Fragmentation of Proteins in Cartilage Treated with Interleukin-1

**SPECIFIC CLEAVAGE OF TYPE IX COLLAGEN BY MATRIX METALLOPROTEINASE 13 RELEASES THE NC4 DOMAIN**

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Degradation of bovine nasal cartilage induced by interleukin-1 (IL-1) was used to study catabolic events in the tissue over 16 days. Culture medium was fractionated by two-dimensional electrophoresis (isoelectric focusing and SDS-PAGE). Identification of components by peptide mass fingerprinting revealed released fragments representing the NC4 domain of the type IX collagen α1 chain at days 12 and 16. A novel peptide antibody against a near N-terminal epitope of the NC4 domain confirmed the finding and indicated the presence of one of the fragments already at day 9. Mass spectrometric analysis of the two most abundant fragments revealed that the smallest one contained almost the entire NC4 domain cleaved between arginine 258 and isoleucine 259 in the sequence -ETCNELPAR258-COOH NH2-ITP-. A larger fragment contained the NC4 domain and the major part of the COL3 domain with a cleavage site between glycine 400 and threonine 401 in the sequence -RGPGGPPGGPSG400-COOH NH2-TIG-. The presence of multiple collagen α1 (IX) N-terminal sequences demonstrates that the released molecules were cleaved at sites very close to the original N terminus either prior to or due to IL-1 treatment. Matrix metalloproteinase 13 (MMP-13) is active and cleaves fibromodulin in the time interval studied. Cartilage explants treated with MMP-13 were shown to release collagen α1 (IX) fragments with the same sizes and with the same cleavage sites as those obtained upon IL-1 treatment. These data describe cleavage by an MMP-13 activity toward non-collagenous and triple helix domains. These potentially important degradation events precede the major loss of type II collagen.

In hyaline cartilage type IX collagen is a minor constituent of the fiber network, and type II collagen is the major constituent. The type IX collagen molecule (1) is a heterotrimer consisting of polypeptide chains α1, α2, and α3 (2). It belongs to the fibril-associated collagens with interrupted triple helix. Each chain contains three triple helical (collagenous) domains, COL1, -2, -3, and -4, surrounded by four non-triple helical (non-collagenous) domains, NC1, -2, -3, and -4 (Fig. 1) (3). The domain numbers are counted from the C terminus. Using electron microscopy, it has been shown that type IX collagen decorates the surface of type II collagen fibrils and that the NC4 domain forms a globular structure, which together with the stalk-like COL3 domain protrudes out from the type II collagen fibril (4). Type IX collagen is covalently cross-linked to the type II collagen fibrils through binding to both type II collagen and other type IX collagen molecules (5–7). These bonds render extraction of type IX collagen from mature cartilage virtually impossible by agents that do not cleave peptide bonds. The NC4 domain has been shown to have an affinity for a number of molecules, for example heparin and cartilage oligomeric matrix protein (8–10), whereas the COL domains interact with matrilin-3 (11).

Mutations in the interstitial cartilage oligomeric matrix protein MATN-3 and COL9 have been found in patients with multiple epiphyseal dysplasia, a rare inherited heterogeneous range of diseases, affecting growth and ossification of the epiphysis (12). Mice lacking collagen α1 (IX) appear normal at birth, but with age they develop a degenerative joint disease with features of human osteoarthritis (13). Interestingly all types of cartilage fibrils found in normal newborn, adolescent, and adult mice were also found in the mice lacking type IX collagen. Thus, type IX collagen is believed to be required for long-term tissue stability rather than fibril formation in cartilage (14).

MMP-13 seems to be a key factor in cartilage degradation appearing in osteoarthritis (15–19) and has also been found in articular cartilage and synovial fluids from rheumatoid arthritis patients (20). Many components of articular cartilage have been shown to be susceptible to degradation by MMP-13 in vitro, e.g. collagen type II, type IX, and possibly type IX (21, 22). Also non-collagenous molecules, e.g. aggrecan (23), fibromodulin (24), and biglycan (25, 26), have been shown to be cleaved by MMP-13.

