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Effects of non-steroidal antiinflammatory drugs and dexamethasone on the activity and expression of matrix metalloproteinase-1, matrix metalloproteinase-3 and tissue inhibitor of metalloproteinases-1 by bovine articular chondrocytes

T. Sadowski* and J. Steinmeyer†

Institute of Pharmacology and Toxicology, University of Bonn, Germany

Summary

Objective: To determine the *in-vitro* effects of several non-steroidal antiinflammatory drugs and the glucocorticoid dexamethasone on the IL-1 altered expression and activity of MMP-1, MMP-3 and TIMP-1 by bovine articular chondrocytes.

Design: Bovine chondrocytes were cultured in alginate gel beads. Cells were treated with IL-1α in the presence of vehicle or drugs at various concentrations. After 48 h mRNA expression of MMP-1, MMP-3, and of the tissue inhibitor of metalloproteinases (TIMP-1) was analysed by RT-PCR-ELISA. The protein synthesis of TIMP-1 and MMP-3 was determined by immunoprecipitation. The activity of enzymes and inhibitors was measured by functional assays.

Results: IL-1 increased the expression and activity of MMPs. In contrast, TIMP activity remained unchanged although TIMP-1 expression was down-regulated. All tested NSAIDs and dexamethasone inhibited collagenase activity induced by IL-1. Transcript levels of MMP-1, however, were only reduced by indomethacin, meloxicam, naproxen and dexamethasone. Proteoglycanase activity was only reduced by indomethacin, meloxicam and dexamethasone. These effects were pre-translational as confirmed by immunoprecipitation. The IL-1 decreased expression of TIMP-1 was further reduced by dexamethasone, which resulted in a significant loss of TIMP activity. No effects on TIMP activity or TIMP-1 biosynthesis were observed after treatment of chondrocytes with NSAIDs.

Conclusion: Our studies clearly demonstrate that marked differences exist between individual NSAIDs with respect to their ability to modulate the imbalance between proteases and inhibitors during OA and RA, suggesting that the respective modes of action are independent of the inhibition of cyclooxygenases. Due to their co-regulation of MMPs and TIMP(s) glucocorticoids should be carefully studied for their overall effect on ECM proteolysis. © 2001 OsteoArthritis Research Society International

Key words: Bovine articular chondrocytes, Matrix metalloproteinases, Tissue inhibitor of metalloproteinases, Interleukin-1, NSAIDs, Glucocorticoids.

Introduction

A common feature of joint diseases like osteoarthritis (OA) and rheumatoid arthritis (RA) is the destruction of articular cartilage. Integrity of a functional matrix, which in the healthy joint serves to distribute loads over bone surfaces and provides a low-friction surface over which bones can move, is impaired by dysregulated production of matrix components and proteolytic enzymes. The matrix metalloproteinases (MMPs) in concert with the recently identified aggrecanases (ADAMTS4 and -11), which are members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family[1,2] are collectively capable of degrading essentially all cartilage extracellular matrix components (ECM). The MMPs, zinc-dependent endopeptidases, comprise an enzyme superfamily of at least 21 members, which can be classified into subgroups of collagenases, stromelysins, gelatinases and membrane-type MMPs. The collagenases (MMP-1, -8, -13) are distinguished from other MMPs by their unique ability to cleave triple helical regions of the major cartilage collagen, i.e. collagen type II. Identification of MMP-specific collagen cleavage products in human RA and OA cartilage has established a significant correlation between collagenase activities and tissue destruction during these diseases[3]. Stromelysin-1 (MMP-3), on the other hand, is involved in the degradation of aggrecan by cleavage at the Asn^{341}-Phe^{342} site resulting in the neoepitope sequence VDIPEN[4]. Although it has been argued that cleavage at the Glu^{373}-Ala^{374} site by aggrecanases is mainly responsible for aggrecan degradation[5], VDIPEN neoepitopes were found in synovial fluid as well as in cartilage of RA and OA patients[6]. Additionally, when experimental RA was induced in MMP-3-deficient mice articular cartilage remained intact whereas in wild-type mice severe erosion was observed[7]. Apart from a pivotal role in cartilage degradation MMP-3 is essential for full activation of proMMP-1[8].

MMP activity is controlled by tissue inhibitors of metalloproteinases (TIMPs) which form inhibitory complexes with

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*Present address: Institute of Biochemistry, University of Kiel, Germany.

†Present address: Orthopaedic Research Laboratories, Department of Orthopaedic Surgery, University of Giessen, Germany.

Address correspondence to: Dr Thorsten Sadowski, Institute of Biochemistry, CAU Kiel, Olshausenstr. 40, D-24098 Kiel, Germany. Tel: +49-431-8802217; Fax: +49-431-8802018; E-mail: thorsten.sadowski@biochem.uni-kiel.de

MMPs in a 1:1 stochiometry. To date four different TIMPs (TIMP-1, -2, -3, -4) are known[9], however, their specific roles are not well understood. TIMP-1 to -3 were found to be expressed by chondrocytes[10,11], and therefore are possibly essential for maintaining an enzyme-inhibitor balance required for physiological ECM turnover. Pathologic proteolysis of cartilage components during OA and RA on the other hand is believed to be the result of an excess of MMPs in comparison to TIMPs[12].

