# The New Collagenase, Collagenase-3, Is Expressed and Synthesized by Human Chondrocytes but not by Synoviocytes

## A Role in Osteoarthritis

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## Abstract

**Recently, a new human collagenase, collagenase-3 has been identified. Since collagen changes are of particular importance in cartilage degeneration, we investigated if collagenase-3 plays a role in osteoarthritis (OA).**

**Reverse transcriptase–PCR analysis revealed that in articular tissues collagenase-3 was expressed by the chondrocytes but not by the synoviocytes. Northern blot analysis of the chondrocyte mRNA revealed the presence of two major gene transcripts of 3.0 and 2.5 kb, and a third one of 2.2 kb was occasionally present. Compared to normal, OA showed a significantly higher (3.0 kb, $P \leq 0.05$; 2.5 kb, $P \leq 0.03$) level of collagenase-3 mRNA expression. Collagenase-3 had a higher catalytic velocity rate (about fivefold) than collagenase-1 on type II collagen. With the use of two specific antibodies, we showed that human chondrocytes had the ability to produce collagenase-3 as a proenzyme and as a glycosylated doublet. The chondrocyte collagenase-3 protein is produced in a significantly higher ($P \leq 0.04$) level in OA ($\sim$ 9.5-fold) than in normal. The synthesis and expression of this new collagenase could also be modulated by two proinflammatory cytokines, IL-1β and TNF-α, in a time- and dose-dependent manner.**

**This study provides novel and interesting data on collagenase-3 expression and synthesis in human cartilage cells and suggest its involvement in human OA cartilage pathophysiology. (*J. Clin. Invest.* 1996. 97:2011–2019.) Key words: cartilage • collagen • chondrocytes • arthritis • cytokines**

## Introduction

Collagens are the major structural proteins of all connective tissues. The most abundant collagens are the types I, II, and III, named interstitial collagens. They consist of three peptide chains, termed α chains, with a unique primary sequence for each interstitial collagen. The expression of the type I and type II collagen genes is tightly regulated during embryonic development with each type of collagen being made in specific tissues at specific times (1, 2). Type I collagen is widely distributed, being present in bone, skin, tendons, and ligaments, whereas type II collagen is located almost exclusively in hyaline cartilage. In addition to the predominant type II collagen fibrils in cartilage, smaller amounts of minor collagens, such as types VI, IX, X, and XI, have also been identified (3–9). Proteoglycans are also major structural components of the extracellular matrix of cartilage. In this articular tissue, the collagen network provides the tensile strength, whereas the proteoglycans are largely responsible for the compressive stiffness (10, 11).

Cartilage loss is one of the dominant features in osteoarthritis (OA).[1] Histopathologically, the earliest lesion is a breakdown of the collagen network around the chondrocytes and a depletion of matrix proteoglycan (12–14). Collagen changes are of particular importance as their breakdown results in the loss of structural integrity of the tissue. Much evidence supports the hypothesis that the early phase of OA involves the loosening of the collagen network and subsequently results in an increase in gross collagen fibril disruption (13, 14). It is also considered that the loss of cartilage matrix reflects the action of proteolytic enzymes released by joint articular cells in response to various factors (15, 16).

Current knowledge points to the involvement of a family of metalloproteases (MMP) in the cartilage destruction. Based on their structure and function, the metalloproteases constitute a single evolutionary protein super family which may be classified into at least three different families of closely related members: collagenases, gelatinases, and stromelysins. They all contain zinc and require calcium for full activity (17). Collagenases are capable of cleaving all three α chains of native triple helical collagens (types I, II, and III) after the Gly residue of the partial sequence Gly-[Ile or Leu]-[Ala or Leu] at a single locus approximately three-quarters from the amino terminus (18–20). The products resulting from this cleavage can be further denatured by a variety of other proteases including gelatinases and stromelysin (21–23). However, it was demonstrated in vitro that the 72-kD gelatinase, free of tissue inhibitors of metalloproteases (TIMP), is able to cleave soluble, triple helical type I collagen or reconstituted type I collagen fibrils (24), and that stromelysin-1 has the ability to cleave the type II collagen in the nonhelical aminotelopeptide domain (25).

Recently, a new human collagenase named collagenase-3 or MMP-13 has been identified from breast carcinomas by Freije et al. (26), which is localized on the chromosome

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1. *Abbreviations used in this paper:* APMA, aminophenylmercuric acetate; MMP, metalloproteases; OA, osteoarthritis; rh, recombinant human; RT, reverse transcriptase; TIMP, tissue inhibitor of metalloproteases.

11q22.3 (27). Amino acid sequence comparison revealed that human collagenase-3 is the homologue of rat and mouse collagenase-1 (rat or mouse MMP-1), and has homology, to a lesser extent, with human collagenase-1 (MMP-1) and neutrophil collagenase (MMP-8). Collagenase-3 has not been found in several normal tissues including liver, placenta, prostate, and breast, but identified in breast carcinomas tissues (26). This finding indicates that the collagenase-3 gene in normal tissues is either expressed at a very low level or repressed, but its expression may be stimulated or derepressed in pathological conditions.

With regards to the present concept of the pathophysiology of OA, it is believed that collagenase-1 is the major enzyme responsible for the degradation of type II collagen in OA cartilage. However, due to the high homology of human collagenase-3 with rat collagenase-1, it is very possible that the latter may also play a role in human cartilage degradation and in particular during the OA process. We, therefore, investigated the expression and synthesis of collagenase-3 in normal and OA human articular joint tissues and studied its modulation in chondrocytes by two proinflammatory cytokines which are most likely involved in this disease, IL-1β and TNF-α.

## Methods

### *Specimen selection*

Articular cartilage specimens (femoral condyle and tibial plateau) and synovial membranes were obtained from OA patients (mean age 67±3 yr) undergoing total knee joint replacement. All OA patients were evaluated by a certified rheumatologist and were diagnosed as having OA, based on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for OA (28). These specimens represented moderate to severe OA as defined according to macroscopical criteria of typical OA cartilage. Surface fibrillation and pitting were prominent along with the eburnation of variable size. Normal control knee specimens were obtained at autopsy within 12 h of death from individuals (mean age 61±4 yr) with no history of joint disease and with macroscopic and microscopic normal cartilage or synovial membrane.