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**The on-line version of this article (available at http://www.jbc.org) contains supplemental data 1 and data 2, which includes Tables A–C.**

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The abbreviations used are: COL, collagenous; NC, non-collagenous; MMP-13, matrix metalloproteinase 13; IL-1, interleukin 1; Chaps, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; Asp-N, endoproteinase Asp-N; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; LC ESI, liquid chromatography on line with electrospray ionization; MS/MS, tandem MS; HPLC, high pressure liquid chromatography.
MMP-13 is a collagenase with additional activity toward other proteins. Its expression is restricted to occasions with elevated extracellular remodeling such as in fetal bone development and in growing chondrosarcomas (27). Active MMP-13 is often found in disease tissue where the enzyme may generate protein fragments characteristic for the degenerative disease.

In the joint diseases of rheumatoid arthritis and osteoarthritis the articular cartilage undergoes a destructive process with degradation of its molecular constituents. Among the factors controlling the degradation are catabolic cytokines such as interleukin-1 (IL-1) and tumor necrosis factor α. These two inflammatory cytokines have been shown to induce cartilage resorption (28–30). IL-1 and tumor necrosis factor α were found to induce MMP-13 gene expression in chondrocytes (31) and were identified in synovial fluids from patients with rheumatoid arthritis (32, 33). Bovine nasal cartilage stimulated with IL-1 released active MMP-13 first after 6 days of culture, suggesting that it might be secondary to other effects (24). The active MMP-13 was suggested to be responsible for the subsequent fibromodulin cleavage.

We have now used a model of events in joint disease cartilage by applying IL-1 stimulation of bovine nasal cartilage to detect release of fragments generated by proteolysis. In the middle to late phase of the tissue culture, we found that a fragment comprising almost the entire collagen α1 (IX) NC4 domain was released from the cartilage. A C-terminal cleavage site close to the COL3 (Fig. 1 III) was identified with differential enzymatic degradation and reversed phase liquid chromatography on line with electrospray ionization mass spectrometry (LC ESI MS). A larger fragment consisting of the NC4 domain and a major part of the COL3 domain represented a triple helical region cleavage (Fig. 1 IV). The same fragments were released from cartilage by treatment of tissue explants with MMP-13.

**EXPERIMENTAL PROCEDURES**

**Bovine Nasal Cartilage Explant Culture**

Bovine nasal cartilage core biopsies were prepared essentially as described previously (24). The cores were rinsed with a final wash solution of cold Dulbecco’s modified Eagle’s medium with 6× PEST (600 units/ml penicillin, 600 μg/ml streptomycin) before use. The cores were wiped dry on filter paper prior to weight determination, and approximately 50 mg of tissue (three cores) was placed in each well (24-well plate Costar) and precultured for 1 day in 500 μl of Dulbecco’s modified Eagle’s medium containing 1× PEST, 100 μg/ml bovine serum albumin (tissue culture grade from Sigma), and 50 μg/ml L-ascorbic acid 2-phosphate (Sigma). The medium was removed at day 0, and new culture medium with (10 ng/ml) or without IL-1α (Roche Applied Science) was added. The medium was changed to an identical fresh medium at days 3, 6, 9, and 12, and the spent medium was stored at −80 °C until processed. Culture was ended at day 16, and the last medium was separated from the remaining cartilage, and both medium and tissue were stored at −80 °C.

**MMP-13 Digestion of Bovine Nasal Cartilage**

Approximately 3 mg of cartilage was washed in phosphate-buffered saline and incubated at 37 °C for 24 h in 80 μl of 150 mM NaCl, 50 mM Tris-HCl, 5 mM CaCl2, 0.01% NaN3, 0.01% Brij 35 with or without 7.5 μg of recombinant human MMP-13 (generously provided by Dr. Peter Mitchell, Pfizer, Groton, CT). Medium was then separated from cartilage, and both tissue and medium were frozen.

**Electrophoresis**

Prior to SDS-PAGE the culture medium and MMP-13 digest solutions were precipitated with 10 volumes of 96% ethanol overnight. Precipitates were recovered by centrifugation at 10,000 × g for 30 min and washed once with 10 volumes of 96% ethanol with 50 mM sodium acetate. All steps were performed at 4 °C. Separations were done with SDS-polyacrylamide gels (either linear 16% or gradient 4–20%) (34).

