MMP-2/gelatinase A is a gene product of human adult articular chondrocytes and is increased in osteoarthritic cartilage

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Abstract

Objective

Collagen fibril degeneration involves initially the cleavage within the triple helix by the collagenases (1 and 3), but then mainly involves also the gelatinases, of which gelatinase A (MMP-2) appears to play a central role in many tissues. The objective of this study was to determine the quantitative expression levels as well as the distribution in normal and osteoarthritic cartilage of gelatinase A and in cultured articular chondrocytes with and without stimulation by Il-1β.

Methods

Conventional and online PCR technology and immunohistochemistry were used to determine MMP-2 expression levels on the mRNA and protein level.

Results

Conventional PCR analysis could demonstrate the presence of MMP-2 mRNA in normal and osteoarthritic chondrocytes. Online quantitative PCR confirmed the presence of MMP-2 mRNA expression in normal articular chondrocytes in vivo *(and* in vitro*). An increase of 5x (p < 0.001) was observed in osteoarthritic cartilage* in vivo*. Of note, no significant up-regulation of gelatinase A was observed by Il-1β* in vitro*. Immunostaining for gelatinase A confirmed the presence of MMP-2 with mono- and polyclonal antibodies in normal and osteoarthritic chondrocytes with somewhat higher levels observed in the latter.*

Conclusions

The presented results confirm the increased expression of gelatinase A by osteoarthritic articular chondrocytes as previously described. Of note, also normal adult articular chondrocytes expressed significant amounts of gelatinase A in vivo *and* in vitro *suggesting gelatinase A as being also involved in physiological collagen turnover in human adult articular cartilage.*

Key words

Metalloproteinases, gelatinase, collagen degradation, osteoarthritis, cartilage.

Introduction

Osteoarthritic cartilage degeneration as well as cartilage destruction in rheumatoid arthritis depend at least to a significant portion on enzymatic degradation of the collagen fibrils. The initial degradation of collagen fibrils depends on the cleavage at the collagenase site, most likely by collagenase 1 (MMP-1) and collagenase 3 (MMP-13) (1) (2, 3). Subsequently, denatured collagen fibrils are thought to be further degraded by gelatinases with gelatinase A being the major player in particular in the pathology of many tissues (4).

Previously, the expression of gelatinase A by human adult articular chondrocytes was reported several times, but only in organ cultures (5) or after cell isolation (6). No systematic investigation exist so far on the *in situ* expression of gelatinase A except an *in situ* hybridization analysis by Chubinskaya and colleagues (7), which could detect weak signals of gelatinase A in normal articular chondrocytes with, however, no increase in osteoarthritic cartilage. In an own gene array analysis we were not able to pick up any expression of gelatinase A in normal adult articular chondrocytes *in vivo* nor *in vitro*, but a significant expression and increase in the osteoarthritic (3).

In this study, we used highly sensitive online quantitative PCR technology as offered by the TAQMAN system (Perkin Elmer) on a series of normal, early degenerative, and late stage osteoarthritic cartilage samples in order to provide data on this important question. Additionally, we compared the *in vivo* levels with the *in vitro* expression in short-term-high density monolayer cultures with and without stimulation by Il-1 . Finally, we localized MMP-2 protein by immunohistochemistry in normal and osteoarthritic cartilage.

Materials and Methods

Cartilage samples

For the study of mRNA expression levels, cartilage from human femoral condyles was processed as described previously (3). Normal articular cartilage (n = 10, 45 to 88 yrs) was obtained from autopsies, within 48 hours of death. Osteoarthritic cartilage was obtained from total knee replacement (n =15, 63 to 85 yrs). Cartilage was considered to be normal if it showed no significant softening or surface fibrillation. Cases of rheumatoid arthritis were excluded from the study. Only primary degenerated and not regenerative cartilage (osteophytic tissue) was used for RNA isolation.

