Enhanced Cleavage of Type II Collagen by Collagenases in Osteoarthritic Articular Cartilage

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Abstract

We demonstrate the direct involvement of increased collagenase activity in the cleavage of type II collagen in osteoarthritic human femoral condylar cartilage by developing and using antibodies reactive to carboxy-terminal (COL2-3/4Cshort) and amino-terminal (COL2-1/4N1) neoeptopes generated by cleavage of native human type II collagen by collagenase matrix metalloproteinase (MMP)-1 (collagenase-1), MMP-8 (collagenase-2), and MMP-13 (collagenase-3). A secondary cleavage followed the initial cleavage produced by these recombinant collagenases. This generated neoepitope COL2-1/4N2E. There was significantly more COL2-3/4Cshort neoepitope in osteoarthritis (OA) compared to adult nonarthritic cartilages as determined by immunoassay of cartilage extracts. A synthetic preferential inhibitor of MMP-13 significantly reduced the unstimulated release in culture of type II collagen (381–95%) and that MMP-13 may play a significant role in this process. (J. Clin. Invest. 1997. 99:1534–1545.) Key words: matrix metalloproteinases • arthritis • chondrocytes • antibodies

Introduction

Type II collagen is composed of a triple helix of three identical α chains. These molecules associate to form a fibril that is stabilized by intermolecular crosslinks (1). The fibrils provide the tensile strength and maintain the integrity of mammalian articular cartilage by forming a network that resists the swelling pressure resulting from the hydration of the polyanionic proteoglycan aggregates in the extracellular matrix (2, 3). Damage to this fibrillar meshwork, made up of primarily type II collagen (∼90–95%), may be a critical event in the pathology of many arthritides, due in part to the very slow rate of collagen turnover within the cartilage (4). In early degeneration in articular cartilage, which may lead to osteoarthritis (OA), there is a loss of the tensile properties, indicative of damage to the fibrillar network (2). Recent studies in this laboratory have provided evidence for increased denaturation of the triple helix of type II collagen in human articular cartilage in OA (5–7).

The extracellular degradation of fibrillar types I, II, and III collagens can occur both in nonhelical sites (8) and through a triple helical cleavage. Only the latter results in denaturation of the triple helix at physiological temperatures. This is achieved by collagenases which belong to the family of zinc-dependent endopeptidases called matrix metalloproteinases (MMPs). Collagenase-1 or MMP-1 (interstitial collagenase), collagenase-2 or MMP-8 (neutrophil collagenase), and the recently cloned and characterized collagenase-3 or MMP-13 (9, 10) are the only mammalian enzymes known to be able to initiate the intrahelical cleavage of triple helical collagen at neutral pH. MMP-3 (stromelysin-1) can cleave type II fibrillar collagen, but only in the nonhelical amino–telopeptide domain (11). These collagenases are thought to define the rate-limiting step in normal tissue remodeling events such as bone resorption and growth, embryonic development, uterine involution, wound healing, and in degradative processes such as arthritis, tumor invasion and metastasis, and periodontitis.

Until now, there has been no direct evidence for their involvement in intrahelical cleavage, except for the inability of murine collagenase to cleave type I collagen in vivo when the intrahelical site is mutated (8). Three collagenases cleave the fibrillar collagens types I, II, and III at a single site (Gly755–Leu/ILE756) within each α chain of the triple helical collagen molecule, approximately three quarters of the distance from the amino-
terminal end of each chain, resulting in the generation of three-quarter (TC\(^4\)) and one-quarter (TC\(^8\)) length collagen fragments (12–14). The cleaved collagen fragments spontaneously denature into nonhelical gelatin derivatives at physiological temperatures, thereby becoming susceptible to further degradation by these collagenases (15) and/or by other proteinases, such as MMP-3, and the gelatinases MMP-2 and MMP-9.

Collagenases have been detected in the synovial fluid of patients with traumatic arthritis, RA, and OA (16–19). In the synovial joint, there are three distinct tissue sources of the collagenases that may be involved in the degradation of articular cartilage. Cells in OA and RA synovium express collagenases (20–23). The expression of MMP-13 in the synovial membrane was identified in RA and OA tissues in one study (24), but in other reports MMP-13 was not found in isolated synoviocytes from OA and RA patients, nor could it be induced in these cells by IL-1 (25, 26). Fibroblasts at the cartilage-pannus interface also secrete a collagenase that can degrade cartilage (27). Chondrocytes express and secrete MMP-1 (22, 25, 26) and MMP-13 (14, 25, 26) in both normal and OA articular cartilages. This is upregulated by IL-1 (1–14). Moreover, two recent studies describe the expression of MMP-8 (neutrophil collagenase) by human articular chondrocytes from normal donors, and its upregulation by IL-1 (29, 30).

Collagenases have been routinely assayed in vitro (31, 32) and in vivo (33, 34) using radiolabeled collagen fibrils or fluorogenic peptides (35). These methods do not allow for the differentiation of the collagenases involved in the cleavage of these substrates. ELISAs have been developed using specific anticollagenase antibodies allowing the detection of collagenase and tissue inhibitor of metalloproteinase (TIMP)-collagenase complexes (36–38). However, the presence of enzyme in body fluids and tissues does not necessarily imply activity, as the collagenases are secreted as zymogens, they require activation, and their catalytic potential is determined by the presence of inhibitors such as tissue inhibitor of metalloproteinase and alpha, macroglobulin.

Using polyclonal and monoclonal antibodies that react specifically to denatured type II collagen \(\alpha\) chains, such as can result from intrahelical cleavage by collagenases, we have shown that increased denaturation of this molecule can be detected in OA articular cartilages (5–7). This implicates collagenases in the denaturation of type II collagen. However, to definitively demonstrate a role for these proteinases in the cleavage of this molecule, a means of specifically identifying the collagenase cleavage site(s) in situ is required. This would be possible if we could prepare and use antibodies that react with the carboxy- (COOH-) and amino- (NH\(_2\)) terminal neoepitopes created by cleavage of collagen by collagenases and which do not react with the intact molecule or \(\alpha\) chains thereof. Antibodies of this kind prepared to cleavage site neoepitopes have already proven valuable in defining MMP activity in aggrecan degradation (39–45).

