage plugs.

Results

Identification of an immunogenic peptide. Five hydrophilic domains (hydrophobicity less than -1.0) were identified from the hydrophobicity profile of the cyanogen bromide peptide 11 of human type II collagen (Fig. 1). A 21-amino acid sequence (-Gly-Lys-Val-Gly-Pro-Ser-Gly-Ala-Hyp-Gly-Glu-Asp-Gly-Arg-Hyp-Gly-Pro-Hyp-Gly-Pro-Gln-) from one of these domains satisfied the four additional criteria for peptide sequence selection described above, in Methods. The peptide, α 1(II)-CB11B, was synthesized with an additional amino-terminal cysteine (for conjugation) and carboxy-terminal tyrosine (for iodination if needed for an RIA), coupled to ovalbumin, and used to immunize mice.

Monoclonal antibody to α 1(II)-CB11B. Hybridoma cells from the most reactive clone were used to produce ascitic fluid containing monoclonal antibody COL2-3/4m, which was reactive with both α 1(II)-CB11B and HDC. It has an IgG₁ (κ) isotype. The specific peptide sequence recognized by the monoclonal was identified by synthesizing short, overlapping peptides from within the sequence of α 1(II)-CB11B and testing these for reactivity with the monoclonal (data not shown). Maximum reactivity was retained with a 13-amino acid pep-

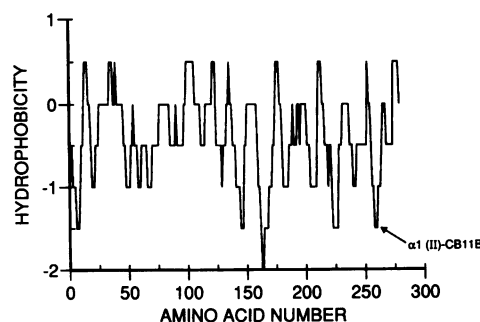


Figure 1. Hydrophobicity profile of the cyanogen bromide peptide 11 (CB11) of human type II collagen. Hydrophobicity was estimated according to the method of Hopp and Woods (15). The domain shown as " α 1(II)-CB11B" was synthesized as a 23-amino acid peptide (see text).

ptide: -Ala-Hyp-Gly-Glu-Asp-Gly-Arg-Hyp-Gly-Pro-Hyp-Gly-Pro-. Shortening of the sequence at the amino terminus by removing one residue reduced reactivity with COL2-3/4m by 70% and shortening by one residue at the carboxy terminus reduced reactivity by 33%, and 92% when two residues were removed. The α 1(II)-CB11B peptide retained full reactivity with antibody COL2-3/4m in an inhibition assay when proline was substituted for any one of the three hydroxyproline residues (data not shown). The antibody reacted (inhibition ELISA assay) with soluble denatured type II collagen isolated from human, bovine, mouse, rat, hamster, rabbit, and horse cartilages (data not shown).

Western blotting analysis confirmed that antibody COL2-3/4m reacts with denatured type II collagen but not with denatured collagen types I, III or X (Fig. 2). None of the collagens reacted with a control antibody, 6B, which has the same isotype as COL2-3/4m (see reference 22 and legend to Fig. 2). COL2-3/4m cross-reacts with the α 3 chain of type XI collagen, the sequence of which is identical to that of the α 1(II) chain, but is more heavily glycosylated (23, 24). It did not react with the α 1(XI) or α 2(XI) chains. The sequence of the 13-amino acid epitope of α 1(II)-CB11B (see above) was compared to those of all the proteins included in release 22 of the SWISS-PROT protein sequence database, using MacMolly Tetra software (Soft Gene GmbH, Berlin, FRG). Collagen chains α 1(I) and α 2(I) each contained a sequence with 77% homology to the 13 amino acid epitope of α 1(II)-CB11B, but the only protein sequences found to contain the entire epitope were those of collagen chains α 1(II) and α 3(XI). Despite the homologous sequences in the type I collagen α -chains, antibody COL2-3/4m does not recognize denatured type I collagen, as shown by immunoblotting (Fig. 2) and immunoassay (see below and Table III).

Characteristics of the α 1(II)-CB11B inhibition ELISA. One sample of HDC was assayed for α 1(II)-CB11B 15 times on each of 10 ELISA plates. From this data, the mean coefficient of variation was calculated as 9% for intraassay analysis and 10% for interassay analysis.

