ADAMTS-1, a Gene Product of Articular Chondrocytes *in Vivo* and *in Vitro*, Is Downregulated by Interleukin 1ß

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ABSTRACT. *Objective.* Osteoarthritic (OA) cartilage degeneration and cartilage destruction in rheumatoid arthritis depends significantly on enzymatic degradation of cartilage proteoglycan aggrecan. A member of the ADAMTS family of proteases, ADAMTS-1 was described to have "aggrecanase" activity. We investigated the quantitative expression and distribution of ADAMTS-1 in healthy and OA cartilage and in cultured articular chondrocytes with and without stimulation by interleukin 1ß (IL-1ß) and insulin-like growth factor-I (IGF-I).

Methods. Conventional and online polymerase chain reaction (PCR) technology was used to determine ADAMTS-1 mRNA expression levels of ADAMTS-1. Protein was localized using immunostaining with different polyclonal antibodies.

Results. Conventional and online semiquantitative PCR showed significant levels of ADAMTS-1 mRNA expression in normal and OA chondrocytes *in vivo* and *in vitro*. Only a slight increase was observed in OA cartilage. After stimulation with IL-1ß a downregulation of ADAMTS-1 was observed, whereas IGF did not appear to change mRNA expression levels *in vitro*. The *in vivo* mRNA expression results were confirmed by the presence of significant protein staining with antibodies for ADAMTS-1 in normal and OA chondrocytes as well as Western blotting analysis. Whereas a significantly stronger stain was seen in normal articular cartilage in the upper zones, in OA cartilage as well the middle zone showed enhanced staining.

Conclusion. Our results confirm the expression and presence of ADAMTS-1 in articular cartilage. However, they also point out that ADAMTS-1 appears to be constitutively expressed by adult articular chondrocytes and overall is not strongly upregulated in OA. Thus our data suggest that ADAMTS-1 is the first matrix-degrading enzyme downregulated by the catabolic factor IL-1ß *in vitro*. (J Rheumatol 2004;31:315–20)

Key Indexing Terms:
METALLOPROTEINASES   AGGRECANASE   COLLAGEN
AGGRECAN   OSTEOARTHRITIS   CARTILAGE

Osteoarthritic (OA) cartilage degeneration as well as cartilage destruction in rheumatoid arthritis (RA) depend to a significant extent on enzymatic degradation of matrix components. Two molecule types represent the major targets in terms of enzymatic activity and functional loss of the cartilage matrix as a result of damage to the molecules, namely, collagen fibrils and the proteoglycan aggrecan. Whereas degradation of the former leads to matrix instability with tissue swelling, degradation of proteoglycans leads to loss of fixed charges and cartilage softening[1]. Both are classical features of cartilage destruction.

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The catabolism of aggrecan is supposed to involve different types of enzymes, largely from 2 protease families: the matrix metalloproteinases (MMP) and the A disintegrin and metalloproteinases (ADAMTS), cutting at 2 major sites of aggrecan in normal and degenerated cartilage[2,3]: the MMP site (Asn[341]–Phe[342])[4], at which cleavage by many MMP and in particular stromelysin (MMP-3) is described[4,5], and the aggrecanase site (Glu[373]–Ala[374])[6]. For the latter, enzymes such as aggrecanase-1 (ADAMTS-4)[7,8] and aggrecanase-2 (ADAMTS-5)[9] were suggested to be responsible[10]. However, aggrecanase-1 is expressed scarcely in articular (and also osteoarthritic) cartilage and neither enzyme is significantly upregulated in OA cartilage. Recently, another member of the ADAMTS family of proteases was described to have "aggrecanase" activity[11], although its aggrecan-degrading capacity is not restricted to the "aggrecanase" site of aggrecan[12]: ADAMTS-1. This molecule was originally identified as METH-1 together with METH-2 (ADAMTS-8) as new members of a family of proteins with angioinhibitory activity[13,14].

A preliminary study based on 2 cases (one healthy and one osteoarthritic) suggested that no major differences in expression levels of ADAMTS-1 are found in OA versus

normal articular cartilage based on conventional polymerase chain reaction (PCR) experiments[15]. We used online quantitative PCR technology as offered by the TAQMAN system (Perkin Elmer, Boston, MA, USA) on a series of normal, early degenerative, and late-stage OA cartilage samples of different disease stages to investigate mRNA expression levels in normal and diseased tissue. Additionally, we compared the *in vivo* levels with the *in vitro* expression in 2 classical *in vitro* culture systems (alginate beads and short-term, high density monolayer culture) with and without stimulation by interleukin 1ß (IL-1ß), a strong inducer of chondrocyte catabolic activity[16,17], and insulin-like growth factor-I (IGF-I), a known anabolic stimulus of chondrocytes[18]. Finally, immunodetection was used to determine the distribution pattern of ADAMTS-1 in normal and OA cartilage on the protein level.

