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Induction of collagenase-3 (MMP-13) in rheumatoid arthritis synovial fibroblasts

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Abstract

There is a growing body of evidence that implicates matrix metalloproteinases (MMPs) as major players in numerous diseased conditions. The articular cartilage degradation that is characteristic of rheumatoid arthritis (RA) is believed to be mediated by the collagenase subfamily of matrix metalloproteinases. The preference of collagenase-3 (CL-3) for collagen type II makes it a likely candidate in the turnover of articular cartilage and a potential target for drug development. In this study, RA synovial membrane tissue was shown to express CL-3 mRNA by reverse transcriptase-polymerase chain reaction (RT-PCR) and protein by immunohistochemistry. Fibroblasts isolated and cultured from RA synovial membrane tissue were induced to express CL-3 mRNA. CL-3 mRNA was detected after PMA treatment in 16 of the 18 RA synovial membrane fibroblast cell lines established for this study. These fibroblasts also expressed mRNA for collagenase-1 (CL-1, MMP-1), membrane type-1 matrix metalloproteinase, gelatinase A, gelatinase B, stromelysin-1, stromelysin-2, TIMP-1, and TIMP-2. They were further shown to express CL-1 mRNA constitutively and CL-3 mRNA only after stimulation with PMA, IL-1, TGF-β1, TNF-α, or IL-6 with IL-6sR. These fibroblasts also expressed after induction both CL-1 and CL-3 at the protein level as determined by Western blot analyses and immunofluorescence.

Keywords: Matrix metalloproteinase; Collagenase-3; Synovial fibroblast

Abbreviations: bp, base pair(s); CL-1, collagenase-1, fibroblast-type collagenase, MMP-1; CL-3, collagenase-3, MMP-13; DAPI, 4,6-diamidino-2-phenylindole; FBS, fetal bovine serum; FITC, fluorescein-5-isothiocyanate; Gel A, gelatinase A, M_r 72 000 gelatinase, MMP-2; Gel B, gelatinase B, M_r 92 000 gelatinase, MMP-9; IL-1, interleukin-1; IL-6, interleukin-6; IL-6sR, interleukin-6 soluble receptor; mAb, monoclonal antibody; MMP, matrix metalloproteinase; MT1-MMP, membrane type-1 MMP, MMP-14; pAb, polyclonal antibody; PBS, phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SL-1, stromelysin-1, MMP-3; SL-2, stromelysin-2, MMP-10; TBS-TX, Tris buffered saline with 0.1% Triton X-100; TGF-β1, transforming growth factor-β1; TIMP, tissue inhibitor of metalloproteinases; TNF-α, tumor necrosis factor-α; TXRD, Texas-red dye

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1. Introduction

The matrix metalloproteinases (MMPs) comprise an expanding family of zinc and calcium dependent enzymes (for reviews see [1,2]), which collectively are able to degrade most of the components of the extracellular matrix. MMPs are powerful enzyme players in tissue remodeling and wound healing. Alterations of the levels of MMP expression, activation, and inhibition by tissue inhibitors of metalloproteinases (TIMPs) may contribute to the progression of numerous diseases.

Most MMPs are secreted as zymogens that require external activation to proteolytically competent forms. In vitro, MMP activation can result from treatment with conformational perturbants such as sodium dodecyl sulfate or (4-aminophenyl)mercuric acetate, as well as by proteolytic activation with trypsin or other proteases. The MMPs can be inhibited by the TIMPs and α_2-macroglobulin. The ratio of inhibitors versus MMPs is only one mechanism that plays a role in controlling the degradation or the accumulation of the extracellular matrix. Regulation also occurs at the transcriptional level and at the level of MMP activation. Improper management of MMP expression and activation has been suggested to play major roles in a number of disease conditions such as osteoarthritis, rheumatoid arthritis, tumor invasion and metastasis, and Alzheimer's disease.

The collagenases, because of their ability to cleave the helical collagens, are believed to play a major role in the collagen degradation associated with arthritic conditions. One of the newest members of the collagenase subfamily is collagenase-3 (CL-3, MMP-13), which was originally isolated from a breast tumor library [3]. Human CL-3 shares only 50.5% amino acid homology with human collagenase-1 (CL-1), while it is 86% homologous to the mouse and rat collagenases [3–5]. CL-3 has been shown to degrade collagen types I, II, and III, as well as being able to degrade the cartilage proteoglycan aggrecan [6]. Unlike CL-1, which has a substrate preference for collagen type III, CL-3 prefers collagen type II which is the primary collagen found in articular cartilage [7]. Gelatinase B (MMP-9) and membrane type-1 MMP (MT1-MMP, MMP-14) have both been shown to be able to activate CL-3 in vitro [7]. In addition, activation of CL-3 by MT1-MMP is augmented by the presence of gelatinase A (MMP-2) [7].

In addition to being detected in breast tumors, CL-3 mRNA has been found in articular cartilage [8] and in the synovial tissue from patients with osteoarthritis or rheumatoid arthritis, but not in normal synovial tissue [9]. The CL-3 protein has been detected in synovial fluid and in cartilage from patients with reactive arthritis or active seropositive rheumatoid arthritis, but no CL-3 protein could be detected in the synovial tissue of these patients [8]. Immunohistochemical staining of rheumatoid arthritis and osteoarthritis synovial tissues identified fibroblast- and macrophage-like cells that were positive for CL-3 [10]. It was originally reported in several studies that cultured human synovial fibroblasts could not express CL-3 mRNA or protein [11,12]. Recently, CL-3 mRNA and protein have been found in pannocytes of the pannus-hard tissue junction of tissue from RA patient samples [13]. Also in this recent study, cultured cells from RA tissue were shown to express CL-1 and CL-3 constitutively and could be stimulated with tumor necrosis factor-α (TNF-α) or interleukin-1 (IL-1β) [13]. However, CL-3 and not CL-1 expression was stimulated by phorbol 12-myristate 13-acetate (PMA) [13]. In contrast in yet another study, the CL-3 mRNA basal level of human synovial fibroblasts was not stimulated with PMA [14]. In addition, CL-3 expressed by synovial fibroblasts isolated from rabbit synovium was stimulated with PMA, IL-1β, or TNF-α [15]. As mentioned above, a clear consensus has not been reached in regard to the expression and induction patterns of CL-3 in synovial fibroblasts.