Typical standard curves, plotted as log % inhibition against log α 1(II)-CB11B and HDC concentrations (μ g/ml), are shown in Fig. 3A. The inhibition profiles for HDC and α 1(II)-CB11B were essentially parallel (Fig. 3A). On a molar basis the concentration ranges of antigen producing linear inhibition were similar for α 1(II)-CB11B and HDC (Fig. 3B), although

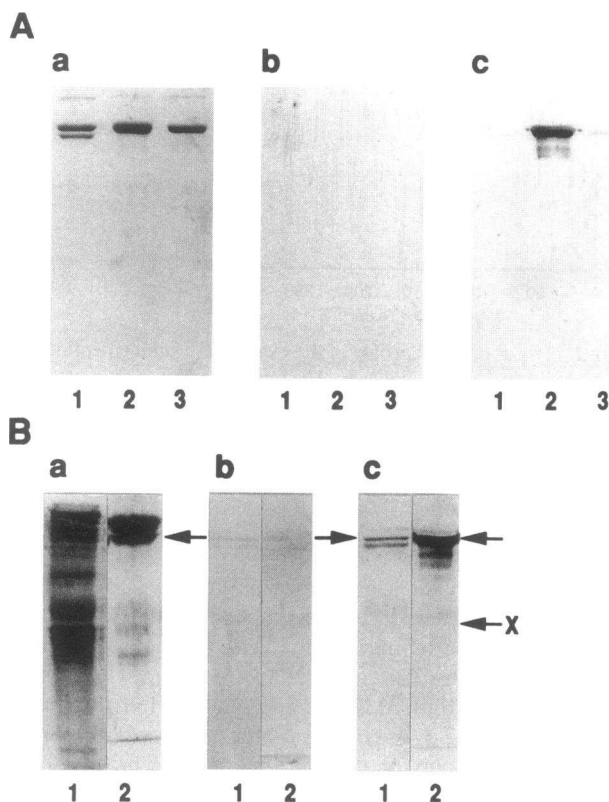


Figure 2. SDS-PAGE and Western immunoblot analysis of bovine collagens. Coomassie Brilliant Blue-stained SDS-PAGE of bovine collagens (panel *a*) and Western immunoblots of the same collagens for reactivity with a control ascitic fluid containing monoclonal antibody 6B (22) that has the same isotype as COL2-3/4m and reacts with unsaturated stubs of Δ di-chondroitin-4-sulfate produced by digestion of chondroitin sulfate with chondroitinase ACII or ABC (panel *b*) or ascitic fluid containing antibody COL2-3/4m (panel *c*) were performed as described in Methods. In *A*, lane 1 is type I collagen; lane 2 is type II collagen; lane 3 is type III collagen. A 7.5% SDS-gel was used. In *B*, lane 1 is medium enriched for type X collagen; lane 2 is type XI collagen. A 10% SDS-gel was used. Unlabeled arrows indicate the position of the α 3(XI) chain. Note that the medium enriched for type X collagen (lane 1 in *B*) contains small amounts of types II and XI collagen, which are detected with COL2-3/4m.

the peptide was slightly more inhibitory. Since proteinase-derived peptides were to be assayed as well as for practical convenience, the α 1(II)-CB11B peptide was used as a standard. Conversion to amounts of intact α 1(II) chains were made as described in Methods. Biological samples being assayed for denatured type II collagen were always diluted such that the level of inhibition in the assay fell within the range 20–70%. This represents 0.35–3.5 μ g/ml α 1(II)-CB11B (15.4–154 μ g/ml denatured type II collagen) in the diluted sample.

The traditional method of estimating collagen concentrations is on the basis of hydroxyproline content. Therefore a comparison was made of the concentrations of α 1(II)-CB11B and hydroxyproline in different HDC samples. There was a good correlation between the two methods (Fig. 4).

It was important to demonstrate that the inhibition ELISA was specific for denatured type II collagen. Accordingly, bovine type II collagen was prepared at various concentrations in the native and heat-denatured forms and assayed for α 1(II)-

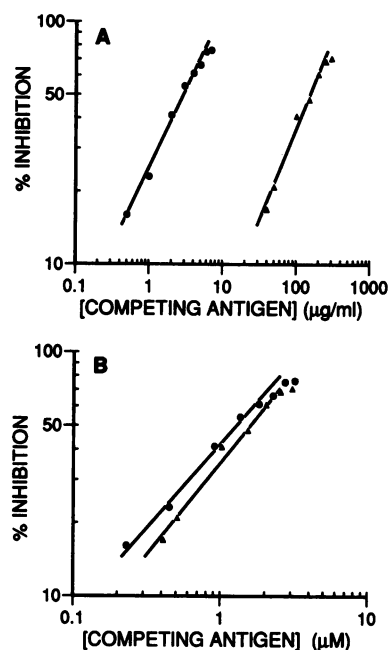


Figure 3. Standard curves for the α 1(II)-CB11B ELISA. α 1(II)-CB11B peptide and HDC were prepared at various concentrations and assayed for α 1(II)-CB11B as described in Methods. A typical α 1(II)-CB11B standard curve (●) and the equivalent HDC dilution curve (▲) are shown plotted as microgram per milliliter (*A*) and micromolar (*B*) concentrations of each antigen.

CB11B. Table II shows that only background levels of denatured α chain, measured as α 1(II)-CB11B could be detected in the native type II collagen preparation. The α 1(II)-CB11B epitope could not be detected by immunoassay in samples of heat denatured types I or III collagen (Table III), confirming the finding from Western blotting that COL2-3/4m does not react with these molecules (see above).