### *Cell cultures*

Specimens were rinsed and dissected and cells released, as previously described (29, 30). Briefly, the specimens were full-thickness strips of tissue cut across the surface, excluding mesenchymal repair tissue and subchondral bone. The normal control cartilage was from the same location as OA. Chondrocytes were obtained from cartilage following a sequential enzymatic digestion performed with 1 mg/ml pronase (Boehringer Mannheim Biochemicals, Indianapolis, IN) succeeded by 6 h with 2 mg/ml collagenase (type IA; Sigma Chemical Co., St. Louis, MO) at 37°C in DME (Gibco-BRL Canadian Life Technologies, Burlington, Canada) supplemented with 10% heat-inactivated FCS (Hyclone, Logan, UT), 100 U/ml penicillin (Gibco-BRL), and 100 μg/ml streptomycin (Gibco-BRL). Synoviocytes were obtained from synovial membranes as for the cartilage but the synovia were first digested with 0.25% trypsin for 1 h. High density seeded chondrocytes or synoviocytes were then plated in tissue culture flasks and cultured in DME supplemented with 10% FCS and antibiotics, at 37°C in a humidified atmosphere of 5% $CO_2$/95% air, until confluence. 24 h before the experiment, the cells were incubated in fresh serum-free medium. Primary or first passage culture cells were used.

### *RNA extraction*

Total RNA was extracted from normal or OA either directly from cartilage or from primary culture of chondrocytes or from first passage synoviocytes. RNA extraction was also carried out on synoviocytes stimulated with human recombinant (rh)IL-β (Genzyme Corp., Cambridge, MA) for 18 h, and on chondrocytes stimulated with rhIL-1β or TNF-α (Genzyme Corp.) for various periods of time (0–18 h) and/or with increasing concentrations (0–100 U/ml) for 18 h.

*Cartilage.* Total RNA from cartilage was extracted as previously described (31). Briefly, each cartilage specimen was homogenized in 6 M guanidine hydroxychloride containing 25 mM sodium citrate, pH 7, 25 mM EDTA, 0.5% sarkosyl, and 100 mM 2-mercaptoethanol, followed by the addition of 0.1 vol of 3 M sodium acetate buffer, pH 5, 0.25 vol of saturated phenol and 0.25 vol of isoamyl alcohol/chloroform (1:49). After being shaken and cooled at 4°C for 1 h, the solution was centrifuged (12,000 *g*, 30 min at 4°C), the pellet resuspended in 4 M guanidine isothiocyanate buffer containing 3.3 ml of cesium trifluoroacetate (2.01 g/ml; Pharmacia Biotech, Baie d'Urfé, Canada), and the solution centrifuged again at 4°C for 24 h at 100,000 *g* in an SW 40 Ti rotor (Beckman Instruments Inc., Mississauga, Canada). The pellet was dissolved in 20 mM sodium acetate, pH 5, 0.5% SDS and 1 mM EDTA. The lysate was then extracted twice with preheated (60°C) phenol in 20 mM sodium acetate, pH 5. The RNA in the resultant aqueous phase was precipitated overnight with 2 vol of ethanol at −20°C. The pellet was resuspended in the acetate buffer and reprecipitated with ethanol. After solubilization of the RNA pellet in sterile water, RNA was quantitated spectrophotometrically and examined by agarose gel electrophoresis.

*Cells.* Chondrocytes or synoviocytes were lysed directly in the sodium acetate buffer (20 mM), pH 5, containing 0.5% SDS and 1 mM EDTA, as above. The solution was put in saturated hot phenol and RNA extraction performed in the aqueous phase and processed as described above.

### *Reverse transcriptase (RT)-PCR*

RNA from chondrocytes or synoviocytes were converted to a single-stranded cDNA using a random hexamer of oligonucleotide. PCR was performed using primers specific for each human metalloprotease and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the latter a housekeeping gene served as a positive control for the methodology. The primers were for collagenase-1 5′-ATTTCT-CCGCTTTTCAACTT-3′ (forward primer) and 5′-ATGCACAGC-TTTCCTCCACT-3′ (reverse primer), stromelysin-1 5′-ATGAAG-AGTCTTCCAATCCT-3′ (forward primer) and 5′-GTCCTT-TCTCCTAACAAACT-3′ (reverse primer), and GAPDH 5′-CCACCCATGGCAAATTCCATGGCA-3′ (forward primer) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (reverse primer). A specific set of primers for collagenase-3 was developed: 5′-TGCT-GCATTCTCCTTCAGGA-3′ (forward primer) and 5′-ATGCATC-CAGGGGTCCTGGC-3′ (reverse primer).

A digoxigenin-11-2′-uridine-5′-triphosphate (DIG-11-UTP) (Boehringer Mannheim Biochemicals, Mannheim, Germany) was added to each reaction mix, and the RT-PCR procedure carried out as instructed by the Perkin-Elmer Cetus Corp. (Foster City, CA). RT-PCR assays were done in a Gene ATAQ Controller (Pharmacia LKB Biotechnology, Sollentuna, Sweden) with 2 μg RNA and 30 cycles. The PCR products were analyzed on 1% agarose/TAE gel, denatured in 0.5 M NaOH, 1.5 M NaCl and 1 mM EDTA for 15 min. Amplified DNA was transferred to Hybond-N (Amersham Corp., Oakville, Canada) and visualized by AP-conjugated anti-DIG antibody (Boehringer-Mannheim). The membranes were exposed to a sensitive Kodak X-AR5 film (Eastman Kodak Ltd., Rochester, NY) at room temperature.

### *Northern blotting*

5 μg of total RNA were resolved on 1.2% formaldehyde-agarose gels and transferred electrophoretically to nylon membranes (Hybond-N; Amersham) in an 10 mM sodium acetate buffer, pH 7.8, containing 20 mM Tris and 0.5 mM EDTA, overnight at 4°C. The RNA was cross-linked to the membranes by exposure to ultraviolet light.

