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Pharmacological Influence of Antirheumatic Drugs on Proteoglycanases from Interleukin-1 Treated Articular Cartilage

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ABSTRACT. The purpose of this study was to examine whether drugs used in the treatment of arthritic disorders possess any inhibitory potential on the proteoglycanolytic activities of matrix metalloproteinases (MMPs), and to determine whether drugs which inhibit these enzymes also modulate the biosynthesis and release of proteoglycans (PGs) from interleukin-1-(IL-1) treated articular cartilage explants. The cartilage-bone marrow extract and the glycosaminoglycan-peptide complex (DAK-16) dose-dependently inhibited MMP proteoglycanases in vitro when tested at concentrations ranging from 0.5 to 55 mg/mL, displaying an IC_{50} value of 31.78 mg/mL and 10.64 mg/mL (1.9×10^{-4} M) respectively. (R,S)-N-[2-[2-(hydroxyamino)-2-oxoethyl]-4-methyl-1-oxopentyl]-L-leucyl-L-phenylalaninamide (U-24522) proved to be a potent inhibitor of MMP proteoglycanases (IC_{50} value 1.8×10^{-9} M). None of the other tested drugs, such as possible chondroprotective drugs, nonsteroidal anti-inflammatory drugs (NSAIDs), disease modifying antirheumatic drugs (DMARDs), glucocorticoids and angiotensin-converting enzyme inhibitors tested at a concentration of 10^{-4} M displayed any significant inhibition. Only U-24522, tested at a concentration ranging from 10^{-4} to 10^{-6} M, significantly inhibited the IL-1-induced augmentation of PG loss from cartilage explants into the nutrient media, whereas DAK-16 and the cartilage-bone marrow extract were ineffective. DAK-16 and the cartilage-bone marrow extract did not modulate the IL-1-mediated reduced biosynthesis and aggregability of PGs by the cartilage explants. The addition of 10^{-5} M U-24522, however, partially maintained the aggregability of PGs *ex vivo*. In our experiments, both possible chondroprotective drugs as well as U-24522 demonstrated no cytotoxic effects on chondrocytes. BIOCHEM PHARMACOL **53**;11:1627–1635, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. matrix metalloproteinase inhibitor; stromelysin; cartilage; antirheumatic drugs; U-24522; interleukin-1

DESTRUCTION of articular cartilage is a common feature in joint diseases such as osteoarthritis and rheumatoid arthritis. Pathophysiologically, a structural breakdown of proteoglycans (PGs) and collagen is observed, which impairs the biomechanical properties of cartilage. The maintenance of a normal, healthy extracellular matrix reflects a balance between the rate of biosynthesis and incorporation of matrix components, and the rate of their degradation and subsequent loss from the cartilage into the synovial fluid. Production and activation of tissue-degrading matrix metalloproteinases (MMPs) are key events in the cartilage breakdown process during chronic inflammatory arthritides and osteoarthritis. The MMPs have been shown to be secreted from chondrocytes in a number of species including humans [1, 2], bovines [3] and rabbits [4]. MMPs are secreted from the cells in a latent form, are activated extracellularly, and are inhibited by tissue inhibitors of metalloproteinases (TIMPs). The balance between the activities of MMPs and TIMPs is thought to be important for the maintenance of an intact cartilage matrix. Under pathological conditions such as osteoarthritis and rheumatoid arthritis, several studies have shown elevated amounts of MMPs, resulting in an imbalance between MMPs and TIMPs that accounts at least in part for the observed cartilage destruction [1, 2, 5–7].

Interleukin-1 (IL-1) seems to be a key mediator by which the chondrocytes and synoviocytes enhance their protease production. This leads to cartilage destruction [8], usually assessed as the loss of sulfated glycosaminoglycans (GAGs) and cleavage of collagen. IL-1, which is present in the joint fluid of patients with arthritic diseases, stimulates chondrocytes to synthesize elevated amounts of enzymes such as stromelysin, fibroblast and neutrophil collagenase as well as

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† *Abbreviations:* PMSF, phenylmethylsulfonyl fluoride; APMA, 4-aminophenylmercuric acetate; DAK 16, glycosaminoglycan-peptide complex; U-24522, (R,S)-N-[2-[2-(hydroxyamino)-2-oxoethyl]-4-methyl-1-oxopentyl]-L-leucyl-L-phenylalaninamide; IL-1, interleukin-1; LDH, lactate dehydrogenase; MMP, matrix metalloproteinase; GAG, glycosaminoglycan; PG, proteoglycan; TIMP, tissue inhibitor of metalloproteinases; NSAID, nonsteroidal anti-inflammatory drugs; DMARD, disease modifying antirheumatic drugs.

