MMP-8 (neutrophil collagenase) mRNA and aggrecanase cleavage products are present in normal and osteoarthritic human articular cartilage

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Osteoarthrosis is characterized by a progressive destruction of articular cartilage resulting from an imbalance of synthesis and degradation of aggrecan and collagen, the major matrix components. The process is governed by inflammatory episodes, thus the disease is called Osteoarthritis (OA). Aggrecan is a large proteoglycan (PG) which is responsible for the biomechanical function of the cartilage to withstand compressive loading. Aggrecan consists of a core protein to which chains of chondroitin and keratan sulfate are attached (Hascall 1988; Heinegård 1992). Cleavage of the core protein between the two globular domains at the animo terminal end results in loss of aggrecan from the cartilage and impaired function. A number of potential cleavage sites have been identified within the interglobular domain for different proteases including members of the family of matrix metalloproteinases (MMPs), lysosomal enzymes, plasmin, elastase and urokinase (Fosang et al. 1991; 1992; 1993). The predominant proteolytic product was identified by amino acid sequencing of the aggrecan core peptides which had been released into synovial fluid from human arthritic cartilage and culture media of bovine cartilage or Swarm rat chondrosarcoma stimulated by interleukin 1 or retinoic acid (Sandy et al. 1991; Ilic et al. 1992; Sandy et al. 1992; Loulakis et al. 1992; Lohmander et al. 1993; Lark et al. 1995). The protease responsible for the cleavage product was called "aggrecanase" and a search began to identify the enzyme. Recently, a MMP synthesized by neutrophils (MMP-8; neutrophil collagenase) was shown to be capable of generating the aggrecanase cleavage product in vitro (Fosang et al. 1994a; b). It is, however, still not known whether chondrocytes express MMP-8 (Hardingham et al. 1994); this protease was thought to be a unique gene product of neutrophils (Mainardi et al. 1991). In preliminary reports we described briefly that mRNA for MMP-8 is detectable by in situ hybridization in normal and osteoarthritic cartilages (Chubinskaya et al. 1995; Huch et al. 1995).

The purpose of the current study was to test the specificity of two oligonucleotide cDNA probes designed by us by comparing mRNA expression in human peripheral blood neutrophils with that of human articular chondrocytes. Secondly, this study identified aggrecanase cleavage products extracted from human arthritic cartilage. These data suggest that MMP-8 is expressed by chondrocytes and that the aggrecanase neoepitope cleavage product is retained within the cartilage.

Material and methods

Cartilage acquisition. Human articular (adult) or epiphyseal (fetal) cartilage was obtained from donors within 24 hours of death through the Regional Organ Bank of Illinois according to their protocol and with institutional approval. Full thickness, non-calcified cartilage was removed from the articular surface of knees which had macroscopically no visible signs of osteoarthritis. Cartilages (knee) were also obtained from patients undergoing joint replacement for either osteoarthritis or rheumatoid arthritis. The cartilages were processed immediately for in situ hybridization or digested to isolate chondrocytes for culture in alginate beads or the tissue was extracted with 4M guanidine chloride to extract PGs according to standard procedures (Hascall and Kimura 1982).

Human blood cell preparation. As a positive control for neutrophil collagenase mRNA, human neutrophils from normal peripheral blood were partially purified following the procedure of Boyum (1968).

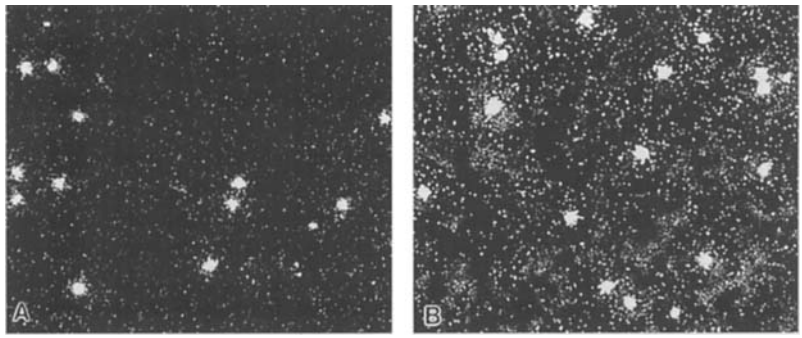


Figure 1. A. Darkfield photomicrograph of an enriched population of neutrophils from normal human blood obtained by venopuncture and hybridized to oligonucleotide probes complementary to bp 1588–1610.
B. Darkfield photomicrograph of same population of cells hybridized to the oligonucleotide probe complementary to bp 796-822. (X 112.5)

Alginate bead culture. Chondrocytes from full-thickness uncalcified cartilage slices were isolated by protease digestion and cultured within alginate gels (beads) as previously described (Aydelotte and Kuettner 1988; Häuselmann et al. 1992). Cultures were fed daily with Ham's medium F-12/DME supplemented with 10% fetal bovine serum and 25 µg/ml ascorbate and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cultures were continued for 2 days at which time the beads were processed for in situ hybridization with fixation in 4% paraformaldehyde containing 0.01 M $BaCl_2$ to maintain bead integrity during histological processing.