**One-dimensional Gel Electrophoresis**—The Bio-Rad Mini-Protein II gel electrophoresis system was used in combination with PowerPac 200. The precipitate pellets were dissolved in sample buffer (modified Laemmli containing 0.125 M Tris-HCl, 2 mM EDTA, 5% SDS, 20% glycerol, and bromphenol blue. The samples were reduced with 2 mM dithiothreitol (Sigma) at 56 °C for 1 h and alkylated for 1.5 h at room temperature (dark) with 5 mM iodoacetamide (Sigma). The samples were applied to SDS-polyacrylamide gels (16%) using the Laemmli buffer system containing 2 mM EDTA.

**Two-dimensional Gel Electrophoresis**—The Protean IEF cell and Protean II xi cell (Bio-Rad) were used for two-dimensional gel electrophoresis (35). The precipitate pellets were dissolved in rehydration buffer containing 6 M urea, 2 M thiourea, 4% Chaps, 2 mM tributylphosphine (Bio-Rad), and 0.2% Bio-Lyte 3/10 (Bio-Rad). The samples were applied through active rehydration and focused on ready made immobilized pH 3–10 gradient strips 17 cm long (Bio-Rad) according to instructions supplied by the manufacturer. Wicks (Bio-Rad) were applied between the gel and the electrodes after rehydration. Gel strips were stored at −20 °C until use. They were first equilibrated for 15 min in 2 ml of equilibration buffer (6 M urea, 0.375 M Tris, 2% SDS, 20% glycerol) containing 2% (w/v) dithiothreitol and then for a further 15 min in 2 ml of equilibration buffer containing 2.5% (w/v) iodoacetamide. Gel strips were quickly washed in running buffer prior to applying strips on top of SDS-polyacrylamide gels (4–20% gradient) using the Laemmli buffer system containing 2 mM EDTA. The molecular weight standard was pipetted onto a wick, allowed to dry briefly, and applied next to the acidic end of the isoelectric focusing strip. Gel strips and standard were sealed with 1% agarose containing bromphenol blue.
Staining and Molecular Weight Marker—Gels were stained with Coomassie Brilliant Blue G-250 (Serva) (36). 5 µl of SeeBlue® Plus2 (Invitrogen) was used to indicate molecular weights.

Collagen α1 (IX) NC4 Peptide Antiserum

A 15-mer peptide sequence of the N-terminal portion of the type IX collagen α1 NC4 domain showing potential as an immunogen and no homology to other sequences found in a Basic Local Alignment Search Tool (BLAST) search was selected for immunization. The sequence is identical in human, mouse, rat, cow, chicken, dog, chimpanzee, and rhesus monkey and was represented in medium from IL-1-stimulated cartilage. An N-terminal cysteine was added for coupling such that the sequence for immunization was NH$_2$-CG$_{50}$QDDLPGFDLISQFQ$_{64}$-CONH$_2$. The peptide coupled to keyhole limpet hemocyanin was used for immunization of a rabbit. Peptide synthesis and antibody production were custom services provided by Innovagen AB (Lund, Sweden). The peptide and the antiserum will be referred to as NC4 peptide and NC4 antiserum, respectively.

Western Blot

Samples of medium from bovine cartilage digested with MMP-13 were separated by SDS-polyacrylamide gel (16%) electrophoresis (as described above) according to standard protocols using the Laemmli buffer system and then transferred to a nitrocellulose membrane (Hybond-C, Amersham Biosciences) (37). Blocking was performed overnight with 3% (w/v) low fat dry milk in Tris-buffered saline, pH 7.4, and 0.2% (v/v) Tween 20 at 4 °C. The membrane was rinsed with the blocking solution (described above) omitting milk and incubated at room temperature with the NC4 antiserum diluted 1:1000 in 2% (w/v) milk, Tris-buffered saline, and 0.2% Tween 20 for 1 h. After rinsing, the nitrocellulose membrane was incubated for 1 h at room temperature with a peroxidase-conjugated secondary antibody diluted 1:6000 (AfiniPure donkey anti-rabbit IgG, Jackson ImmunoResearch Laboratories) in 2% milk, Tris-buffered saline, Tween 20. Blots were activated for 1 min using homemade enhanced chemiluminescence reagents (20 ml of 0.1 M Tris-HCl, pH 8.5, 48 µl of 250 mM Luminol in Me$_2$SO, 48 µl of 40 mM p-coumaric acid in Me$_2$SO, and 14 µl of H$_2$O$_2$). Agfa CRONEX 5 medical x-ray films were exposed to the membrane and automatically developed with an Agfa Curix 60 processor.