In this paper, reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry were utilized to examine the CL-1 and CL-3 expression in RA synovial membrane tissue. RT-PCR was also utilized to examine CL-1 and CL-3 mRNA expression in human RA synovial membrane fibroblasts. Not only did these synovial fibroblasts express mRNA for CL-1, but also CL-3 when induced. Sequencing of the RT-PCR fragment verified that the primers were specific and that these cells do express mRNA for CL-3. CL-3 mRNA was induced with PMA in 16 out of 18 (88.8%) RA cell lines established for this study. CL-3 mRNA was also induced in these synovial membrane fibroblasts with IL-1,

TGF-β1, TNF-α, and IL-6 in the presence of IL-6sR. CL-3 protein expression was also observed in these RA synovial fibroblasts after PMA induction by Western blot analyses and immunofluorescence.

2. Materials and methods

2.1. CL-3 expression in Escherichia coli, extraction, and purification

The CL-3 cDNA was a generous gift from Carlos Lopez-Otin [3]. The cDNA was removed from pEMBL19 by *Hin*dIII digestion. The insert was then ligated into the *Hin*dIII site of pGEMEX-1 (Promega, Madison, WI). The coding sequence for gene 10 of pGEMEX was removed by site-directed mutagenesis using the following oligonucleotide: 5′-ACTTTAAGAAGGAGATATACATATGCTGCC-CCTTCCCAGT-3′.

The deletion and the coding sequence for CL-3 was verified by DNA sequencing. The construct (pGEMEX-CL-3) was transformed into *E. coli* DE3 cells [16] and transcription from the T7 promoter of the expression plasmid was induced after the cells were grown to a density of 10^8 cells/ml by 1 mM isopropyl-β-D-thiogalactopyranoside.

After a 2 h expression period at 37°C, the cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$. Following passage through a French press at 10 000–15 000 psi in the presence of 1 mM phenylmethylsulfonyl fluoride, the samples were centrifuged and the pellets washed with 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$. The washed pellets were extracted with 6 M urea in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$ for 2–3 h at 4°C on an orbital mixer.

The 6 M urea extracts were passed over a Sephacryl S-200 HR (Pharmacia, Piscataway, NJ) column (2.5 cm×88 cm) equilibrated with 6 M urea in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$. Peak fractions (>90% pure) were dialyzed against 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$ to remove the urea. Protein concentrations were determined by the Bradford method [17] using bovine serum albumin as a standard. The purified recombinant CL-3 was then used to make monoclonal antibodies (mAb) according to previously described methods [18]. Anti-CL-3 mAb ID3 was then utilized throughout this study.

2.2. Other antibodies

Polyclonal (pAb 647) and monoclonal (mAb III_{12}) anti-CL-1 antibodies have been previously described and shown to be specific for human CL-1 [19,20]. MT1-MMP pAb 815 was purchased from Chemicon (Temecula, CA).

2.3. Electrophoretic methods

Samples were resolved on SDS-PAGE gels by the methods of Laemmli [21] and transferred to nitrocellulose membrane by electroblotting for Western blot analyses. The membranes were blocked with 5% bovine serum albumin in 50 mM Tris-HCl, pH 7.5, and incubated with primary antibody (5 μg/ml) in 50 mM Tris-HCl, pH 7.5, 0.5% bovine serum albumin for 1 h at 37°C. The membranes were then washed in TBS-TX (50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 1% Triton X-100) and subsequently incubated with peroxidase-conjugated secondary antibody (6.5 μg/ml) as previously described [18]. Alternatively, enhanced chemiluminescence plus kit was utilized as per the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.4. Immunohistochemistry

Human synovial membrane tissue was obtained from RA patients through the University of Alabama at Birmingham tissue procurement facility. Tissue sections were prepared and then fixed in phosphate buffered formalin (Fisher Scientific, Pittsburgh, PA), dehydrated in xylenes, and embedded in paraffin. Tissue blocks were cut into 4 μm sections with a power microtomer and placed on microscope slides. The sections were deparaffinized by heating to 65°C for 1 h and subsequent treatments with xylenes. The tissues were rehydrated by soaking in 100%, 95%, and then 75% ethanol. Endogenous peroxidase quenching was carried out with 3.0% hydrogen peroxide for 10 min. Samples were stained using 3–6 μg/

ml of anti-CL-1 mAb III_{12}, anti-CL-3 mAb ID3, or anti-vimentin mAb 3400 (Chemicon) and with secondary antibodies, horseradish peroxidase enzyme conjugate, and aminoethyl carbazole chromogen and then developed as per the manufacturer's instructions (Zymed Laboratories, San Francisco, CA). The samples were then briefly counterstained with Mayer's hematoxylin and mounted in glycerol vinyl alcohol (GVA) mounting medium (Zymed Laboratories). Images were captured using a SPOT camera and software (Diagnostic Instruments, Birmingham, AL).

2.5. RT-PCR

Total RNA was recovered from homogenized synovial membrane tissue or cells as per the RNeasy spin mini-column manufacturer's instructions (Qiagen, Valencia, CA). RNA (0.4 μg) was added to each RT reaction containing One-Step RT-PCR mix (Qiagen) and the appropriate PCR primers in 50 μl total. RT was carried out for 30 min at 50°C followed by a 15 min step at 94°C. PCR was then performed in a thermocycler programmed for 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The RT-PCR reaction was mixed with 10 μl 40% sucrose with bromophenol blue and 8 μl of sample was loaded per lane on a 1% agarose gel. Gels were stained with ethidium bromide and photographed. The following specific primers were used in the PCR reactions and the size of the PCR product in base pairs (bp) are shown.