Conventional immunohistochemistry

Conventional immunohistochemical studies were performed on paraformaldehyde fixed and paraffin embedded specimens of normal (n = 5) and osteoarthritic (n = 10) articular cartilage as described previously (8). Deparaffinized sections were enzymatically pretreated, incubated with primary antibodies (see below) over-night at 4°C and visualized using a streptavidin-biotin-complex technique (Biogenex, San Ramon, CA, USA: Super Sensitive Immunodetection System for mouse or rabbit primary antibodies) with alkaline phosphatase (as detection enzyme) and 3-hydroxy-2-naphtylacid 2,4-dimethyl-anilid (as substrate). Alternatively, peroxidase-labeling was performed (S-100 protein) with diaminobenzydine as color substrate. Nuclei were counterstained with hematoxylin.

In order to obtain optimal staining results various enzymatic pretreatments including hyaluronidase (Boehringer, Mannheim, FRG; 2 mg/ml in phosphate buffered saline (PBS), pH 5, for 60 minutes at 37°C), pronase (Sigma, Deisenhofen, FRG; 2 mg/ml in PBS, pH 7.3, for 60 minutes at 37°C), or bacterial protease XXIV (Sigma; 0.02 mg/ml; PBS, pH 7.3, for 60 minutes at 37°C) were tested. The mouse monoclonal antibody (clone 42-5D11; Chemicon Temecula, USA) and the sheep polyclonal antibody (Acris Bad Nauheim, FRG) against MMP-2 recognize the precursor and active forms of MMP-2 and were used in a dilution of 1:500 and 1:200 respectively.

For the final staining enzymatic pretreatment with pronase (Sigma, Deisenhofen, FRG; 2 mg/ml in PBS, pH 7.3, for 60 minutes at 37°C) was used for both antibodies, because this allowed to obtain the best results.

As negative control for immunohisto-

chemical stainings, the primary antibody was replaced by non-immune mouse or rabbit serum (BioGenex; San Ramon, CA, USA) or Tris-buffered saline (pH 7.2). No signal was obtained in any sample investigated (Fig. 2c).
Evalutation of the immunostaining results on a semiquantiative level (0: no staining; +: week staining; ++: moderate staining; +++ strong staining) was performed by two observers (S.D. and S.S.). For testing the statistical significance the non-parametric Mann-Whitney test was used. P-values below 0.05 were considered to be significant.

Cell isolation – Stimulation by Il-1β
Normal human knee articular cartilage was obtained from normal donors at autopsy, within 48 hours of death (4 males: 46-84 years of age; 1 female: 63 years of age).
Cells were enzymatically isolated and cultured in high density monolayer cultures (2×10^6 cells/well) in 6 well tissue culture plates with and without serum as described previously (2). After two days without stimulus, chondrocytes were treated with 10 ng/ml rhIL-1 (Biomol, Germany) for 30 min, 6 h, 16h, 48 h, and 72 hrs. Also, different concentrations were tested for 48 hrs (Il-1 : 0.01 / 0.1/1 / 10 ng/ml).

RNA isolation and cDNA synthesis – Conventional PCR
Total RNA from cartilage tissue and cultured chondrocytes was isolated and cDNA synthesized as described previously (9).
For conventional PCR, cDNA equivalent to 50ng total RNA was first denatured and than amplified in a 35 cycle protocol using SilverStart-DNA-Polymerase (Eurogentech, Belgium). Two primers for MMP-2 were selected using the PRIMER EXPRESS TM software (Perkin Elmer) (forward primer: 5´-ACTGCCCCAGACAGGTGATCT-3´; reverse primer: 5´-TCTCCCAAG-GTCCATAGCTCA-3´).

Online quantitative PCR
The primers (MWG Biotech, Germany) and TAQMAN probes (Eurogentech, Belgium) for MMP-1, MMP-2 and MMP-13 were designed using PRIMER EXPRESS TM software (Perkin Elmer). The master-mix contained a final concentration of 200 μM NTPs, 600 nM Roxbuffer, 100 nM TAQMAN probe, cDNA, 1U polymerase (Eurogentech, Belgium) forward and reverse primers, and $MgCl_2$ (Table I). All experiments were performed in triplicates using titrated standard curves as described elsewhere (2). The assay for GAPDH, MMP-1 and MMP-13 were described previously (2, 10).