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plasminogen activator [9–11], to inhibit synthesis of plasminogen activator inhibitor-1 [12, 13] and TIMP [14], and also to inhibit synthesis of matrix constituents such as collagen and PGs [9, 14]. The imbalance between the levels of inhibitors and enzymes leads to an increase in the amount of active proteases. Combined with suppression of matrix biosynthesis, a severe degradation of cartilage is observed [8, 14].

Nonsteroidal anti-inflammatory drugs (NSAIDs), disease modifying antirheumatic drugs (DMARDs), glucocorticoids and possible chondroprotective drugs are commonly used in the treatment of patients with osteoarthritis or rheumatoid arthritis, but their effects on articular cartilage metabolism and the course of human arthritic diseases remains a subject of debate [15]. For instance, the clinical treatment of osteoarthritis and rheumatoid arthritis with NSAIDs is successful in reducing inflammation and providing symptomatic relief. However, it is believed that some NSAIDs, principally salicylates and indomethacin, accelerate osteoarthritic cartilage destruction by impairing PG synthesis by chondrocytes [15, 16], while others are thought to have a somewhat chondroprotective effect by slowing the disease process or stimulating cartilage repair [15, 17–24]. Most studies, however, have shown that NSAIDs have little or no effect on cartilage [15, 18, 25–30]. Due to the widespread use of these drugs in the treatment of rheumatic disorders, a deeper understanding of their effects on the pathophysiological mechanisms that contribute to cartilage destruction is important.

Given the variety of mechanisms that could be imparted by the range of antirheumatic drugs, the aim of this study was to examine for the first time whether: 1) these drugs possess any inhibitory potential on the activity of MMP proteoglycanases *in vitro*; 2) any of the drugs, which proved to be inhibitors of MMP proteoglycanases, can also prevent IL-1-inducible PG degradation *ex vivo*; and 3) these agents have any impact on the PG biosynthesis and viability of IL-1-treated articular cartilage explants.

MATERIALS AND METHODS

Materials

Ham's F-12 and ascorbate were purchased from Gibco (Eggenstein, Germany). Alpha-ketoglutarate, penicillin, streptomycin, L-glutamine, fluorescein diacetate, propidium iodide, optimized lactate dehydrogenase (LDH) test, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), phenylmethylsulfonyl fluoride (PMSF), benzamidine hydrochloride, N-ethylmaleimide, 4-aminophenylmercuric acetate (APMA) and chondroitin sulfate A from bovine trachea were obtained from Sigma (Heidelberg, Germany). 1,9-dimethylmethylene blue was from Aldrich Chemie (Steinheim, Germany). Sephadex® G-25M (PD10) columns and Sepharose CL-2B were obtained from Pharmacia Biotech (Uppsala, Sweden) and [^{35}S]-SO_4 was purchased from Du Pont de Nemours (Bad Homburg, Germany). CR-ITS+™ Premix was obtained from Collaborative Research (Serva, Heidelberg, Germany), IL-1 from Genzyme (Cambridge, MA, U.S.A.).

The following drugs were generous gifts from the pharmaceutical companies listed in parentheses: acetylsalicylic acid and chloroquine (Bayer AG, Leverkusen, Germany), ademethionine (Degussa Pharma GmbH, Frankfurt, Germany), auranofin and aurothiopolypeptide (SmithKline Beecham Pharma GmbH, Munich, Germany), captopril (Schwarz Pharma AG, Monheim, Germany), cilazapril (Hoffmann–La Roche AG, Grenzach–Wyhlen, Germany), L-cysteine (Fluka, Neu Ulm, Germany), dexamethasone and indomethacin (Merck, Darmstadt, Germany), diclofenac-Na and phenylbutazone (Ciba–Geigy GmbH, Wehr, Germany), D-penicillamine (Asta Medica AG, Frankfurt, Germany), flufenaminic acid (Parke–Davis GmbH, Berlin, Germany), glucosamine sulfate (Opfermann Arzneimittel GmbH, Wiehl, Germany), a glycosaminoglycan-peptide complex with an estimated molecular weight of 55 kDa isolated from Rumalon® (DAK-16) [19, 31, 32] and the lyophilized cartilage-bone marrow extract, which is a high molecular weight glycosaminoglycan-peptide complex (Robapharm, Basel, Switzerland), glycosaminoglycan polysulfate (Luitpold Pharma GmbH, Munich, Germany), ketoprofen (Rhone–Poulenc Pharma GmbH, Cologne, Germany), lisinopril (Zeneca GmbH, Heidelberg, Germany), methotrexate (Medac GmbH, Hamburg, Germany), naproxen (Syntex Arzneimittel GmbH, Aachen, Germany), piroxicam (Pfizer GmbH, Karlsruhe, Germany), sodium pentosan polysulfate (bene-Arzneimittel GmbH, Munich, Germany), and tiaprofenic acid (Roussel Uclaf, Romainville, France). (R,S)-N-[2-[2-(hydroxyamino)-2-oxoethyl]-4-methyl-1-oxopentyl]-L-leucyl-L-phenylalaninamide (U-24522) was kindly prepared and provided by Dr. B. De (The Procter & Gamble Company, Cincinnati, OH, USA). Drugs were made up on the day of experiment in sterile distilled water or in dimethylsulfoxide (methotrexate) or in ethanol 96% (auranofine, flufenaminic acid, indomethacin, ketoprofen, naproxen, phenylbutazone, piroxicam, tiaprofenic acid).