For collagenase-3, the RNA probe (Pfizer Central Research, Groton, NJ) transcribed from the full-length cDNA sequence was

used. Degenerate primers from latency domain and Zn binding domain were used to amplify a 350-bp fragment by RT-PCR from IL-1–induced human chondrocytes, which display ~ 65% homology with known metalloproteases. 3′ and 5′ RACE (rapid amplification of cDNA ends) generated 1.6- and 0.8-kb fragments, respectively, which were cloned into pGEM-T (Promega, Fisher Scientific, Montreal, Canada). A partial Apa 1 restriction digest was done on the 5′ RACE clone and a 700-bp fragment containing the 5′ end of the gene was purified. The 3′ RACE clone was digested to completion with Apa 1 restriction endonucleases and the 4.6-kb fragment, containing the pGEM T vector and the 3′ portion of the gene was purified. The products were ligated and transformed into DH5α competent *Escherichia coli* cells. Transformants were screened by restriction analysis. To eliminate the poly G tail from the 5′ end gene, specific primers were used to amplify the complete gene. The reamplified gene was subcloned into pGEM-T, and sequencing of the complete gene was done on both strands. The sequence was identical to that of Freije et al. (26). The probe (1.9 kb) consisted of the total coding sequence of the gene (1.4 kb) as well as the 490 bp of the 3′ untranslated region.

For GAPDH (housekeeping gene), a 780 PstI/XbaI fragment from GAPDH cDNA (1.2 kb, No. 57090; American Type Culture Collection, Rockville, MD) was subcloned into a pGEM-3Z vector (Promega Biotechnology), as described (29). Both sense and antisense probes were tested, and data revealed that the antisense probes used were specific.

The RNA probes were transcribed from these plasmids and labeled with the DIG-11-UTP and were revealed using a luminescent reagent. The films were subjected to laser scanning densitometry (GS300; Hoeffer Scientific Instrument, San Francisco, CA) for mRNA measurement, and when applicable the results calculated as the relative expression of mRNA normalized to GAPDH.

The membranes were first hybridized with one probe then stripped and rehybridized with GAPDH probe. Stripping the probe was performed by incubating the membrane in sterile distilled water for 1 min, then at 75°C for 1 h in 60% formamide, 1% SDS, 50 mM Tris, pH 8, and rinsed in water.

### *Western immunoblot*

Chondrocytes from primary cultures or synoviocytes from first passage of normal or OA individuals were incubated in DME/antibiotics for 72 h at 37°C, and the collagenase-3 protein level determined on the culture medium treated or not with aminophenylmercuric acetate (APMA; 1.5 mM) or N-glycosidase F (Boehringer-Mannheim, 1 U) according to the manufacturer. Furthermore, this enzyme was also determined after stimulation of the first passage culture of cells with either rhIL-1β or rhTNF-α.

The culture medium was collected and proteins concentrated with aquacide II (Boehringer-Mannheim). The protein level was determined (32) for each specimen, and 20 μg of the protein were electrophoresed on a discontinuous 4–12% SDS gel polyacrylamide. The protein were transferred electrophoretically onto a nitrocellulose membrane (Hybond C extra; Amersham) according to the method of Towbin et al. (33). The efficiency of transfer was controlled by a brief staining of the membrane with 0.1% Ponceau S (Sigma Chemical Co.) in 5% acetic acid and destained in water before the immunoblotting.

The membranes were immersed 3 h in a blocking solution consisting of TTBS (Tris, 20 mM, pH 7.4, NaCl, 137 mM, and 0.1% Tween 20) and 5% skim milk and washed twice with TTBS for 15 min. Membranes were incubated in TTBS containing 0.25% skim milk overnight with anti–human collagenase-3. Two antibodies were used: a mAb (No. F17 IB-3-C5; Pfizer Central Research) and a rabbit polyclonal (generously provided by C. Lopez-Otin, 26). These antibodies were used at a dilution of 1:1,000. The membranes were then washed three times with TTBS and incubated for 1 h at 22°C with the second antibody (anti–mouse or anti–rabbit IgG AP conjugate; Promega), respectively, and washed again. Detection was carried out using the BCIP/NBT staining (Fisher Scientific).

The mAb No. F17 IB-3-C5 was generated against a 35 amino acid (257–291) peptide from human collagenase-3. This antibody is of the IgG isotype and produced from ascites. Specificity of the antibodies against other recombinant human metalloproteases, including collagenase-1, stromelysin-1, and gelatinases (72 kD and 92 kD) (all generously provided by Pfizer Central Research) were verified by Western immunoblot. A protein amount as high as 260 ng was used. Results showed no cross-reactivity of the monoclonal anti–collagenase-3 with the other metalloproteases tested.

Band intensity was measured on a negative film, using a laser scanning densitometer (GS 300; Hoefer Scientific Instruments, San Francisco, CA). Data were analyzed on the densitometry software GS 370 Macintosh version (Hoefer Scientific Instruments) using the Quick Integration function. The results are presented as arbitrary units.

### *Electrophoresis*

In evaluating if collagenase-3 plays a role in cartilage degradation, we determined the cleavage rate of this enzyme on soluble type II collagen (bovine; Sigma Chemical Co.) and soluble type I collagen (rat; Sigma Chemical Co.) and compared it to collagenase-1. An increasing amount (2–20 μg) of either type II or type I collagens was incubated in a Tris (50 mM) buffer, pH 7.5, containing 10 mM $CaCl_2$ and 200 mM NaCl, for 3 h at 25°C in the presence of APMA (1.5 mM, 3 h at 37°C)-activated collagenase-3 or collagenase-1, and the $K_m$ and $V_{max}$ determined following the method of Welgus et al. (34, 35). Briefly, after the incubation, the samples were boiled in SDS buffer containing EDTA and dithiothreitol and electrophoresis on a gel of 8% polyacrylamide. After electrophoresis, the protein bands were stained with 1% Coomassie blue. Measurement of the intensity of the bands was done using the laser scanning densitometer of the negative film. The $V_{max}$ and $K_m$ were determined by the Lineweaver-Burk plots; $V_{max}$ was expressed as the number of collagen molecules degraded/molecule of collagenase/h, and $K_m$ as molarity.