Cytokines from synovial membrane like interleukin-1 (IL-1) can markedly alter the metabolic activity of chondrocytes. Elevated levels of IL-1 have been found in synovial fluid from patients suffering OA or RA[13]. IL-1 has been found to contribute to the disease process by stimulating the biosynthesis of proteolytic enzymes[14], and also by inhibiting the production of extracellular matrix constituents such as collagen and PGs from articular cartilage[15,16]. The resulting imbalance between the anabolic and catabolic activities leads to the severe degradation of articular cartilage observed in OA.

Non-steroidal antiinflammatory drugs (NSAIDs) and glucocorticoids belong to the most frequently used drugs in OA and RA therapy. Despite their therapeutic efficacy in modulating pain and inflammation, their influence on cartilage metabolism is still under discussion. For instance, some NSAIDs have been shown to influence the PG and collagen synthesis rates in articular cartilage[17–19]; however, few studies focused on the extent to which various NSAIDs can modulate the proteolytic activities in chondrocytes[20,21]. Suppression of MMP synthesis by glucocorticoids has been reported[22,23], but little is known about the effects of steroids on TIMPs. Here, we report that, first, the NSAIDs acetylsalicylic acid (ASA), diclofenac-Na, indomethacin, meloxicam and naproxen profoundly differ in their ability to modulate proteolytic activities by articular chondrocytes; second, MMP-1 and MMP-3 differ in their responses to NSAIDs, and finally, dexamethasone simultaneously suppresses production of TIMP-1 and MMPs by articular chondrocytes.

Materials and methods

MATERIALS

Pronase®, protein G PLUS/protein A agarose and anti-TIMP-1 antibody were purchased from Calbiochem (Bad Soden, Germany), ascorbate, penicillin, streptomycin, L-glutamine, alpha-ketoglutarate, 6-aminocaproic acid, N-ethylmaleimide, 4-aminophenylmercuric acetate (APMA), phenylmethylsulfonyl fluoride (PMSF), orthophenylenediamine (OPD), bovine type I collagen, antigoat IgG peroxidase conjugate, mouse IgG, and rabbit IgG were obtained from Sigma (Deisenhofen, Germany). Streptavidin-peroxidase conjugate, 72 kDa gelatinase, low range protein molecular weight marker, DIG labeling mix, biotin labeling mix and PCR ELISA were from Boehringer (Mannheim, Germany), alginate from Kelco (London, U.K.), and collagenase CLS-2 as well as trypsin were purchased from Worthington (Freehold, U.S.A.). Ham's F12, fetal bovine serum (FBS), gentamycin, and Trizol® were obtained from Life Technologies (Eggenstein, Germany), human recombinant IL-1α from R&D Systems (Wiesbaden, Germany), and soybean trypsin inhibitor from Fluka Chemie (Deisenhofen, Germany). Goat antitype I collagen antibody was ordered from Southern Biotechnology Association (Birmingham, U.K.), and M-MLV reverse transcriptase as well as Taq polymerase from Promega (Mannheim, Germany). CR-ITS+™ was purchased from Collaborative Research (Bedford, U.K.), anti-MMP-3 antibody from Chemicon International (Hofheim, Germany), gelatine from Bio-Rad (Richmond, U.S.A.) and Easy Taq™ express protein labeling mix [^{35}S] from NEN (Dreieich, Germany). Drugs were generous gifts from the following pharmaceutical companies listed in parentheses: ASA (Bayer AG), diclofenac-Na (Ciba Geigy AG), indomethacin (Merck KG), meloxicam (Boehringer Ingelheim), naproxen (SmithKline Beecham GmbH), dexamethasone (Merck KG).

CULTURE OF CHONDROCYTES

Chondrocytes were isolated from macroscopically healthy metacarpophalangeal joints of 18–24-month-old steers and subsequently encapsulated in alginate beads. Cells were released from the matrix by initial digestion with pronase (0.8% w/v) for 1 h at 37°C followed by collagenase (0.5% w/v) digestion for 4 h at 37°C. Viability of the harvested cells, as assessed by the trypan blue exclusion test, was always greater than 95%. Chondrocytes were suspended in sterile alginate solution (1.2% w/v) to a final concentration of 3×10^6 cells/ml alginate. The preparation of alginate beads was done as described earlier[24]. Twenty beads (approx. 6×10^5 cells) per well were transferred to a 12-well culture dish containing 2.5 ml per well growth medium (Ham's F12 containing 10% (v/v) FBS, 25 mM HEPES, 0.3 mg/ml L-glutamine, 0.03 mg/ml alpha-ketoglutarate, 0.05 mg/ml ascorbic acid, 10 U/ml penicillin and 0.1 mg/ml streptomycine). Media were changed every second day. Cultures were maintained for 8 days at 37°C, 5% CO_2 and 95% humidity. At day 4 serum was reduced to 5% (v/v) and 0.5% (v/v) of the serum substitute CR-ITS+™ was added to the media. During the final 48 h, culture medium containing 1% (v/v) CR-ITS+™ instead of FBS[25], and IL-1α at a final concentration of 0.5 ng/ml were given to the cells either alone or together with NSAIDs (1 μM to 50 μM) or dexamethasone (0.1 μM to 50 μM) or the drug vehicle, respectively. After the incubation period media were collected and stored frozen at −20°C. Cells were harvested by solubilization of the alginate beads in citrate buffer (55 mM sodium citrate and 150 mM NaCl, pH 7.2, 1 ml per 20 beads) with subsequent centrifugation. The alginate supernatant and the chondrocytes were stored frozen at −20°C until analysed.