Sample Preparation for Mass Spectrometry

Protein spots/bands were excised using either a scalpel or a cutting device with three interchangeable cylinders (1.5, 2.5, and 4 mm in diameter), each with a piston for easy release of the gel piece. The cutting devices were thoroughly cleaned prior to and after each cut. Gel pieces were reduced and alkylated as described previously (38). Digestion proceeded overnight at 37 °C with 3–5 µl of trypsin (Promega) or endoproteinase Asp-N (Asp-N; Roche Applied Science) both at 20 µg/ml in 25 mM NH$_4$HCO$_3$, pH 7.8. Five minutes after the addition of enzyme, 10–20 µl of buffer was added to cover the gel pieces. Samples were immediately processed or frozen after digestion. Peptides in gel digests were purified and concentrated using reversed phase tips either with commercially available ZipTip™-µ-C18 (Millipore) or homemade Stop and Go extraction tips (39). The peptides retained for samples for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis were eluted onto dried 2,5-dihydroxybenzoic acid (Bruker Daltonik GmbH, Bremen, Germany) spots on Anchorchip™ target plates (Bruker Daltonik GmbH) using 1 µl of 50% acetonitrile, 0.1% trifluoroacetic acid. Samples for reversed phase LC ESI MS were purified exclusively with Stop and Go extraction tips (39) using formic acid instead of trifluoroacetic acid. Retained peptides were eluted using 10 µl of 50% acetonitrile, 0.1% formic acid into autosampler glass vials (Qsert, Waters). The organic solvent was evaporated using N$_2$ gas and replaced with 0.1% formic acid.

**Mass Spectrometry**

Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS)—The Bruker Reflex III MALDI-TOF MS instrument was run in reflective mode with delayed extraction and an acceleration voltage of 25 kV. To improve signal-to-noise ratio 50–100 spectra were summed. Trypsin autolysis peaks at 842.5100 and 2211.1046 m/z were used for internal calibration. Spectra were annotated, and the peptide mass lists were exported to Mascot Peptide Mass FingerPrint (40) for protein identification, matching data to the National Center for Biotechnology Information non-redundant (NCBInr) data base (www.ncbi.nlm.nih.gov/).

Reversed Phase LC ESI MS—Two LC ESI-MS instruments were used, a Qtof (quadrupole time-of-flight) micro (Micromass) and an Esquire HCT (high capacity trap) IonTrap (Bruker Daltonik GmbH).

The Qtof was coupled to a CapLC (Waters) with a PepMap™ nano-precolumn (LC Packings, C$_{18}$, 300-µm inner diameter, and 5 mm long) and a Waters Symmetry analytical column (C$_{18}$, 3.5-µm particles, 150 mm × 75 µm). The analytical column was coupled to a PicoTip needle (New Objective Inc., Woburn, MA). The inlet was further equipped with a Nano-lock spray infusing [Glu$^1$]fibrinopeptide B (Sigma) for continuous calibration. LC and MS were controlled from MassLynx software running on the instrument computer. Generated data were processed to peak lists (pkf files) using ProteinLynx GlobalServer and exported to Mascot MS/MS Ions Search (40) and used for searches in the NCBInr data base. The accuracy for both precursor ion and fragment ion mass was set to 0.1 Da. Enzyme specificity was set to trypsin, semitrypsin (representing peptides where one end matched cleavage by trypsin and the other could represent any specificity), or Asp-N (cleavage N-terminal of both glutamic acid and aspartic acid in NH$_2$HCO$_3$ buffer at pH 7.8). Oxidation of methionine residues, presence of hydroxyproline residues, and deamidation were set to variable, whereas all cysteine residues were required to be carbamidomethylated. MS/MS spectra of specific interest were imported to BioLynx PepSeq software for de novo sequencing and manual interpretation.

The HCT IonTrap was equipped with an Ultimate HPLC system (LC Packings) with the same type of precolumn as described above and an Atlantis™ analytical column (Waters, C$_{18}$, 3-µm particles, 150 mm × 150 µm). The analytical column...
was coupled to the MS instrument through a microflow nebulizer. The equipment was controlled by HyStar software (Bruker Daltonik GmbH), generated spectra were processed using DataAnalysis (Bruker Daltonik GmbH), and data base searches were performed via web-based Mascot MS/MS Ions Search (40) and the Global Proteome Machine (Global Proteome Machine Organization). The accuracy for both precursor ion and fragment ion mass was set to 0.4 Da. All other settings were as for the Qtof (described above). MS/MS of selected spectra were checked using BioTools (Bruker Daltonik GmbH).

