Pannus invasion and cartilage degradation in rheumatoid arthritis: Involvement of MMP-3 and interleukin-1β

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Abstract

Objective

Synovial inflammation in rheumatoid arthritis (RA) leads to pannus tissue invasion and destruction of cartilage/bone matrix by proteinases. Our intention was to analyze some of the key matrix metalloproteinases (MMPs) in pannus tissue overlying evolving cartilage erosions in RA.

Methods

Frozen tissue samples of pannus and synovium from advanced RA and synovium from osteoarthritic patients were used for immunohistochemical, western blotting and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of MMP-1, -3, -13 and -14. Synovial fibroblast cultures, stimulated with tumour necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β), were analyzed with enzyme-linked immunosorbent assays (ELISA) and quantitative RT-PCR.

Results

MMP-3 was highly expressed in pannus tissue compared with significantly lower expression levels of MMP-1, –13 and –14. In fibroblast cultures IL-1β was a potent stimulus for MMP-3, whereas TNF-α was more potent for MMP-1.

Conclusion

This is the first study to demonstrate quantitatively in real time that MMP-3 mRNA expression is clearly higher in advanced RA pannus tissue compared to parallel RA or osteoarthritic synovium. MMP-3 mRNA levels were also clearly overexpressed in RA pannus compared to MMP-1, -13 and -14. Advanced RA has previously been found to overexpress IL-1β. The high expression of MMP-3 in pannus and IL-1β mediated stimulation of MMP–3 suggest that MMP-3 plays a significant role in the progression of erosions through the proteoglycan-rich cartilage matrix.

Key words

Rheumatoid arthritis, cartilage, inflammation, cytokines, matrix metalloproteinases.

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Introduction

Rheumatoid arthritis (RA) is the most common inflammatory arthritis, which in Finland affects 1.9% of the population [2.7% of women and 1.0% of men; (1)]. Inflammatory synovial membrane expands on cartilage leading to pannus formation. Synovial hyperplasia involves proliferation of synovial fibroblast-like lining cells (2, 3) and accumulation of macrophages and lymphocytes (4). The leading edge of pannus is composed of fibroblast-like cells (5, 6), which produce proteolytic enzymes able to cause destruction of hyaline articular cartilage, e.g. collagenase-3 or MMP-13 and MT1-MMP or MMP-14 (7, 8). Fibroblasts also express the receptor activator of the nuclear factor kappa B ligand, which may induce osteoclastogenesis leading to erosion of the underlying bone (9, 10).

Proteolytic pathways play an important role in the development of RA. Several matrix metalloproteinases (MMPs) have been shown to increase during cell invasion (11, 12). In our recent analysis of 16 different MMPs in RA synovium, the main enzymes were MMP-1, -3, -13 and -14 (13). MMP-1 (collagenase-1 or "fibroblast collagenase") and MMP-3 (stromelysin-1) are considered to play an important role in collagen degradation and their increased expression has been observed in RA synovial membrane (14,15). These enzymes have also been found in increased concentrations in rheumatoid synovial fluid (16, 17). However, apart from synovial membrane and fluid, also pannus tissue contains some of the key pro-inflammatory cytokines, in particular tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (18-20, 8), which are able to upregulate MMP-1 and -3 production (21-25). Therefore, due to their supposed role in tissue destructive events, in particular degradation of cartilage, our aim was to characterize the pannus-hard tissue junction in terms of their expression of MMP-1, -3, -13 and -14 and their regulation in synovial fibroblasts *in vitro* by the pro-inflammatory cytokines TNF-α and IL-1β.

Materials and methods

Patients and samples

All tissue samples were collected from well-characterized osteoarthritic and advanced RA patients. The study plan was approved by the Ethics Committee of Helsinki University Central Hospital. Nine synovial membrane and eight pannus tissue samples were collected parallel from advanced RA patients undergoing primary total hip replacement, knee arthroscopy or synovectomy. The mean age of these patients was 54 years (range 34-80 years). Nine synovial membrane samples were collected from osteoarthritis patients undergoing primary total hip replacement, with a mean age of 73 years (range 58-85 years). None of the patients included in the present study had any clinical or microbiological signs of infection.