Statistical analysis
Statistical evaluation of significant differences in expression levels was done by the non-parametric Wilcoxon-Mann-Whitney test for the *in vivo* investigations. For the *in vitro* probes the t-test for pairwise comparison was used due to the limited number of cases to be compared.

Results
Determination of expression levels of MMP-2 in normal and osteoarthritic human articular chondrocytes *in vivo*
Because previous cDNA-array experiments did not reveal positive signals (3), the expression of MMP-2 mRNA was checked by conventional PCR (Fig. 1a). This showed detectable mRNA for MMP-2 in both, normal and osteoarthritic, cartilage samples, but clearly stronger amplification products were observed in osteoarthritic samples.
In order to be able to quantify mRNA expression levels, a quantitative PCR-assay was developed. The TAQMAN assay confirmed the presence of MMP-2 mRNA in normal articular chondrocytes (Fig. 1b), but only at a low level (0.03 molecules/molecules GAPDH). In osteoarthritic cartilage, a significant increase of MMP-2 mRNA expression was found (5x; $p < 0.001$) confirming the conventional PCR measurements.

Immunohistochemical demonstration of MMP-2 in normal and osteoarthritic articular cartilage
For immunostaining monoclonal and polyclonal antibodies to MMP-2 were used. In normal (Fig. 2a) and osteoarthritic cartilage (Fig. 2b-d) signals for

Table I. Sequences of primers and probes for quantitative online quantitative PCR experiments.

