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MMP-9/gelatinase B is a gene product of human adult articular chondrocytes and 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 (MMP-1) and 3 (MMP-13), but then mainly involves also the gelatinases A (MMP-2) and B (MMP-9). The objective of this study was to determine the quantitative expression levels as well as the distribution in normal and osteoarthritic cartilage of gelatinase B and in cultured articular chondrocytes with and without stimulation by Il-1β.*

Methods. *Conventional and real-time quantitative PCR technology and immunohistochemistry were used to determine gelatinase B expression on the mRNA and protein level.*

Results. *Conventional PCR analysis could demonstrate the presence of gelatinase B mRNA only in osteoarthritic chondrocytes. Real-time quantitative PCR confirmed the increased expression of gelatinase B mRNA expression in osteoarthritic chondrocytes. No significant up-regulation of gelatinase B was observed by Il-1β. Immunostaining for gelatinase B showed the presence of gelatinase B in a subset of normal and in a large portion of osteoarthritic chondrocytes with a more extended distribution in the latter.*

Conclusion. *In osteoarthritic cartilage destruction, gelatinase B is involved in collagen destruction though still at a very much lower level than gelatinase A. Only a very small subset of normal adult articular chondrocytes express gelatinase B* in vivo *suggesting that gelatinase B unlike gelatinase A is hardly or only very focally involved in physiological collagen turnover.*

Introduction

Osteoarthritic cartilage degeneration as well as cartilage destruction in rheumatoid arthritis depend to a significant portion on enzymatic degradation of the collagen fibrils by collagenases and gelatinases.

Previously, we could show the expression and presence of gelatinase A in normal and significantly increased in osteoarthritic chondrocytes (1). Also, the expression of gelatinase B by human adult articular chondrocytes was reported (2-4). In this study, we used highly sensitive and quantitative real-time PCR technology on a series of normal, early degenerative, and late stage osteoarthritic cartilage samples in order to investigate *in vivo* and *in vitro* expression levels of gelatinase B by normal and osteoarthritic chondrocytes. Additionally, we localized gelatinase B 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 obtained at autopsies within 36 hrs of death (normal: n = 8, 48 to 83 yrs; early degenerative: n = 8; 59 to 86 yrs) or total knee replacement (n = 9, 60 to 83 yrs) was processed as described previously (1). For histological and immunohistochemical analysis, cartilage was obtained after hip and knee replacement surgery (osteoarthritic: n = 12; 54 - 82 yrs) and autopsies (normal: n = 5; 65 - 81 yrs), fixed in 4% paraformaldehyde and embedded into paraffin wax.

Immunohistochemistry

Sections were incubated overnight with a monoclonal antibody against gelatinase B (MAB3309, detects active and inactive gelatinase B; Chemicon, Chandlers Ford, UK) and antibody binding was visualized as described previously (1). For negative controls, the primary antibody was replaced by non-immune mouse serum or Tris-buffered saline (pH 7.2).

Cell isolation - stimulation by Il-1β

Normal human knee articular cartilage was obtained from normal donors at autopsy, within 48 hours of death (n = 7; 37 to 79 yrs). Cells were enzymatically isolated and cultured in high density monolayer cultures ($2x10^6$ cells/-well in 6-well plates) as described previously (5). After two days without stimulus, chondrocytes were treated with 10 ng/ml rhIL-1β (Biomol, Germany) for 48 hrs with and without 10% fetal calf serum (Biochrom, FRG).

RNA isolation and cDNA synthesis - Conventional PCR

Total RNA from cartilage tissue and

cultured chondrocytes was isolated, cDNA synthesized, and PCR performed using a cDNA equivalent to 50 ng total RNA as described previously (6) (forward primer: 5'-ATGGTTACACTCGGGTGGCA-3'; reverse primer: 5'-CACGCGAGTGAAGGTGAGC-3').

Real-time quantitative PCR

The primers (MWG Biotech, Germany) and TAQMAN probe (Eurogentech, Belgium) for gelatinase B was designed using PRIMER EXPRESS TM software (Perkin Elmer): forward primer: 5'-TTCCAGTACCGAGAGAAAGCCTAT-3'; reverse primer: 5'-TAGGTCACGTAGCCCACTTGGT -3'; probe: 5'-CTACTGGCGCGTGAGTTCCCGGA-3'. All experiments were performed in triplicates using titrated standard curves as described elsewhere (5). The assays for GAPDH, gelatinase A and -13 were described previously (1, 5).