Cyclophilin (274 bp)	5′-CCGTGTTCTTCGACATT (sense)
	5′-GCCAGGACCCGTATGCT (antisense)
CL-1 (721 bp)	5′-GAAGGTGATGAAGCAGCCCAGATGT (sense)
	5′-CAGTTGTGGCCAGAAAACAGAAGTG AAA (antisense)
CL-3 (517 bp)	5′-GCTTAGAGGTGACTGGCAAC (sense)
	5′-CCGGTGTAGGTGTAGATAGGAAC (antisense)
MT1-MMP (670 bp)	5′-GATAAACCCAAAAACCCCACCTA (sense)
	5′-CCCTCCTCGTCCACCTCAATG (antisense)
Gel A (364 bp)	5′-ATCCGTGGTGAGATCTTCTTCTT (sense)
	5′-AGCCAGGATCCATTTTCTTCTT (antisense)
Gel B (519 bp)	5′-TGCCAGTTTCCATTCATCTTCCAA (sense)
	5′-CTGCGGTGTGGTGGTGGTT (antisense)
TIMP-1 (534 bp)	5′-CCTTCTGCAATTCCGACCTCGTC (sense)
	5′-CGGGCAGGATTCAGGCTATCTGG (antisense)
TIMP-2 (433 bp)	5′-TGGAAACGACATTTATGGCAACC (sense)
	5-ACAGGAGCCGTCACTTCTCTTGAT (antisense)
SL-1 (659 bp)	5′-GACACCAGCATGAACCTTGTT (sense)
	5′-GGAACCGAGTCAGGTCTGTG (antisense)
SL-2 (863 bp)	5′-TGCCCAGCAATACCTAGAAAAGTA (sense)
	5′-GGTTCCAGTGGGATCTTCG (antisense)

2.6. Tissue culture

RA synovial membrane tissue was washed with 70% ethanol and minced in phosphate buffered saline (PBS). The dissected tissue was digested with 1.5 mg/ml Dispase (Roche, Nutley, NJ) in serum free Dulbecco's modified Eagle's (DME) medium (Irvine Scientific, Santa Ana, CA). The cells were pelleted, resuspended in DME medium supplemented with 10% fetal bovine serum (FBS), plated, and grown in a 5% CO_2 incubator at 37°C. The cells were treated with PMA (Sigma) or cytokines IL-1, IL-6, IL-6 with IL-6sR, TNF-α, or TGF-β1 (R&D Systems, Minneapolis, MN). SW1353 chondrocytes were obtained from American Type Culture Collection (Rockville, MD) and maintained in DME medium supplemented with 10% FBS.

2.7. Sequencing of the collagenase-3 PCR fragment

The PCR fragment from RA synovial fibroblasts amplified with the CL-3 specific primers was purified using the Qiagen Gel Extraction Kit (Qiagen) and ligated into the modified TA vector, pGEM-T Vector (Promega). The plasmid was amplified in *E. coli* DH5α competent cells. Qiagen Mini-Prep Kits were used for plasmid purification. A primer specific for the T7 promoter (5′-AATACGACTCACTATAGGG-3′) and the CL-3 PCR primers were used in

modified Sanger sequencing reactions [22] to sequence the CL-3 fragment.

2.8. Western blot of conditioned medium

For detection of CL-3 in the conditioned medium, SW1353 chondrocytes or synovial membrane fibroblasts were treated with 1.6×10^{-7} M PMA in serum free medium for 15 h. The conditioned medium was removed and 10 ml were lyophilized overnight in a Freezemobile Lyophilizer (Vitris, Gardiner, NY). It was then resuspended in 400 μl of 4 M urea. Equal volumes of sample and of sample buffer were mixed and 20 μl total was loaded per lane on 10% SDS-PAGE gels and analyzed by Western blots.

2.9. Crude membrane extracts

Membrane fractions were extracted using a modified protocol described by Wrenn et al. [23]. In brief, after a 15 h treatment with PMA in serum free medium, the cells were washed thoroughly with cold PBS and suspended in isolation buffer (20 mM HEPES pH 7.4, 250 mM sucrose, 2 mM benzamidine, 2 mM ε-amino-*n*-caproic acid, 1 mM $CaCl_2$, 1 mM $MgCl_2$). The cells were then lysed with a tight fitting dounce and drawn repeatedly through a 25 g needle and syringe. The cell lysates were then centrifuged at $500\times g$ to pellet the nuclei. Post-nuclear supernatants were ultracentrifuged at $30\,000\times g$ for 45 min. The resulting pellet was resuspended in SDS-PAGE loading dye for Western blot analyses.

2.10. Immunofluorescence

Cells were seeded on glass coverslips and grown to 80% confluence. Cells were treated with PMA for 15 h. Cells were then washed with PBS and subsequently incubated in serum free medium with 10^{-6} M monensin for 3 h. All the samples were immediately fixed with phosphate buffered 10% formaldehyde for 1 h at 25°C. After fixation, the cells were washed with PBS and permeabilized for 1 min at 25°C with Tris buffered saline with 0.1% Triton X-100 (TBS-TX). The cells were blocked with 10% normal goat serum (Zymed Laboratories) in TBS-TX for 30 min at 25°C. Primary antibodies to CL-1 and CL-3 were diluted in 10% normal goat serum

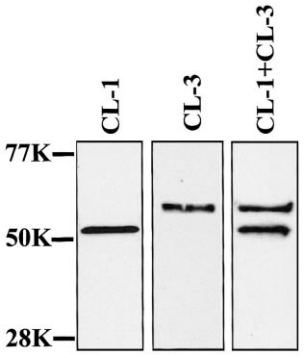

Fig. 1. Characterization of the anti-CL-3 mAb ID_3. Western blot analyses of PMA stimulated SW1353 chondrocyte conditioned medium demonstrated that these cells express and secrete CL-1 and CL-3, as well as demonstrated the specificity of these antibodies. Conditioned medium was concentrated, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with either 5 μg/ml anti-CL-1 mAb III_{12}, 5 μg/ml anti-CL-3 mAb ID_3, or 5 μg/ml of each antibody combined.

in TBS-TX and incubated for 1 h at 25°C. Fluorescein-5-isothiocyanate (FITC) conjugated goat anti-rabbit and Texas-red dye (TXRD) conjugated goat anti-mouse secondary antibodies (Zymed Laboratories) were incubated in TBS-TX with 10% normal goat serum and incubated for 1 h at 25°C. During incubation with the secondary antibodies, 5 μg/ml of 4,6-diamidino-2-phenylindole (DAPI) was used to counterstain the nuclei. Slides were mounted in Prolong Antifade Medium (Molecular Probes, Eugene, OR) and stored at −20°C. Images were captured using an Olympus IX70 microscope equipped with digital confocal capabilities and IP Lab software (Signal Analytics, San Jose, CA). Three images were captured per sample using filters for FITC, TXRD, and UV. The DAPI images were overlaid with the FITC or TXRD to yield the final images.