HPLC—Samples (1–10 µl) were applied onto the precolumn, which was rinsed with 0.1% formic acid for 5 min. Bound peptides were eluted using a gradient consisting of solution A (3% acetonitrile, 0.1% formic acid) and solution B (80% acetonitrile, 0.1% formic acid). The elution gradient was 5–95% solution B in 60 min.

RESULTS

Note about the Bovine Collagen α1 (IX) Amino Acid Sequence—The amino acid sequence of bovine collagen α1 (IX) that matched to mass spectrometric analysis in this study can be found in the NCBI RefSeq database (www.ncbi.nlm.nih.gov) under the accession number gi119901059 (December 22, 06). Alignment of this sequence from NCBI with the human α1 (IX) sequence (Swiss-Prot accession number P20849, www.expasy.ch/sprot/) reveals major discrepancies N- and C-terminally (supplemental Data 1). The bovine sequence shows 90% similarity to the first 642 amino acids of the human counterpart, whereas it contains an additional 332 amino acids N-terminal of the first methionine. This additional bovine sequence is likely to represent an artifact, which is likely to be corrected when more sequences become available. In support, we did not obtain mass spectrometric data that matched any parts of this “additional” sequence. We have therefore chosen to number annotations in this text referring to the human sequence. Data base search results maintain their bovine sequence numbers. Furthermore the bovine sequence shows no coverage of the C-terminal 279 amino acids in the human variant as a result of the only partial sequence available, hampering search for cleavage in the part of the molecule that is primarily triple helical.

Bovine Nasal Cartilage Explant Culture—Proteins released into the medium from IL-1-stimulated cartilage explants (3, 6, 9, 12, and 16 days) were separated using two-dimensional SDS-PAGE and identified through peptide mass fingerprinting. Gels from days 3 and 6 showed irregular separations particularly at the top part, typical of samples containing large amounts of intact and fragmented aggrecan that is too large to enter the gel. In this project focus was on the subsequent degradation after aggrecan release but before the major collagen release (24). Fragments of collagen α1 (IX) were identified at different positions on two-dimensional gels of media from days 12 and 16 (a typical gel is shown in Fig. 2 with collagen α1 (IX) spots encircled). Corresponding spots were not present in the control samples incubated without IL-1 (data not shown). A set of faster migrating proteins had an apparent mass of 28 kDa, and the sequence coverage as found by peptide mass fingerprinting (data from spot 1 in Fig. 2 matched 13 peaks to peptides; supplemental Data 2, Table A) indicated that they represented a major portion of the NC4 domain of collagen α1 (IX). Peptide mass fingerprinting of the collagen α1 (IX) fragment migrating with an apparent mass of ~50 kDa (spot 2 in Fig. 2) revealed 23 peaks that matched peptides derived from collagen α1 (IX) (supplemental Data 2, Table B) whereof 10 were from the COL3 domain and the remaining were from NC4. The peptide coverage of the slowest migrating spot (spot 3 in Fig. 2) identified as collagen α1 (IX) (apparent mass, 100 kDa) included, in addition to NC4 and COL3, parts of the COL2 domain (25 peaks matched peptides; supplemental Data 2, Table C). This spot might represent cross-linked parts or the full size collagen α1 (IX) and was not further studied (the bovine C-terminal sequence is not known).

Peptide Antibody—To confirm and to facilitate the study of the identified NC4 fragment a unique peptide (NH2-CG6QQQQQLPGDFDSLISIQFQ544-CONH2) representing a portion of the collagen α1 (IX) NC4 domain, close to its N terminus, was synthesized with an added cysteine for coupling to keyhole limpet hemocyanin and used to immunize a rabbit. The antiserum showed binding to the NC4 peptide (coated at 1 µg/ml) in solid state enzyme-linked immunosorbent assay (titer, 1:50,000). The binding was inhibited by addition of the NC4 peptide. A peptide concentration of 0.3 ng/ml showed inhibition of binding.