|	Accession No.	Primers	[nM]	Probes	$MgCl_2$ [mM]
GAPDH	NM_002046	fw: GAAGGTGAAGGTCGGAGTC	50	CAAGCTTCCCGTTCTCAGCC	5,5
		rv: GAAGATGGTGATGGGATTTC	900		
MMP-1	NM_002421	fw: CTGTTCAGGGACAGAATGTGCT	300	ACGGATACCCCAAGGACATCTACAGCTCC	6,5
		rv: TCGATATGCTTCACAGTTCTAGGG	900		
MMP-2	NM_004530	fw: TCACTCCTGAGATCTGCAAACAG	300	TTGATGGCATCGCTCAGATCCGTG	6
		rv: TCACAGTCCGCCAAATGAAC	900		
MMP-13	NM_002427	fw: TCCTCTTCTTGAGCTGGACTCATT	900	TCCTCAGACAAATCATCTTCATCACCACCAC	7
		rv: CGCTCTGCAAACTGGAGGTC	50		
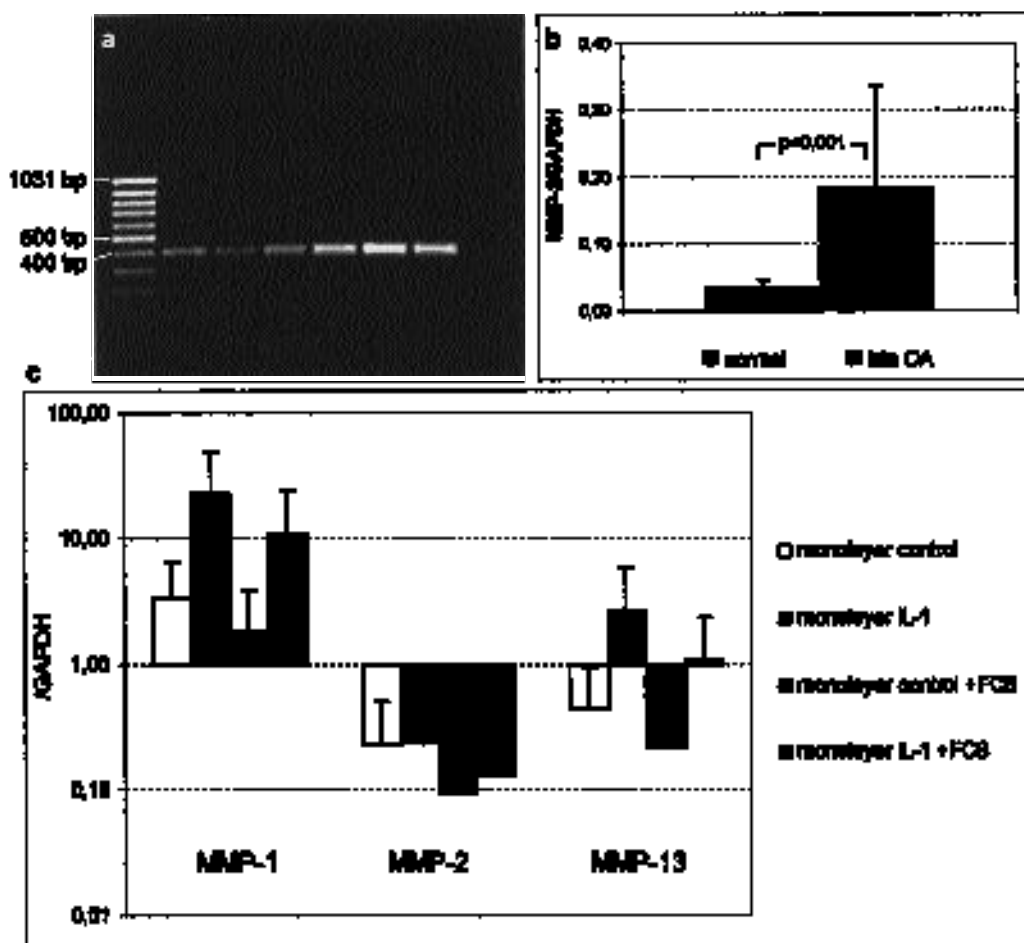
Fig. 1. (**a**) Demonstration of MMP-2 in normal (lanes 2-4) and osteoarthritic (lane 5-7) articular chondrocytes by conventional PCR (lane 1: 100 bp-DNA-ladder, MBI Fermentas, Heidelberg, Germany; lane 8: negative control, no cDNA added; length of amplification product: 405 bp).
(**b**) Quantificative TAQMAN-analysis for mRNA expression levels of MMP-2 in chondrocytes of normal and late-stage osteoarthritic cartilage (given are the values normalized to GAPDH).
(**c**) Quantificative TAQMAN-analysis for mRNA expression levels of MMP-1, MMP-2 and MMP-13 in isolated normal articular chondrocytes with and without stimulation with Il-1ß (given are the GAPDH/ratios of five independent experiments in short term high density monolayer cultures with and without serum).

MMP-2 could be detected with both antibodies within the chondrocytes with a stronger staining observed in the osteoarthritic specimens (difference of staining intensity on a semiquantitative level as described in Materials and methods p < 0.05). Also secondary osteoarthritic cartilage, i.e. newly formed cartilage covering the surface of osteophytes (Fig. 2e,f), was strongly positive.

Determination of expression levels of MMP-2 in normal human articular chondrocytes in vitro *with and without stimulation by Il-1*β
Subsequently, the expression of MMP-2 mRNA was investigated in cultured human adult articular chondrocytes maintained in short-term high-density monolayer cultures with and without stimulation with Il-1 and serum (Fig. 1c). It was significantly more expressed *in vitro* than *in vivo* (p < 0.05). Of note, no significant up-regulation of MMP-2 expression was observed after Il-1 treatment in any concentration of Il-1 tested (0.01 - 10 ng/ml). This was also not time dependent (tested were 30 min up to 48 hrs). In contrast, MMP-1 and -13, analyzed in parallel, were significantly induced by Il-1 confirming the activity of the cytokine and the responsiveness of the chondrocytes used in the assays.