DETERMINATION OF TIMP ACTIVITY

The activity of TIMP(s) was quantitated by its ability to inhibit the 72kDa gelatinase (MMP-2) in a solid-phase assay employing biotin labeled gelatin adsorbed onto microtiter plate wells as substrate[26]. Briefly, gelatin was biotinylated by the use of a biotin labeling kit (Boehringer) according to the manufacturer's instructions. Biotin-labeled gelatin was diluted in carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) to a final concentration of 25 ng/ml. The wells of a 96-well microtiter plate were coated with 50 μl of this solution and unbound gelatin was removed by repeated washing with PBS (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 137 mM NaCl and 2.7 KCl, pH 7.2). Wells were then blocked with unlabeled gelatin, and the plates were stored at 4°C until used. MMP-2 was activated by addition of 1 mM APMA and then diluted in Tris buffer (50 mM Tris, 5 mM $CaCl_2$ and 0.05% Triton X-100, pH 7.5) to a final

concentration of 250 mU/ml. MMP-2 was then incubated with or without conditioned media and solubilized alginate samples. This mixture was subsequently added in duplicate to the wells of the prepared microtiter plate. After 75 min incubation at 37°C, the enzyme reaction was stopped by repeated washing with PBS-Tween (PBS with 0.05% Tween 20). The remaining amount of undigested gelatin was measured with a streptavidin–peroxidase conjugate. Conversion of the peroxidase substrate OPD was measured in an ELISA photometer at 492 nm. One unit of inhibitor activity was defined as 1% inhibition of gelatinolytic activity. A standard curve of MMP-2 was included in each experiment to reduce variability due to coating differences between individual microtiter plates.

DETERMINATION OF MMP ACTIVITY

Media samples were assayed for collagenolytic activity by their ability to degrade collagen type I coated on microtiter plate wells as previously described[27]. Briefly, proenzymes were activated by treatment with trypsin (0.1 mg/ml) for 20 min at 37°C. Soybean trypsin inhibitor was subsequently added to stop the reaction (0.5 mg/ml for 10 min at 37°C). The samples were then added in duplicate to the wells of microtiter plates coated with bovine type I collagen. After 2 h of incubation at 37°C the reaction was stopped by addition of EDTA (25 mM). The remaining amount of undigested collagen was detected with a goat antitype I collagen antibody, followed by the addition of a peroxidase-conjugated antigoat IgG antibody. Conversion of OPD was measured in an ELISA photometer at 492 nm. One unit of MMP collagenolytic activity was defined as the digestion of 1% collagen as compared with the undigested control. A linear relationship between the amount of enzyme and substrate digestion was confirmed in preliminary experiments (data not shown).

The MMP proteoglycanolytic activity was determined as previously described[28]. Briefly, MMPs in the media were activated by addition of 1 mM APMA for 6 h at 37°C. The samples were then diluted in a reaction mixture containing 60 mM Tris, 5 mM $CaCl_2$, 80 mM NaCl and 0.02% Brij-35®, pH 7.5 and proteinase inhibitors (100 mM 6-aminocaproic acid, 2 mM PMSF, 10 mM N-ethylmaleinimide). The mixture was added to a glass scintillation vial containing ~2 mg [^{3}H]proteoglycan (PG) monomers entrapped in polyacrylamid gel beads. PG monomers were purified from bovine nasal septum cartilage and labeled with [^{3}H]acetic anhydride. The radiolabeled PG monomers were entrapped in polyacrylamide gel beads with a pore size that retains the monomers but permits the escape of digestion products smaller than ~200 000 Da[29]. After incubation for 16 h at 37°C in a rocking water bath, 6 ml scintillation cocktail was added and the radioactivity was determined by using a liquid scintillation counter. One unit of MMP proteoglycanolytic activity was defined as one count per minute of tritium released per mg PG beads. A linear relationship between the amount of enzyme and substrate digestion was confirmed in preliminary experiments (data not shown). Control experiments, performed in the presence of EDTA or 1,10-phenanthroline, revealed that both assays measured only MMP-dependent proteolytic activities. No activity could be detected in solubilized alginate fractions, possibly because of the Ca^{2+}-binding properties of the citrate buffer used for dissolving the alginate beads.

RNA ISOLATION AND REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT–PCR)

Total RNA was isolated from chondrocytes using Trizol® according to the manufacturer's instructions. For the generation of single stranded cDNA, 0.25 μg RNA was incubated with 50 pmol oligo dT primer and 200 units M-MLV reverse transcriptase for 90 min at 42°C with a final denaturation step at 95°C for 15 min. The cDNA was diluted in sterile water and then stored at −20°C. The following primer pairs were chosen from the published sequences and used for amplification[30–34].