2.11. PMA and cytokine induction

Cells were washed with PBS and incubated with either PMA (1.6×10^{-7} M), IL-1 (5 ng/ml), IL-6 (5 ng/ml), IL-6 (5 ng/ml) with IL-6sR (250 ng/ml), TNF-α (10 ng/ml), or TGF-β1 (10 ng/ml) in serum free DME. The cells were then washed and RT-PCR performed as described above. Equal volumes of each sample were analyzed in a 1% agarose gel and visualized with ethidium bromide staining.

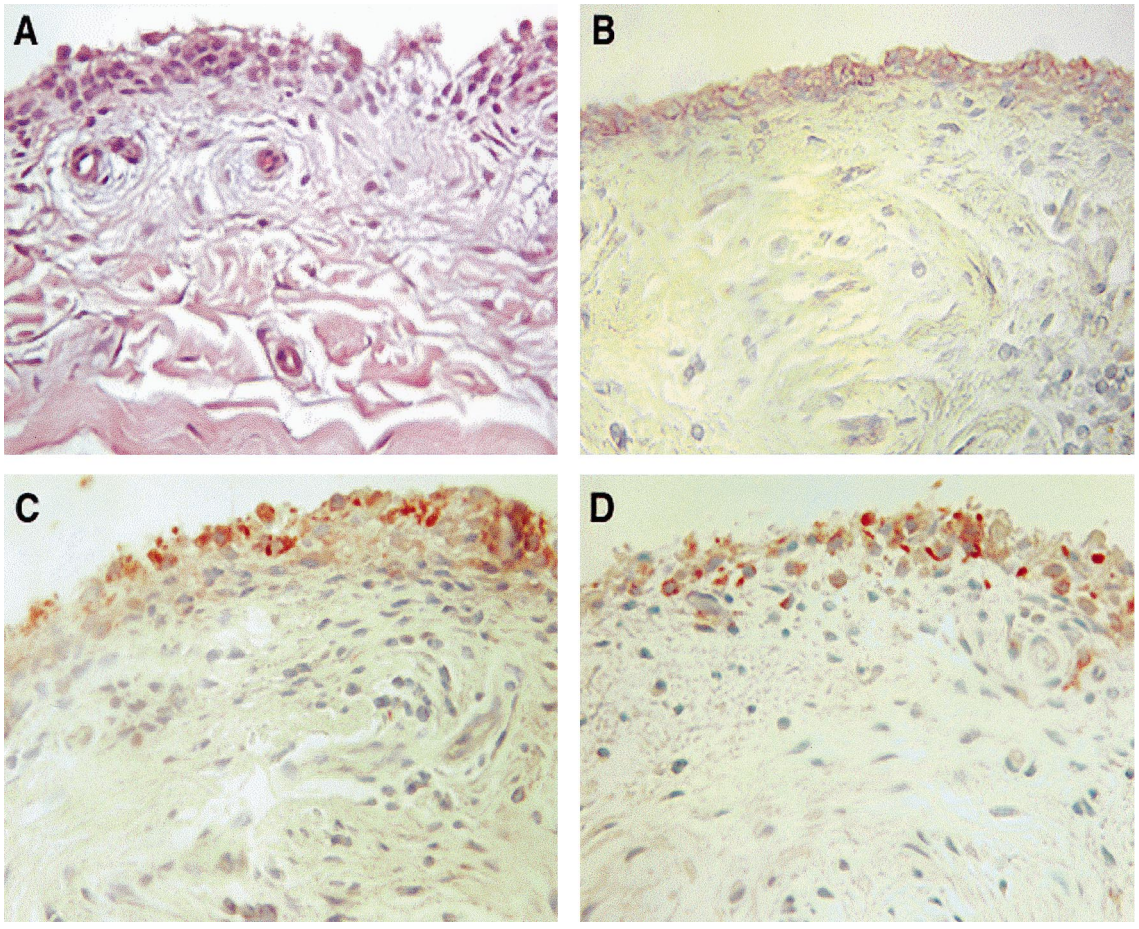

Fig. 2. Immunohistochemistry of human RA synovial membrane tissue. Panel A presents the overall morphology of human synovial membrane with a hematoxylin and eosin stain. Panels B, C, and D were probed with monoclonal antibodies specific for vimentin, CL-1, and CL-3, respectively. Cells which expressed CL-1 and CL-3 protein were found in the periphery of the RA synovial membrane and were fibroblast-like cells, which where also vimentin positive.

3. Results

3.1. CL-3 antibodies

CL-3 was expressed in *E. coli*, extracted, and purified by size-exclusion chromatography to greater than 90% purity (data not shown). This purified recombinant CL-3 was then used as the antigen to produce antibodies that recognized CL-3 and did not cross-react with other MMPs (Fig. 1 and data not shown). Medium from SW1353 chondrocytes was concentrated and analyzed by Western blot analyses. Anti-CL-1 mAb III_{12} detected CL-1 (Fig. 1, lane CL-1) and anti-CL-3 mAb ID_3 detected CL-3 protein expression by these cells (Fig. 1, lane CL-3). When both antibodies were used together, CL-1 and CL-3 were readily visualized at slightly different molecular weights (Fig. 1, lane CL-1+CL-3). Anti-CL-3 mAb ID3 was then utilized in this study for Western blots, immunofluorescence, and immunohistochemistry.