MATERIALS AND METHODS

Cartilage samples. Cartilage from human femoral condyles of the knee joints was used for study of mRNA expression levels within the tissue. Samples of normal articular cartilage (n = 13; from patients 39 to 76 years of age, mean age 58.6 yrs) and early degenerated cartilage (n = 14; patients 49 to 91 yrs, mean 69.1 yrs) were obtained from donors at autopsy, within 48 h of death. OA cartilage samples from late stage OA joint disease were obtained from patients undergoing total knee replacement surgery (n = 11; 61 to 76 yrs, mean 70.7 yrs). The cartilage was frozen in liquid nitrogen immediately after removal and stored at –80°C until required for RNA isolation. Cartilage was considered normal if it showed no significant softening or surface fibrillation. Early degenerated cartilage was defined as moderate fibrillation and softening, but no advanced erosion of the articular cartilage. Only this cartilage was taken for study and not (peripheral) areas showing no obvious signs of degeneration. Late stage OA cartilage was always derived from patients undergoing knee arthroplasty due to complete destruction of cartilage in major portions of the joints. Patients with RA were excluded from the study. Only primary degenerated and not regenerative cartilage (osteophytic tissue) was used for mRNA expression analysis.

Histomorphology and histochemistry. H&E and safranin O staining was performed for all slices to evaluate matrix abundance, cellularity, and content of glycosaminoglycans[19].

Immunohistochemistry. Conventional immunohistochemical studies were performed on paraffin embedded specimens of normal (n = 6) and late stage OA (n = 6) articular cartilage using a streptavidin-biotin-complex technique (Biogenex, Mainz, Germany) with alkaline phosphatase as detection enzyme as described[20]. The specimens were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, immediately after removal.

To obtain optimal staining results we tested various enzymatic pretreatments including hyaluronidase (Boehringer, Mannheim, Germany; 2 mg/ml in PBS, pH 5, for 60 min at 37°C); pronase (Sigma, Deisenhofen, Germany; 2 mg/ml in PBS, pH 7.3, for 60 min at 37°C); chondroitinase ABC (Sigma; 0.25 U/ml in 0.1 M Tris-HCl, pH 8, for 60 min at 37°C); and bacterial protease XXIV (Sigma; 0.02 mg/ml in PBS, pH 7.3, for 60 min at 37°C). The optimal pretreatment used for all staining for all 3 antibodies was chondroitinase ABC 0.25 U/l, 60 min at 37°C.

Three different polyclonal goat antibodies against ADAMTS-1 were used. One (Santa Cruz sc-5468) was raised against an internal peptide region, the second (Santa Cruz sc-5467) against the amino-terminus, and the third (Santa Cruz sc-5463) against the C-terminus of the ADAMTS-1 protein. All antibodies were tested and used at optimal dilution of 1:200.

Negative controls using nonspecific goat antiserum (Biogenex) or replacing the primary antibodies with PBS revealed no positive stainings.

Western blot analysis. Two million cells each of normal and OA primary human chondrocytes and Jurkat cells as a negative control were lysed in 100 µl of 1× Laemmli buffer and run on 10% acrylamide slab gels according to Laemmli[21]. Proteins were electrotransferred to a PVDF membrane (Pall Gelman, Dreieich, Germany), then the membrane was blocked in 2% (w/v) bovine serum albumin in Tris buffered saline (TBS; Sigma). Primary antibody [Santa Cruz anti-ADAMTS-1 (A-19), Santa Cruz Biotechnology, Heidelberg, Germany] was diluted 1:1000 in TBS; the secondary antibody was diluted 1:10,000 (anti-goat IgG, horseradish peroxidase-coupled; Santa Cruz, Heidelberg, Germany) in TBS. Immunodetection was performed by means of ECL detection reagents according to the manufacturer's protocol (Amersham Pharmacia Biotech, Freiburg, Germany).

RNA isolation from articular cartilage. Total RNA from cartilage tissue was isolated as described[22]. Isolated RNA was controlled for quality by electrophoresis and by spectrophotometry.