### *Statistical analysis*

Data are expressed as mean±SEM. When applicable, statistical significance was assessed by the Student's two tailed $t$ test; $P$ values equal or less than 0.05 were considered significant.

## Results

*Collagenase-3 mRNA in articular tissue.* RT-PCRs for collagenase-1, stromelysin-1, and collagenase-3 were performed on RNA from primary culture of chondrocytes and from first passage synoviocytes of normal or OA conditions or stimulated with rhIL-1β. Results (Fig. 1) revealed that although both collagenase-1 and stromelysin-1 are expressed by chondrocytes and synoviocytes, collagenase-3 was expressed in chondrocytes

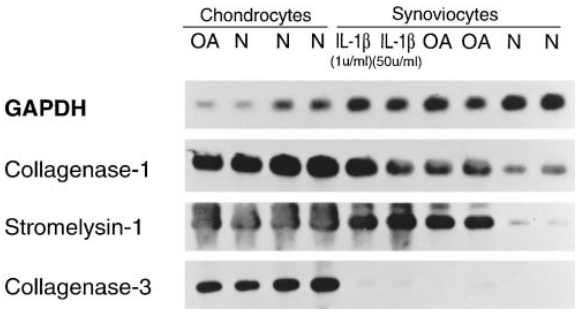


*Figure 1.* RT-PCR of GAPDH, collagenase-1, stromelysin-1, and collagenase-3 of RNA from normal and osteoarthritic (*OA*) chondrocytes and synoviocytes stimulated (18 h at 37°C) or not with rhIL-1β. The experiment was processed with 2 μg RNA for each specimen and 30 cycles for the PCR.

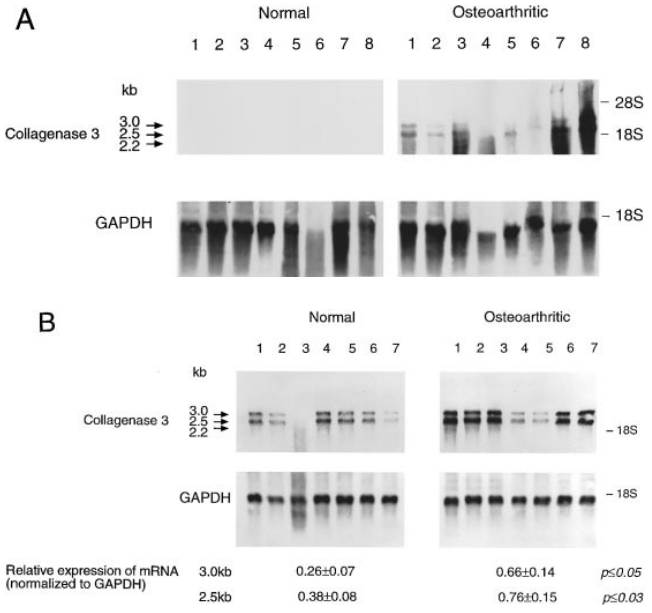


*Figure 2.* Northern blot of collagenase-3 and GAPDH mRNA of normal and OA human (*A*) cartilage and (*B*) chondrocytes in primary culture. Briefly, cells were released by sequential enzymatic digestion, plated and cultured in DME supplemented with 10% FCS and antibiotics. 24 h before the experiment, the cells were incubated in fresh serum-free medium. The positions of 28S and/or 18S ribosomal RNA are indicated. The relative expression of collagenase-3 mRNA normalized to GAPDH is expressed as mean ± SEM. *P* values were calculated using the Student's *t* test.