3.2. Immunohistochemistry

After embedded RA synovial membrane tissue was deparaffinized and rehydrated, sections were then stained with hematoxylin and eosin or probed with monoclonal antibodies specific for vimentin, CL-1, or CL-3 and lightly counterstained with hematoxylin. Fig. 2 shows tissue sections from a patient in which isolated fibroblasts were able to express CL-3 mRNA and protein. Fig. 2A shows the morphology of the

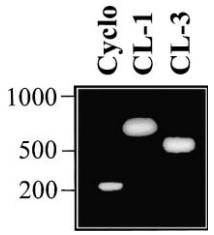

Fig. 3. RA synovial membrane tissue RT-PCR. mRNA was isolated from fresh RA synovial membrane tissue and RT-PCR was performed to specifically amplify the positive control cyclophilin (Cyclo, 274 bp), collagenase-1 (CL-1, 721 bp), or collagenase-3 (CL-3, 517 bp). Products were separated and visualized in an ethidium bromide stained 1% agarose gel. The tissue expressed mRNA for both CL-1 and CL-3.

synovial membrane tissue with hematoxylin and eosin staining. At the periphery of the tissue is a compact yet not contiguous layer of fibroblasts-like cells ranging from one to five cells deep. Blood vessels, macrophages, and muscle fibers were also visible throughout the tissue. The section in Fig. 2B was stained with anti-vimentin monoclonal antibody and shows staining primarily at the periphery of the tissue. The CL-1 staining pattern is shown in Fig. 2C and is again primarily at the periphery of the tissue. Fig. 2D shows the presence of CL-3 protein also at the periphery of the tissue. Similar staining patterns were observed in eight of the nine specimens checked and only one specimen stained negative for CL-3 (data not shown). Control experiments using nonspecific mAbs or no primary antibodies showed no significant staining (data not shown).

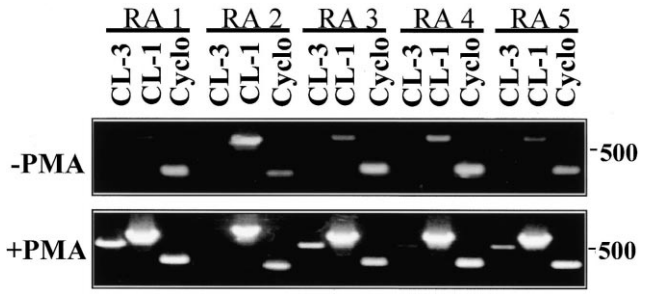

Fig. 4. RT-PCR of synovial membrane fibroblasts with and without PMA. Specific primers amplified cyclophilin (Cyclo), collagenase-1 (CL-1), or collagenase-3 (CL-3). CL-1 was constitutively expressed in all the RA synovial membrane fibroblasts cell lines and further enhanced by PMA stimulation, whereas all of the RA synovial membrane fibroblast cell lines that had detectable levels of CL-3 mRNA required PMA stimulation.

3.3. RT-PCR of human RA synovial membrane tissue

RNA was isolated directly from synovial membrane tissue from which RA synovial fibroblasts were also isolated. Specific RT-PCR products for cyclophilin, CL-1, and CL-3 were detected in the synovial membrane tissue (Fig. 3). This demonstrated that CL-3 mRNA, as well as CL-1 mRNA, as expected from the immunohistochemistry data was present in this human RA synovial membrane tissue.

3.4. MMP and TIMP mRNA expression by RA synovial fibroblasts

RA synovial fibroblasts were isolated from synovial membrane tissue excised from patients with clinical rheumatoid arthritis. RT-PCR was performed on mRNA isolated from PMA treated or untreated

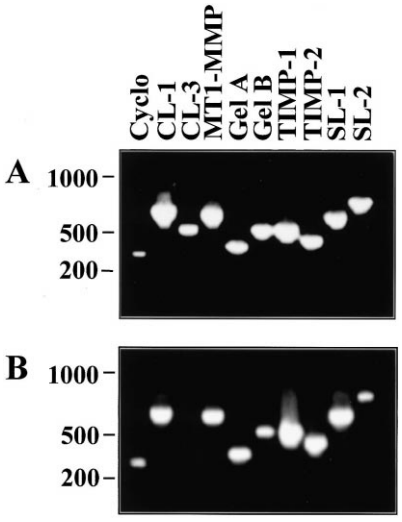

Fig. 5. RT-PCR of MMPs and TIMPs in PMA treated synovial membrane fibroblasts. RT-PCR products from PMA treated RA synovial membrane fibroblasts were resolved in 1% agarose gels and stained with ethidium bromide. (A) A synovial membrane fibroblast cell line in which mRNA for CL-1 and CL-3 was detected. (B) A synovial membrane fibroblast cell line in which CL-1 mRNA, but no CL-3 mRNA, was visualized. mRNA expression was also examined for cyclophilin (Cyclo, 274 bp), membrane type-1 matrix metalloproteinase (MT1-MMP, 670 bp), gelatinase A (Gel A, 364 bp), gelatinase B (Gel B, 519 bp), tissue inhibitor of matrix metalloproteinase-1 (TIMP-1, 534 bp), tissue inhibitor of matrix metalloproteinase-2 (TIMP-2, 433 bp), stromelysin-1 (SL-1, 659 bp), and stromelysin-2 (SL-2, 863 bp).