Cell isolation. For the *in vitro* culture studies, normal human knee articular cartilage was obtained from healthy donors at autopsy, within 48 h of death or from amputation (n = 3, age range 52–79 yrs), and OA specimens were from knee arthroplasty (n = 7, range 58–80 yrs).

Cartilage pieces were finely chopped and chondrocytes were enzymatically isolated from associated matrix. Cells were first digested with 1 mg/ml pronase (Roche, Reinach, Switzerland) in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco BRL, Germany) with 10% fetal calf serum (FCS; Biochrom, Germany) for 30 min and subsequently with 1 mg/ml collagenase P (Boehringer) in Ham's-F12 (Gibco BRL) with 10% FCS. Finally, cells were washed several times in Ham's-F12 and counted and checked for viability using the trypan blue staining method.

High density monolayer culture stimulation with IL-1ß and IGF-I. After isolation, chondrocytes were seeded at 2×10^6 cells/well in 6-well tissue culture plates and maintained for 48 h in DMEM/F12 medium supplemented with 10% FCS, 50 µg/ml penicillin/streptomycin solution (Gibco BRL), and 50 µg/ml ascorbate (Sigma).

Then chondrocytes were stimulated with 0.1 ng/ml, 1 ng/ml, and 10 ng/ml recombinant human IL-1ß (Biomol, Germany) with 10% FCS or cultivated in 10% FCS alone for 3 days. Alternatively, chondrocytes were cultured with 100 ng/ml rhIGF-I (Biomol) in medium without FCS and in medium with no supplement. The medium was changed every day. At the end of the stimulation period the cells were washed in sterile PBS, lysed in 70 µl lysis RLT buffer/10^6 cells (Qiagen GmbH, Hilden, Germany), and stored at –80°C.

RNA isolation. RNA was isolated from cultured cells using the RNeasy mini-kit (Qiagen GmbH) with an on-column DNAse digestion step, according to the manufacturer's instructions. Briefly, cells were passed through a Qiashredder (Qiagen GmbH) and the eluted lysate was mixed 1:1 with 70% ethanol. The lysate was applied to a mini-column and after washing and DNAse digestion, the RNA was eluted in 30–50 µl of RNAse-free water. The quantity and quality of RNA was assessed by ethidium bromide staining of RNA separated on 1.2% agarose gels.

cDNA synthesis. First-strand cDNA was synthesized using 2 µg of total RNA, 400 U M-MLV reverse transcriptase, RNase H Minus (Promega, Mannheim, Germany), 2 mM dNTPs (Roth, Germany), and 200 ng random primers (Promega) in a total volume of 40 µl.

Conventional PCR. For conventional PCR, cDNA equivalent to 50 ng total RNA was first denatured (94°C, 4 min) and then amplified in a 35 cycle protocol (94°C/30 s, 60°C/30 s, 72°C/60 s) using SilverStart-DNA-Polymerase (Eurogentech, Liege, Belgium). Buffers and nucleotides were purchased from Roth, Germany, and used in standard concentrations. $MgCl_2$ was added at 1.5 mM.

Primers were selected using the Primer ExpressTM software (Perkin Elmer); forward primer: 5'-AAGCTGGTTGTGATCGCATCA-3'; reverse primer: 5'-CATTCGCCCCACTCTTCAA-3'.

Automated DNA sequencing of PCR products. PCR products were purified

for sequencing using the QIAquick gel extraction method (Qiagen) and sequenced using the ABI Prism® BigDye cycle sequencing kit (Version 2, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's specifications. Electrophoresis was performed on an ABI Prism 310 Genetic Analyzer.

TAQMAN PCR. First-strand synthesis was performed with the First Strand Synthesis Kit (Boehringer) using 1 µg RNA. TAQMAN PCR was used to detect human ADAMTS-1 in human articular cartilage RNA samples. The primers (MWG Biotech, High Point, NC, USA) and the TAQMAN probe (Eurogentech, Seraing, Belgium) were designed using Primer Express software. A separate master mix was made up for each of the primer pairs and contained a final concentration of 200 µM NTP, 450 nM Roxbuffer, and 100 nM TAQMAN probe. For ADAMTS-1 (Accession number NM-006988) the final reaction mix contained, in addition to cDNA and 0.5 U polymerase (Eurogentech), 900 nM forward (GCCAAAGGCATTGGC-TACTTC) and 900 nM reverse (GTGGAATCTGGGCTACATGGA) primers, 100 nM of corresponding probe (CGTTTTGCAGCCCAAG-GTTGTAGATGGT), and 7 mM MgCl$_2$. All experiments were performed in triplicate.