Cell stimulation

Synovial fibroblasts from osteoarthritic patients were established using explant culture method (26). For stimulations the cells were grown to confluence in 6 well plates with 4 coverslips and 200,000 cells per well at passages 2-4. The cells were stimulated with 0.05 ng/ml TNF-α (R&D Systems, Minneapolis, MN) or 0.01 ng/ml IL-1β (R & D Systems) for 72 hours before the media were collected. All fibroblast stimulations were made in duplicates.

Cloning of PCR fragments

For quantitative PCR standard curve, the gene of interest was amplified, extracted from agarose gel and cloned with TOPO TA cloning kit (Invitrogen, Paisley, UK) into a pCRII-TOPO vector. Plasmid DNAwas isolated and the concentrations of the plasmids were analyzed spectrophotometrically. The plasmids were identified by gel electrophoresis after restriction enzyme digestions and sequenced from 1 μg of isolated DNA. The acquired sequence was verified with NCBI blastn program.

Quantitative reverse transcriptase polymerase chain reaction (quantitative RT-PCR)

Total RNA from frozen tissue and cultured cells were isolated by TRIzol re-

agent (Invitrogen). Messenger RNA (mRNA) was isolated from 50 µg total RNA from frozen tissue using magnetic $(dT)_{25}$-polystyrene beads (Dynal, Oslo, Norway). Total RNA from cell culture was DNase treated using RQ1 RNase-Free DNase (Promega, Madison, WI). 100 ng mRNA or 5 µg DNase treated total RNA was transferred to cDNA with SuperScript Preamplification System (Invitrogen). Quantitative PCR amplification was performed from 9.5 ng cDNA in SYBR Green I PCR mix (Roche, Mannheim, Germany) by LightCycler™ PCR machine (Roche). PCR amplification was performed using 0.5 µM of target specific primers for β-actin (accession X00351): sense 5'-TCACCCACACTGTGCCCATCTACGA -3' and antisense 5' -CAGCGGAACCGCTCATTGCCAATGG -3' producing a 295 bp band, for PBGD (accession X04217): sense 5'- ACATGCCCTGGAGAAGAATG -3' and antisense 5'- AGATGCGGGAACTTTCTCTG -3' producing a 237 bp band, for MMP-1 (accession X05231): sense 5'- CTGAAGGTGATGAAGCAGCC -3' and antisense 5'- AGTCCAAGAGAATGGCCGAG -3'producing a 428 bp band, for MMP-3 (accession J03209): sense 5'- ctcacagacctgactcggtt -3' and antisense 5'- cacgcctgaaggaagagatg -3' producing a 294 bp band, for MMP-13 (accession X75308): sense 5'-CTATGGTCCAGGAGATGAAG -3' and antisense 5'- AGAGTCTTGCCTGTATCCTC -3' producing a 390 bp band and for MMP-14 (accession D26512): sense 5'- CAACACTGCCTACGAGAGGA -3' and antisense 5'-GTTCTACCTTCAGCTTCTGG -3' producing a 380 bp band. The identity of the product was verified by size, sequence and a melting curve analysis. Serial dilutions of cloned PCR fragments were used to determine the mRNA copy number of the amplicon per housekeeping genes, because of the different cellular quantity of the samples, in tissue samples per 1000 β-actin and in cell culture samples per PBGD. Different housekeeping genes were used to get amplified genes within the range of standard curve. Each individual sample was amplified at least two times for all genes of interest.

Enzyme-linked immunosorbent assay

Cell culture media of fibroblast (n = 3) cultures, non-stimulated or stimulated for 72 hours with TNF-α or IL-1β were centrifuged at 10.000 g for 5 minutes. Supernatants were diluted 1:2, 1:5 and 1:10 and the amount of total MMP-3 (pro- and/or active) was measured using Human MMP-3 Quantikine ELISA Kit according to the manufacturer's protocol (R&D Systems). The absorption of quadruple samples and standards were measured at 450 nm and wavelength correction at 540 nm.