Denaturation of type II collagen after cleavage by interstitial collagenase. Cleavage of purified native type II collagen by recombinant human interstitial collagenase produced the characteristic three-quarter and one-quarter products, seen by SDS-PAGE, but only the three-quarter product was detected by immunoblotting with COL2-3/4m (Fig. 5 *A*). When the collagenase-cleaved collagen was assayed for α 1(II)-CB11B, it was found that 56% of the collagen had been denatured compared to just 11% denaturation in the native collagen control which had been incubated under the same conditions but without collagenase. The partial denaturation of the control collagen was due to the prolonged incubation (20 h) at 30°C. There was no evidence to indicate that the α 1(II)-CB11B epitope is cleaved by interstitial collagenase.

Location of α 1(II)-CB11B on cyanogen bromide peptide CB11. In that the α 1(II)-CB11B epitope amino acid sequence only occurs in the CB11 peptide of type II collagen, the antibody should only recognize CB11. Cleavage of type II collagen

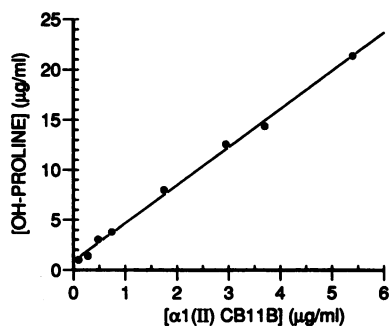


Figure 4. Correlation of α 1(II)-CB11B content with hydroxyproline content of HDC.

Table II. Specificity of Antibody COL2-3/4m for Denatured Rather than Native Type II Collagen

[Collagen]	HDC	Native
$\mu\text{g/ml}$		$\mu\text{g/ml}$
62.5	1.3	0.2
125	2.1	0.0
250	5.8	0.1

Values for $\alpha 1(\text{II})$ -CB11B by inhibition ELISA are shown.

with cyanogen bromide produced a number of peptides, including CB8, CB10, and CB11, as seen by SDS-PAGE and silver staining (Fig. 5 B). The only peptide to be detected by immunoblotting with monoclonal COL2-3/4m was, as expected, based on the location of the epitope, CB11 (Fig. 5 B).

Epitope in peptide $\alpha 1(\text{II})$ -CB11B is cleaved by clostridial collagenase. When clostridial collagenase alone was inhibited with 100 mM EDTA and assayed for $\alpha 1(\text{II})$ -CB11B it produced a small amount of inhibition, equivalent to a background level of 0.85 $\mu\text{g/ml}$ $\alpha 1(\text{II})$ -CB11B (Table IV). This inhibition was probably due to cleavage of some of the HDC bound to the ELISA plate, by residual clostridial collagenase activity. HDC or $\alpha 1(\text{II})$ -CB11B alone incubated in Tris with CaCl_2 at 37°C for 24 h produced good inhibition in the assay for $\alpha 1(\text{II})$ -CB11B, but the amount of $\alpha 1(\text{II})$ -CB11B detected was reduced to the background level when HDC or $\alpha 1(\text{II})$ -CB11B were treated with clostridial collagenase (Table IV), demonstrating hydrolysis of the epitope by clostridial collagenase. Therefore this property can be used to confirm the identity of the epitope in ELISA inhibition assays of samples containing it.

Proteolytic cleavage of HDC. HDC was extensively cleaved into small, undetectable peptides by both α -chymotrypsin and proteinase K, as judged by SDS-PAGE (data not shown) but without loss of the epitope (Table V). The inhibited enzymes themselves did not have any inhibitory activity in the assay. These results again demonstrate a small increase in immunoreactivity on cleavage of the HDC.

Total type II collagen contents and contents of denatured type II collagen in full-depth samples of human femoral condylar cartilages. The mean type II collagen concentration for the normal cartilages was 139.5 $\mu\text{g/mg}$. Thus for normal cartilage the total type II collagen accounts for a mean (range) of 14.0% (9.2–20.8%) of the wet weight of the tissue. For OA cartilage

Table III. Specificity of Antibody COL2-3/4m for Denatured Type II Collagen

[Collagen]	Denatured collagen type		
	I	II	III
$\mu\text{g/ml}$		$\mu\text{g/ml}$	
62.5	0	1.53	0
125	0	2.19	0
250	0	5.34	0

Values for $\alpha 1(\text{II})$ -CB11B by inhibition ELISA are shown.