In Vitro *Effects Of Drugs On The Activity Of MMP Proteoglycanases*

Drugs were tested at concentrations ranging from 10^{-6} to 10^{-4} M or in the case of the cartilage-bone marrow extract from 0.55 to 55 mg/mL for their inhibitory potential on the activity of MMP proteoglycanases found in the media of IL-1 alpha-treated bovine articular cartilage explants. The activity of MMP proteoglycanases was determined by the [^{3}H]-proteoglycan-bead assay, as previously described [33, 34]. Briefly, PG monomers were purified from bovine nasal septum cartilage and labeled with [^{3}H]-acetic anhydride. The labeled PG monomers (33000 cpm/mg) were entrapped in polyacrylamide gel beads having a pore size that retains the monomers but permits escape of digestion products smaller than 200 kDa.

To prepare an enzyme solution containing proteoglycanases, articular cartilage from the metacarpophalangeal

joints of 18–24-month-old steers were removed in full thickness under sterile conditions and incubated for 4 days with DMEM containing 100 U/mL IL-1. After 4 days in culture, the medium was collected and combined. MMPs were activated by the addition of 1.0 mM APMA for 6 hr at 37°. The medium was then dialyzed (MW cut off: 2 kDa) at 4° for 48 hr with 3 buffer changes using 100 volumes of sterile buffer containing 50 mM Tris/HCl, pH 7.2, 10 mM $CaCl_2$, 200 mM NaCl, 0.05% (w/v) Brij 35® and 0.02% (w/v) NaN_3. The resulting APMA-free medium was aliquoted, frozen at −70° for a maximum period of 8 weeks and used as an enzyme solution.

For *in vitro* assay, 300 µL enzyme solution, 100 µL buffer containing 100 mM Tris/HCl, pH 7.2, 400 mM NaCl, 20 mM $CaCl_2$, 0.04% (w/v) NaN_3 and 0.1% (w/v) Brij35®, 60 µL of drug solution (or drug vehicle or solution containing 1,10-phenanthroline, or EDTA) and 40 µL of proteinase inhibitor mixture were combined and incubated for 30 min. This mixture contained a final concentration of the following proteinase inhibitors: 100 mM 6-aminocaproic acid to inhibit cathepsin D, 2 mM PMSF to inhibit all serine proteinases, and 10 mM N-ethylmaleimide which inhibits thiol proteases and also prevents disulphide exchanges. The mixture was then added to a 6 mL glass scintillation vial (Packard Instruments, Groningen, Netherlands) containing ~2 mg [^{3}H]-PG monomers comprising polyacrylamide gel beads [33, 34] and incubated for 18 hr at 37° in a rocking water bath. At the end of the incubation period, 5.0 mL scintillation cocktail (Emulsifier 299™, Packard Instruments, Groningen, The Netherlands) was added to the vials and the radioactivity was determined using a liquid scintillation counter (LS 6000 TA, Beckman Instruments, Munich, Germany). Enzyme activity is expressed as counts per minute of tritium released per mg beads. Instead of the enzyme solution, blanks contained the same buffer, which was used to dialyze the media for the removal of APMA. As a negative control, we included in some in vitro assays 10 mM EDTA, 1 mM 1,10-phenanthroline or 0.001 to 100 µM of the hydroxamate U-24522, a broad spectrum MMP inhibitor, in order to inhibit all MMPs present. The variability of the assay lies in the range of ±8%.