TIMP-1: 5′-ATGGCCTCTGGCATCCTGTTG-3′, 5′-AAAG GTGGGAGTGGAAACACG-3′, MMP-1: 5′-AACTCTGGA GCAATGTCACAC-3′, 5′-CCTCATAATCAGCTTGAAGTC-3′, MMP-3: 5′-GAAATGCA GAAGTTCCTTGG-3′, 5′-GTG AAAGAGACCCAGGGAGTG-3′, GAPDH: 5′-GAGATGAT GACCCTTTTGG-3′, 5′-GTGAAGGTCGGAGTCAACG-3′.

Depending on the cDNA to be amplified, PCR was performed for 22–28 cycles (denaturing at 95°C for 45 sec, annealing for 60 sec, extension at 72°C for 120 sec) to keep the reaction in the exponential range of amplification and thus obtain semiquantitative results (data not shown). PCR products were labeled with digoxigenin (DIG) by addition of a DIG labeling mix to the reaction mixture. This mixture further contained 50 mM KCl, 10 mM Tris, 0.1% Triton® X-100, 1.5 mM MgCl2, 20 pmol primer, 1 unit Taq polymerase and 2.5 μl cDNA, respectively, negative control in a total volume of 25 μl. PCR products were commercially sequenced (MWG Biotech, Ebersberg, Germany), which revealed their identity to the predicted sequences (28–35).

PCR-ELISA

DIG-labeled PCR products were quantitated by the use of a PCR ELISA kit according to the manufacturer's instructions. Briefly, DIG-labeled PCR products were immobilized to streptavidin-coated microtiter plate wells by hybridization to a biotinylated oligonucleotide that was complementary to a ~20 bp spanning sequence in the inner part of the DIG-labeled PCR product. The bound hybrids were detected by an antidigoxigenin peroxidase conjugate and by the use of the colorimetric substrate ABTS®. Absorbance was read in an ELISA photometer at 405 nm and was taken as a measure for the amount of amplified product. Expression of GAPDH was taken as internal control.

TIMP-1 AND MMP-3 IMMUNOPRECIPITATION

TIMP-1 and MMP-3 protein synthesis was analysed by metabolic labeling of chondrocytes followed by immunoprecipitation. Cells were radiolabeled with 50 μCi/ml [^{35}S]methionine/cysteine during the final 18 h of the incubation period with IL-1 and drugs. Media and alginate samples were pooled and pre-cleared by incubation with non-immunne mouse IgG (TIMP-1) or non-immune rabbit IgG (MMP-3) and protein G plus /protein A agarose suspension for 1 h. Mouse anti-TIMP-1 antibody (0.5 μg/ml), which detected free TIMP-1 as well as TIMP-1 complexed with MMPs, or rabbit anti-MMP-3 antibody (2 μg/ml), which detected the active and latent form of the enzyme, was added to the samples. After overnight incubation at 4°C, immune complexes were precipitated by the addition of a

Table I
Activity and expression of MMPs and TIMP(s) after treatment of chondrocytes with 0.5 ng/ml IL-1α for 48 h

	Control	IL-1α
Activity (U/10^6 cells)		
Collagenase	86.2±102.4	1054.9±675.9[c]
Proteoglycanase	392.7±526.1	2935.6±1261.2[c]
TIMP	933.7±473.4	928.3±412.0
mRNA expression (arbitrary units)		
MMP-1	1.2±2.2	6.9±5.4[b]
MMP-3	1.7±1.5	8.1±5.5[b]
TIMP-1	14.7±10.5	9.1±6.7[a]
Protein synthesis (cpm/10^6 cells)		
MMP-3	1.1±3.8	51.3±25.8[c]
TIMP-1	131.5±35.4	65.1±26.8[c]

Experiments were performed between culture days 6 and 8 in the presence of the serum substitute CR-ITS+™. MMP activity was assayed in conditioned media, whereas the activity of TIMP was determined in conditioned media as well as in solubilized alginate samples. The definition of units is described under Materials and methods. mRNA expression was determined by quantitating PCR products with a PCR-ELISA. Protein expression was quantitated by immunoprecipitation. Values are mean±S.D. (N=6). Groups of data were analysed using Student's one-tailed paired t-test. Statistical significance different from untreated control values: [a]$0.01<P\leq0.05$, [b]$0.001<P\leq0.01$, [c]$P\leq0.001$.

protein G plus /protein A agarose suspension. Precipitates were washed three times in Tris buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100, pH 7.4), boiled for 5 min in Laemmli buffer and were then separated on 10% SDS-polyacrylamide gels under reducing conditions. Gels were stained with Coomassie Brilliant Blue R 250 for visualization of molecular weight marker proteins, fixed and dried. The radioactivity of the single protein bands at 27kDa (TIMP-1) and 58kDa (proMMP-3) was measured with the Automatic TLC-Linear Analyser LB284/LB285 (Berthold, Wildbad, Germany). Peaks were analysed with the Chroma 1D software (Berthold).