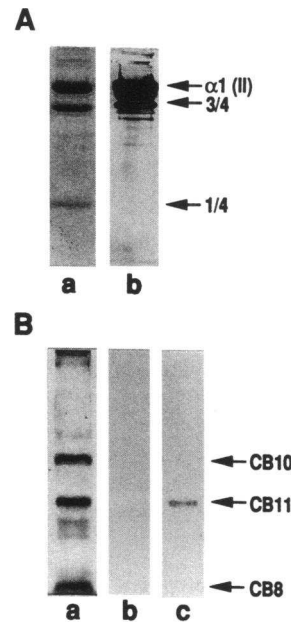


Figure 5. SDS-PAGE and Western immunoblot analysis of bovine type II collagen cleaved by human collagenase or cyanogen bromide. (A) Coomassie Brilliant Blue-stained 7.5% SDS-PAGE gel (panel a) and Western immunoblot with antibody COL2-3/4m (panel b) of type II collagen after cleavage by human recombinant collagenase. Arrows indicate un-cleaved α chain and the three-quarter and one-quarter cleavage products. (B) Silver-stained 12% SDS-PAGE gel (panel a) and Western immunoblot with a control ascitic fluid (see legend to Fig. 2) (panel b) or ascitic fluid containing antibody COL2-3/4m (panel c) of cyanogen bromide peptides of type II collagen. Arrows indicate the major cyanogen bromide peptides. Staining with Coomassie or silver stain and the Western immunoblots were performed as described in Methods.

the equivalent mean (range) value was 10.3% (7.4–15.0%). The total amount of type II collagen/mg wet weight was significantly decreased in OA compared to normal cartilage (Fig. 6).

The proportion of denatured type II collagen was significantly increased in OA compared to normal cartilage (Fig. 6). The mean values for the percentage of denatured collagen in normal and OA cartilages were 1.1% and 6.0% respectively.

There were no correlations between either total or percent denatured type II collagen and the degree of cartilage degradation recorded as the Mankin grade of OA cartilages (Fig. 7).

Digestion of extracted collagen with clostridial collagenase. It was important to demonstrate that inhibition observed in the $\alpha 1(\text{II})$ -CB11B ELISA by α -chymotrypsin and proteinase K

Table IV. Clostridial Collagenase Cleaves the $\alpha 1(\text{II})$ -CB11B Epitope

Sample	Treatment	[$\alpha 1(\text{II})$ -CB11B] by immunoassay
		$\mu\text{g/ml}$
Tris	Clostridial collagenase	0.85
HDC	Tris/ CaCl_2	3.80
HDC	Clostridial collagenase	0.84
$\alpha 1(\text{II})$ -CB11B	Tris/ CaCl_2	4.44
$\alpha 1(\text{II})$ -CB11B	Clostridial collagenase	0.83

HDC and $\alpha 1(\text{II})$ -CB11B were dissolved in Tris containing 5 mM CaCl_2 and 14 U/ml chromatographically purified clostridial collagenase. Control tubes contained HDC or $\alpha 1(\text{II})$ -CB11B in Tris with 5 mM CaCl_2 but no clostridial collagenase or bacterial collagenase in Tris with 5 mM CaCl_2 but no HDC or $\alpha 1(\text{II})$ -CB11B. The samples were incubated overnight at 37°C and the clostridial collagenase was inactivated by addition of EDTA to a final concentration of 100 mM (this concentration was required to ensure maximal inhibition of the proteinase).

Table V. α -Chymotrypsin and Proteinase K Do Not Cleave the $\alpha 1(II)$ -CB11B Epitope

Proteinase	$\alpha 1(II)$ -CB11B by immunoassay
	$\mu\text{g/ml}$
None	5.0
α -Chymotrypsin	5.7
Proteinase K	6.0

Aliquots of HDC at 0.25 mg/ml were prepared in Tris containing 0.5 mg/ml of type VII α -chymotrypsin or proteinase K. Control tubes contained only HDC, or 0.5 mg/ml α -chymotrypsin or 0.5 mg/ml proteinase K. The samples were incubated overnight at 37°C (α -chymotrypsin) or 56°C (proteinase K). The α -chymotrypsin was inactivated by addition of 115 $\mu\text{g/ml}$ TPCK. Proteinase K was inactivated by boiling for 10 min.

extracts of human cartilage was due entirely to the $\alpha 1(II)$ -CB11B epitope. Since the epitope is destroyed by treatment with clostridial collagenase (see above and Table IV), some extracts were treated with this enzyme as a control for specificity. There was a loss of immunoreactivity in each case (Table VI), demonstrating the specificity of the assay for the $\alpha 1(II)$ -CB11B epitope in the cartilage extracts.

Variation in total and denatured type II collagen with depth of cartilage. From cartilage in which specimens could be obtained (minimum thickness, 2 mm), plugs were divided into the upper 1 mm (articular zone) and lower 1 mm (deeper zone; these are approximate thicknesses) using a scalpel and a specially constructed slicing bed. The total type II collagen concentration was higher in the deeper (lower 1 mm) compared to more superficial (upper 1 mm) cartilage for four of five normal and five of eight OA specimens (Figs. 8, A and C). The percentage of denatured collagen did not vary significantly with depth in normal cartilage (Fig. 8 B). In one sample there was a high level of denatured collagen in the more superficial cartilage and increased denaturation in the deeper cartilage too. In two of the four other specimens, denaturation was enhanced in the more superficial cartilage, otherwise it was similar in each zone or higher in the deeper cartilage. For OA cartilage the percentage