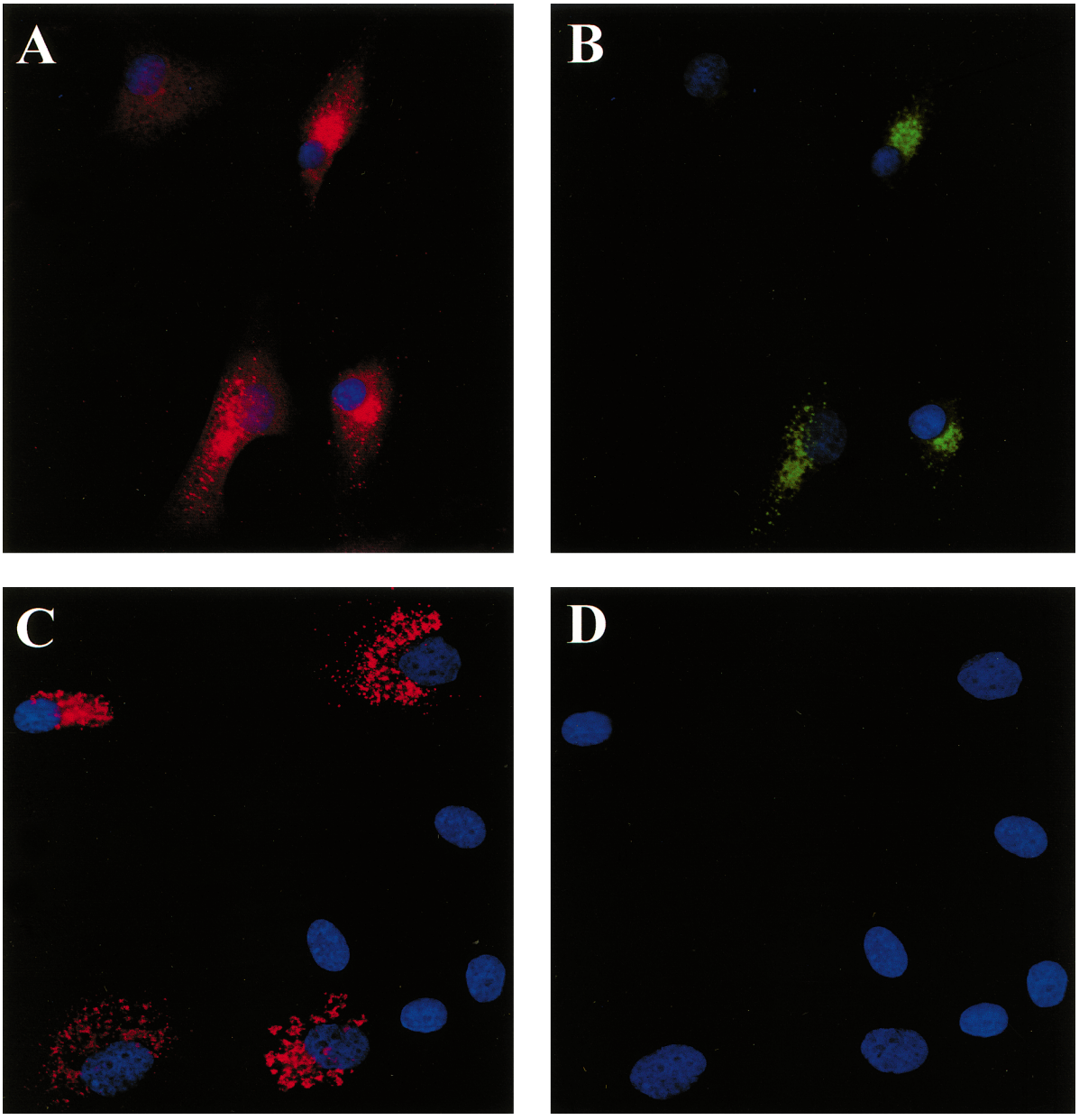

Fig. 6. Immunofluorescence of CL-1 and CL-3. Two distinct human synovial membrane fibroblast cell lines were PMA stimulated and monensin treated prior to fixation. All slides were probed with anti-CL-1 pAb 647 and anti-CL-3 mAb ID3 followed by fluorescent secondary antibodies and then the nuclei were counterstained with DAPI. Panels A and B represent a CL-3 positive RA synovial membrane fibroblast cell line, whereas panels C and D represent a CL-3 negative cell line. Images for panels A and C were captured with a TXRD filter to visualize CL-1. Panel B and D images were captured with the FITC filter to visualize CL-3.

early passage (1–3) RA synovial membrane fibroblasts (Fig. 4). It was routinely observed that these cells expressed mRNA for CL-3, but only upon induction with PMA (Fig. 4). CL-1 on the other hand was constitutively expressed and could be stimulated with PMA (Fig. 4).

RT-PCR was then performed on a total of 18 PMA treated RA synovial fibroblast cell lines (cell lines 1–18) established for this study. The control cyclophilin mRNA was detected in 100% of the samples as expected. mRNA for MT1-MMP, gelatinase A, gelatinase B, TIMP-1, TIMP-2, and stromelysin-1 were also detected in 100% of these RA samples. Stromelysin-2 mRNA was amplified in 78.9% of

the samples. CL-1 mRNA was detected in 100% of the cell lines, while CL-3 mRNA was found in 88.8% of these samples by RT-PCR. Similar values were recently obtained for CL-1 (100%) and CL-3 (90%) from RT-PCR of RA synovial membrane tissues directly [24]. As seen in Fig. 5A and B, representative agarose gels illustrate the presence (Fig. 5A) or absence (Fig. 5B) of mRNA for the MMPs and TIMPs in RA synovial fibroblast cell lines 1 and 11, respectively.

3.5. Sequencing of the CL-3 RT-PCR fragment

To ensure the specific amplification of CL-3, the PCR product was ligated into a modified TA vector, amplified, and plasmid DNA purified. The PCR product was sequenced using a modified Sanger method and yielded sequence identical to the published sequence (data not shown) for human CL-3 originally isolated from a breast tumor library [3].

3.6. Immunofluorescence

Immunofluorescence was used to investigate CL-3 protein expression by individual RA synovial membrane fibroblasts (Fig. 6). Synovial membrane fibroblasts from a CL-3 positive cell line (cell line 1) and a CL-3 negative cell line (cell line 11) were grown to 90% confluence and induced overnight with PMA. The cells were then treated with monensin for 3 h before fixation and staining. After treatment with monensin, the CL-1 and CL-3 staining patterns were typical of proteins trapped in the golgi apparatus. However, fluorescent detection of the CL-3 protein yielded a reproducible though weaker signal than for CL-1. Fig. 6 shows the two different RA synovial fibroblasts cell lines fixed and stained for CL-1 or CL-3 and counterstained with DAPI. Fig. 6A shows the presence of CL-1 in synovial fibroblast cell line 1 and Fig. 6C shows that cell line 11 also expressed CL-1. Fig. 6B depicts the presence of the CL-3 protein in cell line 1 while Fig. 6B shows that cell line 11 did not express the CL-3 protein. A hundred cells from cell line 1 were counted in three samples and an average of 67 cells per sample were CL-1 positive (67%), while 30 cells per sample were CL-3 positive (30%). Individual cells were seen expressing both CL-1 and CL-3, yet some cells expressed only CL-1 or CL-3 (data not shown). The protein expression of CL-1 and CL-3 seen in Fig. 6 correlates well with the RT-PCR performed on these two cell lines (Fig. 5). Fig. 6A and B represent the same cells in Fig. 5A (cell line 1) and Fig. 6C and D represent the cells shown in Fig. 5B (cell line 11).