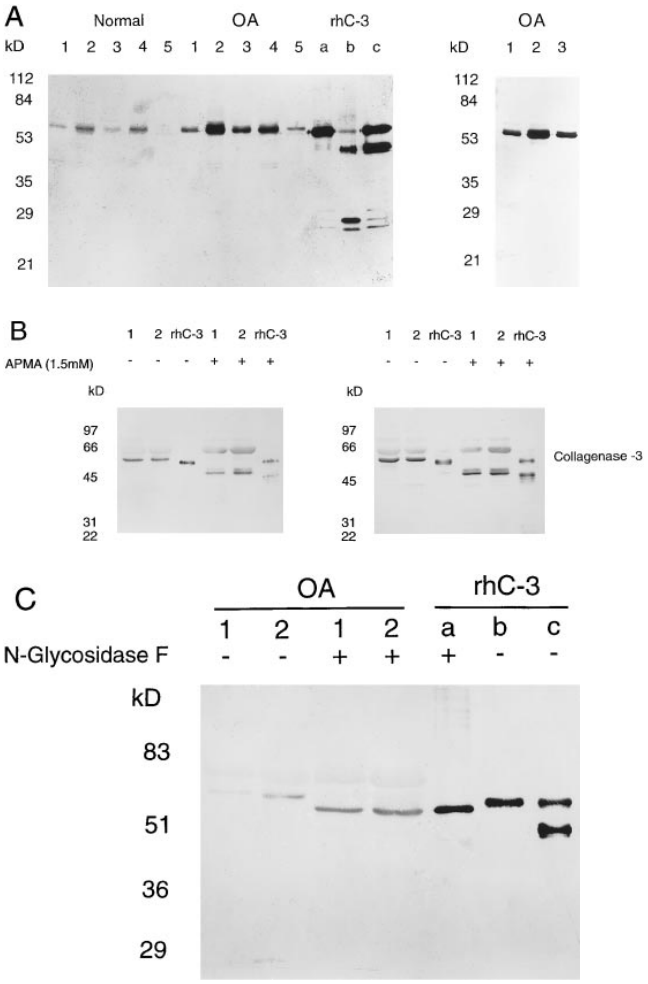


*Figure 3.* Western immunoblot of collagenase-3 (*A*) of culture medium from normal and OA chondrocytes. Controls were the human recombinant collagenase-3 (*rhC-3*) alone (lane *a*, 20 ng), and (lanes *b* and *c*) APMA-treated (30 ng; 100 ng, respectively), in which the first band corresponded to the proform (lane *a*) and the second to the APMA-activated form (lanes *b* and *c*). Specific antibodies were the mAb (*left*) and the polyclonal (*right*) as described in Methods. In the latter (*right*) are represented the first three OA samples. (*B*) Two OA chondrocyte culture medium treated or not with APMA. The controls were the human recombinant collagenase-3 treated with APMA (*rhC-3+*). Specific antibodies were the mAb (*right*) or the polyclonal (*left*). (*C*) Two OA chondrocyte culture medium treated or not with N-glycosidase F. Controls were the rhC-3-treated (lane *a*) or not with N-glycosidase-F (lane *b*) and with APMA (lane *c*). The specific antibody was the mAb. *kD*, represents the molecular weight standards. Of note, in addition to the bands characteristic of collagenase-3, higher bands (~ 66 and 60 kD) were sometimes present (*B* and *C*); further experiments revealed that the latter bands represented nonspecific binding.

and barely detectable in synoviocytes stimulated with IL-1β and from one OA sample.

Prompted by the previous finding, we investigated the in vivo expression of collagenase-3 gene in human articular cartilage from normal ($n = 8$) and OA ($n = 8$) patients. Interestingly, and as illustrated in Fig. 2 *A*, no collagenase-3 mRNA could be detected in normal cartilage, but the enzyme was highly expressed in OA. This cannot be related to the loading of RNA, as the GAPDH mRNA (housekeeping gene) showed little difference between the normal specimens. The only exception was specimen No. 6 where the RNA appeared to be degraded. However, when the film exposition time was increased (fourfold) and combined with the use of more sensitive chemiluminescent reagent, we detected collagenase-3 expression from the normal cartilage (data not illustrated), suggesting that in this normal tissue, the mRNA for the collagenase-3 is expressed in very low abundance. In OA, bands corresponding to 3.0 and 2.5 kb were found in all specimens examined, with some variation in the amount among different individuals. For some specimens, a third band corresponding to 2.2 kb was also detected.

The level of collagenase-3 mRNA in primary culture chondrocytes (Fig. 2 *B*) showed that indeed normal chondrocytes expressed this enzyme gene. Similar to the cartilage mRNA findings, the two highest bands of 3.0 and 2.5 kb were always detected, and the third of 2.2 kb appeared only in some specimens. A mean higher level of collagenase-3 expression was found for OA chondrocytes; the relative expression of collagenase-3 mRNA normalized to GAPDH, as scanned by densitometry, showed significant differences ($P \leq 0.05$ and $P \leq 0.03$) between normal and OA chondrocytes for the 3.0- and 2.5-kb bands, respectively. Of note is that the relative expression of two OA specimens (lanes *4* and *5*) appeared to be similar to some of the normal specimens. Although the present work does not allow us to draw a definite conclusion, one has to remember that this study was carried out with human OA specimens and variations among specimens could result from the medication taken by the patient. For example, it has been

shown that drugs such as corticosteroids downregulate the expression of metalloproteases (36).

Similar experiments were carried out with human synoviocytes isolated from normal and OA patients. With the Northern blot, we were not able to find collagenase-3 expression neither from normal nor pathological conditions (data not illustrated). This was true even if the film exposition time was stretched to its maximum.

*Collagenase-3 protein synthesis level.* The capability of human chondrocytes to produce the collagenase-3 protein was also investigated. Primary cells were incubated for 72 h at 37°C, and Western immunoblotting performed on the culture medium using two different antibodies: an mAb (F17 IB-3-C5) and a rabbit polyclonal anti–collagenase-3 (26). Preliminary experiments examining metalloprotease cross-reactivity were carried out with the mAb, and no cross-reactivity was noted when 20–260 ng of the protein were used for human collagenase-1, stromelysin-1, and gelatinases 72 kD and 92 kD. Results with both antibodies showed that human chondrocytes were able to synthesize and secrete collagenase-3 (Fig. 3 *A*). Comparison between normal and OA revealed that the synthesis of collagenase-3 was low in normal chondrocytes, whereas high levels were found for OA cells (Fig. 3 *A*). Densitometric analysis (arbitrary unit) showed that the levels of enzyme in OA cells (6,889±2,428) was statistically higher ($P \leq 0.04$) that those from normal (728±290) chondrocytes. The rabbit polyclonal antibody revealed the same band as the monoclonal, and results from the first three OA samples (Nos. 1, 2, and 3) are illustrated in Fig. 3 *A* (*right panel*).

To assess if collagenase-3 secreted by chondrocytes could be activated similarly by other metalloproteases, culture medium from OA chondrocytes were treated with APMA (1.5 mM) for 18 h at 37°C. Western immunoblotting demonstrated (Fig. 3 *B*) that collagenase-3 from chondrocytes could be activated by APMA with a reduction of the molecular mass of ~ 10 kD. This was noted with both antibodies.

Of note was the presence of a doublet for the proenzyme as well as the APMA-activated collagenase-3 when the polyclonal antibody was used (Fig. 3 *A*, *right*). Although only one band could be seen for the proenzyme form when revealed with the mAb, the doublet was detected on the APMA-activated collagenase-3 (Fig. 3 *B*), indicative of its presence as a doublet but the epitope hidden in the proform. In addition, one can note the slightly higher molecular weight of the chondrocyte collagenase-3 proform when compared to the human recombinant (Fig. 3, *A*, *B*, and *C*). Given that the enzyme primary sequence had three potential sites of N-glycosylation, we were suspicious about a higher level of glycosylation on the chondrocyte collagenase-3. Indeed, the treatment of the culture medium and the rh collagenase-3 with the N-Glycanase F yielded both enzymes to an identical molecular weight (Fig. 3 *C*).