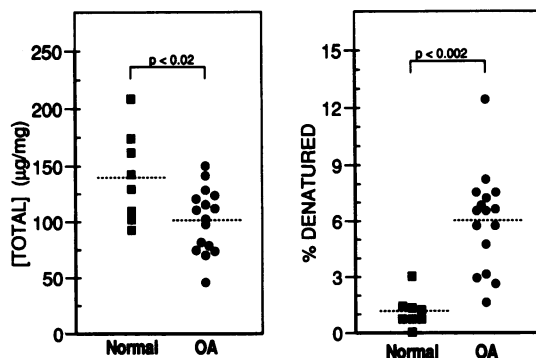


Figure 6. Total and denatured type II collagen in human femoral condylar cartilage from different patient groups. Each point is the result for cartilage obtained from one patient. Total collagen is shown as micrograms per milligram wet weight and denatured collagen is expressed as a percentage of total collagen in each specimen. Bars indicate the mean value for each patient group. Statistical analysis is by Mann-Whitney U-test.

of denatured collagen was significantly higher in the upper 1-mm zone (Fig. 8 D). This difference was seen in six of eight specimens. Of the other two specimens one showed the opposite trend and the other exhibited similar denaturation in the upper and lower levels. There was no correlation between total or percent denatured collagen in either the upper or lower zones and Mankin grade of the full-depth cartilage for normal and OA specimens (data not shown). The mean values of total and denatured collagen in the upper and lower 1-mm zones are shown in Table VII, for comparison of each zone in OA with the same zone in normal cartilage. In OA, total collagen content was only significantly reduced in the deeper cartilage of OA compared to normal. Similarly, type II collagen denaturation was only significantly increased in the lower zone of OA compared to normal cartilage. The differences in the percentage of denatured collagen in either region were not as marked as the changes seen in full-depth plugs. However it should be noted that for the depth-study, cartilage was taken from the thickest region of OA tissue and this may have been less degraded than cartilage from the thinner tissue sites.

Guanidinium chloride extracts of OA cartilage. In order to determine if the type II collagen extracted from cartilage by α -chymotrypsin was mostly derived from fibril-associated, cross-linked collagen or from nonfibrillar, non-cross-linked α chains, a comparison was made of extraction by α -chymotrypsin and 4 M guanidinium chloride. The latter is a chaotropic reagent which can only extract the nonfibrillar, non-cross-linked collagen molecules. Adjacent plugs of cartilage from five OA patients were extracted with α -chymotrypsin or guanidinium chloride. Far less type II collagen was extracted with 4 M guanidinium chloride than with α -chymotrypsin. For the five OA specimens examined the mean \pm SD total (native + denatured) type II collagen extracted by guanidinium chloride and α -chymotrypsin was 0.019 ± 0.003 and 0.164 ± 0.012 $\mu\text{g/mg}$ wet weight, respectively. Therefore the amount of type II collagen extracted by 4 M guanidinium chloride is 11.6% of that extracted by α -chymotrypsin, suggesting that most of the denatured material extracted with α -chymotrypsin is derived from cross-linked fibrils rather than a pool of newly synthesized, non-cross-linked α chains or peptides thereof. Of the type II collagen extracted with guanidinium chloride or α -chymotrypsin, only a small proportion (10.5% and 7.3%, respectively) was native collagen (detected after digestion with proteinase K). However, we have found that incubation of purified native type II collagen for 72 h at 4°C in 4M guanidinium chloride, followed by dialysis into Tris, causes over 90% of the collagen to denature, as judged by assaying for $\alpha 1(II)$ -CB11B (data not shown). Therefore, it is likely that most of the $\alpha 1(II)$ -CB11B extracted with guanidinium chloride is contained within newly synthesized molecules that are denatured by the extraction procedure.

Of the collagen extracted with α -chymotrypsin, the amount of native type II collagen was too small to be detected by immunoblotting of α -chymotrypsin extracts (Fig. 9). Moreover, peptides bearing the epitope recognized by antibody COL2-3/4m were too small to detect under the conditions of electrophoresis.

Discussion

The degeneration of cartilage, which is an integral feature of both OA and RA, involves localized loss of the proteoglycan

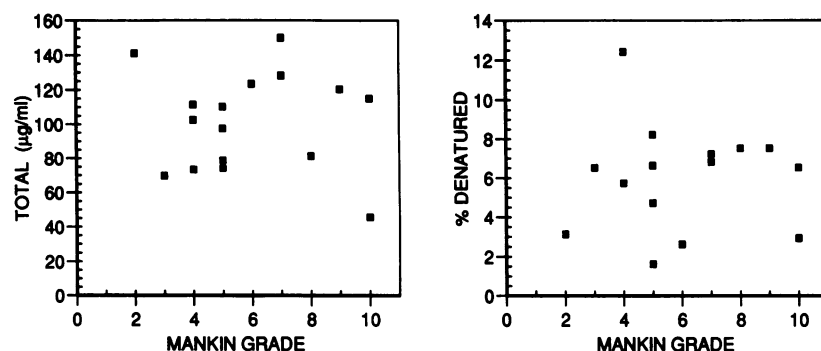


Figure 7. Lack of any correlation between Mankin grade and total or denatured type II collagen in OA cartilage. Total collagen is shown as micrograms per milligram wet weight and denatured collagen is expressed as a percentage of total collagen in each specimen.