Cartilage Explant Cultures

The cartilage-bone marrow extract and DAK-16 were tested for their effects on the synthesis and release of proteoglycans from IL-1-treated bovine articular cartilage explants. Under sterile conditions, five macroscopically healthy articular cartilage explants were removed in full thickness from one condyle of the metacarpophalangeal joints of 18–24-month-old steers. Twenty–thirty mg of cartilage explants were cultured in 3.0 mL of supplemented and serum-free Ham's F-12 nutrient media in the presence of the serum substitute CR-ITS^{+}™ Premix [35]. Cultures were maintained for 10 days at 37°, 5% (v/v) CO_2 and 95% humidity. Media were changed every second day and stored frozen at −20° in the presence of 10% volume of a protease inhibitor mixture (0.1 mM PMSF, 200 mM EDTA, 5 mM benzamidine hydrochloride and 10 mM N-ethylmaleimide). Fresh media, IL-1 and drugs were added to the cartilage explants. One cartilage explant was untreated and received 20 µL of sterile drug vehicle while another was treated with 50 U/mL IL-1 and 20 µL of sterile drug vehicle. The other 3 cartilage explants were each treated with 50 U/mL IL-1 and 20 µL of solution containing the cartilage-bone marrow extract or DAK-16 at a final concentration of 0.55, 5.5 or 55.0 µg/mL. In order to verify whether this cartilage explant system is adequate to test drugs for their inhibitory potential on IL-1-induced MMP proteoglycanases *ex vivo*, explants were also treated with 1, 10 or 100 µM of the MMP inhibitor U-24522.

Measurement of Proteoglycan Synthesis

The effects of the cartilage-bone marrow extract as well as of DAK-16 on the biosynthesis of PGs was determined by radiolabeling cartilage explants with 10 or 20 µCi/mL [^{35}S]-SO_4 for 18 hr on day 10 of the culture period. At the end of the radiolabeling period, media were harvested and stored frozen at −20° in the presence of 10% (v/v) protease inhibitor mixture until analyzed. Cartilage explants were washed three times with Gey's balanced salt solution and frozen at −20° together with 10% (v/v) protease inhibitor mixture until further analysis. Explants were digested for 4 hr at 65° with 1 mL of 0.5 mg/mL papain digestion solution, pH 6.5 containing 50 mM monosodium phosphate, 2 mM N-acetylcysteine and 10 mM EDTA. [^{35}S]-SO_4-labeled PGs within the papain-digested explants and within the media were determined by separation of free [^{35}S]-SO_4 from macromolecular [^{35}S]-SO_4 labeled PGs by size exclusion chromatography on Sephadex® G-25 (PD-10) columns.

Analytical Gel Chromatography

In experiments in which cartilage explants were radiolabeled with 20 µCi/mL [^{35}S]-SO_4 on day 10 of the culture period, explants were frozen, pulverized and extracted on a rocker for 48 hr at 4° with 1 mL of 4 M guanidinium chloride dissolved in extraction buffer containing 50 mM acetate buffer, pH 5.8, 100 mM 6-aminocaproic acid, 5 mM benzamidine/HCL, 10 mM EDTA, 10 mM *N*-ethylmaleimide and 1 mM PMSF [36]. The pulverized explants were then separated from the extracts by centrifugation at 15800 g for 5 min. The extracts were concentrated according to the manufacturer's instructions using Microcon-10® ultrafiltration membranes (molecular weight cut off: 10 kDa, Amicon, Witten, Germany) and diluted to 0.2 mL with the extraction buffer supplemented with 0.1% (v/v) Triton X-100. The PG aggregates were allowed to reassemble for 18 hr at 4°. Samples were then applied to a Sepharose CL-2B column (1.6 cm × 80 cm) and eluted under associative conditions at a flow rate of 4 mL/h using the extraction buffer [36]. Fractions of 1.0 mL were col-

lected and assayed for radioactivity. In order to verify whether the analysis of the aggregability of newly synthesized PGs is applicable to test drugs for their potential to inhibit the activity of MMP proteoglycanases *ex vivo*, the effect of 10 μM U-24522 on this biochemical parameter was identically determined as for the cartilage-bone marrow extract and DAK-16.