Western Blot—Western blots using the polyclonal NC4 antiserum confirmed the presence of collagen α1 (IX) fragments at 28 and 50 kDa in medium from IL-1-stimulated cartilage cultures separated by SDS-PAGE. An additional band at a position corresponding to 40 kDa indicated the presence of another collagen α1 (IX) entity initially released somewhat earlier than the...
The 28-kDa fragment was cut out from a two-dimensional gel, in-gel digested with Asp-N, and analyzed using LC ESI-Qtof MS. This represents data from the same experiment as shown in Fig. 4. \( M_{\text{exp}} \) is the experimentally measured monoisotopic mass of the peptide. "Delta" means the difference between the measured and the theoretically calculated mass of a matching peptide. "Miss" shows how many missed cleavages that are present in a matched peptide sequence. "Sequence" shows the sequence of the matching peptide with one extra amino acid on both the N- and the C-termini separated by a hyphen. Ion score is based on the probability that an observed match is a random hit (40). A higher value indicates a better hit. The matched peptides are tabulated with start and end positions relating to the bovine sequence gi|19901059. Allowed modifications (variable) were oxidation of methionines, deamidation (asparagine and glutamine), and hydroxyproline. Cysteine residues were assumed to be carbamidomethylated. Data base, NCBI nr.

### TABLE 1

| Start–end | \( M_{\text{exp}} \) | Delta | Miss | Sequence | Ion score |
|-----------|---------------------|-------|------|----------|-----------|
| 457–466   | 1353.6852           | 0.0023| 0    | L-EKHMSIWQI-Q-D | 8         |
| 547–556   | 1017.5858           | 0.0000| 0    | V-DGFAVGLKLV-D  | 13        |
| 557–574   | 2212.0394           | -0.0050| 1   | V-DNPQVSYFPEQLQNLTHC-D | 13        |
| 566–574   | 1228.5726           | -0.0006| 0   | F-ELQQMLTHC-D    | 24        |
Collagen 9 Cleavage

TABLE 2
Peptides matching collagen α1 (IX) in the 28-kDa fragment

The 28-kDa band was cut out from a two-dimensional gel, in-gel digested with trypsin, and analyzed using LC ESI-QQTOF MS, and data were used to search the NCBI RefSeq database with Mascot MS/MS ions search. The matched peptides are tabulated with start and end positions relating to the bovine sequence gi|199001059. Most notations are described in Table 1 legend. "Modifications" describes modifications in a peptide sequence required to match it to a Mexp obtained with mass spectrometry. Allowed modifications (variable) were oxidation of methionines, deamidation (asparagine and glutamine), and hydroxylation of proline residues to form hydroxyprolines. Cysteine residues were assumed to be carboxamidomethylated.

| Start–end | Mexp | Delta | Miss | Sequence | Modifications | Ion score |
|-----------|------|-------|------|----------|---------------|-----------|
| 363–378   | Da   | −0.0003| 0    | R-FFVNSNSNGENELCPR-V | 2 deamidation (NQ) | 13 |
| 364–378   | Da   | −0.0049| 0    | P-NSNSNGENELCPR-V  | 2 deamidation (NQ) | 11 |
| 367–378   | Da   | −0.0002| 0    | N-NSNSNGENELCPR-V  | Deamidation (NQ) | 63 |
| 381–399   | 2148.0814 | 0.0076| 0    | R-IQDDLPGLQDSQFOIDR-A | | |
| 409–420   | 1234.6974 | 0.0054| 0    | R-VGGSTALQAVYK-L  | | |
| 412–428   | 933.4652  | −0.0015| 0    | K-LGGNFDPR-I      | | |
| 421–428   | 934.4522  | 0.0015| 0    | K-LGGNFDPR-I      | | |
| 433–450   | 2184.0481 | −0.0046| 0    | R-HLYPMGLPEEYSFLTFR-M | Deamidation (NQ) | 22 |
| 459–471   | 1570.7518 | −0.0010| 0    | K-HWSIHGQDSSK-E   | | |
| 491–514   | 2618.7285 | −0.0060| 0    | K-QLGSGVQTAAPSNLPELFDSQMK-I | | |
| 533–541   | 1051.6430 | 0.0041| 0    | R-IESLPIKPR-G      | | |
| 542–554   | 1317.6970 | 0.0043| 0    | R-QQDVGDFAVLGK-L  | | |
| 581–590   | 1244.5958 | 0.0027| 1    | R-ETCNELPAR-I     | | |
| 582–590   | 1088.4734 | −0.0186| 0    | R-ETCNELPAR-I     | | |