Immunohistochemistry

Serial 3 µm thick paraffin tissue sections were stained using DAKO ChemMate™ Reagent System and Automated Immunostainer 500 (TechMate™, Dako, Clostrup, Denmark) with the MSIPE protocol were 30 minutes enzymatic digestion in 1mg/ml pepsin. The concentrations of the antibodies were as follows: 0.67 µg/ml for mouse anti-human MMP-1 $IgG_{2a/κ}$ (Chemicon, Temecula, CA) and the same concentration for the negative control mouse $IgG_{2a/κ}$ (Dako), 2.5 µg/ml for mouse anti-human MMP-3 $IgG_{1/κ}$ (Chemicon) and the same concentration for the negative control mouse $IgG_{1/κ}$ (Dako).

Immunofluorescence

Cultured fibroblasts were fixed with 3 % paraformaldehyde, incubated for 30 minutes in primary mouse anti-human MMP-3 $IgG_{1/κ}$ (Chemicon) and for 30 minutes with secondary Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Leiden, Netherlands). Normal mouse $IgG_{1/κ}$ (Dako) at the same concentration was used as a negative control. Nuclei were stained for 5 minutes with DAPI (Sigma, Steinheim, Germany).

Western blotting

Synovium (n = 3) and pannus (n = 3) of RAand synovium of osteoarthritis (n = 3) were homogenized in RIPA Buffer Set (Boehringer Mannheim, Germany), sonicated, centrifuged and filtrated. Trypsin (DIFCO, Detroit, MI) was added to a final concentration of 10 µg/ml for 30 minutes at 37°C. Electrophoresis was performed using 50µg total protein, blotted onto nitrocellulose membrane, blocked overnight in 3% bovine serum albumin (BSA) and incubated for 90 minutes with 0.2 µg/ml polyclonal goat anti-human MMP-1 IgG (R&D Systems) or 0.2 µg/ml polyclonal goat anti-human MMP-3 IgG (R&D Systems) in 2% BSA. Detection was performed with 0.12 µg/ml alkaline phosphatase-conjugated rabbit anti-goat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) with colour development solution (Alkaline Phosphatase Conjugate Substrate Kit, Bio-Rad).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 3.02 for Windows (GraphPad Software Inc., San Diego, CA). Paired t test was used for compairing paired samples between two groups. One-way ANOVA and nonparametric methods were used for multiple group comparison combined with Dunnett's post-hoc test for compairing against control column and Tukey's for compairing all columns. $p < 0.05$ was considered to be significant.

Results

MMPs in pannus and synovial tissue

MMP-3 mRNA levels in RA pannus tissue (4138 ± 1751, n = 8), RAsynovial tissue (865 ± 552, n = 9) and osteoarthritic synovial tissue (727 ± 262, n = 9) were different ($p = 0.044$). In particular, the mRNAcopy numbers of MMP-3 in RA pannus tissue were high compared to RA($p < 0.01$) or osteoarthritis ($p < 0.001$) synovial tissue (Fig.1). Furthermore, in RApannus tissue the copy numbers of MMP-3 were high ($p < 0.001$) when compared to MMP-1, -13 or –14 pannus tissue. MMP-1, -13 and –14 did not show significant differences between the pannus and synovial tissue compartments or between RA and osteoarthritis.

MMPs in cytokine stimulated cell cultures

The eventual effect of TNF-α and IL-1β on fibroblast-mediated MMP-3 synthesis was studied in a small (n = 3) number of samples, which did not show statistically significant differences at

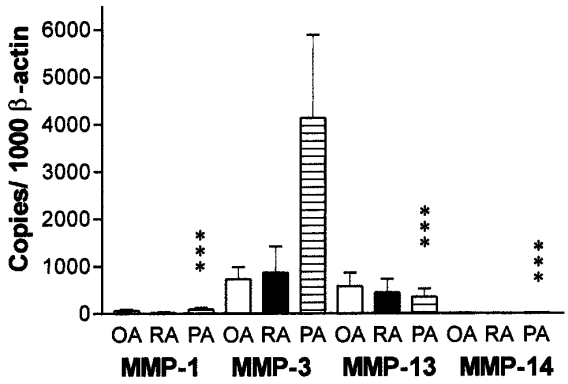
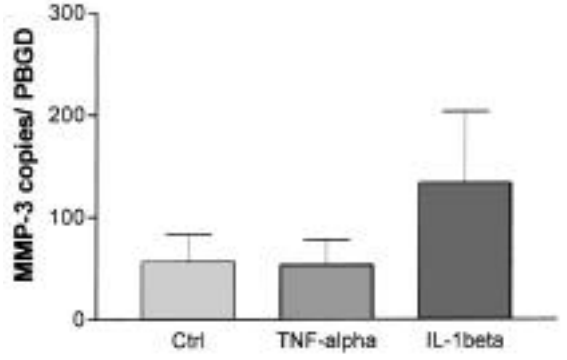