In this study, we have developed, for the first time, antibodies to the neoepitopes that represent the COOH- and NH\(_2\) termini of TC\(^{\alpha}\) and TC\(^{\delta}\), respectively, of human type II collagen \(\alpha\) chains, produced by cleavage of the triple helical collagen molecule by any one of the three collagenases: MMP-1, MMP-8, and MMP-13. Here we describe how these antineoepitope antisera were used to provide direct evidence for the involvement of collagenases in the cleavage of type II collagen in osteoarthritic human articular cartilages. Moreover, we show that there is a direct association between this cleavage and the denaturation of type II collagen. Finally, through the use of a synthetic preferential inhibitor of MMP-13, we provide evidence that indicates that the in vitro release of collagenase-specific type II collagen cleavage site neoepitopes from OA articular cartilage is usually mediated by MMP-13.

**Methods**

**Tissue.** Human adult and fetal articular cartilages, and human fetal skin were collected at autopsy (within 15 h postmortem) and from therapeutic abortions. Full-depth human osteoarthritic articular femoral condylar cartilage was obtained at arthroplasty from the knee joints of 26 patients with OA, as diagnosed using the criteria of the American College of Rheumatology for OA (46). Osteophytic cartilages were never examined. Macroscopically normal full-depth nonarthritic articular cartilages were obtained within 15 h of postmortem from the femoral condylar regions of 19 individuals from sites where there were no observable joint abnormalities. None of these persons had recent chemotherapy.

**Collagen purification.** Human type II collagen was prepared by pepsin digestion and differential salt precipitation, using the method of Miller (47). Human types I and III collagen were prepared by pepsin digestion and differential denaturation and renaturation (48). Bone type X collagen was provided by Dr. A. Marriott (Joint Diseases Laboratory, Montreal, Canada). Cyanogen bromide (CNBr) peptides of human type II were prepared as described by Dodge and Poole (5).

**Preparation of recombinant human proMMP-1, proMMP-8, and proMMP-13.** Recombinant human proMMP-1 and proMMP-13 were purified from the culture medium of S9 cells that were infected with recombinant baculovirus, as described recently (14). Briefly, proMMP-1 was affinity purified using a monoclonal antibody column and proMMP-13 was purified using heparin-agarose and SP-Sepharose Fast Flow columns. Both the enzymes were >95% pure as determined by Coomassie blue staining of SDS-PAGE gels. Recombinant human proMMP-8 was expressed in Escherichia coli as inclusion body protein, refolded, and purified as previously described (49).

**Identification and synthesis of type II collagen cleavage site neoepitopes.** For both the neoepitope peptides COL2-3/4C\(_{\text{short}}\) (Gly-Pro-Hyp-Gly-Pro-Gln-Gly), which corresponds to the COOH terminus of TC\(^{\alpha}\), and COL2-1/4N1 (Leu-Ala-Gly-Gln-Arg-Gly), corresponding to the NH\(_2\) terminus of TC\(^{\delta}\) of the primary cleavage of native triple helical human type II collagen produced by collagenase-1 (MMP-1), amino acid sequences for their synthesis were based on published amino acid sequences (50) of fibroblast collagenase (MMP-1) cleaved human type II collagen alpha 1 (II1) chains (Fig. 1), with the following exception. The assignment of the third residue of the COL2-3/4C\(_{\text{short}}\) peptide (hereafter referred to only as COL2-3/4C), as a hydroxylated proline (Hyp) was based on the assumption that proline residues in the Y position of the repeating Gly-X-Y triplets that make up the helical portions of collagen molecules are potential hydroxylation sites within the collagen \(\alpha\) chains. Moreover, we have shown that antisera generated to peptides containing a nonhydroxylated proline at this position react poorly to the cleaved TC\(^{\delta}\) of type II collagen and in turn, these peptides lacking hydroxylated proline, are recognized less effectively by antisera generated from the COL2-3/4C peptide containing the hydroxylated proline residue (unpublished observations). The sequence for neoepitope peptide COL2-1/4N2 (Gln-Arg-Gly-Ile-Val-Gly), representing a secondary (N2) collagenase cleavage site at the NH\(_2\) terminus of the human \(\alpha1(II)\) TC\(^{\delta}\), was determined using the methodology described recently (12). These peptides (COL2-3/4C, COL2-1/4N1, and COL2-1/4N2) and those peptides used for epitope analyses and antisera characterization, as described below, were synthesized at a 0.25-mmol scale, using standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry, on a solid-phase peptide synthesizer (model 431A; Applied Biosystems, Foster City, CA). A cysteine was added to the NH\(_2\) terminus of pep-
tide COL-2/3/4C and to the COOH termini of peptides COL-2/1-4N1 and COL-2/1-4N2 for conjugation of the peptides to the carrier proteins ovalbumin (OVA) and BSA using the bifunctional reagent N-hydroxy-succinimidyld bromoacetate (Sigma Chemical Co., St. Louis, MO), as described by Hughes et al. (39). A glycine spacer was included in the synthesis of the COL-2/1-4N1 peptide, between the added COOH terminal cysteine and the neoepitope sequence, to improve both the immunogenicity of this peptide when conjugated to OVA and the sensitivity of the assays employing the peptide conjugated to BSA.

Preparation of polyclonal antibodies from rabbit antisera. For neoepitope antisera production, female New Zealand White rabbits were obtained by pepsin digestion of each antiserum, as previously described (51). The Fc portion and undigested IgG were removed by AH-Sepharose-Protein A chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden).