DISCUSSION

This model of tissue breakdown in joint disease where cartilage degradation is induced by IL-1 stimulation has been used frequently in studies of release of tissue macromolecules. It has been shown that aggrecan fragments are initially released followed by cartilage oligomeric matrix protein, fibromodulin, and last collagen (24, 43, 44). In medium from cartilage cultured in the presence of IL-1 we found, using two-dimensional gel electrophoresis followed by MALDI-TOF MS, release of collagen α1 (IX) fragments. The most abundant fragment detected on two-dimensional gels, with an apparent molecular mass of 28 kDa, represented large parts of the globular NC4 domain. The presence of the 28-kDa fragment in spots on the two-dimensional gel over a large interval of isoelectric points might be due to deamidation of asparagine residues (45). Indeed one of the peptides sequenced appeared to exhibit deamidation (Fig. 6). This phenomenon was not further investigated. The larger
TABLE 3
Peptides matching the collagen α1 (IX) chain

The 50-kDa MMP-13-generated band was cut out from a one-dimensional gel, in-gel digested with trypsin, and analyzed using the LC ESI-IonTrap MS setup. Data were further analyzed by a data base search using Mascot MS/MS ions search (database, NCBI nr). These data are from the same experiment as in Fig. 5c. The matched peptides are tabulated with start and end positions relating to the bovine sequence gi|119901059. See Tables 1 and 2 legends for details.

| Start–end | M_exp | Delta | Miss | Sequence | Modification | Ion score |
|-----------|-------|-------|------|----------|--------------|-----------|
| 381–399   | 2148.0514 | −0.0224 | 0 | R-IQDDLPQFDLISQPQDDPK-A | Deamidation (NQ) | 84 |
| 409–420   | 1234.7914 | 0.0994 | 0 | R-VVGSTALQVARY-L | 62 |
| 433–450   | 2184.0082 | −0.0445 | 0 | R-HLYPNGLPEEYSLFLTF-M | 76 |
| 522–532   | 1268.6014 | 0.0195 | 0 | R-SSATLFPDGR-I | 53 |
| 581–590   | 1244.7394 | 0.1463 | 1 | R-RETCELNLPA-R | 38 |
| 719–732   | 1273.6574 | 0.0524 | 1 | R-RETCNELPAR258- | 35 |

Collagen 9 Cleavage

Western blots with our new NC4 antisera clearly indicated the presence of three different type IX collagen fragments in medium from days 9, 12, and 16 (Fig. 3). The apparent masses of two of them match earlier identified spots from two-dimensional gels corresponding to ~28 and 50 kDa. The third fragment with an apparent mass of 40 kDa was observed already in day 9 medium. Upon trypsin digestion and mass spectrometry of the 40-kDa fragment found in medium from IL-1-stimulated cartilage at day 12, the NC4 domain of collagen α1 (IX) was identified. The apparent mass suggests a fragment larger than the NC4 domain, but data from mass spectrometry showed limited coverage and did not include any peptides from the COL3 domain. The most abundant 28-kDa fragment was initially selected for more extensive characterization.

Digestion with trypsin and Asp-N combined with LC ESI-IonTrap MS revealed a new cleavage terminus in the C-terminal part of NC4, a few amino acids N-terminal to the COL3 domain (Fig. 1 III). The most C-terminal fragment found with both enzymes was ETCELNLPA258. Asp-N itself will not produce a cleavage between the PAR and the following isoleucine, suggesting that cleavage at this site had occurred in the cartilage subsequent to IL-1 stimulation. The Asp-N digest did not contain any other peptides that could have been generated by trypsin, excluding any contamination by this enzyme. As another control, Asp-N digestion of the longer 50-kDa collagen α1 (IX) fragment did not produce an ETCELNLPA258 peptide detectable with LC ESI-IonTrap MS, whereas trypsin indeed cleaved at the arginine residues to produce this fragment (data not shown). Tryptsin digestion of the 50-kDa collagen α1 (IX) fragment and subsequent analysis by LC ESI-IonTrap MS identified two peptides in the COL3 domain in medium from IL-1 day 16. The most C-terminal one has a C-terminal non-tryptic cleavage (-RPQPPGPPGPGPGS490-COOH NH2-TIG-), suggesting that a cleavage between glycine and threonine had occurred in the tissue during IL-1 stimulation (Table 3, Fig. 1 IV, and 5c). The 28- and the 50-kDa fragments are released simultaneously from the tissue (Fig. 3) and are thus likely cleaved in parallel with formation of the 40-kDa fragment preceding. Whether more than one of these cleavages occurs in the same molecule, resulting in a fragment containing primarily the COL3 domain, is not known.