The assay for GAPDH was as described[23].

To obtain quantifiable results for ADAMTS-1 and GAPDH, specific standard curves using sequence-specific control probes were constructed in parallel to the analyses. Thus, gene-specific cDNA fragments were amplified by the gene-specific primers and cloned into pGEM-T Easy (Promega). The cloned amplification product was sequenced for confirmation of correct cloning. Cloned standard probes were amplified using the Qiagen amplification kit, then linearized and used after careful estimation of the concentration [gel electrophoresis, UV photometry, and a fluorometric assay for RNA (Picogreen; Molecular Probes, Eugene, OR, USA)]. Concentrations of 10, 100, 1000, 10,000, 100,000, and 1,000,000 molecules per assay were used for the standard curves (all in triplicate).

RESULTS

Conventional RT-PCR analysis. By conventional PCR, mRNA expression of ADAMTS-1 was clearly detectable in 3 samples of normal and 3 samples of OA cartilage, with a stronger signal detected in the latter (Figure 1A).

TAQMAN assay development. A TAQMAN assay was developed for exact quantification of mRNA expression for ADAMTS-1. The computer-selected primers and probes (Primer Express software) and optimal assay conditions were tested according to the manufacturer's recommendations (Perkin Elmer).

For standardization of gene expression levels as determined by TAQMAN analysis, mRNA ratios relative to GAPDH as housekeeping gene were calculated.

Quantification of gene expression levels: expression of ADAMTS-1 in normal and OA chondrocytes in vivo (Figure 1B). ADAMTS-1 showed low constitutive expression in all samples investigated (0.032–0.047/GAPDH). A slight, but not significant increase in early degenerative and late stage OA samples compared to normal samples was detectable.

Immunohistochemical demonstration of ADAMTS-1 in normal and OA cartilage. All 3 antibodies showed a similar staining pattern in normal and OA cartilage specimens, thus confirming each other. Overall, a cellular staining pattern sparing the nuclei (Figures 2C, 2D, 2G) was observed in the majority of chondrocytes in normal as well as OA cartilage
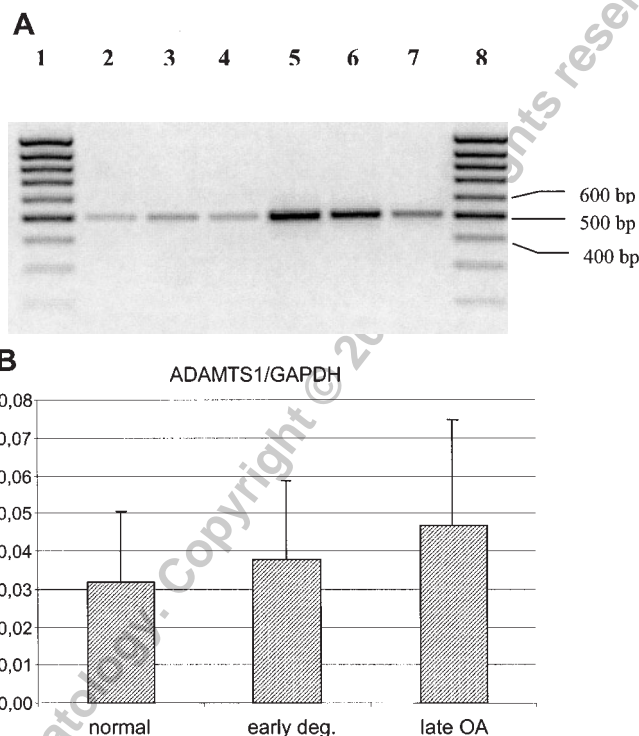
Figure 1. A. ADAMTS-1 mRNA expression in normal (lanes 2–4) and OA (lanes 5–7) articular cartilage by conventional PCR. Lanes 1 and 8: 100 bp DNA-ladder; MBI Fermentas, Germany). B. Quantification by TAQMAN analysis for mRNA expression levels of ADAMTS-1 in normal (n = 13), early degenerative (n = 14), and late stage OA (n = 11) cartilage. Ratios per GAPDH are illustrated.

samples including chondrocyte clusters (Figure 2G), with a significantly lower level of staining seen in the middle and deep zone in normal articular cartilage (p < 0.01; Figures 2B and 3). In OA cartilage the middle zone, which also showed loss of proteoglycan staining (Figure 2E), showed enhanced staining compared to the normal cartilage (Figures 2F and 3), although the immunohistochemical protocol applied cannot be considered fully quantitative. In both normal and OA cartilage samples negative cells were always observed as well (Figure 2C). Scarcely any staining was seen in the calcified zone of the 2 sample groups, presumably because this zone contains a high number of empty lacunae or dead cells[24].