The preparation of a mouse monoclonal antibody (COL-2/3/4m) to an intrachain epitope (CB11B) in the TC chain of human type II collagen was described by Hansen (50) while the secondary collagenase cleavage site was obtained from Birkedal-Hansen (50) at the University of California, Champaign-Urbana, IL. The primary collagenase cleavage sites were obtained from Birkedal-Hansen (50) and the secondary collagenase cleavage site was obtained by NH2-terminal sequencing of electrophoretically separated and transferred collagen fragments obtained after MMP-13 (hHuMMP-13) digestion of triple helical human type II collagen, as described in Methods. The assignment of hydroxylation of the proline residues in the immunizing peptides indicated as Pro(OH), is also described in Methods.

Inhibition ELISAs for analyses of type II collagen neoepitopes produced by collagenase cleavage. Linbro 96-well round bottom microtiter plates (ICN Flow) were precoated with 100 μl/well of PBS-1% BSA for 30 min at room temperature and washed once with PBS-Tween. Four nonspecific binding wells each contained 100 μl PBS-1% BSA and 50 μl PBS-1% BSA-Tween. Each polyclonal F(ab’)2 antisera preparation was diluted 1:200 in PBS-1% BSA-Tween (as determined by checkerboard analyses of antisera and peptide-BSA titrations, data not shown) and 50 μl was added to each of the remaining wells of these preincubated plates. Four wells with antisera were mixed with 50 μl/well of PBS-1% BSA to determine maximum binding in the absence of the inhibitory epitopes. To the remaining wells containing 50 μl of diluted antisera F(ab’2), were added 50 μl/well of appropriate dilutions of the standard peptides COL-2/3/4C, COL-2/1-4N1, or COL-2/1-4N2; native, heat-denatured (80°C for 20 min) or collagenase-cleaved type II, I, and III collagen solutions; concentrated (threefold) culture media from human OA articular cartilage explants; or α-chymotrypsin extracts of human OA articular cartilage. For antibody specificity analyses, standard peptides containing the epitope that had been used for immunization were added, as described above, but with amino acid residues either added to (+1, +2, +3) or removed from (−1, −2, −3) the end of the immunizing peptide corresponding to the cleaved terminus of the α chain fragment. Also, two overlapping 16mer peptides representing the amino acid sequence bridging the primary and secondary cleavage sites of native type II collagen (Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val-Gly and Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-Gly-Ile-Val-Gly-Leu-Hyp-Gly) were used to demonstrate the specificity of the immunoreactivities of the antisera to the cleaved terminus and not to intact α chains at the cleavage sites. All standards and samples were tested in duplicate wells. Analyses were always repeated at least once.

After incubation for 1 h at 37°C, 50 μl of each preincubated sample was transferred to the equivalent wells of Immulon-2 ELISA plates, precoated, as described above, with neoepitope peptide-BSA conjugates diluted to 50 ng/well for COL-2/3/4C, 150 ng/well for COL-2/1-4N1, and 120 ng/well for COL-2/1-4N2, all in PBS, pH 7.2. Note that for the COL-2/3/4C assay, the BSA-conjugate used to coat the plates contained the peptide Cys-Gly-Gly-Glu-Gly-Pro-Hyp-Gly-Pro-Gln-Gly (COL-2/3/4C), which included the natural in-sequence amino acid residue (Glu) and two glycine spacers added to the NH2 terminus of the immunizing COL-2/3/4C peptide. In preliminary experiments, the use of this longer peptide was found to increase the sensitivity of the COL-2/3/4C assay over that obtained by using the shorter immunizing peptide-BSA conjugate (data not shown). These plates were incubated for 30 min at 4°C and then washed three times with PBS-Tween. Goat anti–rabbit IgG F(ab’2) fragment conjugated to alkaline phosphatase (Sigma Chemical Co.) was diluted 1:20,000 in...
PBS-1% BSA-Tween and added at 50 μl/well. After incubation for 1 h at 37°C, the plates were washed three times with PBS-Tween and three times with distilled water. From an ELISA amplification system kit (GIBCO BRL, Gaithersburg, MD), 50 μl of amplifier substrate (NADPH) was added to each well and after 15 min at room temperature, 50 μl of amplifier (alcohol dehydrogenase and diaphorase) solution was added to each well. After a final 15 min at room temperature, the color development was halted with 50 μl of 0.3 M H₂SO₄ and the absorbance was measured at 490 nm. For each plate the mean absorbance from the four non-specific binding wells was subtracted from the absorbance values of each of the other wells. The percentage inhibition of binding by standards or samples were calculated relative to the mean absorbance from the four maximum binding wells which represented 0% inhibition (100% binding). Results were also expressed on a molar basis using a molecular weight for type II collagen α chain of 98,291 D (6) and for the COL2-3/4C peptide of 608 D.

Proteolytic cleavage of native type I, II, and III collagens by human recombinant collagenase MMP-1, MMP-8, and MMP-13. Lyophilized human type I, II, and III collagens were dissolved in 0.5 M acetic acid and then diluted to a final concentration of 2.5 mg/ml in digestion buffer consisting of 50 mM Tris, 10 mM CaCl₂, and then diluted to a final concentration of 2.5 mg/ml in digestion buffer with 2 mM (final concentration) aminophenyl mercuric acetate (APMA; Sigma Chemical Co.) in the same digestion buffer. After necessary, the pH was adjusted to 7.5. Controls contained collagen in digestion buffer with 2 mM APMA but with no collagenase.