In previous experiments (24) it was shown that MMP-13 appeared in an active form and cleaved fibromodulin starting around day 9 when cartilage was treated with IL-1. This...
Collagen 9 Cleavage

occurred in the same time frame as the type IX collagen cleavages. Thus, we decided to test whether digestion of cartilage with MMP-13 could generate the cleavage of collagen α1 (IX) NC4 that we found in medium from IL-1-stimulated cartilage explants.

Bovine nasal cartilage explants were digested with MMP-13, and indeed the enzyme cleaved collagen α1 (IX). The appearance of NC4-containing fragments in the medium as shown by Western blot using the NC4 antisera was very similar to those from IL-1-induced cleavage (Fig. 5a). The two fragments, 28 and 50 kDa, from collagen α1 (IX) NC4 previously observed upon IL-1 stimulation were confirmed by LC ESI-IonTrap MS to be released from the cartilage into the medium upon MMP-13 digestion. The 40-kDa band that was seen in IL-1-treated cartilage at days 9, 12, and 16 could be seen as a faint band (Fig. 5b). It is likely that the enzyme causing the cleavages in the IL-1-stimulated cartilage indeed is MMP-13 because one substrate site defined represents one for a collagenease and a second is for a different enzyme, such as a gelatinase. MMP-13 represents an enzyme known to have these two activities. Our present study demonstrates cleavage of collagen IX by MMP-13 in the tissue. Previously published data used digestion of pepsin-extracted collagen IX, representing a substrate not necessarily available in vivo. Indeed the cleavages we have demonstrated are quite different from cleavage of the COL1 domain implicated by these authors (21). Another collagenase, MMP-1, was identified in IL-1-stimulated bovine nasal cartilage medium at day 16. Whether there is any active MMP-1 is unknown. Using a selective collagenase inhibitor sparing MMP-1 it was shown that the type II collagen cleavage was only partly inhibited in bovine nasal cartilage but completely inhibited in bovine articular cartilage (50). Still it is not known whether active MMP-1 actually would cleave collagen IX in vivo. Okada et al. (51) were not able to show any activity of MMP-1 against type IX collagen in vitro.

Removal of type IX collagen as a step in tissue maturation has been discussed in earlier studies (42, 52). The latter reported that the NC4 domain was removed at the initiation of mineralization, and as the mineralization progressed, both type II and IX collagen were removed from the matrix. The cleavage was not identified, and it is not possible to conclude whether it was the same as any of those observed here. The presence of MMP-13 in hypertrophic chondrocytes has been observed previously using in situ hybridization (20, 53). Combined with the results reported in our present study, it seems plausible that collagen IX is removed from growth plate fibrils by MMP-13 as a step in endochondral bone formation.

Hagg et al. (14) extracted fibrils from cartilage (yield, <3%) and showed that fetal bovine cartilage type II collagen fibrils of all thicknesses were decorated with type IX collagen, whereas in adult bovine cartilage only thin fibrils (15–26 nm) had this decoration. They noted that proteolytic activities would be necessary if removal of type IX collagen from thin fibrils is required for thick fibrils to form. The now presented cleavage sites could represent steps in this potential removal of type IX collagen from thin fibrils.

The cleavage removing the 50-kDa fragment could disrupt the potential binding of type IX collagen to cells, mediated by collagen receptor integrins (54), as well as to heparan sulfate proteoglycans at the cell surface.

The presented data elucidate the degradation of an important cartilage component in a model system and pinpoint MMP-13 as a protease with the ability to accomplish different types of specific cleavages in collagen IX (Fig. 1). Sites of non-triple helix as well as sites for triple helical parts were found. The new sites can be used to develop antibodies only recognizing cleaved molecules and could be used in the development of new diagnostic procedures by molecular marker technology (55).

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