Osteophytic cartilage was strongly positive for ADAMTS-1 in most layers (Figure 2H).

Western blot analysis. In Western blot analysis the specificity of the antibodies used for ADAMTS-1 (Figure 4) was confirmed. It revealed the presence of ADAMTS-1 in normal and OA chondrocytes at the protein level.

Expression of ADAMTS-1 in normal and OA chondrocytes in vitro — modulation by (catabolic) IL-1ß and (anabolic) IGF-I. ADAMTS-1 was significantly more highly expressed by articular chondrocytes *in vitro* than *in vivo* (p < 0.01).

After stimulation with IL-1ß a downregulation of
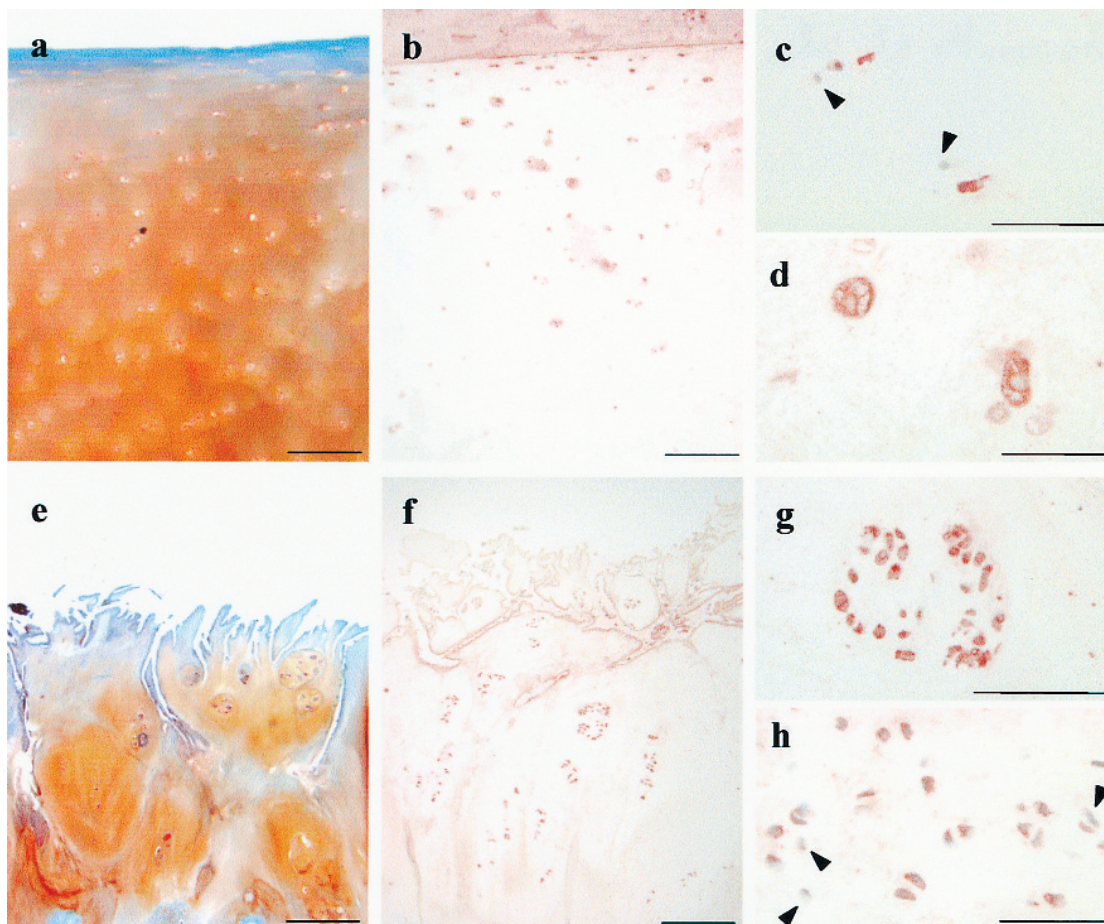
Figure 2. Immunohistochemical expression of ADAMTS-1 (panels B–D, F–H) in normal (B, C) and OA (D, F–H) cartilage using the anti-ADAMTS-1 antibody (A-19; sc-5468). Cellular staining pattern spared the nuclei (C). In both normal and OA cartilage negative cells were observed as well (C). Staining was correlated to the corresponding presence of glycosaminoglycans (A, E: safranin O staining). Chondrocytes in cell clusters (G) and osteophytes (H) showed prominent positive immunohistochemical staining. Arrowheads in C and H indicate cells negative for ADAMTS-1. Magnification bars = 50 µm.