Fig. 1. MMP-1, -3, -13 and -14 mean mRNA copy numbers in synovial tissue (ST) from osteoarthritis (OA) (n = 9) and rheumatoid arthritis (RA) (n = 9) patients, and pannus tissue of rheumatoid arthritis (PA) (n = 8) by quantitative real-time RT-PCR per 1000 β-actin copies. ***p < 0.001 for MMP-1, -13 and -14 vs. MMP-3 in pannus tissue.



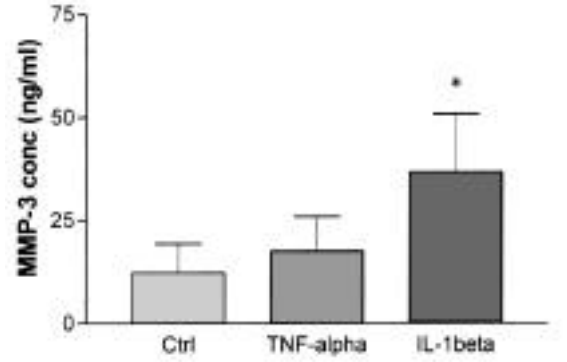


Fig. 2a. MMP-3 mRNA expression in nonstimulated (negative control, n = 3) and stimulated (TNF-α and IL-1β, n = 3) fibroblast cell culture extracts by quantitative real-time RT-PCR per PBGD copy numbers, **b**) MMP-3 total protein concentrations (ng/ml) in nonstimulated (negative control, n = 3) and stimulated (TNF-α and IL-1β, n = 3) fibroblast cell culture media by ELISAmethod. *p < 0.05 vs. control.

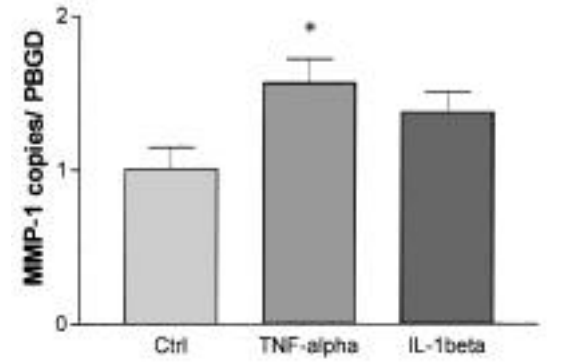


Fig. 3. MMP-1 mRNA expression in nonstimulated (negative control, n = 3) and stimulated (TNF-α and IL-1β, n = 3) fibroblast cell culture extracts by quantitative real-time RT-PCR per PBGD copy numbers. *p < 0.05 difference to control.

the mRNA level but a clear trend for IL-1β stimulated cells (for TNF-α 53 ± 24 and for IL-1β 133 ± 70 vs. 56 ± 27 in control cultures, Fig. 2a). By paired test this was in line (p = 0.0027) with MMP-3 protein levels from the same fibroblast cultures, which thus showed a significant difference (p = 0.0244, Fig. 2b) between stimulated and control supernatants. In detail, MMP-3 protein concentration in IL-1β stimulated fibroblast supernatants was 36.6 ± 14.2 ng/ml, which was 3 fold higher (p < 0.05) than in non-stimulated supernatants 12.23 ± 7.13 ng/ml. TNF-α stimulated fibroblast MMP-3 levels were 17.53 ± 8.40 ng/ml. These results indicate that in particular IL-1β effectively up-regulates MMP-3 synthesis. In addition, MMP-1 mRNA expression in TNF-α (1.57 ± 0.15 ng/ml), IL-1β stimulated (1.37 ± 0.14 ng/ml) and non-stimulated (negative control) (1.01 ± 0.14 ng/ml) fibroblasts were different (p = 0.0194). More specificially, TNF-α stimulated fibroblasts expressed 1.5 times more MMP-1 mRNA than non-stimulated fibroblasts (p<0.05) (Fig. 3). However, the copy numbers of MMP-1 compared to MMP-3 were extremely low (p = 0.0078), only approximately 1-2 copies of MMP-1/ PBGD compared with 50-200 copies of MMP-3/ PBGD.