aggrecan, its degradation (5, 6) and eventual loss of function of this tissue (25–27). The tissue is composed of a fibrillar organization of type II collagen (3) which endows cartilage with its tensile strength (1, 2). With increasing age there is a progressive reduction of the tensile properties of femoral head cartilage (28). This is not observed in the talus of the ankle joint where the incidence of osteoarthritis is relatively low compared to that observed in the hip or knee (28). In the present study we show that damage to type II collagen, measured as denaturation (unwinding) of the triple helix, is detectable in adult human femoral condylar cartilage and is increased in OA cartilage. This damage in OA is accompanied by a net reduction in the total type II collagen content. Previously, a loss of tensile properties was observed in OA cartilage, indicative of damage to type II collagen (25–27). The present studies reveal that the reduced tensile strength in part relates to damage to the triple helix of type II collagen leading to denaturation (unwinding). It also correlates with an apparent net reduction in the content of this molecule in OA cartilage, previously indicated by a reduction in total cartilage collagen content measured as hy-

droxyproline (10). This reduction may result from an increase in tissue hydration, caused by increased swelling of proteoglycans permitted by damage to collagen fibrils. Increases in water content in OA are well recognized (27). This decrease in collagen content is not observed in RA femoral condylar cartilage, although there is an increase in type II collagen denaturation similar to that observed in OA (A. Hollander, T. Heathfield, I. Pidoux, W. Fisher, E. Bogoch, and A. R. Poole, manuscript in preparation).

We also show that the damage to type II collagen of the femoral condylar cartilage in OA is most pronounced in the superficial and upper-mid zone (upper 1-mm slice): this is where the proteoglycan aggrecan is first lost from the matrix in OA (3). Such a difference between upper and lower zones is not observed in normal cartilage. If we compare damage to the lower zone in normal and OA cartilages we find that there is a significant increase in denaturation in OA and this increased lower zone damage is accompanied by a significant loss of type II collagen compared to normal cartilage. When, however, we compare damage to the upper zone in normal and OA cartilages, we find that the more pronounced collagen denaturation observed in the upper layers of OA cartilage is not significantly different from the level of denaturation in the same zone of normal cartilage. This result is probably due to the fact that denaturation in the upper zone was very variable within the normal group (one specimen in particular), which may reflect early, pre-clinical, OA-like changes in normal cartilage (see Fig. 6 B). Indications from preliminary studies are that there is less denaturation in younger (skeletally mature) cartilages, suggesting that type II collagen denaturation may in part be a function of ageing. This may lead to excessive degenerative changes in some cases (A. Hollander, T. Heathfield, and A. R. Poole, unpublished observations).

These findings on sites of damage to type II collagen confirm and extend our earlier work, using an immunohistochemical approach coupled with a polyclonal rabbit antiserum to epitopes in the CB8 and CB11 peptides of type II collagen. Those studies also revealed that there is increased damage in OA cartilage, which is first observed in the upper region (11). In separate immunohistochemical studies with monoclonal antibody COL2-3/4m we confirm that denaturation in normal and OA cartilages usually starts at and close to the articular surface and progressively extends down into the cartilage with increasing Mankin grade (A. P. Hollander, I. Pidoux, R. Bourne, C. Rorabeck, and A. R. Poole, manuscript in preparation).

The mechanisms responsible for the denaturation (unwinding) of the triple helix have not been definitively identi-

Table VI. The Inhibition Observed in Cartilage Extracts Is Lost on Treatment with Clostridial Collagenase

[$\alpha 1(\text{II})$ -CB11B] by immunoassay			
Sample	Proteinase digestion	Digested sample treated with Tris/CaCl ₂	Digested sample treated with Tris/CaCl ₂ + collagenase
$\mu\text{g/ml}$			
Tris	None	0.00	0.46
OA-(i)	α -chymotrypsin	1.39	0.47
OA-(ii)	α -chymotrypsin	3.22	0.45
OA-(iii)	α -chymotrypsin	2.11	0.43
OA-(iv)	α -chymotrypsin	3.53	0.48
OA-(v)	α -chymotrypsin	2.86	0.47
OA-(i)	Proteinase K	53.4	0.49
OA-(ii)	Proteinase K	93.2	0.47
OA-(iii)	Proteinase K	82.5	0.47
OA-(iv)	Proteinase K	148.9	0.40
OA-(v)	Proteinase K	61.7	0.56

OA cartilages (i–v) were extracted/digested with α -chymotrypsin and proteinase K. The digested samples were then treated with Tris/5 mM CaCl₂ with or without 14 U/ml clostridial collagenase at 37°C. Clostridial collagenase activity was inhibited with 100 mM EDTA. The samples were assayed for $\alpha 1(\text{II})$ -CB11B.