ADAMTS-1 compared to controls was observed ($p < 0.05$; Figure 5). Interestingly, no downregulation was found at 0.1 ng/ml in normal chondrocytes, but downregulation was observed in OA chondrocytes ($p < 0.05$).

IGF-I did not influence the expression of ADAMTS-1 (data not shown).

DISCUSSION

The first significant finding of our study is the (low) constitutive expression and presence of ADAMTS-1, a recently described new member of the ADAMTS family with aggrecanase activity[11], in articular chondrocytes of knee joints. However, there appears to be only a slight increase of ADAMTS-1 mRNA expression levels in OA chondrocytes compared to normal specimens. The only slightly enhanced expression level argues against ADAMTS-1 being the protease centrally responsible for the highly increased aggrecanolytic activity in the diseased tissue, although mRNA levels do not necessarily reflect protein and in particular proteolytic activity, as discussed below.

Moreover, ADAMTS-1 is widely distributed throughout the body and is not restricted to articular cartilage[9,14]. Interestingly, however, our immunostaining showed a more pronounced presence of ADAMTS-1 in the upper zones of normal and the upper and middle zones of OA cartilage, which also show the process of the loss of proteoglycans. Whether this implies an increased activity and thus involvement of ADAMTS-1 in proteoglycan degradation specifically in this zone cannot be confirmed based on our data, and further investigation is required. A somewhat strange finding was that the antibody staining was primarily within the cytoplasm of the cells: whether this represents actual synthesis or storage by the cells remains unclear from our study.

Similarly to other proteases such as MMP-3, MMP-1, and MMP-13[25], ADAMTS-1 showed a highly significant increase of mRNA expression levels *in vitro* compared to the *in vivo* situation, which might be due to derangement of the pericellular environment after cell isolation. However, in contrast to many other proteases including MMP-1, MMP-
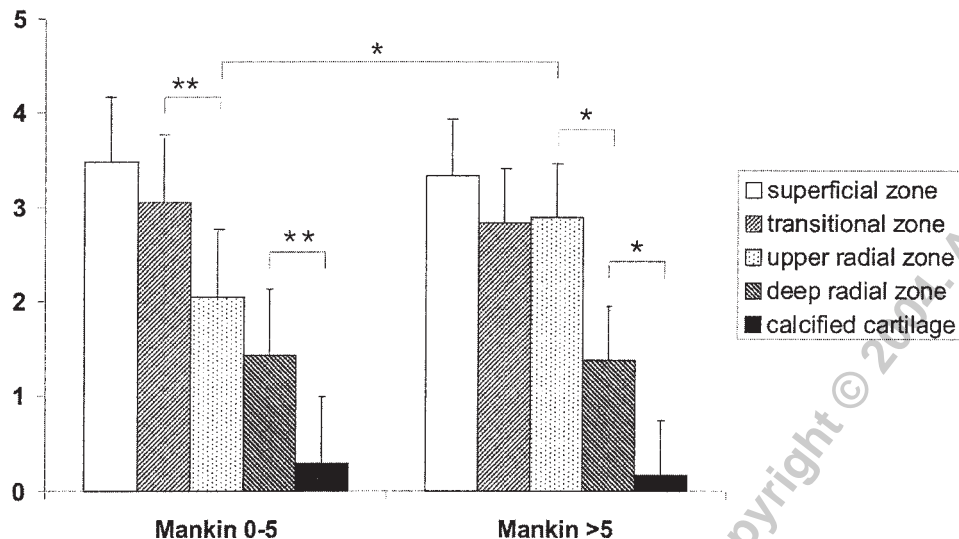
Figure 3. Distribution of ADAMTS-1 staining (y-axis) in the different cartilage zones related to the total Mankin score. A cutoff was set at Mankin grade 5 (up to 5: not or only mildly degenerated cartilage; over 5: severely damaged cartilage). ADAMTS-1 staining in the different zones of articular cartilage was graded on a 5-point scale (according to intensity reported by 2 observers independently. Staining intensities: 0: no staining; 1: least positive staining; 2: weak; 3: moderate; 4: strong; 5: very strong staining). Asterisks indicate statistically different immunohistochemical grades between different layers within one group, and between the upper radial zone of normal and degraded cartilage samples; *p < 0.05, **p < 0.01.
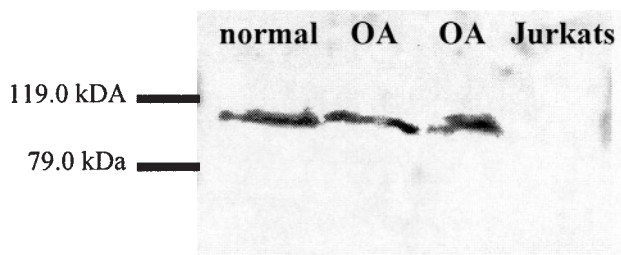