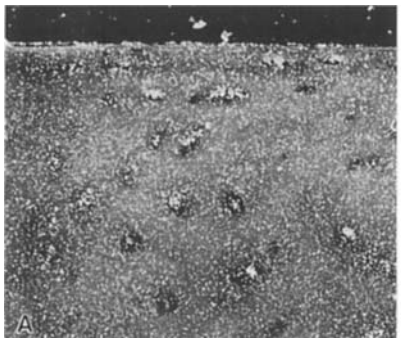
In situ hybridization. The cartilage specimens and chondrocytes cultured in alginate beads were fixed in paraformaldehyde, embedded in paraffin and sectioned at 6 µm. The neutrophils were allowed to adhere to chamber slides (Tissue-Tek). Sections and adherent cells were hybridized with cDNA probes using the technique of Sandell et al. (1991). Two cDNA probes specific for neutrophil collagenase were designed with sequences complementary to bp 1588–1610 [5'-GGT- AGA-ATG-GAT-ACA-GTG-ATG-GG-3'] and to bp 796–822 [5'-GAG-GGA-GTG-AGT-AGT-TGC-TGG-TTT-CCC-3'] according to the published sequence of human neutrophil collagenase gene (Hasty et al. 1990). Both probes were synthesized and purified by Research Genetics (Huntsville, AL). The specificities of these probes were compared with sequence data available from PC/GENE on the human DNA data base. The oligonucleotide probes were 3' end labeled with 5'-[α-thiol-^{35}S]-dCTP using terminal deoxynucleotidyl transferase. The radiolabeled probes were hybridized to tissue sections under conditions of high stringency.

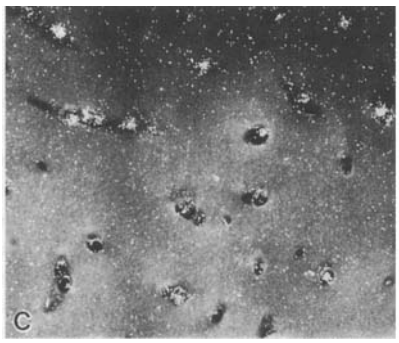
Cartilage extract. Select specimen of cartilage were extracted in 4 M guanidine HCl in the presence of protease inhibitors according to the procedure of Hascall and Kimura (1982). The extract was dialyzed against water, lyophlyzed, resolubilized in water at an equal weight/volume. Samples were digested with chondroitinase ABC, dialyzed against water and lyopholyzed prior to SDS-PAGE and immunoblotting with BC-3. This is a monoclonal antibody which recognizes the new N-terminus (ARGSV) on core protein produced by 'aggrecanase' (Hughes et al. 1995).

Results

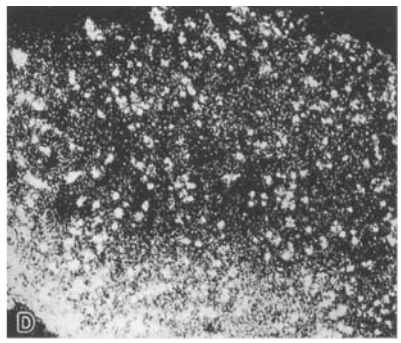
Oligonucleotide probes complementary to MMP-8 mRNA were hybridized to an enriched population of neutrophils, normal human knee articular cartilage, osteoarthritic knee cartilage, and cultured chondrocytes from normal knee (Figures 1 and 2). The enriched population of neutrophils were positive with both probes for MMP-8 (Figure 1). The dark field photomicrographs are shown to emphasize the cells that are positive with the radiolabeled probes complementary to bp 1588–1610 (Figure 1A) and to bp 796–822 (Figure 1B).