For the determination of the synthesis of total protein, cells were first released from alginate beads by solubilization in citrate buffer and then resuspended in lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton® X-100, 2 mM PMSF, pH 7.4). After centrifugation, proteins in the supernatant were precipitated by the addition of 0.5 M perchloroacetic acid (PCA). Precipitates were applied to Whatman GF/C-filters and extensively washed with 0.25 M PCA. Filters were dried and the filter-associated radioactivity was quantitated by liquid scintillation counting.

STATISTICAL ANALYSIS

Data were collected from four to six independent experiments. Samples were determined in duplicate and groups of data were analysed using Student's one-tailed paired t-test. Significance was set $P\leq0.05$. Data are presented as mean±standard deviation (S.D.).

Results

EFFECTS OF IL-1 ON THE PRODUCTION OF PROTEASES AND INHIBITORS

Initially, we stimulated chondrocytes with various concentrations of IL-1 (data not shown). The concentration of the cytokine (0.5 ng/ml) that was used in our study caused submaximal effects on the expression and activity of enzymes and inhibitors (Table I). Unstimulated chondrocytes produced detectable but low levels of enzymes. Incubation with IL-1 for 48 h led to a substantiated rise of metalloproteolytic activities with a concomittant increase of MMP-1 and MMP-3 expression. No effects were observed on TIMP activities in the culture supernatants, although mRNA expression and protein synthesis of TIMP-1 was significantly reduced by cytokine action. These data indicate that treating chondrocytes with IL-1 induced an excess of MMPs in comparison with TIMP(s).

Table II(a)
Effects of NSAIDs and dexamethasone on collagenase activity and mRNA expression of MMP-1

Condition	Collagenase activity	MMP-1 mRNA expression
IL-1	100	100
+ASA	65.93±17.2[b]	101.02±35.9
+diclofenac-Na	56.37±16.5[c]	80.74±38.3
+indomethacin	68.22±16.9[b]	42.19±33.1[a]
+meloxicam	40.15±8.0[c]	62.06±20.9[b]
+naproxen	90.01±25.4	59.40±27.9[c]
+dexamethasone	6.96±17.0[c]	21.84±29.6[b]

Experiments were performed between culture days 6 and 8 in the presence of the serum substitute CR-ITS+™. Drugs were given to chondrocytes to a final concentration of 10 μM. MMPs in media samples were activated with trypsin and analysed for enzymatic activities against collagen type I. MMP-1 mRNA expression was determined by RT-PCR-ELISA. Values are expressed in mean±S.D. (N=4–6). Data were analysed using Student's one-tailed paired t-test. Statistical significance different from control values (IL-1+vehicle=100%): [a]$0.01<P\leq0.05$, [b]$0.001<P\leq0.01$, [c]$P\leq0.001$.

DRUG EFFECTS ON COLLAGENASE ACTIVITY AND mRNA EXPRESSION OF MMP-1

In a first set of experiments we incubated chondrocytes with IL-1 and drugs at a concentration of 10 μM and, after 48 h, determined collagenase activity and transcript levels of MMP-1. This primary screen revealed that all tested NSAIDs and dexamethasone significantly reduced enzyme activity and/or mRNA expression of MMP-1 [Table II(a)]. Drugs were then tested at additional concentrations to obtain pharmacokinetic data. In these studies a clear concentration-dependent reduction of collagenase activity and transcript levels of MMP-1 was observed for indomethacin, meloxicam and naproxen. A 50% inhibition of enzyme activity (IC_{50}) and MMP-1 transcript level was achieved by meloxicam at calculated IC_{50} values of 7.3 μM and 25.1 μM, respectively. However, we did not reach a 50% inhibition even at an exceedingly high concentration of 50 μM of indomethacin and naproxen. On the other hand, after treating chondrocytes with ASA and diclofenac-Na, enzyme activities were lowered by 40% and 60%, respectively, at all concentrations tested. Surprisingly, these drugs did not alter the steady-state level of MMP-1 mRNA, indicating that their inhibitory action might be exerted post-transcriptionally. Finally, the IL-1 increased collagenase activity and MMP-1 expression was completely reversed by dexamethasone at all concentrations tested [Table II(b)].

Table II(b)
Collagenolytic activity and mRNA expression of MMP-1 after treatment of chondrocytes with 0.5 ng/ml IL-1 and various concentrations of NSAIDs and dexamethasone for 48 h

	Collagenolytic activity	mRNA expression
ASA		
50 μM	53.91±10.4[c]	115.86±28.9
5 μM	59.15±18.8[b]	103.28±35.2
1 μM	58.89±17.6[a]	107.94±34.7
Dexamethasone		
50 μM	15.98±13.3[c]	7.37±3.9[c]
1 μM	9.30±8.4[c]	6.48±4.1[c]
0.1 μM	3.67±8.2[c]	6.38±1.9[c]
Diclofenac-Na		
50 μM	37.75±34.8[c]	93.81±24.4
5 μM	33.15±15.2[c]	118.39±35.4
1 μM	36.54±21.1[c]	105.81±26.4
Indomethacin		
50 μM	60.76±15.2[b]	55.14±24.5[b]
5 μM	79.84±26.7[a]	90.10±27.7
1 μM	80.89±33.1	88.20±23.4
Naproxen		
50 μM	51.35±15.1[c]	76.79±17.7[a]
5 μM	66.54±21.9[b]	99.22±32.2
1 μM	89.27±21.5	94.94±27.2
Meloxicam		
50 μM	31.63±19.9[c]	45.23±18.4[c]
5 μM	63.75±20.1[b]	105.31±35.2
1 μM	108.77±26.2	108.17±23.4