Discussion
The two major results of our study are that MMP-2 is expressed in articular chondrocytes of the knee and that there is a significant increase in expression levels in osteoarthritic cartilage *in vivo*, but not after Il-1 treatment *in vitro*. The very low mRNA expression levels of MMP-2 in adult normal articular chondrocytes *in situ* explain previous contradicting results as these low levels are readily detectable by highly sensitive technologies such as PCR, but not by cDNA arrays (3). This confirms in a quantifying manner previous reports by Mohtai and colleagues (6) and Chubinskaya and colleagues (7) showing MMP-2 expression in normal chondrocytes *in situ* or directly after isolation from articular cartilage. This rejects other reports by ourselves (3) and Tellow *et al.* (11) which were not able to show expression of MMP-2 in normal chondrocytes. However, in our work
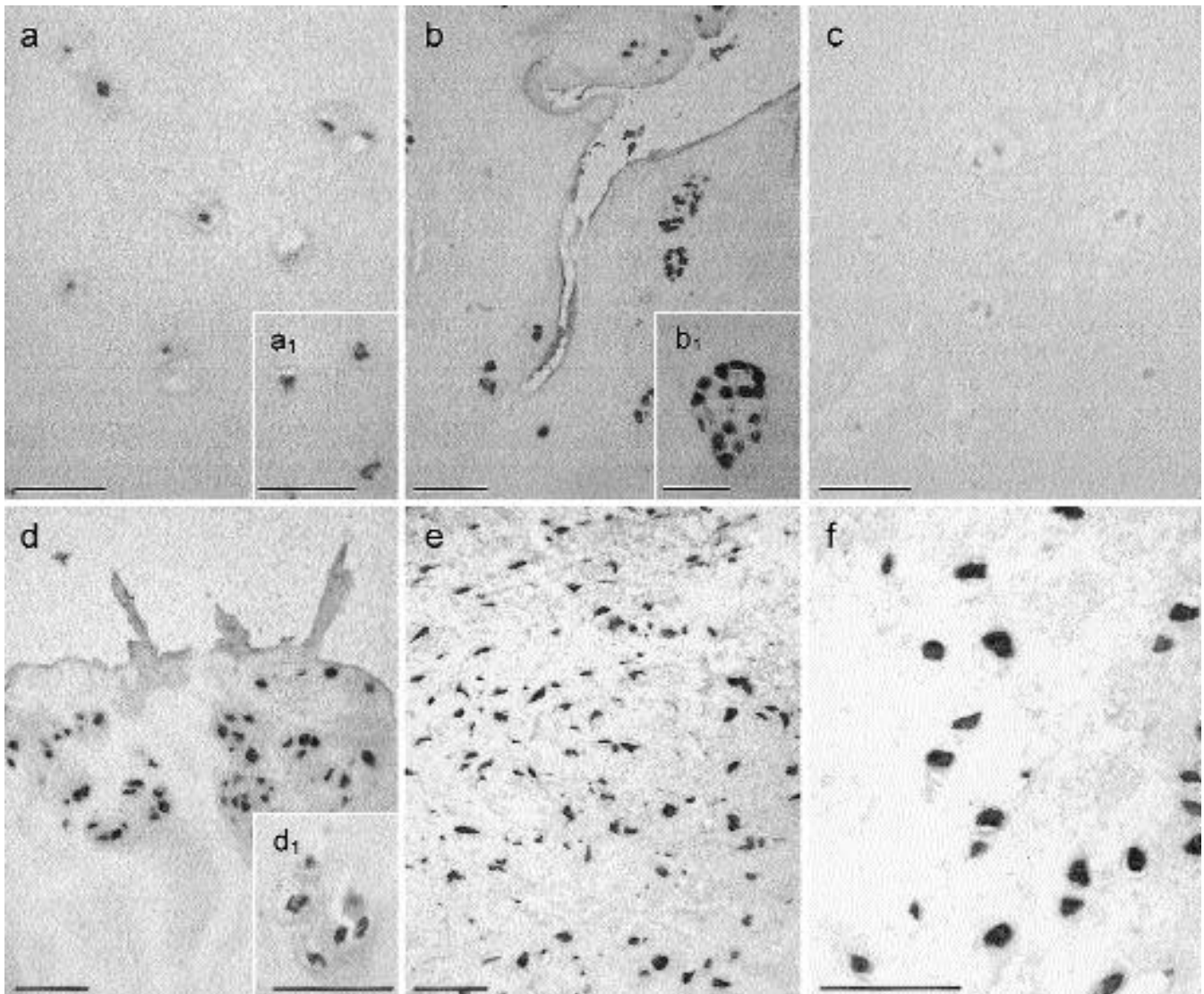
Fig. 2. Immunolocalization of MMP-2 using monoclonal (**a**, **a1**, **d1**; clone 42-5D11; Chemicon Temecula, USA) and polyclonal (**b**, **d**, **e**, **f**; Acris Bad Nauheim, FRG) antibodies against the inactive and activated form in normal (**a**; **a1**: detail) and osteoarthritic (**b**, **1**: typical chondrocyte cluster, **d**, **d1**: detail) as well as osteophytic (**e**, **f**) cartilage (**c**: negative control). (magnification bars: a, a₁, b₁, c, d₁, e, f: 50 μm; b, d: 100μm)