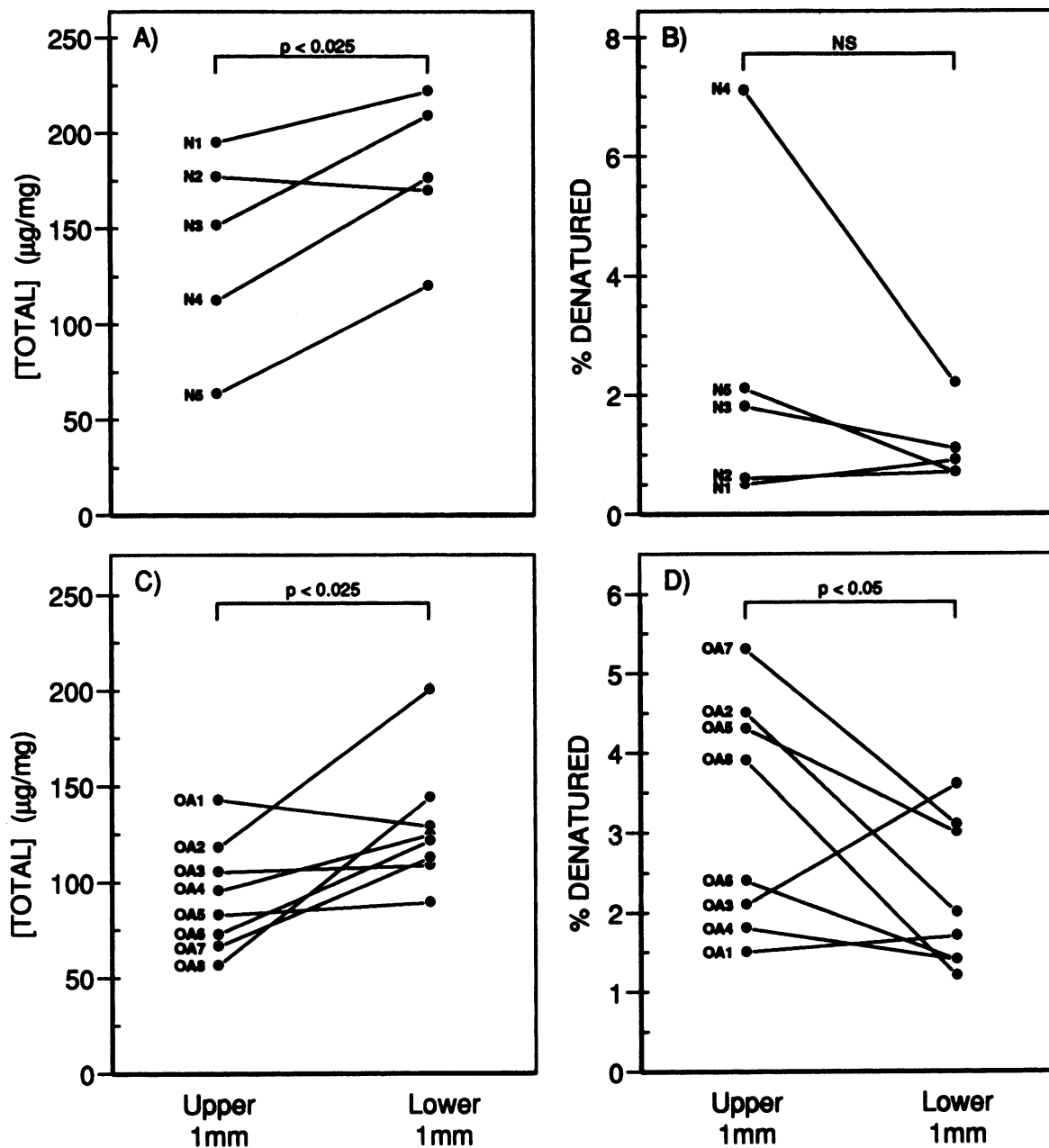


Figure 8. Variation in total and denatured type II collagen with depth in normal and OA human femoral condylar cartilage. Plugs of cartilage were divided into the more superficial zone (upper 1 mm) and deeper zone (lower 1 mm) using a scalpel. Each line connects upper and lower cartilages obtained from one patient. Total collagen is shown as micrograms per milligram wet weight and denatured collagen is expressed as a percentage of total collagen in each specimen. Individual patients are identified by the numbers N1–N5 for normal cartilage and OA1–OA8 for OA cartilage. A comparison of the mean values for each patient group is shown in Table VII. Statistical analysis is by Mann-Whitney U-test.

fied. These studies are currently in progress. But the only known proteinase capable of causing unwinding of the triple helix of type II collagen is interstitial collagenase (matrix metalloproteinase-1; MMP-1). This cleaves the triple helix between residues 906 and 907 to produce the characteristic one-quarter and three-quarter pieces shown here, of which our monoclonal antibody recognizes the three-quarter piece. The use of antibody COL2-3/4m will hopefully assist us in identifying any other cleavage sites within the collagen molecule that may accompany the unwinding of the triple helix. Suffice it to say that the unwinding of the triple helix is not accompanied by such rapid secondary cleavage as to remove all α chain frag-

ments bearing the CB11B epitope and render them nondetectable. Such secondary trimming can occur by proteinases such as the 72- and 92-kD gelatinases, the expressions of which are increased in OA cartilage as well as collagenase (3, 5, 29).