Determination of Proteoglycan Content of Cartilage Explant Cultures

Papain-digested cartilage explants and culture media (25 μL portions) were assayed for sulfated GAGs by reaction with 0.25 mL 1,9-dimethylmethylene blue dye solution in polystyrene 96-well plates and spectrophotometry at 523 nm using an ELISA-plate reader. Chondroitin sulfate A from bovine trachea was used as the standard [37]. Appropriate blanks were included to correct for any interference of the drugs with this spectrophotometric assay.

Quantitation of DNA Content

The DNA content of papain-digested cartilage explants was determined fluorometrically using the bisbenzimidazol dye Hoechst 33258 as previously described in detail [38].

Assessment of Chondrocyte Viability

The activity of the cytoplasmatic enzyme LDH (EC 1.1.1.27) in the culture media was measured as a biochemical indicator of cell viability. An optimized LDH test (Sigma, Heidelberg, Germany) was used for the quantitative kinetic determination of LDH activity within the media. In addition, the viability of chondrocytes within the cartilage explants was quantified histochemically using the vital stains fluorescein diacetate and propidium iodide. At the end of the culture period, three cartilage slices, each 50 μm thick, were immediately cut perpendicular to the joint surface from treated and untreated explants. Cartilage slices were placed in a small drop of sterile PBS on a glass slide, and 200 μL Ham's F-12 containing 0.1 mM fluorescein diacetate and 0.3 mM propidium iodide were added and subsequently incubated in the dark at 37° and 95% humidity for 5 min. Chondrocytes were viewed using a 450–490 excitation filter and a 510–520 barrier filter. At least 50 chondrocytes were counted in each of the three chondrocyte layers (superficial, intermediate and deep zone) under a fluorescence microscope. Cells that have an intact plasma membrane, a specific active transport system and active cytoplasmatic esterases can take up fluoresceine diacetate and convert it intracellularly to a fluorescent form. Propidium iodide penetrates only into the cytoplasma of cells with damaged cell membranes where it binds to DNA. Both procedures are standard tests of plasma membrane integrity and cellular viability in several isolated cell lines, including chondrocytes as well as in cartilage explants [39–41].

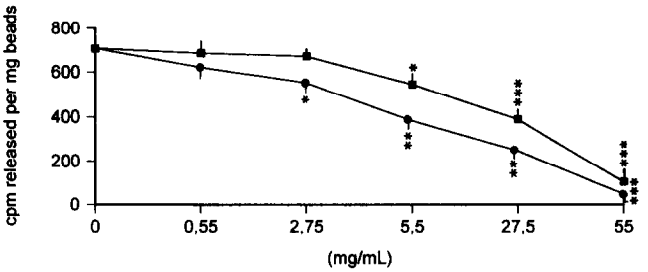

FIG. 1. The inhibitory effect of the cartilage-bone marrow extract (■) and DAK-16 (●) on the activity of MMP proteoglycanases. The activity of these enzymes was determined in vitro using the [^{3}H]-proteoglycan-bead assay as described under Methods. Enzyme activity is expressed as counts per min (cpm) of [^{3}H]-labeled fragments of PG monomers released per mg beads. Each value was determined in triplicate and the whole experiment was repeated 3 times. Data are means ± SD. *0.01 < $P \leq 0.05$, **0.001 < $P \leq 0.01$, ***$P \leq 0.001$, significantly different from control (no drug value), using student's two tailed paired *t*-test.

Statistical Analyses

Each value was determined in duplicate. The whole experiment was repeated three times using different specimens. Groups of data were evaluated using the student's two-tailed paired *t*-test. Significance was set at $P \leq 0.05$.

RESULTS

In Vitro *Effect of Drugs on the Activity of MMP Proteoglycanases*

Of the antirheumatic drugs tested, only the lyophilized cartilage-bone marrow extract (Rumalon®) and DAK-16 dose-dependently, albeit weakly, inhibited the proteoglycanolytic activity *in vitro* (Fig. 1). DAK-16 and the cartilage-bone marrow extract displayed an IC_{50} value of 10.64 mg/mL (1.9×10^{-4} M) and 31.78 mg/mL respectively. Only at the highest concentration tested was the inhibition significant. None of the other possible chondroprotective drugs (ademethionine, L-cysteine, glucosamine sulfate, glycosaminoglycan polysulfate, and sodium pentosan polysulfate), DMARDs (auranofine, aurothiopolypeptide, aurothiomalate, chloroquine, D-penicillamine, and methotrexate), NSAIDs (acetylsalicylic acid, diclofenac-Na, flufenaminic acid, indomethacin, ketoprofen, naproxen, phenylbutazone, piroxicam, and tiaprofenic acid), glucocorticoid (dexamethasone) and ACE inhibitors (captopril, cilazapril, and lisinopril) tested at a concentration of 100 μM displayed any significant inhibition. Addition of 10 mM EDTA or 1 mM 1,10-phenanthroline in the presence of the proteinase inhibitor cocktail blocked any proteoglycanolytic activity. The synthetic MMP inhibitor U-24522 dose-dependently inhibited stromelysin (Table 1), displaying an IC_{50} value of 1.8×10^{-9} M.