Immunocytochemistry

Immunocytochemical staining disclosed both MMP-1 (Fig. 4a) and MMP-3 (Fig. 4b) in synovial lining layer, cartilage-pannus junction and endothelium of blood vessels. MMP-3 staining in synovium was slightly more intense than MMP-1, but clearly overexpressed in pannus. Also chondrocytes contained both MMP-1 and MMP-3, MMP-1 staining being more intense. However, these were not really quantitatively assessed for staining intensity as this is not only dependent on the concentration of the immunoreactive epitopes but also the concentration and avidity of the antibody used for staining. MMP-3 staining was more specifically located into vesicles in cytoplasm of synovial fibroblast (data not shown). Negative staining controls with normal mouse IgG confirmed the specificity of the staining.

Western blotting

The same pattern of MMP-3 and MMP-1 protein expression was found in all osteoarthritic, RAsynovial and RApannus tissue samples studied. MMP-3 was seen as a proenzyme (57 kDa), intermediate (53 kDa) and active (45 kDa) form in RA, both in synovial and pannus tissue (Fig. 5a). In contrast, osteoarthritic synovial tissue contained only the pro-

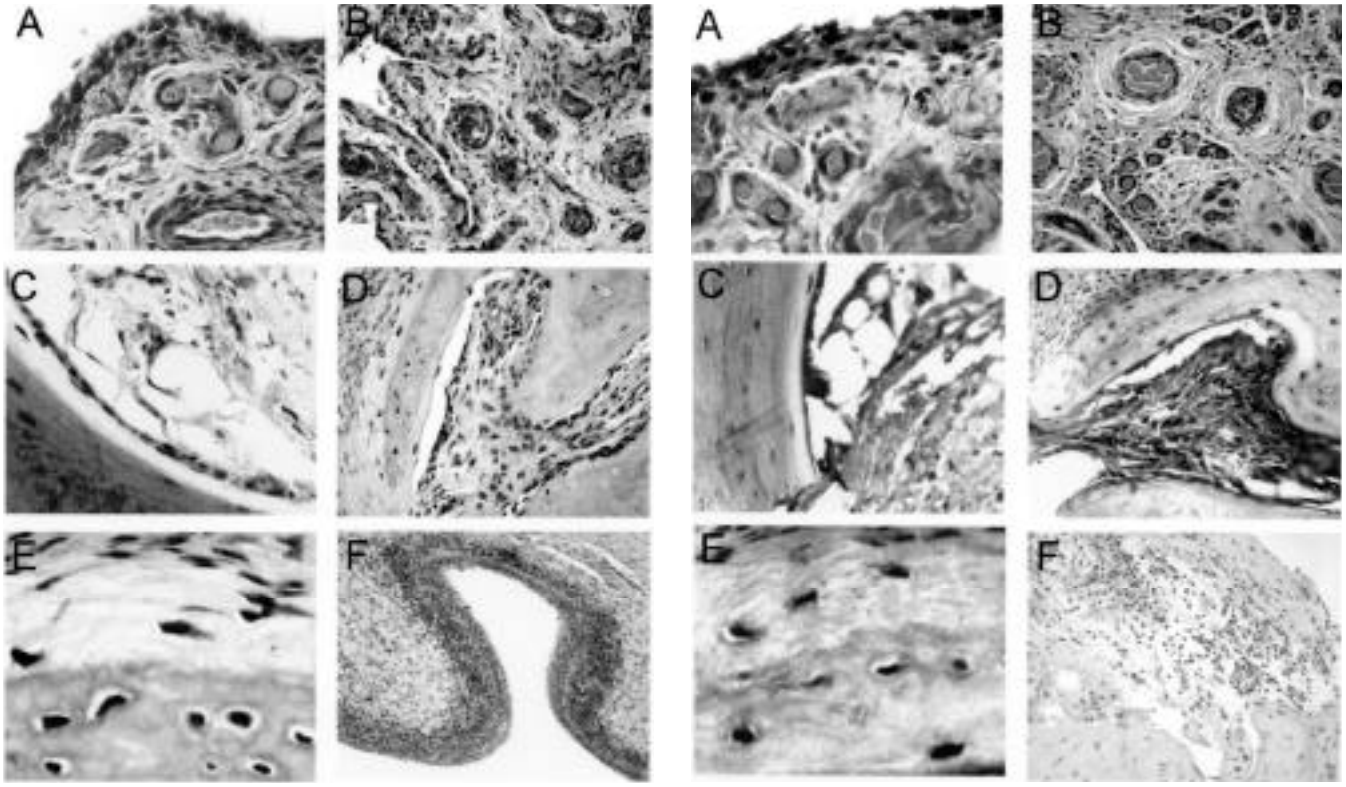


Fig. 4. Immunohistochemical localization of **a**) MMP-1 and **b**) MMP-3 in synovial lining (**A**), endothelium of blood vessels (**B**), bone-pannus junction (**C**), cartilage-pannus junction (**D**), chondrocytes (**E**) and negative control with mouse IgG (**F**).

enzyme and intermediate forms of MMP-3, which in addition were much weaker than the corresponding bands in RA synovial and pannus samples. Trypsin digestion of representative pannus tissue samples was done to clarify the active form of MMP-3 present. The results showed that 57 kDa proform and 53 kDa intermediate form of MMP-3 were transformed into the corresponding 45 kDa active form (Fig. 5b). In contrast, MMP-1 bands were weak. The 52 kDa proform of MMP-1 was very weak and no 43 kDa active form of MMP-1 was seen in any of the samples tested (data not shown).