3.7. Western analyses of CL-1 and CL-3

To further examine CL-1 and CL-3 protein expression, conditioned culture medium and membrane extracts from cell line 1 (CL-3 positive) were examined by Western blot analyses. Synovial fibroblasts were grown to 90% confluence and induced with PMA in serum free medium for 15 h. The medium was then removed and concentrated. The remaining cell monolayer was used to isolate cell membrane extracts as described in Section 2. Anti-CL-3 mAb ID3 and anti-CL-1 mAb III_{12} were then used for Western blot analyses. The conditioned medium was shown to contain both CL-1 and CL-3 (Fig. 7A). The association of CL-3 with the cell membrane and/or with MT1-MMP was examined since MT1-MMP has been shown to activate CL-3 in vitro [7]. Also in recent studies in the lab, CL-3 has been observed to be associated with crude membrane extracts from a squamous cell carcinoma (SCC-25)

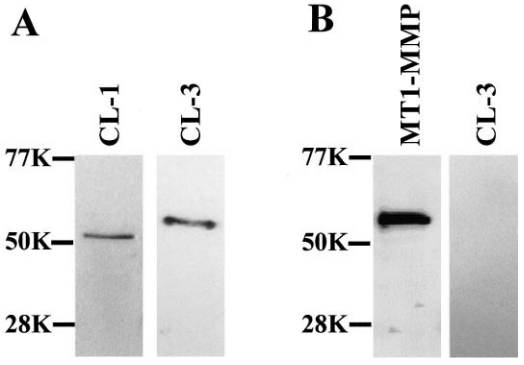

Fig. 7. Western blot analyses of conditioned medium (A) and membrane extracts (B) from PMA stimulated RA synovial membrane fibroblasts. (A) Concentrated conditioned medium was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-CL-1 mAb III_{12} or anti-CL-3 mAb ID_3. Both CL-1 and CL-3 were found in the conditioned medium from PMA stimulated cells. (B) Crude membrane extracts were also resolved by SDS-PAGE, blotted, and probed with anti-CL-3 mAb ID_3 or anti-MT1-MMP pAb 815. The MT1-MMP protein was detected in the crude membrane extracts, yet no CL-3 protein was detected.

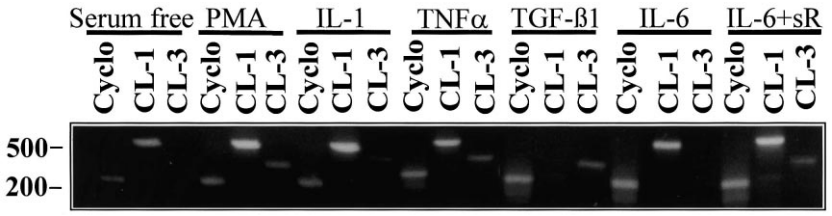

Fig. 8. PMA and cytokine induction in RA synovial membrane fibroblasts. RT-PCR products cyclophilin (Cyclo), collagenase-1 (CL-1), and collagenase-3 (CL-3) were amplified from RA synovial fibroblasts treated with serum free medium only, PMA, human IL-1, human TNF-α, human TGF-β1, human IL-6, or human IL-6 with the IL-6sR. Products were resolved and visualized in an ethidium bromide stained 1% agarose gel. CL-3 mRNA was readily induced with PMA, IL-1, TNF-α, TGF-β1, or IL-6 with IL-6sR. Whereas, CL-1 mRNA was stimulated with PMA, IL-1, and IL-6 with IL-6sR.

from American Type Culture Collection. However, in the cell membrane extracts from the RA synovial fibroblasts, a strong signal for MT1-MMP was visualized while no CL-3 protein was seen (Fig. 7B).

3.8. PMA and cytokine induction

CL-3 positive cells (cell line 1) were treated with either PMA (10^{-7} M), IL-1 (5 ng/ml), IL-6 (5 ng/ml), IL-6 (5 ng/ml) with IL-6sR (250 ng/ml), TNF-α (10 ng/ml), or TGF-β1 (10 ng/ml) in serum free DME. After RT-PCR was performed as described above, equal aliquots were analyzed in a 1% agarose gel and visualized with ethidium bromide staining. In cells not stimulated with PMA or cytokines, CL-1 and not CL-3 was constitutively expressed by the RA synovial membrane fibroblasts (Fig. 8). CL-3 mRNA in these cells was induced by PMA, IL-1, TNF-α, TGF-β1, or IL-6 with IL-6sR. IL-6 alone did not induce CL-3 mRNA expression. As noted before, CL-1 was constitutively expressed and only PMA, IL-1, or IL-6 with IL-6sR increased CL-1 mRNA levels above the basal level. TNF-α had little effect on CL-1 mRNA expression, while TGF-β1 appeared to down regulate CL-1 expression.

4. Discussion

The degradation of articular cartilage is a characteristic of rheumatoid arthritis. Since MMPs are extremely efficient in cleaving cartilage components, they have been implicated in the pathogenesis of rheumatoid arthritis. The collagenase MMP subfamily members, CL-1 and CL-3, have both been suggested to play a role in this degradation. In addition, it has recently been shown that CL-3 has a higher activity than CL-1 against type II collagen [7], which is the major component of articular cartilage.

CL-3 mRNA has been detected in human articular cartilage [8], as well as in human synovial membranes [9]. In early studies, no CL-3 mRNA could be detected in cultured human synovial fibroblasts but could be detected in cultured human chondrocytes [11,12,25]. In contrast, CL-3 mRNA and protein could be detected and induced with PMA in cultured rabbit synovial fibroblasts [15]. Recently, it was shown that synovial fibroblasts did express CL-3 mRNA constitutively and that PMA could stimulate CL-3 above basal levels [13]. However, another recent study showed that in synovial fibroblasts CL-3 mRNA levels was unaffected by PMA treatment [14]. Therefore, the purpose of this study was to further examine the expression and/or induction of CL-3 in human RA synovial membrane fibroblasts.