For immunohistological and immunoassays, SDS-PAGE of MMP-1, MMP-8, and MMP-13 cleaved purified native human collagens were performed as previously described (52) under denaturing conditions using 10%, 1-mm thick, 7 x 8 cm mini-Protean gels stained with Coomassie Blue R-250 (Bio-Rad Laboratories, Mississauga, Ontario, Canada) in 40% vol/vol methanol and 10% vol/vol acetic acid in distilled water. Electrophoretic transfers to nitrocellulose membranes (Bio-Rad) were performed as previously described (5), with the following exceptions. The PBS-3% BSA blocked membranes were incubated overnight at 4°C with either anti-COL2-3/4C or anti-COL2-3/4N (anti-COL2-3/4N F(ab)²) preparations diluted 1:200 in PBS-3% BSA-Tween. After three 10-min washes in PBS-1% BSA-Tween, the membranes were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (F(ab)²) fragment diluted 1:3,000 with PBS-3% BSA-Tween. The membranes were cut 3 x 10 mm washes in PBS-1% BSA-Tween and then rinsed well in distilled water, before adding alkaline phosphatase substrate solution from a commercial kit (Bio-Rad) using 5-bromo-4-chloro-3-indolyolphosphate and nitroblue tetrazolium. After optimal color development (10-20 min), the reaction was stopped by washing off the substrate solution with distilled water.

Extraction of human articular cartilage for the measurement of collagenase-cleaved, denatured, and total type II collagen contents. Preliminary studies using MMP-1 and MMP-13 cleaved human type II collagens (not shown) established that the levels of the COL2-3/4C neoepitope, as detected by ELISA, were not significantly affected by α-chymotrypsin treatment under the conditions previously described to extract denatured collagen from articular cartilage (6). Therefore, femoral condylar articular cartilages from 19 normal and 26 osteoarthritic joints were diced and incubated overnight at 37°C with 1.0 mg/50–75 mg cartilage of α-chymotrypsin in 50 mM Tris-Cl, pH 7.6 (with the protease inhibitors: 1 mM EDTA, 1 mM iodoacetamide, and 10 μg/ml pepstatin A; Sigma Chemical Co.). The α-chymotrypsin activity was inhibited with 20 μl (160 μg/ml final concentration) of N-tosyl-L-phenylalanine-chloromethyl ketone (TPCK; Sigma Chemical Co.). After 20 min at 37°C, the samples were centrifuged and the supernatants were removed and assayed for the COL2-3/4C neoepitope (as described above) and COL2-3/4m hidden epitope (6). The percent cleavage cartilage residues were digested overnight at 56°C with 1.0 mg/ml proteinase K in Tris solution containing the same protease inhibitors and then boiled for 20 min to denature the enzyme. Total type II collagen content of each sample was determined from the collective amount of COL2-3/4m epitope in both the α-chymotrypsin extract and the proteinase K digestion. The percent denatured collagen was determined from the amount of COL2-3/4m epitope in the α-chymotrypsin extract expressed as a percentage of the total collagen content. The percent cleaved collagen was calculated from the amount of COL2-3/4C neoepitope assayed in the α-chymotrypsin extract expressed as a percentage of the total collagen content.

Mankin grading. Frozen sections that were prepared for extraction immunoassay were stained with Safranin O and Fast Green and were graded for degenerative changes as described by Mankin et al. (53). The maximum grade was 13, as there was no calcified cartilage in the specimens used.

Human OA articular cartilage explant cultures. Full-depth human femoral condylar articular cartilages in serum-free culture media (see below) were removed from the subchondral bone within 6 h of its removal from three OA patients undergoing joint replacement surgery. The cartilage was diced into ~1-mm² pieces and placed for 1 h in basic culture medium consisting of DME with 3.6 mg/ml of Hepes (GIBCO BRL), 100 U/ml penicillin and 100 μg/ml streptomycin to which was added 2.5 μg/ml amphotericin B (Fungizone; GIBCO BRL). It was then transferred to the same basic culture medium (DME-Hepes) containing 1,000 U/ml penicillin and 1 mg/ml streptomycin for 30 min. After a final wash in basic culture medium, 40–50 mg wet weight of cartilage was transferred to each well of a 24-well tissue culture plate (Falcon Labware, Becton Dickinson & Co., Lincoln Park, NJ) containing 1 ml/well of basic culture medium supplemented with 1 mg/ml BSA (Sigma Chemical Co.), 50 μg/ml L-aspartic acid (Sigma Chemical Co.), and 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite (ITS; Boehringer Mannheim, Mannheim, Germany). The cartilage was precultured for four days at 37°C, 5% CO₂ and 95% relative humidity. The media were removed and quadruplicate samples were cultured either in the basic culture medium (+ BSA, ascorbic acid, ITS; Boehringer Mannheim) supplemented with 10, 2.5, and 1.0 mM of RSH2481 (Roche Biosciences, Palo Alto, CA), a synthetic, preferential MMP-13, carboxylate inhibitor (Kᵢ [mM]: 1,100, MMP-1; 32, MMP-2; 19, MMP-3; 18, MMP-8; 0.08, MMP-13) in 0.1% DMSO, or in medium with only 0.1% DMSO, the inhibitor vehicle. Media and inhibitors were replaced every 2 d of culture and the conditioned media were frozen at −20°C until assayed for the COL2-3/4C neoepitope (as described above). Cultures were terminated after 12 d. The experiment was repeated twice with the articular cartilage from two other patients.

The measurement of total collagen synthesis. To determine the effect of inhibitor RS 102,481 on type II collagen synthesis, explants of human OA articular cartilage were cultured as described above, with or without the highest concentration (10 nM) of inhibitor. On days 14, 16, and 18 of culture, 15 μCi of [³H]proline was added to each ml of medium. The conversion of radiolabeled proline into tritiated hydroxyproline and total explant, as an indicator of type II collagen synthesis, was determined after day 20 in two independent cultures, using the method described by Tyler and Benton (54).