TABLE 1. The effect of U-24522 on the activity of MMP proteoglycanases

Drug	Final concentration	% Inhibition
U-24522	100 (μM)	98.6 ± 5.7†
	10.0 (μM)	102.3 ± 3.4†
	1.00 (μM)	100.7 ± 5.6*
	100 (nM)	97.9 ± 8.3*
	10.0 (nM)	78.7 ± 5.6†
	1.00 (nM)	32.5 ± 8.8†

The percentage values indicate the proportion by which the activity of MMP proteoglycanases was inhibited by this compound. Data are means ± SD (N = 3). *$P < 0.05$, †$P < 0.001$, different from control (no drug value), using student's two tailed paired *t*-test.

The Effect of the Cartilage-Bone Marrow Extract and DAK-16 on the Synthesis and Release of Proteoglycans from IL-1-treated Bovine Articular Cartilage Explants

In order to determine whether the *in vitro* inhibition of the MMP proteoglycanases can also be demonstrated *ex vivo*, cartilage explants were treated with 50 U/mL IL-1 alone or together with the drugs at various therapeutically achieved concentrations. IL-1 induced a 2.64 ± 0.13-fold (N = 9; $P \leq 0.001$) significant increased release of PGs from articular cartilage explants into the culture media compared to unstimulated explants (Fig. 2). Both drugs were unable to modulate the IL-1-induced augmentation of PG loss from cartilage explants into the nutrient media (Fig. 2). In comparison, the hydroxamate U-24522 dose-dependently inhibited the IL-1-induced increased release of PGs into the media when tested at concentrations ranging from 1 to 100 μM (IL-1-treated cultures: 100%; IL-1 and with 1, 10 or 100 μM U-24522-treated cultures: 90.4 ± 14.2%; 80.1 ± 13.1%; 61.7 ± 15.4%) (Fig. 2).

Furthermore, a significantly reduced incorporation of

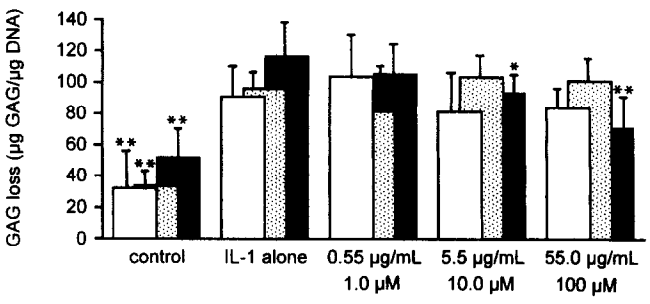

FIG. 2. GAG loss from IL-1-treated bovine articular cartilage explants into the nutrient media as modulated by the cartilage-bone marrow extract, DAK-16 and U-24522. Cartilage explants were cultured for 10 days without any additions (control) or in the presence of 50 U/mL IL-1 alone (IL-1 alone), with 50 U/mL IL-1 together with 0.5, 5.5 or 55.0 μg/mL DAK-16 (open bars) and cartilage-bone marrow extract (dotted bars) or with 1.0, 10.0 or 100 μM U-24522 (dark bars). Each bar represents the total amount of GAGs lost during the 10 day culture period. The results are expressed as mean values ± SD (N = 3) from three cartilage explant cultures each done in duplicate. *$0.01 < P \leq 0.05$, **$P \leq 0.01$, different from cartilage explants treated with IL-1 alone, using student's two tailed paired *t*-test.