Immunohistochemistry demonstrated that the CL-3 protein was present in RA synovial membrane tissue (Fig. 2). As seen in Fig. 2C and D, CL-1 and CL-3 proteins were detected at the periphery of this tissue in the synovial membrane lining cells. These cells were also vimentin positive cells. Previously, in situ hybridization of RA synovial tissue showed the presence of CL-1 and CL-3 mRNA in these peripheral fibroblast-like cells [14]. It was observed by RT-PCR that CL-3 mRNA was present as expected in this RA synovial membrane tissue (Fig. 3). This observation that CL-3 mRNA was present in RA synovial membrane tissues allowed for the further examination of CL-3 expression and induction by cells isolated from these tissues.

RT-PCR of PMA stimulated early passage human RA synovial fibroblasts demonstrated that these cells

could express mRNA for CL-3. The cloning and sequencing of the CL-3 PCR fragment verified that these cells do express mRNA for CL-3 and that it was not the product of mistaken amplification of another mRNA. It was observed that 16 of the 18 (88.8%) established synovial membrane fibroblast cell lines could express mRNA for CL-3 only when induced, whereas 18 of these cell lines expressed mRNA for CL-1 (100%) whether induced or not. mRNA for MT1-MMP, gelatinase A, gelatinase B, TIMP-1, TIMP-2, and stromelysin-1 was detected in 100% of the samples after PMA induction. Stromelysin-2 mRNA was amplified in 78.9% of the PMA treated cells. The presence of these MMPs known to be activators of CL-3 such as MT1-MMP and gelatinase B suggest that this environment is conducive to CL-3 activation in vivo and thus CL-3 may be an important mediator in RA pathogenesis. It was found that 16 of 18 cell lines expressed CL-3 mRNA only upon stimulation with PMA or various cytokines. The mRNA expression profiles of these MMPs from these stimulated RA synovial membrane fibroblasts were similar to the expression profiles of these MMPs observed in RA synovial membrane tissues [24]. CL-1 mRNA was detected in this study in 100% of the cell lines checked and similarly detected in 100% of the RA membrane tissues investigated in the Konttinen et al. study [24]. They detected CL-3 mRNA in 90% of the RA membrane tissues checked [24], while CL-3 mRNA was detected in 88.8% of the RA synovial membrane fibroblast cell lines established for this study. This correlation between CL-3 expression in RA tissue and in the cultured cells of this study suggests that RA synovial membrane fibroblasts could be a source of the CL-3 found in vivo. It was also observed that the two cell lines that failed to express CL-3 did express all the other MMPs and TIMPs checked (data not shown).

Since RT-PCR is an extremely sensitive procedure, the ability of these cells to make detectable levels of the CL-3 protein was addressed. Immunofluorescence was utilized to demonstrate that these cells could express the CL-3 protein and to examine the expression of CL-3 by individual cells. Fig. 6 shows the two different RA synovial membrane fibroblasts cell lines that were examined for CL-1 and CL-3 protein expression. CL-1 and CL-3 were detected in cell line 1 and their expression is shown in Fig. 6A and B, respectively. The CL-3 protein staining appeared to be less intense than the staining of the CL-1 protein. Also, fewer cells stained positive for CL-3 (30%) than the number of cells that stained positive for CL-1 (67%). It was observed that some of these cells stained positive for both CL-1 and CL-3, while other cells stained positive for either CL-1 or CL-3 (data not shown). Staining of RA synovial membrane fibroblast cell line 11 detected the CL-1 protein (Fig. 6C), whereas no CL-3 protein was detected (Fig. 6D). Fig. 6C and D demonstrated further that the anti-CL-3 mAb ID_3 did not cross-react. Western blots of conditioned medium from RA synovial fibroblast cell line 1 detected the CL-1 protein and CL-3 protein (Fig. 7A). Further analyses by Western blots of membrane extracts failed to show any CL-3 protein associated with the membrane and/or MT1-MMP (Fig. 7B). This was in contrast to recent results in the laboratory in which Western blot analyses of crude membrane extracts from SCC-25 cells detected CL-3 associated with the membranes along with MT1-MMP (unpublished data). Although it is possible that CL-3 might be associated with the membrane or with MT1-MMP and be activated by it, this interaction may be very transient and difficult to detect.

PMA and cytokine induction of CL-3 mRNA has been studied in several cell lines. Cultured human chondrocytes have been shown to express CL-3 mRNA upon induction with a combination of IL-1β and TNF-α [11,25]. In the embryonic fibroblast cell line KMS-6, CL-3 mRNA expression had to be induced by TGF-β1, IL-1, or PMA [26]. In endometrial fibroblasts, CL-3 mRNA was not detected even after treatment with TNF-α, IL-1, or IL-6 [27]. Additionally in vivo, IL-6 and TNF-α have been localized to the synovial lining cells in the same location [28] in which CL-3 was visualized by immunohistochemistry (Fig. 3). The RA synovial membrane fibroblasts in this study responded to PMA, IL-1, TNF-α, TGF-β1, or IL-6 with IL-6sR with increased CL-3 mRNA expression. These cells did not constitutively express CL-3, but required stimulus for CL-3 expression. This suggests that RA synovial membrane fibroblasts could be a CL-3 source in vivo since in normal synovial tissue CL-3 is not found and the inflammatory cytokines are in much lower doses than in RA tissue [8].

It should be emphasized that the ability of collagenases, as well as other matrix metalloproteinases, to cleave substrate is contingent on their activation. Therefore, even a small amount of active enzyme may be detrimental, whereas a large amount of unactivated enzyme has no degrading activity. The CL-3 produced by these synovial membrane fibroblasts appeared to be present with other MMPs in an environment that is conducive to CL-3 activation. Therefore, CL-3 production by RA synovial membrane fibroblasts requires further investigation to determine its activation status and exact role in the pathogenesis of rheumatoid arthritis.

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