Statistical evaluation of differences in expression levels was done by the non-parametric Wilcoxon-Mann-Whitney test for the *in vivo* investigations and the t-test for pairwise comparison for the *in vitro* probes.

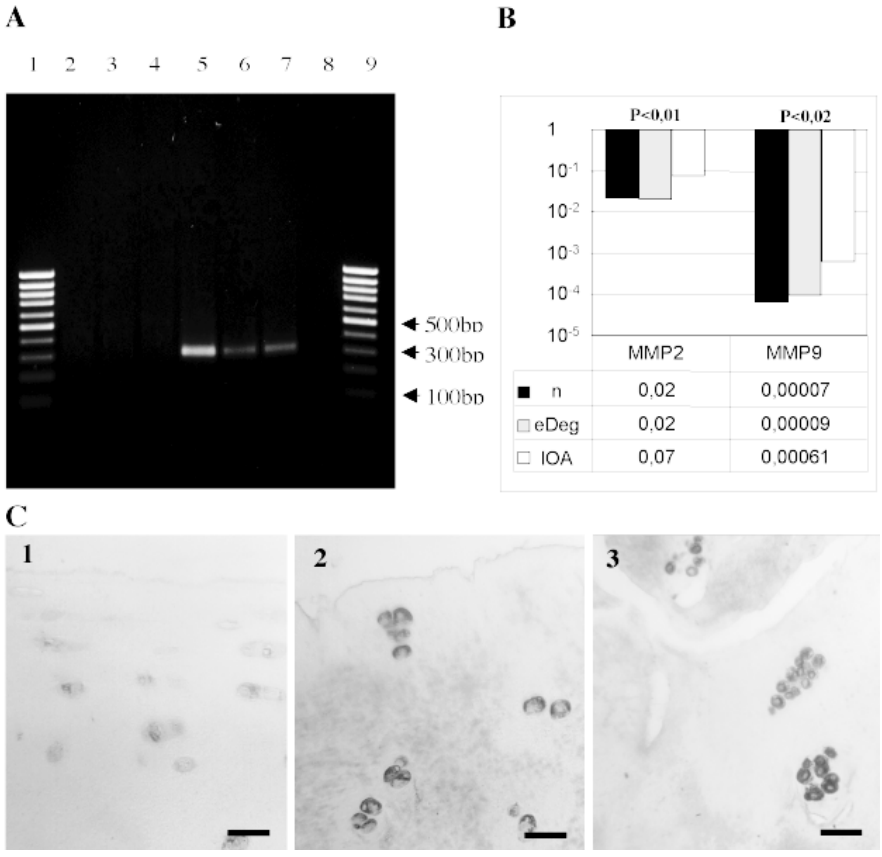

Fig. 1. Expression analysis of MMP-9 in normal and osteoarthritic articular cartilage on the mRNA and protein level: **A**: Demonstration of gelatinase B in normal (lanes 2-4) and osteoarthritic (lane 5-7) articular chondrocytes by conventional PCR (lane 1 and 9: 100 bp-DNA-ladder, MBI Fermentas, Heidelberg, Germany; lane 8: negative control, no cDNA added; length of amplification product: 323 bp).

B: Quantificative PCR-analysis for mRNA expression levels of gelatinase B in chondrocytes of normal ("n": n = 8), dearly degenerative ("eDeg": n = 8) and late-stage osteoarthritic ("lOA": n = 9) cartilage (given are the values normalized to GAPDH).

C: Immunolocalization of gelatinase B: (**1**) In normal cartilage only very occasional gelatinase B positive cells are present in the superficial zone. (**2**) In early stage OA cartilage many positive cells are found in superficial zone, whereas the cells in intermediate/deep zone are mostly negative (not shown). (**3**) In fibrillated late-stage OA cartilage all chondrocytes are gelatinase positive. (Scale bars = 50 μm).