Experiments were performed between culture days 6 and 8 in the presence of the serum substitute CR-ITS+®. Enzyme activities were determined in media samples. Total RNA was isolated and analysed by RT-PCR-ELISA. All analyses were performed as described under Materials and methods. Values are mean±S.D. (N=4–6). Statistical significance different from control values (IL-1+vehicle=100%): [a]$0.01<P\leq0.05$, [b]$0.001<P\leq0.01$, [c]$P\leq0.001$.

DRUG EFFECTS ON PROTEOGLYCANASE ACTIVITY AND THE BIOSYNTHESIS OF MMP-3

Again, drugs were initially given to chondrocytes at a concentration of 10 μM. In contrast to the results obtained on the inhibition of collagenase activity only meloxicam and dexamethasone could reduce the IL-1 stimulated proteoglycanase activity [Table III(a)]. However, indomethacin significantly lowered the transcript level and protein synthesis of MMP-3 and was therefore tested at additional concentrations. These experiments revealed that at concentrations below 10 μM indomethacin failed to reduce the expression of MMP-3 whereas with the maximum concentration tested (50 μM) an inhibition of enzyme activity by ~45% could be achieved. Similar results were obtained for meloxicam, although we still observed significantly reduced proteoglycanase activity at a drug concentration of 5 μM. In comparison, dexamethasone completely abolished the IL-1 induction of enzyme activity and expression of MMP-3 even at the lowest concentration tested [Table III(b)].

DRUG EFFECTS ON TIMP ACTIVITY AND THE BIOSYNTHESIS OF TIMP-1

TIMP(s) plays a pivotal role in the control of metalloproteolytic activites. An increase or decrease of the production of inhibitor(s) would ultimately lead to an altered net proteolysis of cartilage components. Therefore, we examined the influence of NSAIDs and dexamethasone on TIMP activity and the biosynthesis of TIMP-1. Drugs were initially tested at 10 μM in the presence of IL-1. Whereas none of the tested NSAIDs reversed the IL-1 decreased expression of TIMP-1, Table IV demonstrates that TIMP activity and expression of TIMP-1 were further lowered in the presence of dexamethasone. Further experiments with additional concentrations revealed that inhibition was concentration-independent. Protein and mRNA expression of TIMP-1 was always diminished by ~60% and ~40%, respectively. Meloxicam reduced TIMP activity at concentrations of 10 μM and higher, although this drug did not alter the production of TIMP-1 (Table IV).

DRUG EFFECTS ON THE VIABILITY AND PROTEIN SYNTHESIS OF IL-1α TREATED ARTICULAR CHONDROCYTES

The viability of chondrocytes after treatment with IL-1 in the absence or presence of NSAIDs and dexamethasone was assessed by the trypan blue exclusion test. Neither the cytokine nor the drugs significantly altered the untreated control values which were always above 90% viable cells, indicating that these drugs have no cytotoxic effects on chondrocytes. Furthermore, the addition of NSAIDs to IL-1 treated chondrocytes did not alter the synthesis of total

Table III(a)
Effects of NSAIDs and dexamethasone on proteoglycanase activity and biosynthesis of MMP-3

Condition	Enzyme activity	MMP-3 protein synthesis	MMP-3 mRNA expression
IL-1	100	100	100
+ASA	115.75±53.6	93.78±8.6	98.33±39.1
+diclofenac-Na	90.77±21.5	127.95±32.7	77.52±23.9
+indomethacin	93.49±12.9	77.98±16.4[a]	52.76±34.2[b]
+meloxicam	61.82±26.1[b]	59.94±17.5[b]	71.37±19.0[a]
+naproxen	101.81±29.3	104.01±12.4	96.47±68.1
+dexamethasone	19.87±17.2[c]	6.71±7.4[c]	15.19±18.7[c]

Experiments were performed between culture days 6 and 8 in the presence of the serum substitute CR-ITS+®. Drugs were given to chondrocytes to a final concentration of 10 μM. MMPs in media samples were activated with APMA and analysed for enzymatic activities against PG monomers. MMP-3 mRNA and protein expression was determined by RT-PCR-ELISA and immunoprecipitation, respectively. Values are expressed in mean±S.D. (N=4–6). Data were analysed using Student's one-tailed paired t-test. Statistical significance different from control values (IL-1+vehicle=100%): [a]$0.01<P\leq0.05$, [b]$0.001<P\leq0.01$, [c]$P\leq0.001$.