Discussion

MMP-3, an enzyme that cleaves e.g. aggrecan, laminin, collagens III, IV, IX and X, is believed to play a major role also in the pathologic degradation of proteoglycans. In our recent RT-PCR analysis of 16 MMPs suggested that in particular MMP-1, -3, -13 and -14 would be of interest in RA(13). Therefore, in this study we have performed quantitative real time RT-PCR for these selected MMPs and shown MMP-3 to be clearly the predominant MMP in advanced RA pannus tissue compared to synovium of advanced RA or osteoarthritis. Earlier MMP-3 has been immunolocalized in the joints of rats with collagen-induced arthritis (14) and present immunohistochemistry localized the corresponding MMP-3 enzyme protein at the pannus-cartilage junction, at the tissue destructive interface of the soft and hard tissues. These findings are in line with the earlier studies where MMP-3 levels are increased in serum and synovial fluid of RA patients compared to osteoarthritis (27, 28, 17). However, recently Kane *et al.* (29) showed no clear-cut differences in MMP-1 and -3 expressions in RAdistal and proximal to cartilage-pannus junction in their competitive quantitative PCR.

MMP-3 is an established sign of inflammation and the finding of high MMP-3 levels in inflamed tissues was predictable. Also other MMPs have been indicated in ongoing inflammation and this work demonstrates quantitatively that particularly mRNA levels of MMP-3 were high in advanced RA pannus tissue, being 12 times higher than those of MMP-13, 46 times higher than those of MMP-1 and over 400 times higher than those of MMP-14 levels.

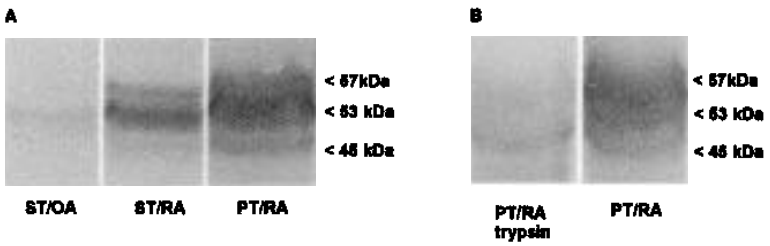


Fig. 5. Western blots showing proactive (57 kDa), intermediate (53 kDa) and active (45 kDa) forms of MMP-3 **A**) in synovial tissue (ST) of osteoarthritis (OA) and rheumatoid arthritis (RA) and RApannus tissue (PT); **B**) and in trypsin digested pannus tissue of RAversus normal non-digested.