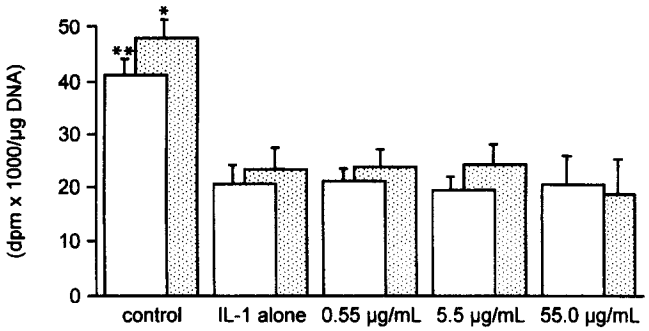

FIG. 3. Effect of the cartilage-bone marrow extract and DAK-16 on PG synthesis of bovine articular cartilage explants. Cartilage explants were untreated (control) or treated with 50 U/mL IL-1 alone (IL-1 alone), with 50 U/mL IL-1 together with 0.5, 5.5 or 55 μg/mL DAK-16 (open bars) or cartilage-bone marrow extract (dotted bars). Explants were pulsed on day 10 with 10 μCi/mL $Na_2[^{35}S]$-SO_4. $[^{35}S]$-SO_4-labeled proteoglycans in the cartilage explants and nutrient media were analyzed as described under Methods. Each bar represents the total amount of $[^{35}S]$-SO_4-labeled proteoglycans found in the media and cartilage explants. The results are expressed as mean values ± SD from three cartilage explant cultures each done in duplicate (N = 3). *$0.001 < P \leq 0.01$, **$P \leq 0.001$, different from IL-1-treated cartilage explants (no addition of drug), using student's two-tailed paired *t*-test.

radiolabeled precursor into PGs was observed on day 10 in cartilage explants treated with IL-1 alone as compared to untreated cultures (IL-1-treated cultures: 49.0 ± 6.3%; untreated cultures: 100%) (Fig. 3). Treatment of IL-1-exposed cartilage explants with the cartilage-bone marrow extract or with DAK-16 did not alter the IL-1 induced reduced biosynthesis of PGs (Fig. 3).

Pharmacological Modulation of the Aggregability and Hydrodynamic Size of Newly Synthesized Proteoglycans

The aggregability and hydrodynamic size of radiolabeled PGs from treated and untreated articular cartilage explants were determined under associative conditions by gel filtration on Sepharose CL-2B columns. The elution profile of $[^{35}S]$-SO_4-labeled PGs from untreated cartilage explants showed the presence of three distinct PG species: a large aggregated PG eluted at the void volume, non-aggregating PG monomers (Kav: 0.26) and small PG fragments (Kav: 0.7). Only two distinct PG species could be identified in cartilage explants treated with 50 U/mL IL-1 alone or together with U-24522, cartilage-bone marrow extract or DAK-16: a large aggregated PG eluted at the void volume, and PG monomers and fragments eluted as a broad peak over a Kav range of 0.12–0.82. The addition of 55 μg/mL of both drugs to IL-1-treated cartilage explants resulted, in contrast to the effect of the MMP inhibitor U-24522, in no significantly increased percentage of newly synthesized PGs able to form macromolecular aggregates (data are normalized to the percentage found for untreated cartilage explants: untreated cartilage explants: 1.0; IL-1-treated cartilage explants: 0.68 ± 0.02; IL-1 and with 10 μM U-24522,

55 μg/mL DAK-16 or cartilage-bone marrow extract treated cultures: 0.89 ± 0.02 ($P \leq 0.01$), 0.71 ± 0.03; 0.73 ± 0.02; N = 3).

The Viability of Cartilage Explants Treated with the Cartilage-Bone Marrow Extract, DAK-16 or U-24522

The activity of the cytoplasmatic enzyme LDH as an indicator of chondrocyte viability in all media changes was determined. We were not able to detect any activity of LDH in the incubation media of cultures treated with both possible chondroprotective drugs, U-24522 or with IL-1 alone, indicating that these compounds have no cytotoxic effects on the chondrocytes. Preliminary experiments showed that the media of articular cartilage explants, which were killed by 3 freeze-thawing cycles, contained LDH at an enzymatic activity of 30 ± 6.8 U/L per mg wet cartilage explant. Neither the 3 freeze-thawing cycles nor the drugs influenced the enzymatic activity of LDH.

In addition, staining of cartilage sections with propidium iodide and fluorescein diacetate revealed that IL-1 alone or given together with the cartilage-bone marrow extract, DAK-16 or U-24522 demonstrated no cytotoxic effects on the chondrocytes of the three different layers (superficial, intermediate and deep zone) of the explants.

DISCUSSION

In an attempt to better understand the mechanisms involved in the reported possible cartilage protective or destructive activities of some antirheumatic drugs, we have determined whether these drugs can directly inhibit MMP-mediated PG degradation *in vitro* and *ex vivo*, and whether drugs with inhibitory potential on MMP proteoglycanases have any impact on PG biosynthesis and viability of articular cartilage explants.