Table III(b)
Proteoglycanolytic activity and biosynthesis of MMP-3 after treatment of chondrocytes with 0.5 ng/ml IL-1 and various concentrations of NSAIDs and dexamethasone for 48 h

Condition	Enzyme activity	MMP-3 protein synthesis	MMP-3 mRNA expression
Dexamethasone			
50 μM	17.48±19.5[c]	16.08±19.2[c]	26.28±31.9[b]
1 μM	8.76±17.8[c]	22.67±27.4[c]	25.64±16.6[c]
0.1 μM	14.06±19.6[c]	14.16±16.2[c]	14.58±25.0[b]
Indomethacin			
50 μM	55.45±20.8[b]	56.81±20.0[b]	53.92±10.2[c]
5 μM	77.49±34.2	84.86±31.9	116.72±7.3
1 μM	73.77±28.1	86.13±27.4	70.43±49.4
Meloxicam			
50 μM	60.71±29.0[a]	77.74±16.2[a]	31.93±14.4[b]
5 μM	83.65±32.1[b]	115.31±41.6	n.d.
1 μM	86.12±22.7	97.00±52.9	76.00±37.9

Experiments were performed between culture days 6 and 8 in the presence of the serum substitute CR-ITS+™. Enzyme activities were determined in media samples. Total RNA was isolated and analysed by RT-PCR-ELISA. Protein synthesis of MMP-3 was quantitated by immunoprecipitation. All analyses were performed as described under Materials and methods. Values are mean±S.D. (N=4–6). Statistical significance different from control values (IL-1+vehicle=100%): [b]$0.001<P\leq0.01$, [c]$P\leq0.001$.
n.d.: not determined.

Table IV
TIMP activity and biosynthesis of TIMP-1 after treatment of chondrocytes with 0.5 ng/ml IL-1 and dexamethasone or NSAIDs for 48 h

	Activity	Protein synthesis	mRNA expression
Dexamethasone			
50 μM	39.01±31.0[b]	32.64±30.9[b]	60.62±15.24[c]
10 μM	60.93±30.3[a]	25.19±11.2[c]	55.20±26.5[b]
1 μM	42.86±33.4[a]	34.55±17.7[c]	68.46±19.0[b]
0.1 μM	39.46±19.1[c]	30.63±20.7[c]	72.01±21.9[a]
Meloxicam			
50 μM	77.90±15.4[a]	108.39±42.4	81.53±24.9
10 μM	80.86±21.9[a]	131.63±45.8	96.31±20.6
5 μM	82.58±68.4	125.34±55.3	84.26±42.2
1 μM	83.33±45.4	112.60±23.4	97.09±33.7
ASA	104.14±17.3	72.12±36.4	78.92±13.3[b]
Diclofenac-Na	113.65±30.1	101.33±24.3	88.74±24.8
Indomethacin	127.32±33.8	89.15±24.1	99.85±25.8
Naproxen	92.45±29.3	139.76±92.0	94.02±32.4

Experiments were performed between culture days 6 and 8 in the presence of the serum substitute CR-ITS+™. Media and solubilized alginate samples were harvested and analysed for TIMP acitivity and TIMP-1 protein. For determination of the mRNA expression, total RNA was isolated and transcript levels were quantitated by RT-PCR-ELISA. All analyses were performed as described under Materials and methods. NSAIDs were tested at a concentration of 10 μM. Values are mean±S.D. (N=4–6). Statistical significance different from control values (IL-1+vehicle=100%): [a]$0.01<P\leq0.05$, [b]$0.001<P\leq0.01$, [c]$P\leq0.001$.

protein and expression of GAPDH, whereas treatment of chondrocytes with dexamethasone resulted in reduced levels of protein synthesis with dose-dependent inhibition ranging from 40% to 13% (data not shown).

Discussion

Glucocorticoids, and especially NSAIDs, belong to the most commonly used drugs for OA and RA treatment. The therapeutic efficacy of NSAIDs in modulating pain and inflammation is generally believed to be the result of prostaglandin inhibition. However, many NSAIDs have been reported also to influence cartilage metabolism to varying degrees. For instance, with regard to their *in vitro* action on glycosaminoglycan synthesis, NSAIDs could be divided into types with stimulatory, inhibitory and neutral properties[35,36]. Further identification of differences between currently used antirheumatic drugs could thus help to choose an appropriate pharmacotherapy of OA and RA. The goal of our study was therefore to compare the effects of various NSAIDs and dexamethasone on the regulation of catabolic activity by articular chondrocytes.

Our data indicate that all tested NSAIDs significantly reduced the IL-1 stimulated collagenolytic acitivity. In the case of indomethacin, meloxicam and naproxen this was achieved by down-regulating the gene expression of MMP-1, whereas reduction of enzyme activity by ASA and diclofenac-Na appears to be not mediated via reduced

expression of MMP-1. As enzyme activity was assayed in media samples with drugs still present, inhibition by the latter drugs might have occurred by direct interference with collagenase activity. Alternatively, ASA and diclofenac-Na could have also reduced expression of MMP-8 and MMP-13 and thereby diminished total collagen degrading activity. Differences between the three mammalian collagenases with respect to pharmacologic inhibition has recently been demonstrated for tetracyclines[37], and could also exist in the case of other drugs. Futhermore, the failure of ASA and diclofenac-Na to regulate expression of MMP-1 makes it unlikely that inhibition by indomethacin, meloxicam and naproxen depends solely on suppression of prostaglandin synthesis, although the prostaglandin PGE_2 is increased by IL-1 and PGE_2 is known to regulate MMP-1 production[38,39]. These findings therefore rather suggest that each NSAID may have unique attributes with respect to inhibition of MMP-1, which corroborates earlier studies in which no correlation between inhibition of PGE_2 and collagenase activity has been observed[18].