Of note, in addition to the bands characteristic of collagenase-3, higher bands were also present on the Western immunoblot (Fig. 3, *B* and *C*). Further experiments, in which the first antibody was substituted by a mouse IgG (Nordic Immunology, Tilburg, The Netherlands), revealed that the higher bands represented nonspecific binding and were not related to collagenase-3.

To ensure that collagenase-3 was not expressed by synoviocytes, Western immunoblotting was also performed on the culture medium of these cells from first passage and treated as above for chondrocytes. Results showed that no specific collagenase-3 protein could be detected from the synoviocytes either from normal or OA cells by this method (data not illustrated).

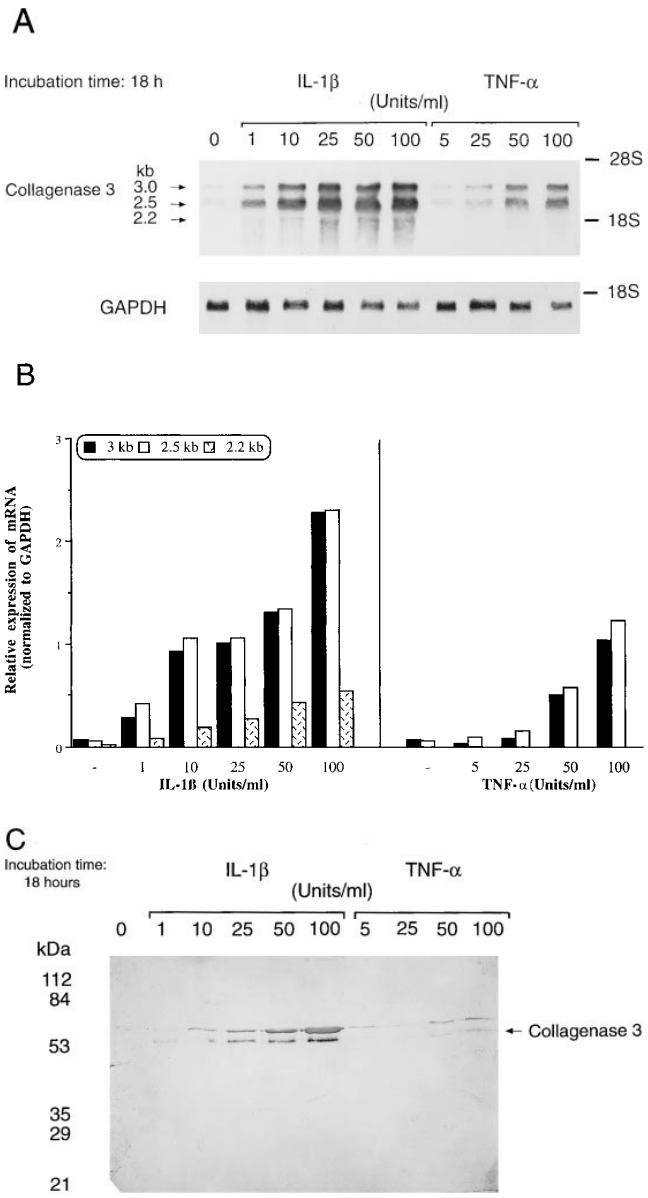


*Figure 4.* Collagenase-3 dose-response of IL-1β- and TNF-α–stimulated chondrocytes. (*A*) Northern blot of collagenase-3 and GAPDH mRNA. The position of 28S and 18S ribosomal RNA are indicated. (*B*) Histogram of the relative expression of mRNA normalized to GAPDH. (*C*) Western immunoblot of collagenase-3 chondrocyte culture medium. Of note is the presence of a nonspecific higher band.

*Cytokine chondrocyte stimulation.* By examining both mRNA and protein levels, we further investigated whether collagenase-3 could be modulated by two proinflammatory cytokines, IL-1β and TNF-α.

In the first set of experiments, first passage chondrocytes were incubated for 18 h with increasing concentrations of each cytokine (0–100 U/ml). Northern blot was performed on cells and Western immunoblotting on the culture medium. Both IL-1β and TNF-α exhibited dose-dependent increases in the expression of collagenase-3 (Fig. 4, *A* and *B*). IL-1β stimulated

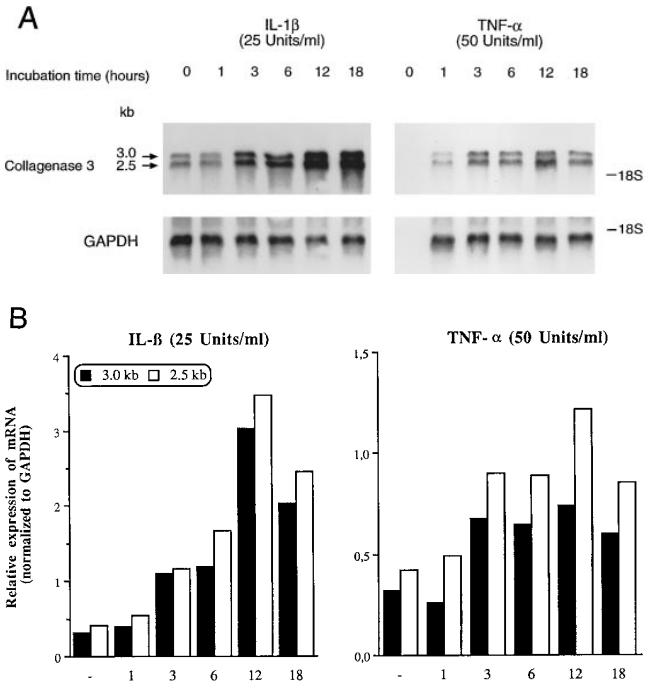


*Figure 5.* Collagenase-3 time course of IL-1β- and TNF-α–stimulated chondrocytes. (*A*) Northern blot of collagenase-3 and GAPDH mRNA. The position of 18S ribosomal RNA is indicated. (*B*) Histogram of the relative expression of mRNA normalized to GAPDH.