Statistical analysis. Spearman rank correlations were used for analyzing relationships between different parameters used in the study. Mann-Whitney U tests were used to compare the nonarthritic (normal) and OA groups, in terms of the collagenase-generated neoepitopes detected by immunoassay.
Results

Polyclonal antibodies to mammalian collagenase-generated neoepitopes of α1 (II) collagen TCβ3 and TCβ8

Direct-binding ELISAs for determination of antibody titers. Sera obtained from rabbits immunized with OVA conjugates of the neoepitope peptides and F(ab')2 preparations of these sera were initially characterized by direct binding ELISAs. In the immunochemical analyses, the F(ab')2 preparations were used to ensure specific binding. Each of the antineoepitope antibodies reacted similarly with its own immunizing peptide (Fig. 2) which was conjugated to BSA and bound to the microtiter plates to improve immunoreactivity. None of the antibodies showed any significant immunoreactivity to BSA, native or heat-denatured human type II collagen (Fig. 2), or native or heat-denatured human type I, III, and X collagens (data not shown).

Inhibition ELISAs for neoepitope characterization. A panel of nonconjugated peptides with amino acid additions to (+1, +2, +3) and deletions from (−1, −2, −3) either the COOH terminus of COL2-3/4C or the NH2 termini of COL2-1/4N1 and COL2-1/4N2 immunizing peptides were prepared. These were used in competitive ELISAs to determine the specific amino acid sequences recognized by each antibody and thereby verify that they were indeed antineoepitope antibodies. Removal or addition of one residue resulted in a loss of inhibition ranging from 80 to 100%, depending upon the peptide concentration, for the COL2-3/4C antibody (Fig. 3 A) and complete loss of inhibition for the COL2-1/4N2 antibody (Fig. 3 B). For the COL2-1/4N1 antibody, it was only after three amino acids were deleted from the NH2 terminus (which is then the NH2 terminus of the COL2-1/4N2 peptide) that there was a complete loss of inhibition (Fig. 3 C). This is significant in that it shows that the COL2-1/4N1 antibody will not recognize the amino acids comprising the NH2 terminus of the secondary cleavage site produced by mammalian collagenases (Fig. 1).

The two 16mer overlapping peptides designed to represent the amino acid sequences bridging both the primary and secondary collagenase cleavage sites of type II collagen α1 chains did not significantly inhibit any of the antibodies (data not shown).

Inhibition ELISAs using antibodies to COL2-3/4C neoepitope demonstrating comparative reactivity to immunizing peptide and collagenase-cleaved and uncleaved fibrillar collagens

To further validate the specificity of the antibodies to the COL2-3/4C neoepitope, complete digests by MMP-1 and MMP-13 of collagen (verified by Coomassie staining of SDS-PAGE gels) and heat-denatured type I, II, and III collagen solutions at concentrations of 29, 58, 115, 230, 460, and 920 μg/ml were examined in inhibition ELISAs along with the nonconjugated COL2-3/4C immunizing peptide. Typical standard curves plotted as the percent inhibition against log of the competing antigen concentration (μg/ml) are shown in Fig. 4 A. Heat-denatured type I and II collagens showed no reactivity. Otherwise, positive immunoreactivity always exhibited excellent parallelity for each analysis. On a molar basis, the inhibition was very similar for MMP-1 and MMP-13 cleaved type II collagens and the COL2-3/4C peptide (Fig. 4 B). The μM concentration of the COL2-3/4C epitope was calculated as described in Methods. Although there was parallelity between both the MMP-1 and MMP-13 cleaved type I collagen solutions and the COL2-

Figure 2. Immunoreactivities of collagenase antineoepitope F(ab')2 antibody subunits with immunizing peptides and human type II collagen as determined by direct-binding ELISAs. Immulon-2 microtiter plates were coated with 1 μg/well of each of the immunizing peptides conjugated to BSA (●). As well, plates were coated with 1 μg/well of BSA (○) or 2 μg/well of either triple helical (■) or heat-denatured (80°C for 20 min) (□) human type II collagens. After blocking unbound sites with BSA, serial dilutions of the collagenase antineoepitope F(ab')2; preparations (in PBS-1% BSA-Tween [from 1:5 to 1:1280]) were added in duplicate to the wells of their respective plates and processed as described in Methods. Shown are the results of the ELISAs for the COL2-3/4C (A), COL2-1/4N1 (B), and COL2-1/4N2 (C) F(ab')2 preparations.
The cleaved type II collagen was much more inhibitory than the cleaved type I collagen (Fig. 4B). This may in part be due to the fact that the sequence in human type I and II collagen for the five residues amino-terminal to the cleavage site is identical. We do not yet know how important hydroxylation is at Pro771, nor do we know how important the threonine residue is at position 770 in the type I collagen and 1 chain, which is a proline residue in the type II 1 chain. Studies are in progress to address these issues. Neither the MMP-cleaved nor the heat-denatured type III collagen solutions showed inhibition (data not shown). The COL2-3/4C peptide was used as the standard in all subsequent ELISAs.

Figure 3. Epitope specificities of the anti-neoepitope antisera determined using competing synthetic peptides in inhibition ELISAs. Varying dilutions of the non-conjugated immunizing peptides (●) were added to 1:200 dilutions of their respective F(ab')2 preparations in 96-well round bottom microtiter plates for 1 h at 37°C. Then 50 µl was transferred from each well to the equivalent wells of Immulon-2 microtiter plates precoated with the respective immunizing peptides conjugated to BSA. The plates were then processed as described in Methods for the development of a standard inhibition curve for each neoepitope antiserum. For the COL2-3/4C (A) and the COL2-1/4N2 (B) antibodies, shown are the results of ELISAs performed with synthetic peptides that had either 1 amino acid added to (○) or deleted from (□) the end of the immunizing peptide corresponding to the cleaved termini of the alpha chain fragments. The sequences of these peptides are shown in the individual legends for each antineoepitope antiserum. For the COL2-1/4N1 antibody (C), residues were either added to (open circles) or deleted from (open squares) the NH2-terminus of the immunizing peptide. It was only when three residues were deleted from the NH2-terminus (△) of the COL2-1/4N1 peptide and not one residue, as for the COL2-3/4C and COL2-1/4N2 peptides, that there was a complete loss of inhibition with the COL2-1/4N1 antibody.

