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 upregulation 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-1β. Finally, we localized MMP-2 protein by immunohistochemistry in normal and osteoarthritic cartilage.

**Materials and Methods**

**Cartilage samples**

For the study of mRNAexpression 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-naphthyl acid 2,4-dimethyl-anilid (as substrate). Alternatively, peroxidase-labeling was performed (S-100 protein) with diaminobenzidine 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). Evaluation of the immunostaining results on a semiquantitative level (0: no staining; +: weak 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 II-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 x 10⁶ 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-1β (Biomol, Germany) for 30 min, 6 h, 16 h, 48 h, and 72 hrs. Also, different concentrations were tested for 48 hrs (II-1β: 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 then 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’-ACTGGCCCAGACAGGTGATCT-3’; reverse primer: 5’-TCTCCAAAGGTCCATAGCTCA-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 MgCl₂ (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 cartilage 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.

<table>
<thead>
<tr>
<th>Accession</th>
<th>No.</th>
<th>Primers</th>
<th>[nM]</th>
<th>Probes</th>
<th>MgCl₂</th>
<th>[mM]</th>
</tr>
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<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>fw: GAAGGTGAAGGTCGGAGCTC</td>
<td>50</td>
<td>CAAGCTTCCCGTTCAGCC</td>
<td>5.5</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>rv: GAAGATGGTGATGGGATTTC</td>
<td>900</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-1</td>
<td>NM_002421</td>
<td>fw: CGTGGTCAGGACAGAAGTGTCTC</td>
<td>300</td>
<td>ACGGATACCCCAAGGACATCTACAGCTCC</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv: TCGATATGCTCACAGGTCTAGGG</td>
<td>900</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>NM_004530</td>
<td>fw: TCACCTCTGAGATCTGCAAAACAG</td>
<td>300</td>
<td>TTGATGCGTCTCCAGACATCGCT</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv: TCACAGTCCGCAAATGGAAC</td>
<td>900</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-13</td>
<td>NM_002427</td>
<td>fw: TCCTCTTCTTGTAGCTGAGACTCATT</td>
<td>900</td>
<td>TCCTAGACAAATCATCTTCACTACCACAC</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv: CGCTCTGCAAATCAGAGGTTC</td>
<td>50</td>
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</table>
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-1β

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-1β 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-1β treatment in any concentration of IL-1β 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-1β 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-1β 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 Telloy et al. (11) which were not able to show expression of MMP-2 in normal chondrocytes. However, in our work
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 suggested 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 II-1β in vitro independent of the II-1β concentration used. This is in line with previous findings reported for II-1α (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 mRNA expression 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.

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 ostoiphic (e, f) cartilage (e: negative control). (magnification bars: a, a1, b, c, d, e, f: 50 µm; b, d: 100 µm)
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 plains its strong presence in osteophyt -16). 13 in pathological degradation of colla -

Susanne Fickenscher for excellent collagenases.

Thus, in degenerating cartilage, nase A might be centrally involved in pert graphical assistance. We are grateful to Freya Boggasch and technical and Pia M. Gebhard for ex -

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