The fact that most (88.4%) of the denatured collagen extracted by α -chymotrypsin is not extractable with 4 M guanidinium chloride indicates that it does not represent newly synthesized collagen. Rather, it reflects denatured collagen bound to the cartilage matrix. Previously we showed that antibodies of the kind described here react primarily with denatured collagen fibrils (12). Thus based on those earlier studies, the majority of the denatured α chains extracted with α -chymotrypsin most

Table VII. Total and Denatured Collagen in Different Depths of Normal and OA Cartilage

Disease group	Total type II collagen		Percentage of denatured type II collagen	
	Zones		Zones	
	Upper	Lower	Upper	Lower
	$\mu\text{g/ml}$		%	
Normal	139.9	179.5	2.42	1.12
OA	92.4 ^{NS}	128.9*	3.2 ^{NS}	2.2 [‡]

Zones are 1 mm thick. Values are the mean result for cartilage from five normal and eight OA patients. NS = not significant; * $P < 0.03$; ‡ $P < 0.02$ v. normal cartilage; Mann-Whitney U-test. Results for the individual patients and statistical comparisons of data for upper versus lower zones in each patients group are shown in Fig. 9.

likely reflects denaturation of type II collagen present within cross-linked fibrils, often remote from chondrocytes. If the guanidinium chloride extractable pool represents denatured, newly synthesized collagen then the amount of this which is present in the cartilage accounts for a very small proportion of the denatured collagen. Studies are in progress to examine this question further.

Until we developed this new methodology, starting with our work on the polyclonal antiserum (4), unwinding of the triple helix of type II collagen could not be detected in situ. To accurately quantitate degradation of collagen fibrils in cartilage it was necessary to develop an assay based on the use of a monoclonal antibody to a defined epitope of known location and which can be extracted intact, using selected proteinases to extract it first from nonhelical α -chains and then from helical molecules. We identified and synthesized such an epitope located in the CB11 peptide and produced a monoclonal antibody to the synthetic peptide. The epitope is recognized in denatured but not native type II collagen α chains. This has made possible development of an immunoassay, used in these studies and the detection of total type II collagen. Monoclonal COL2-3/4m cross-reacts with denatured type II collagen from all mammalian species so far studied (including mouse), indi-

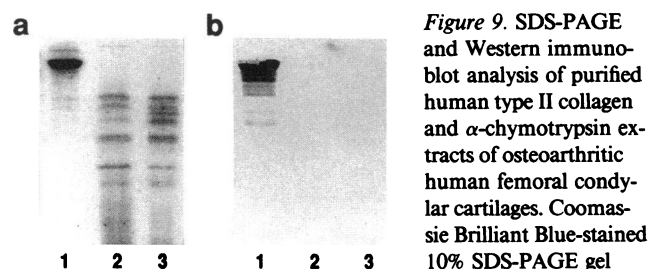


Figure 9. SDS-PAGE and Western immunoblot analysis of purified human type II collagen (lane 1) and two osteoarthritic cartilages (lanes 2 and 3). Approximately 5 μg of collagen II were loaded per lane, determined from epitope content by immunoassay. Note that chymotrypsin-produced peptides could be detected by Coomassie staining (panel a, lanes 2 and 3). But immunoreactive peptides were too small to be detectable on Western blotting. Also, there were no residual type II collagen α chains detectable after α -chymotrypsin extraction.

cating that the epitope contained within peptide $\alpha 1(\text{II})$ -CB11B is highly conserved. Importantly, binding of the antibody to the collagen α -chain is not dependent on hydroxylation of the "Y" position proline residues in the repeating Gly-X-Y triplet. This is important since it is possible that the extent of hydroxylation could change in pathological situations. Also, the epitope does not contain any potential cross-linking residues. Antibody COL2-3/4m cross-reacts with the $\alpha 3(\text{XI})$ chain, which is considered to be the same gene product as the $\alpha 1(\text{II})$ -chain, although it is more heavily glycosylated (23, 24). This cross-reactivity should not interfere with the quantitation of native or denatured type II collagen since there is only one $\alpha 3(\text{XI})$ -chain per molecule of type XI collagen, which represents no more than 1% of total collagen α chains in cartilage (30). The antibody does not cross-react with any other collagen or protein examined. Moreover, the epitope sequence is not present in any other known protein sequence.

Continuation of these studies should permit an improved understanding of the mechanisms involved in the denaturation of type II collagen in arthritis and may lead to a means of regulating this pathogenic process. Antibody COL2-3/4m will be used to detect collagen fragments released from articular cartilage in vitro and in vivo. The latter may provide a means of investigating collagen II degradation in patients with arthritis.

Acknowledgments

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