Results

Determination of expression levels of gelatinase B in normal and osteoarthritic human articular chondrocytes in vivo

Conventional PCR (Fig. 1A) showed detectable mRNA for gelatinase B in osteoarthritic cartilage samples (n = 3), but not normal samples (n = 3) in the conditions used.

In order to be able to quantify mRNA expression levels, a quantitative PCR-assay was developed. The TAQMAN assay showed the presence of gelatinase B mRNA in normal articular chondrocytes (Fig. 1B), but only at a very low level (< 0.0001 molecules/molecules GAPDH; n = 8). In osteoarthritic cartilage (n = 9), a significant increase of gelatinase B mRNA expression was found (about 8,7x; $p < 0.02$). However, also in osteoarthritic chondrocytes much less expression was found for gelatinase B compared to gelatinase A (>100x; $p < 0.005$).

Immunohistochemical demonstration of gelatinase B in normal and osteoarthritic articular cartilage

Normal articular chondrocytes did not synthesize gelatinase B with the exception of some chondrocytes in the very superficial layer (Fig. 1C1). Also cells from the intermediate and deep zones of the cartilage taken from the non-weight bearing regions of OA patients were negative. In contrast, the chondrocytes present in the regions of proteoglycan loss (either in the superficial zone of early degenerative cartilage (Fig. 1C2) or of the non-weight bearing regions of OA patients or throughout OA cartilage (Fig. 1C3)) were immuno-positive for gelatinase B. Thus, in late-stage OA cartilage, characterized by surface fibrillation and the presence of chondrocyte clusters, almost all cells were gelatinase B positive and the enzyme could be detected in the matrix.

Determination of expression levels of gelatinase B in normal human articular chondrocytes in vitro *with and without stimulation by Il-1β*

Subsequently, the expression of gelati-

nase B 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 (data not shown). Again, only very low levels of gelatinase B could be detected similar to the *in vivo* situation. Of note, similar to gelatinase A no significant up-regulation of gelatinase B expression was observed after Il-1β treatment in any concentration of Il-1β tested (0.01 - 10 ng/ml). In contrast, MMP-13 analyzed in parallel (1), was 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 gelatinase B is expressed only in a very minor subpopulation of normal articular chondrocytes and that there is a slight, but significant increase in expression levels in osteoarthritic cartilage *in vivo*. Also, similar to gelatinase A (1), gelatinase B appears in contrast to the collagenases (5, 7) not be induced by IL-1β *in vitro* at least in our culture conditions though others reported differently (8).

Our study is the first to show gelatinase B mRNA expression based on the analysis of RNA isolated directly out of the cartilage tissue and not after previous isolation of the chondrocytes (2, 8). The expression of gelatinase B was increased significantly in osteoarthritic cartilage as suggested previously in freshly isolated chondrocytes (2, 3, 8). Our immunolocalization studies confirmed the absence of gelatinase B in most normal articular chondrocytes and the presence of gelatinase B in the majority of osteoarthritic chondrocytes (3, 4) including strong signals obtained in areas of chondrocyte proliferation (clustering); the latter further documents that this is a process involving active matrix remodeling including matrix degrading proteases such MMP-9. From the data presented in this and previous studies the exact stimuli inducing the up-regulation in osteoarthritic chondrocytes remain unclear. One intriguing speculation is this might 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 (9). Alternatively, genomic demethylation within the promoter region of gelatinase B might lead to increased expression of this gene in osteoarthritic chondrocytes (Roach *et al.*, unpublished results). Also, gelatinase B up-regulation was reported to occur after mechanical loading in the chondrocytic cell line HCS-2/8 (10), maybe suggesting that increased mechanical stress of osteoarthritic chondrocytes might lead to increased expression levels of gelatinase B within the cells.

Altogether, our results suggest that gelatinase B shows not a constant low basic expression level in normal articular chondrocytes like gelatinase A. Thus, gelatinase B is presumably not involved in physiological collagen turnover as it might be the case for gelatinase A (1). In osteoarthritic cartilage, similar to MMP-13 (collagenase 3) and gelatinase A, gelatinase B is induced in the chondrocytes. Our study suggests that gelatinase B might be the partner of gelatinase A and MMP-13 in pathological degradation of collagen fibrils in degenerating cartilage, though it appears to be also in this situation much less expressed than gelatinase A.

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