Figure 4. Western blot analysis of cell lysates using normal (lane 1) and OA chondrocytes (lanes 2 and 3), with Jurkat cells as negative control (lane 4). One major band compatible with the estimated molecular weight of ADAMTS-1 of about 100 kDa was detected[34].

3, MMP-13, and ADAMTS-4[25-32], ADAMTS-1 is not upregulated but downregulated by IL-1ß in monolayer cultures (similar results were obtained in alginate cultures; unpublished results). This makes ADAMTS-1 less likely to be involved in IL-1ß mediated cartilage matrix degradation[33].

One obvious limitation of studies such as ours is that protease activity might not be primarily related to mRNA expression levels, but to molecular activation or presence of inhibitors or for other reasons. However, ADAMTS family members are probably constitutively active after secretion from the cells into the extracellular matrix and do not need further processing as MMP. Thus, our findings argue against the concept of ADAMTS-1 being a major aggrecan-degrading protease during the disease process or after IL-1ß stimulation. Interestingly, OA chondrocytes appeared to be more sensitive to low IL-1ß concentrations compared to
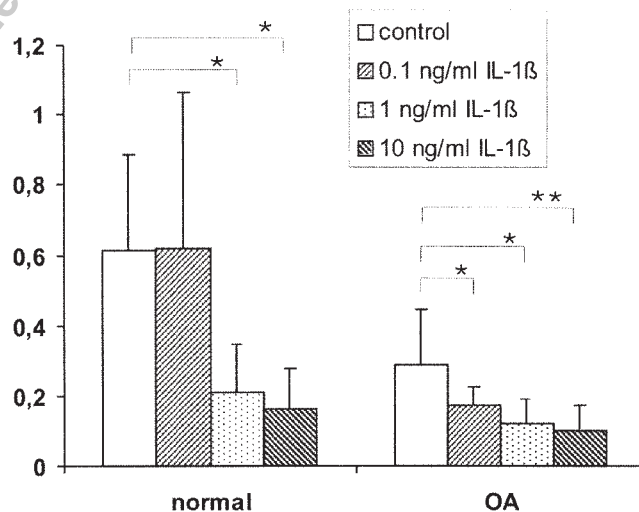

Figure 5. ADAMTS-1/GAPDH ratios in normal and OA chondrocytes determined by quantitative PCR experiments. Monolayer cultures were stimulated with different concentrations of IL-1ß. Asterisks indicate statistically different (p < 0.05) ratios of control group vs respective IL-1ß concentrations (0.1, 1, 10 ng/ml). * p < 0.05, ** p < 0.01.

their normal counterparts. This is presumably of low relevance for the regulation of ADAMTS-1, which gets downregulated, but might be very detrimental for cartilage in respect to other catabolic proteases such as MMP-3 and MMP-13, which are strongly upregulated by IL-1ß *in vitro* and presumably *in vivo*.

Our results confirm the expression and presence of ADAMTS-1 in articular cartilage. They suggest that ADAMTS-1 is constitutively expressed by most adult articular chondrocytes and is not strongly upregulated in osteoarthritis. Of note, ADAMTS-1 is to our knowledge the first matrix-degrading enzyme downregulated by the catabolic factor IL-1ß *in vitro*.

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