Figure 4. ELISA to show specificity of the COL2-3/4C antibody for the COOH terminal neoepitope of α1(II) collagen chains created by the digestion of triple helical type II collagen by the collagenases MMP-1 and MMP-13. Heat-denatured and collagenase-cleaved (MMP-1 and MMP-13) human type I, II, and III collagens (digestions were complete as shown by Coomassie staining of SDS-PAGE gels) 3/4C peptide, the cleaved type II collagen was much more inhibitory than the cleaved type I collagen (Fig. 4B). This may in part be due to the fact that the sequence in human type I and II collagen α chains for the five residues amino-terminal to the cleavage site is identical. We do not yet know how important hydroxylation is at Pro771, nor do we know how important the threonine residue is at position 770 in the type I collagen α1 and α2 chains, which is a proline residue in the type II α1 chains. Studies are in progress to address these issues. Neither the MMP-cleaved nor the heat-denatured type III collagen solutions showed inhibition (data not shown). The COL2-3/4C peptide was used as the standard in all subsequent ELISAs. Similar analyses for the antibodies to the COL2-1/4N2 epitope are not shown because they were not used in immunoassays in this study.

MMP-1, MMP-8, and MMP-13 produce sequential cleavages in type II collagen

Acid-soluble human type II collagen was cleaved with MMP-1 for up to 72 h, with aliquots removed and inactivated by the addition of EDTA at time 0, 0.5, 1, 3, 5, 24, 48, and 72 h. There was almost complete cleavage of the α1(II) chains by 5 h, as shown by Coomassie staining of SDS-PAGE separated digests (Fig. 5A). All the antineoepitope antibodies reacted with their were assayed in inhibition ELISAs at concentrations of 20, 58, 115, 230, 460, and 920 µg/ml of digestion buffer. A standard curve for the COL2-3/4C peptide (●) was also constructed using doubling dilutions from 50 to 0.1 µg of the peptide/ml of buffer in the same inhibition ELISA. Shown are typical standard curves plotted as percent inhibition against the log of the competing antigen concentrations as µg/ml (A) and on a µM basis (B). Symbols show MMP-1 (●, ○) and MMP-13 (●, □) cleaved type I and type II collagens, respectively, and heat-denatured type I (I HDC; ▲) and type II (II HDC; ▼) collagens.
respective cleaved fragments but showed no reactivity with £ chains on Western blot analyses. The COL2-3/4C antibody stained both the single (€TC\textsuperscript{A}) and dimeric (€TC\textsuperscript{B}) £ chain fragments created by MMP-1 cleavage of type II collagen with increasing intensity up to 48 h (Fig. 5 B). The COL2-1/4N1 antibody stained both the single (€TC\textsuperscript{B}) and dimeric (€TC\textsuperscript{B}) forms of TC\textsuperscript{B} alpha chain fragments produced by MMP-1 cleavage of intact type II collagen. Moreover, the COL2-1/4N2 antibody first stained TC\textsuperscript{B} weakly at 3 h and then with increasing intensity up to 72 h as the neoepitope COL2-1/4 N1 disappeared (Fig. 5 D). This demonstrated a loss of the primary collagenase cleavage site neoepitope (COL2-1/4N1) and the appearance of the secondary cleavage site neoepitope (COL2-1/4N2) with MMP-1 digestion of type II collagen. This was confirmed by microsequencing (data not shown).

Type II collagen cleavage by MMP-8 was generally slower under the conditions of this study and the complete cleavage of 1(II) chains occurred only after 24 h of digestion (Fig. 6 A).
The COL2-3/4C antibody stained TC\textsuperscript{A} on Western blot analysis with increasing intensity up to 72 h (Fig. 6 B). The COL2-1/4N1 antibody stained TC\textsuperscript{B} with increasing intensity up to 24 h and then progressively declined thereafter (Fig. 6 C). The COL2-1/4N2 antibody stained TC\textsuperscript{C} with increasing intensity from 5–72 h, as the staining intensity for the COL2-1/4N1 neoepitope weakened (Fig. 6 D).

The primary cleavage of the \( \alpha_1(II) \) chains by MMP-13 was much faster than that by MMP-1 or MMP-8 and was almost complete after only 30 min (compare Fig. 7 A with Fig. 5 A and Fig. 6 A), confirming the results of recent studies comparing the rates of cleavage of type II collagens by MMP-1 and MMP-13 (10, 14, 26). The primary cleavage site neoepitope (COL2-3/4C) was reduced in content after only 5 h digestion indicating further cleavage of TC\textsuperscript{A} (Fig. 7 B). Moreover, the NH\textsubscript{2} terminal primary cleavage site neoepitope (COL2-1/4N1) increased up to 1 h but was then reduced and had disappeared after 3 h (Fig. 7 C). In contrast, the secondary cleavage site neoepitope (COL2-1/4N2) was detectable within 30 min and increased up to 5 h before decreasing. After 24 h, it was no longer detectable (Fig. 6 D).

This secondary cleavage produced by MMP-13 was recently described through the NH\textsubscript{2} terminal sequencing of MMP-13 cleaved human type II collagen TC\textsuperscript{D} (14). To confirm this loss of the COL2-1/4N1 neoepitope (Fig. 7 C), the MMP-13 digestion samples used for this immunoblot were assayed in an inhibition ELISA and no detectable COL2-1/4N1 epitope was found after 5 h of MMP-13 cleavage (data not shown). It is worth noting that the percentage recovery of this neoepitope, based on the amount of epitope present in the intact collagen, reached a maximum of only 21% after 60 min. This suggests a rapid removal of the NH\textsubscript{2} terminal Leu-Ala-Gly tripeptide from TC\textsuperscript{D}. Further studies are in progress to compare, by immunoassay, the relative rates of digestion of type II and other collagens by MMP-1, MMP-8, and MMP-13.