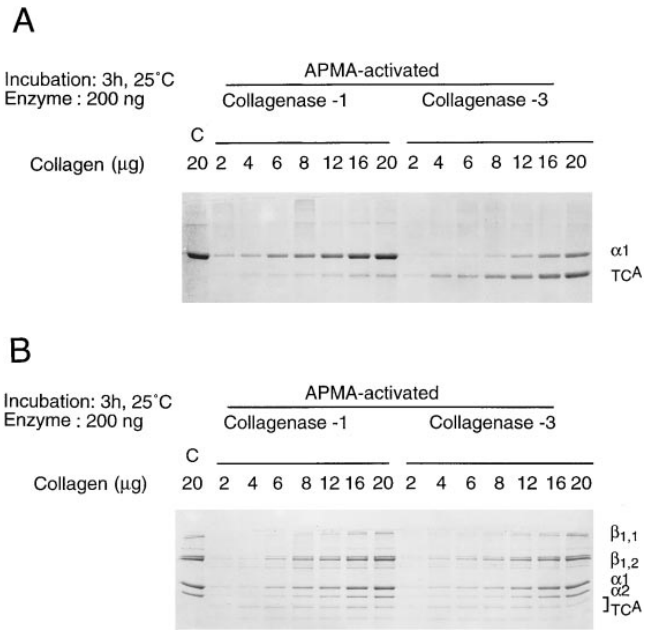


*Figure 6.* Electrophoresis of collagens (*A*) type II and (*B*) type I incubated with APMA-activated collagenase-1 or collagenase-3. Controls (*C*) represent preparations of type II or type I collagen without added enzymes.

simultaneously and proportionally the three collagenase-3 transcripts. Compared to IL-1β, TNF-α induction produced a lower level of collagenase-3 mRNA expression, and only the two higher collagenase-3 transcripts (3.0 and 2.5 kb) were detected.

Western immunoblotting showed that the synthesis of chondrocyte collagenase-3 protein was, as for the mRNA, stimulated by both cytokines and increased in a dose-dependent manner (Fig. 4 *C*). As for the expression, TNF-α was a less potent stimulating factor in the production of the collagenase-3 protein.

In the second set of experiments, chondrocytes were incubated in the presence of rhIL-1β at 25 U/ml or TNF-α at 50 U/ml for different time periods (0–18 h), and the level of collagenase-3 mRNA measured by Northern blot. Treatment of chondrocytes with IL-1β increased the collagenase-3 mRNA level by 3 h, it peaked at 12 h and decreased thereafter (Fig. 5, *A* and *B*). TNF-α also stimulated the collagenase-3 mRNA expression with maximal stimulation observed at 3 h of treatment, which was sustained thereafter. The two major transcripts (3.0 and 2.5 kb) were induced proportionally.

*Collagenase-3 cleavage rate on collagen.* To assess the collagen cleavage rate activity of collagenase-3, values for the $K_m$ and $V_{max}$ were determined on type II and type I collagens and compared with those from collagenase-1. As illustrated in Fig. 6, both collagenases cleaved collagen types II and I at a 0.75: 0.25 locus, typical of catalytic behavior of the collagenase (37). Values for $K_m$ and $V_{max}$ were obtained from the Lineweaver-Burk plots. Results showed that on type I collagen, the $K_m$ and $V_{max}$ values for the collagenase-1 (0.9 μM; 5.2 $h^{-1}$, respectively) were similar to those obtained for collagenase-3 (1.1 μM; 6.1 $h^{-1}$). Interestingly, on the type II collagen, the $V_{max}$ of collagenase-3 (20.8 $h^{-1}$) was increased by about fivefold compared to collagenase-1 (4.3 $h^{-1}$), whereas, the $K_m$ of collagenase-3 (3.2 μM) was only slightly higher than that of collagenase-1 (1.1 μM). Moreover, when the ratio $K_{cat}(V_{max})/K_m$ was used as an index of substrate specificity, results suggested that the type II collagen was a better substrate for collagenase-3. The $K_{cat}/K_m$ value of collagenase-3 (6.5 $\mu M^{-1}$ $h^{-1}$) for type II collagen was ~ 1.7-fold higher than collagenase-1 (3.9 $\mu M^{-1}$ $h^{-1}$).

## Discussion

This is the first study demonstrating the involvement of a new collagenase, collagenase-3, in the pathophysiology of human OA. Supporting this premise are the findings that collagenase-3 expression and synthesis are upregulated in OA cartilage, and that when compared to collagenase-1, the collagenase-3 demonstrated a higher level of cleavage activity on type II collagen. The participation in the pathophysiology of OA of a new member of the collagenase class is of the utmost importance as damage to cartilage collagen structure appears to play a pivotal role in matrix degradation and in the functional loss of matrix physiologic properties.

The present work shows that collagenase-3 is not expressed or synthesized by synoviocytes from normal or OA individuals. Although the latter cells are widely assumed to produce the majority of the metalloproteases in arthritic synovium (37–40), it is therefore improbable that the enhancement of collagenase-3 in OA cartilage reflects a diffusion of this enzyme from the synovial membrane. The present work raises a new insight into this human disease as until now the major enzyme implicated in collagen destruction was the collagenase-1 which can be produced by the chondrocytes but also by the synoviocytes. Moreover, this study provides additional arguments to

the fact that cartilage may cause destruction of its own matrix via an intrinsic pathway.

It is only recently that in addition to the two interstitial human collagenases, collagenase-1 (or fibroblast collagenase) and neutrophil collagenase, a third member belonging to this subgroup was identified and termed collagenase-3 (26). This human metalloprotease presents 86% amino acid sequence similarity with rat and mouse collagenase-1, the latter assumed to be the murine counterparts of human collagenase-1. Collagenase-3 was first identified on pathological tissues (26); the authors reported that a variety of normal tissues did not express collagenase-3.