The idea that marked differences exist between individual NSAIDs is further underscored by our evaluation of proteoglycanase activities and expression of MMP-3 after treatment of chondrocytes with these drugs. Thus, when tested at a concentration of 10 μM, only meloxicam and indomethacin inhibited MMP-3 expression. The latter finding is in agreement with a previous study by Yamada *et al*. in which indomethacin reduced MMP-3 production by human chondrocytes[21]. Interestingly, this study also demonstrated that addition of exogenous PGE_2 did not reverse the effect of indomethacin, which confirms our notion that MMP inhibition by this drug is independent of PGE_2 synthesis. Meloxicam, on the other hand, is a novel NSAID for which data on regulation of MMP expression have been lacking so far. Our data show that, albeit at high concentrations, this drug could inhibit production and activity of both MMPs investigated. Furthermore, as a major component of PG degradation measured by our assay was possibly mediated by the recently identified aggrecanases ADMATS4 and -11[1,2], it would be of special interest to determine whether meloxicam can regulate the expression and/or activity of these enzymes. Our data on MMP-3 regulation further indicate that naproxen action seems to be confined to modulating MMP-1 expression, at least at a concentration of 10 μM. This again emphasizes that NSAIDs have specific properties which cannot be explained by a common mode of action. In contrast to the variations observed with NSAIDs, dexamethasone acted on the IL-1 induced expression of both MMPs in an uniform fashion. IC_{50} values, which were well below our tested concentrations, have been reported and are in good agreement with our findings[23].

Comparing synovial fluid concentrations of the tested drugs after therapeutical dosing with their effective concentrations revealed in our experiments[40,41], inhibition of collagenase activity by ASA, diclofenac-Na and naproxen might be of clinical significance. A beneficial effect of ASA on cartilage integrity, though, could be impaired by its inhibitory potential on the collagen synthesis[42]. In contrast, naproxen and diclofenac-Na have been shown to have some chondroprotective effects in two experimental animal models[43,44]. Our data on dexamethasone once more suggest that glucocorticoids, even at low doses, are potent inhibitors of MMP synthesis and might therefore delay or slow the course of cartilage degradation. However, care must be taken as the design of our experiments essentially equals a prophylactic treatment which does not reflect the clinical situation. Thus, we cannot know how the efficacy of drug treatment might be influenced by the disease stage.

Extracellular MMP activity is tightly controlled by members of the TIMP family of specific MMP inhibitors. Thus, enhanced net proteolysis of cartilage components during OA and RA is likely to be the result of an excess of MMPs in comparison with TIMPs. According to our data, IL-1 can induce this imbalance by stimulating MMP production and by down-regulating expression of TIMP-1, which binds to MMP-1 and MMP-3 with high affinity[45]. Increasing the biosynthesis of TIMP-1 could therefore be an alternative pharmacological approach to reduce the articular cartilage matrix destruction during OA nd RA. As chondrocytes also express TIMP-2 and -3[10,11], which probably participate in the inhibition of MMPs, we also determined total TIMP activities in the presence of drugs. It should be mentioned, though, that our assay possibly measures predominantly TIMP-2 which is an especially strong inhibitor of MMP-2[46]. This might also be the reason why TIMP activity remained unchanged in the presence of IL-1 although the biosynthesis of TIMP-1 was markedly reduced.

Despite the rather high concentration of 10 μM none of the tested NSAIDs reversed the inhibitory effect of IL-1 on the expression of TIMP-1 or increased TIMP activity. On the other hand, dexamethasone enhanced down-regulation of protein and mRNA expression of TIMP-1 by IL-1, which resulted in a significant loss of TIMP activity. Of note is the fact that protein synthesis of TIMP-1 was much more strongly inhibited than mRNA expression, which possibly reflected the general suppression of protein synthesis caused by dexamethasone. To our knowledge, this is the first time an inhibitory effect of dexamethasone on TIMP-1 expression and TIMP activity by chondrocytes has been observed. Previous studies have already shown that expression of TIMP-3 by chondrocytes is reduced by dexamethasone[11,47]. Thus, simultaneous suppression of TIMPs and MMPs might question the cartilage-preserving function which has been proposed for glucocorticoids[48].

In conclusion, our studies clearly demonstrate that NSAIDs such as naproxen, diclofenac-Na and ASA act not only against the joint disease symptoms of pain and inflammation but can also interfere with collagenase activity that underlies the destruction of articular cartilage during OA and RA. Furthermore, the marked differences of the tested NSAIDs with respect to their ability to modulate the imbalance between proteases and inhibitors suggests that the respective modes of action are independent of the inhibition of cyclooxygenases. Due to their co-regulation of MMPs and TIMP(s), glucocorticoids should be carefully studied for their overall effect on ECM proteolysis.

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