The serum MMP-3 level seems to be an indicator for the development of radiological damage in patients with early RA (30-33) and time integrated values of serum MMP-3 correlate with time integrated values of other markers of disease activity such as CRP (34) but there seems to be no difference between serum MMP-3 and CRP with regard to the monitoring of the progression of radiological damage (35). MMP-3 serum levels do not necessarily correlate with the local tissue destructive events if active MMP-3 binds tightly to its substrate and can then not be measured in synovial fluid or serum.

MMP-1 levels are higher in RAthan in osteoarthritis synovial fluid (16). It has been suggested that there is no correlation between MMP-1 and disease activity in RA, but MMP-1 levels seem to correlate with the degree of synovial inflammation (36). In our study, MMP-1 mRNAlevels in advanced RApannus tissue samples were remarkable low compared to MMP-3. Earlier studies have shown an important role for MMP-1 in cartilage collagen degradation in RA (37-39) and increased MMP-1 levels in cartilage (40). In spite of the low MMP-1 mRNA copy numbers in RA pannus tissue, the present immunohistochemical staining results show MMP-1 expression also in chondrocytes in RA tissue. MMP-1 has also been described in cultured chondrocytes (41). All these observations point to an eventual role of MMP-1 in degradation of cartilage collagen II. However, MMP-1 relevant from this point of view does not seem to be derived from pannus tissue.

Primary cultures of fibroblasts produce both MMP-1 and MMP-3 (42). Present quantitative RT-PCR, immunohistochemical and cell culture analysis support those previous studies demonstrating that synovial resident fibroblasts are the main cellular source of MMP-3. IL-1 has been shown to stimulate fibroblast-mediated MMP-3 synthesis (22, 25). Inhibition of IL-1 reduces joint inflammation and cartilage/bone erosion, but does not prevent RA (43, 44). Recombinant IL-1 injected intraarticularly induces minor joint inflammation, but increased proteoglycan loss (45) and IL-1β gene transfer to knee joint of rabbit induces aggressive pannus formation and cartilage/bone erosions (46). In RA, expression of TNF-α may precede IL-1β (20). Knock-out conditions have verified the role of TNF-α in inflammation and joint swelling, whereas IL-1 has not been shown to be a dominant cytokine in early arthritis, however it has been pointed out for its role in cartilage and bone degradation (47, 48). In stimulated fibroblasts MMP-1 and MMP-3 are not always co-ordinately expressed (22). Our quantitative results of cultured fibroblasts showed that MMP-1 mRNA levels were more elevated by TNF-α than by IL-1β stimulation whilst MMP-3 mRNA and protein levels were more effectively stimulated by IL-1β. Interestingly, MMP-1 mRNA copy numbers in fibroblasts were extremely low compared to MMP-3, a finding that may further suggest that MMP-1 may be involved in the initiation of cartilage destruction.

Therefore, Western blots were used to confirm and extend the results of quantitative RT-PCR by demonstrating upregulation of MMP-3 compared with MMP-1. Western blots of pannus tissue extracts demonstrated proteolytic processing and activation of proMMP-3 to its intermediate and active forms, whereas all MMP-1 present was in the latent zymogen form and no band corresponding with the active MMP-1 could be detected. The abundance of apparently proteolytically activated MMP-3 further points out to its potential role as a matrix degrading enzyme in the pannus-cartilage junction.

Early stages of RA may show different histopathology and MMP expression pattern (49). Samples from the present study were obtained from arthroscopy, synovectomy and arthroplastic surgery. Such tissues might represent different stages of diseases. We did not find any histopathological or immunohistochemical or other differences between samples obtained using different methods, so it seems that the method used for sample collection was not a confounding factor. Although the number of samples available for analysis was limited, the statistical methods used for testing showed significant differences between groups.

In summary, this study shows that in particular MMP-3 is highly expressed at both mRNA and protein level in advanced rheumatoid pannus tissue. MMP-3 appears to derive mainly from fibroblasts and is upregulated by IL-1β and to a lesser extent by TNF-α. IL-1β has been shown to be involved more in progressive RAthan in early inflammation, which also connects pannus derived MMP-3 to cartilage destruction. These, together with MMP-3 apparent activation, indicate an important role for this proteinase in the proteoglycan degradation of cartilage matrix in rheumatoid arthritis.

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