Despite the similarities between each member of the metalloprotease family, (among which they are processed as inactive zymogens, they require activation before exerting their effect; they are inhibited by TIMP; and their expression and synthesis are regulated similarly by various factors [22]) differences may exist between closely related members. For instance, opposite effects were observed between the known human gelatinases, the 92 and 72 kD (41–44). Differences were found not only in their gene expression when target cells were treated with factors such as IL-1β and TNF-α, but also in substrate specificity and in the activation process where the known activator, trypsin, was inactive on 72-kD gelatinase. Furthermore, changes of target cell shape resulted in a specific loss of the production of only the 92-kD gelatinase (45).

Therefore, the first part of our study on collagenase-3, was to examine if marked differences existed between this enzyme and the other known collagenase found in the cartilage, collagenase-1. Results showed that one contrast between collagenase-3 and the other metalloproteases, and more particularly collagenase-1, was the absence of the former in synoviocytes. The latter finding could have been indicative of a preferential or an exclusive substrate specificity of collagenase-3 for type II collagen. However, this appeared not to be the case as collagenase-3 also cleaved the type I collagen (26, Fig. 6). Although this corroborates with the findings of Welgus et al. (34, 35), lower values of $V_{max}$ were found when the effect of collagenase was assayed on the type I collagen. One explanation may be that this study, instead of using purified collagenases (34, 35), used human recombinant ones. The latter could therefore lack some posttranslation modification than when it is processed in vivo. This possibility is likely, as we showed that the differences in glycosylation occurred between the human chondrocyte and recombinant collagenase-3.

The presence of an additional collagenase in human cartilage raised the question about the relative importance of collagenase-1 in this tissue, as only a low level of expression of the latter is found in cartilage compared to other metalloproteases (29, 46, 47). As both collagenases are able to cleave the type II collagen, one may speculate that both enzymes are important in the pathophysiology of OA but they may play different roles. The higher $K_{cat}/K_m$ value of collagenase-3 compared to collagenase-1 on type II collagen, indicates that this collagen type was a better substrate for the collagenase-3. Differences in the amino acid sequence of the activated enzymes could be of importance in this regard. Indeed, as demonstrated for the collagenase-1 by site-directed mutagenesis of the cDNA experiments, the truncation of its COOH-terminal domain of the active enzyme results in losses of collagenolytic activity but gains in gelatinolytic activity (48). In that regard, a recent study by Abramson et al. (49) showed differences in the autolytic activation between rat collagenase-1 and human collagenase-1 after APMA or trypsin treatments (50). In contrast with collagenase-1, which required another proteolytic cleavage to be activated to its full potential, rat collagenase-1 was capable of maximal autoactivation. Alternatively, but not exclusively, it may be that the inhibition process by TIMP occurred differently, or as suggested by Freije et al., (26) that other TIMPs, such as TIMP-3, were involved in the inactivation process. Interestingly, although TIMP-3 regulation is not completely known, this inhibitor appeared to be tightly bound to the extracellular matrix macromolecules (51).

All OA cartilage specimens studied demonstrated a measurable amount of collagenase-3 mRNA, whereas Northern blot detection of this message in normal cartilage required a longer exposition time with a higher sensitive chemiluminescent reagent. However, additional experiments showed the presence of collagenase-3 mRNA on those extracted from normal isolated chondrocytes. Although the reason for this discrepancy is not clear at the present time, there are many possible explanations. It may be that the metabolism of the chondrocytes in monolayer culture is higher than the autologous ones in the cartilage, due for example to the change of cell shape. Indeed, the cell shape and the organization of the cytoskeleton have been identified as factors that may influence the activity of some cells types, including chondrocytes (52–54). Moreover, and as mentioned above, MacDougall et al. (45) recently demonstrated that cell shape alterations profoundly affect the production of one of the metalloproteases, the 92-kD gelatinase. Another possibility may be that interaction in the cartilage of chondrocytes with specific components of the extracellular matrix controls specific synthetic events. Nonetheless, all the chondrocyte extractions and culture procedures were carried out in an identical manner for each specimen, and significant differences between normal and OA were obtained with the isolated cells.

Two transcripts of 3.0 and 2.5 kb were always apparent when RNA was extracted either directly from OA cartilage or from isolated chondrocytes, and a third band of 2.2 kb was found occasionally, albeit at a relatively low level. These observations correlate well with the findings of Freije et al. (26) in which three different mRNA species were found with the full-length probe. Although the physiological significance of these mRNA transcripts is not yet known, it is suggested (26) to be the result of different polyadenylation sites in the 3′-flanking region of the gene. Even if further studies are required to determine their significance and translation process, our findings showed that at least the two highest species, the 3.0 and 2.5 kb, are modulated proportionally by the cytokines IL-1β and TNF-α in a time- and dose-dependent manner.

The present work also demonstrated that the chondrocyte mRNA level was parallel with those of secreted protein, as the profile of the collagenase-3 protein level was similar to that of the mRNA. A significantly higher level (9.5-fold) of this enzyme was found when OA was compared to normal. Moreover, IL-1β and TNF-α induced the collagenase-3 protein in a dose-dependent manner. Characterization of collagenase-3 by immunoblot showed, for both normal and OA, that the secreted protein was typical of the metalloprotease family, in which it is released as a proenzyme form and found as a doublet. The former may indicate that this enzyme is not activated in the culture medium. Furthermore, as the other metalloproteases are, the secreted chondrocyte collagenase-3 is glycosy-

lated. This process could be of importance, since the glycosylation could, for example, serve as a prevention against proteolytic cleavage by stearic hindrance (55) and/or increase the enzyme activity for its substrate (56).

It has now been established that synovial inflammation plays an important role in the progression of OA, primarily by its secretion of proinflammatory mediators such as catabolic cytokines. Two of these cytokines, IL-1β and TNF-α, are widely assumed to play a major role in the pathological process of cartilage degeneration (16). They participate in the promotion of matrix degradation by their ability to stimulate chondrocytes to express and synthesize metalloproteases. By the demonstration that these two cytokines are able to upregulate in a dose-dependent fashion not only the expression of collagenase-3 (mRNA) but also its production (protein), it is conceivable to speculate that these cytokines may be responsible for the high level of collagenase-3 in OA cartilage.

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