None of the antibodies recognized the cyanogen bromide generated CB10 peptides of human type II collagen, which contain the intact collagenase cleavage site of triple helical collagen \( \alpha_1(II) \) chains (data not shown). This supports the ELISA results described above showing nonreactivity of the neoepitope antisera for 16mer synthetic peptides representing the amino acid sequences bridging the primary and secondary collagenase cleavage sites in human type II collagen. Together these results confirm the classification of the COL2-3/4C and COL2-1/4N2 antibodies as antineoepitope antibodies recognizing only the termini of \( \alpha_1 \) chains of TC\textsuperscript{A} and TC\textsuperscript{B} produced by the action of mammalian collagenases on type II triple helical collagens.

None of the antibodies reacted to cleaved human type III collagen fragments produced by either MMP-1, MMP-8, or MMP-13, but all reacted to similarly cleaved fragments of human type I collagen (data not shown), confirming the results of the immunoassays that were mentioned above. Type X collagen is a minor cartilage collagen that has recently been demonstrated in OA articular cartilage (35) and it is susceptible to cleavage by the mammalian collagenases. None of the antibodies showed reactivity to intact or MMP-1 cleaved alpha chain fragments of this collagen (data not shown).

The collagenase-generated neoepitope (COL2-3/4C) is significantly elevated in OA articular cartilage and is correlated to the amount of type II collagen denaturation

Human articular cartilage from the femoral condyles of 26 OA (Mankin grade 3–12) and 19 nonarthritic (Mankin grade 1–5) joints were treated with \( \alpha \)-chymotrypsin to extract denatured type II collagen and to assay for COL2-3/4C neoepitope content. The median of the levels of COL2-3/4C epitope extracted from the OA specimens was significantly higher \((P = 0.0002)\) than that of the nonarthritic articular cartilages (Fig. 8). Moreover, when the collagenase-generated neoepitope (expressed as a percentage of total collagen present) was compared to the percentage denatured collagen, based on COL2-3/4m intrachain epitope content in each extract, there were significant correlations found for both the nonarthritic \((r = 0.503, P = 0.028;\ Fig. 9 A)\) and the OA \((r = 0.536, P = 0.0048;\ Fig. 9 B)\) samples. The content of intrachain epitope was about twice that of the collagenase-generated neoepitope (by immunoas-
say) suggesting an increased half-life of the former in both groups of cartilages.

No significant correlations (by Spearman rank analyses) were noted between Mankin grade and either percentage cleaved or percentage denatured collagen (data not shown). However, it is interesting to note that there was a tendency towards an increase in percentage collagenase cleaved collagen with increasing age ($P = 0.054$), that may reach significance with the sampling of more specimens.

Preferential inhibition of MMP-13 abrogates the release of the collagenase-generated COL2-3/4C neoepitope from human OA articular cartilage explants

Explant cultures of human condylar articular cartilages from three different patients were analyzed for the release of COL2-3/4C neoepitope into culture media. There was a steady release of COL2-3/4C into culture media over the 12 d of culture. An example of one of the three studies (OA sample 15) is shown (Fig. 10). In an attempt to determine if and which of the collagenases MMP-1, MMP-8, and MMP-13 may be responsible for the generation and/or release of the COL2-3/4C neoepitope, explants from the same specimens were also incubated with different concentrations (1–10 nM) of a preferential inhibitor of MMP-13, RS 102,481 (see Methods for inhibition profile). In all these studies, the inhibitor produced a significant reduction in the release of the COL2-3/4C neoepitope into the culture media over the 12 d of culture (Fig. 10).

That RS 102,481 did not have a detrimental effect on chondrocyte viability was determined by the measurement of total collagen synthesis. In two separate experiments there were no significant differences between the levels of tritiated hydroxyproline synthesized in OA cartilages cultured with or without the inhibitor at the highest concentration, namely 10 nM (Table I).

Discussion

We have previously shown that OA, which involves the progressive degeneration of articular cartilages, is characterized

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**Figure 8.** Levels of COL2-3/4C neoepitope in α-chymotrypsin extracts of human articular cartilage. Articular cartilage was removed from the femoral condyles of 19 nonarthritic (patient ages: 20–69 yr; median, 60 yr) and 26 OA (patient ages: 44–85 yr; median 71 yr) joints and digested overnight at 37°C with 1 mg/50–75 mg wet weight of α-chymotrypsin in 50 mM Tris-HCl, pH 7.6 (+ the protease inhibitors: 1 mM EDTA, 1 mM iodoacetamide, and 10 μg/ml pepstatin A). After inhibition of enzyme activity with 160 μg/ml TPCK, the samples were centrifuged and the supernatants were assayed for COL2-3/4C neoepitope as discussed in Methods. The levels of the neoepitope are expressed as pmole of peptide (based on a molecular weight of 608 D) per mg wet weight of cartilage. The absolute values of the two highest neoepitope concentrations for the OA extracts are shown in the figure. A significant difference between the median of neoepitope content in the nonarthritic (○) and the OA (●) cartilages was determined by Mann-Whitney analysis and the $P$ value is shown in the figure.