we used only the cDNAarray technology, which is clearly less sensitive than PCR.

Our study is the first to show MMP-2 mRNA expression based on the analysis of RNA isolated directly out of the cartilage tissue and not after previous isolation of the chondrocytes (6). The expression of MMP-2 was increased significantly in osteoarthritic cartilage as suggest by the studies by Mohtai *et al.* (6) looking at freshly isolated chondrocytes. As shown by Mohtai and colleagues, besides an overall increase in expression of MMP-2 an increased activation of MMP-2 might even accelerate this effect in osteoarthritic cartilage

(5). Our immunolocalization studies confirmed the presence of MMP-2, both in normal and osteoarthritic chondrocytes. The fact that we and others (11, 12) observed MMP-2 within the cells might reflect actual synthesis by the cells whereas the secreted molecule may be masked by the extracellular cartilage matrix.

MMP-2 appears to be induced in articular chondrocytes after isolation form the tissue, but appears in contrast to many other proteases such as MMP-1, MMP-3, and MMP-13 (1, 2, 13) not be induced by Il-1ß *in vitro* independent of the Il-1 concentration used. This is in line with previous findings reported

for Il-1 (6) and in tissue culture (7). Still, articular chondrocytes are able to regulate MMP-2 expression as documented in this study by the up-regulation of MMP-2 mRNAexpression after cell isolation or in osteoarthritic cartilage, which appears to be different from synovial fibroblasts which were found to show rather constant MMP-2 expression levels (14). From the data presented in this and previous studies the exact stimuli inducing the up-regulation in both conditions remain unclear. One intriguing speculation is that both would be triggered via the appearance of matrix degradation fragments such as collagen and/or fibronectin

fragments, both of which are known to be able to induce a strongly catabolic response in articular chondrocytes (15, 16).

Altogether, our results suggest that MMP-2/gelatinase A shows a constant low basic expression level in normal articular chondrocytes. Thus, MMP-2 might be in normal articular cartilage together with MMP-1 responsible for the (pericellular) physiological collagen turnover (17). A strong general collagen turnover presumably also explains its strong presence in osteophytic tissue. In osteoarthritis, similar to MMP-13 (collagenase 3), MMP-2/gelatinase A is induced in the chondrocytes. Thus, in degenerating cartilage, MMP-2 might be the partner of MMP-13 in pathological degradation of collagen fibrils. In both conditions, gelatinase A might be centrally involved in the advanced degradation of collagens fibrils cleaved first by one of the two collagenases.

Acknowledgements

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