Normal cartilage as well as osteoarthritic cartilage was examined for MMP-8 mRNA expression. Chondrocytes in the normal cartilage expressed MMP-8 mRNA in the superficial, middle and deep layers of the normal cartilage with strongest expression in the superficial layer and the upper region of the middle layer (Figure 2A). Expression in the deep layer was limited to scattered chondrocytes and appeared to be less than that in the superficial and upper region of the middle layer. In the cartilage from the osteoarthritic patients (Figure 2B), the level of expression was much higher in the superficial and middle layers. In sections of the cartilage where the





Deep layer from same section shown in B (X 112.5).



Normal chondrocytes cultured in alginate beads. (X 45).

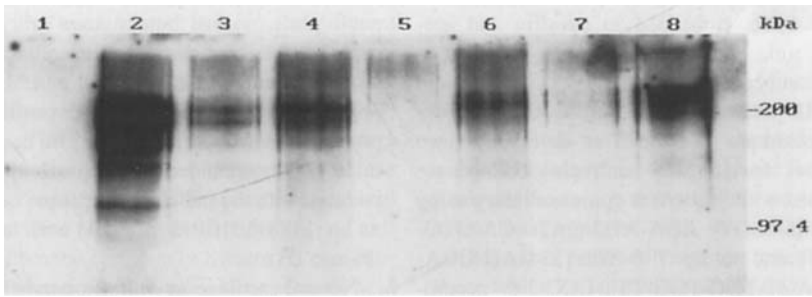


Figure 3. Analysis of human cartilage extract loaded at an equal volume of lyophlyzed sample using BC-3. Localization of the BC-3 neoepitope was performed on proteoglycans extracted from Lane 1 human fetal cartilage; Lanes 2–4 patients diagnosed with rheumatoid arthritis; Lane 5 normal donor; Lanes 6–8 patients diagnosed with osteoarthritis. Molecular weights of reduced globular standards are indicated.

superficial layer was disrupted, the level of expression was higher in the deep layer (Figure 2C). Chondrocytes derived from normal tissues and cultured in alginate beads for 2 days under conditions shown to maintain chondrocytic phenotype also expressed MMP-8 mRNA (Figure 2D).

Aggrecanase degradation products were identified in the proteoglycans extracted from 8 different human cartilages samples. The neoepitope was present as immunopositive bands with different molecular weights; the number and intensity of the bands differed among the samples (Figure 3). The

extract from fetal epiphyseal cartilage contained only a single, weak band (Figure 3, Lane 1); the cartilage extract from the normal adult donor (Figure 3, Lane 5) also contained a single but weak band, which had migrated on the gel more slowly than the band derived from the fetal tissue. The extracts from the rheumatoid arthritic (Figure 3, Lanes 2-4) and osteoarthritic cartilages (Figure 3, Lanes 6-8) contained multiple bands; the intensity of the bands varied with the strongest bands present in the extract from the rheumatoid arthritic samples.

Discussion

We were able to detect expression of mRNA for MMP-8 in human articular chondrocytes using two different oligonucleotide probes. These probes were complementary to bp 1588–1610 and to bp 796–822. The two different probes from two regions of the gene were synthesized to increase the specificity of the probes for MMP-8. Hasty et al. (1990) reported there is significant homology between MMP-8 and MMP-1 with 57% identity with the deduced protein sequence for MMP-1 with 72% chemical similarity; however, MMP-8 is a distinct gene product. The two probes were designed to recognize regions of the MMP-8 gene which have no known identity to any other gene sequence in the data available from PC/GENE on the human DNA data base. Neutrophils were used as a positive control to identify the mRNA of MMP-8.

Previously, MMP-8 was thought to be expressed exclusively by neutrophils and was referred to as neutrophil collagenase (Mainardi et al. 1991). The results of our study however show that chondrocytes also express the MMP-8 gene. We were able to localize mRNA expression in normal and osteoarthritic cartilages as well as in normal chondrocytes cultured in alginate beads.

Proteoglycan core protein extracted from both rheumatoid arthritic and osteoarthritic cartilages also contains the neoepitope of the aggrecanase cleavage product. Compared to fetal or normal adult cartilage with an equal volume of extract, the arthritic cartilages contained multiple bands representing multiple degradation products of the core protein. The results of our study are similar to other studies (Hughes et al. 1995) which have reported the presence of multiple products in media of bovine cartilage explants or retinoic acid-treated Swarm rat chondrosarcoma cells stimulated by either interleukin-1 or retinoic acid.

Our data show that human chondrocytes express MMP-8 mRNA recognized by two different oligonucleotide probes designed to be specific for MMP-8. In addition, our data demonstrates that aggrecanase generated proteoglycan fragments are retained in arthritic cartilages. These data complement the growing body of evidence that MMP-8 from human articular chondrocytes may be the same protease as aggrecanase.

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