**Figure 9.** Correlation between the percent cleaved and the percent denatured type II collagen in α-chymotrypsin extracts of human articular cartilage. The same articular cartilage specimens digested with α-chymotrypsin and described in the legend of Fig. 8, were further digested overnight at 56°C with 1 mg proteinase K in Tris-HCl, pH 7.6 (+ proteinase inhibitors) and then boiled for 20 min to inactivate the enzyme. The total type II collagen content was determined from the amount of COL2-3/4m epitope assayed in both the α-chymotrypsin extracts and the proteinase K digests, as described in Methods. The percent denatured collagen represented the amount of COL2-3/4m and the percent cleaved collagen was the amount of COL2-3/4C in the α-chymotrypsin extracts and both are expressed as a percentage of the total type II collagen. Statistically significant relationships between percent cleaved and percent denatured type II collagen in (A) the nonarthritic (○) and (B) the OA (●) articular cartilage specimens were determined by Spearman rank correlational analysis and the corresponding $n$, $r$ and $P$ values are shown in the figure.
These studies show that there is continued processing at the carboxy-terminus (neoepitopes) of this cleavage site in type II collagen. We used these antibodies to further characterize the amin- and carboxy-termini (neoepitopes) of this cleavage site in type II collagen. We have produced antibodies that recognize the amino-terminal neoepitope of TC\textsuperscript{A} and TC\textsuperscript{B}, respectively. We have produced antibodies that recognize the amino- and carboxy-termini (neoepitopes) of this cleavage site in type II collagen. We used these antibodies to further characterize the cleavage of type II collagen by MMP-1, MMP-8, and MMP-13. These studies show that there is continued processing at the amino terminus of TC\textsuperscript{B} by all these collagenases producing first a secondary cleavage site at Gly\textsubscript{270}–Gln\textsubscript{273}, and then a further cleavage(s) leading to the release or cleavage of these neoepitopes. An antibody was prepared against the secondary amino-terminal neoepitope of TC\textsuperscript{B} and was used to demonstrate its production by MMP-1, MMP-8, and MMP-13. There was evidence for continued processing at the COOH terminus of TC\textsuperscript{B} by MMP-13, albeit at a much slower rate compared to TC\textsuperscript{A} NH\textsubscript{2}-terminal processing. Moreover, the apparent greater resistance of the COOH terminus of TC\textsuperscript{B} to secondary proteolysis, as compared to the NH\textsubscript{2} terminus of TC\textsuperscript{B}, suggested to us that it would be more useful to assay for the COL2-3/4C neoepitope as an indicator of collagenase cleavage of type II collagen, as was done for the immunochemical analyses in this study.

Significant correlations were noted between percentage cleaved collagen (as determined by immunoassay for the COL2-3/4C neoepitope) and percentage denatured collagen (calculated from immunoassay for the COL2-3/4m intrachain epitope) in the immunoassays of extracts of human OA articular cartilage. These correlations were evident in both nonarthritic and OA specimens. These findings support the dogma that collagenase activity plays a key role in the denaturation and subsequent degradation of type II collagen in normal and OA articular cartilages. However, in view of the differences in epitope content being usually lower for the collagenase-generated epitope, it would seem that this epitope is preferentially lost from the cartilage. This loss of the epitope could be detected in culture. Because it was inhibited by a preferential MMP-13 inhibitor, there is thus evidence presented here to suggest that MMP-13 may play a significant role in the cleavage of type II collagen.

Recent studies of normal and OA cartilages have provided evidence for the expression of MMP-1 in normal and OA cartilages (26) and the increased expression and protein content of MMP-8 (29) and MMP-13 (14, 26) in OA cartilages. With the new technology described in this paper, we can now show that the increased expression of MMP-13 and MMP-8 is associated with increased cleavage by collagenase(s) of type II collagen in OA. Other collagenases responsible for this cleavage remain to be identified in human OA articular cartilage, but clearly all three are candidates. By demonstrating the production of the cleavage site that is only produced by collagenases, our study is the first to definitively implicate the activity of a collagenase in the cleavage of type II collagen in human articular cartilage and its increased activity in OA. Whether MMP-13 does in fact play a major role in this cleavage, as is suggested by the inhibitor studies, is presently being investigated by us. In view of the K\textsubscript{i} values, it is conceivable that the inhibitor may also block MMP-8 activity at the higher concentrations. However, it is unlikely that MMP-1 would be affected. In separate studies of cultured bovine articular cartilage explants stimulated with IL-1, we can show a similar significant reduction in the detection of the COL2-3/4C neoepitope in media when these cultures included the same collagenase inhibitor (Billinghurst, R.C., M. Ionescu, A. Reiner, W. Wu, J. Chen, H. Van Wart, and A.R. Poole, manuscript in preparation). Thus, combined with its capacity to preferentially cleave type II collagen as demonstrated here and by others (10, 14), MMP-13, and possibly MMP-8, are identified as potential targets for therapeutic intervention in OA.

In parallel studies that will be published separately, we have shown that in cultured OA cartilage there is an increased release over normal tissue of the COL2-3/4C neoepitope. This combined with our ability to detect type II collagen degradation products in body fluids (Billinghurst, R.C., M. Ionescu, and A.R. Poole, manuscript in preparation) means that the increased degradation of type II collagen by collagenase in OA

Table I. The Lack of an Effect of the Synthetic Collagenase Inhibitor RS 102,481 (10 nM) on Total Collagen Synthesis in Explant Cultures of Human OA Articular Cartilage

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<tr>
<th></th>
<th>Control</th>
<th>RS 102,481</th>
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<tr>
<td></td>
<td>cpm/mg wet weight</td>
<td>cpm/mg wet weight</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>1968.3 (534.6)</td>
<td>1911.4 (770.7)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>941.4 (133.8)</td>
<td>1131.9 (38.7)</td>
</tr>
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The amount of [\textsuperscript{3}H]hydroxyproline synthesized from [\textsuperscript{3}H]proline was determined in cartilage that had been labeled with [\textsuperscript{3}H]proline on days 14–20 of culture, as described in Methods. Values are expressed as mean (SD).
is a fundamental feature of this arthritis, and that its measurement in vivo may be of value in studying disease activity and its clinical management.

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