We chose a crude enzyme solution containing several MMP proteoglycanases in order to see whether antirheumatic drugs possess any inhibitory potential on the activity of these enzymes. The enzyme solution used in our *in vitro* assay did contain MMP-1, MMP-2 and MMP-3, as previously shown by Williams *et al.* [42] and by us (data not shown) and probably also included other MMP proteoglycanases such as MMP-8 and MMP-9 [11, 43–45]. The enzyme solution of our stromelysin assay did not contain any serum, so that cartilage was the only source for enzymes in our *in vitro* assay. By measuring the effect of pH on the activity of the proteoglycanases, we found a broad pH-optimum with a maximum lying at pH 7.1 (data not shown). Since no enzyme activity within our *in vitro* assay was detected in the presence of EDTA or 1,10-phenanthroline given together with the above-described proteinase inhibitor mixture, and since APMA inhibits cathepsin B and any other cysteine proteinase (e.g. cathepsin L), we conclude that our *in vitro* enzyme assay measures only the activity of neutral MMP proteoglycanases.

In our study, the *in vitro* inhibitory effect of DAK-16 as well as of the cartilage-bone marrow extract, shown to inhibit human collagenase [32], on the activity of MMP proteoglycanases might be related to the proteinic part of the complex reacting directly or by competition with the enzyme substrate. The results of our study are consistent with those of Vignon *et al.* [46], who reported a dose-dependent inhibitory effect of the cartilage-bone marrow extract on the metal dependent proteoglycanolytic activities in vitro from human osteoarthritic cartilage. Using our cartilage explant system, the cartilage-bone marrow extract as well as DAK-16, when tested at therapeutically achieved concentrations of 0.55 to 55 μg/mL, did not show any inhibitory potential on IL-1-inducible proteoglycanases. Our results are consistent with data reported by Arsenis *et al.* [26] in which 120 μg/mL of the cartilage-bone marrow extract was also unable to reduce the IL-1-induced increased PG loss from bovine and rabbit articular cartilage explants cultured for a period of 2 days. Studies in which DAK-16 was administered to a human chondrocyte cell culture system [47] or intraarticularly once or twice a week to an *in vivo* animal model of osteoarthritis [19] have shown that the therapeutic and effective dosages lie in the range of 10 to 100 μg/mL cell culture media μg per joint, respectively. Taken together, our results show that an inhibitory effect of these drugs on MMP proteoglycanases can only be found at relatively high concentrations lying in the range of 5500 to 55000 μg/mL enzyme assay, which can not be achieved after therapeutic dosages.

However, the prophylactic and therapeutic administration of the cartilage-bone marrow extract in a lapine model of osteoarthritis resulted not only in a decreased level of neutral metalloproteinase activities but also in an increased level of uncomplexed TIMP within articular cartilage [48]. Since in our experiments the cartilage-bone marrow extract displayed no inhibitory effect on the activity of MMP proteoglycanases *in vitro* at low concentrations and was also ineffective in reducing the IL-1-induced PG loss from cartilage explants during the 10-day culture period, the results obtained in the study done by Dean *et al.* [48] point to the possibility that the pharmacological effects of the cartilage-bone marrow extract on the level of active MMPs and TIMPs are likely to be indirect such as the modulation of the biosynthesis of MMPs and TIMP and/or the blocking of the release and/or physiological activation of latent MMPs.

The knowledge concerning the effects of drugs, especially in terms of their usefulness for treatment of joint diseases, on viability and anabolism of cartilage is important, since any impact of drugs on the viability as well as any stimulatory or inhibitory effect on the biosynthesis of extracellular matrix components can be beneficial or detrimental to cartilage. The cartilage-bone marrow extract has been reported to stimulate the biosynthesis of PGs and type II collagen *in vitro* by unstimulated human chondrocytes at concentrations ranging from 2.3 to 230 μg/ml [47]. However, we report here for the first time that the cartilage-bone marrow extract and DAK-16, while not

cytotoxic, are unable to specifically reverse the IL-1-induced suppression of PG biosynthesis by articular cartilage.

Only limited information is available on the effects of NSAIDs and glucocorticoids regulating MMP activity and/or biosynthesis. For example, naproxen, tiaprofenic acid, dexamethasone, hydrocortisone and methylprednisone have been shown to reduce the level of a neutral MMP activity *in vivo* [21], *in situ* [17, 30, 49] and *in vitro* [50], which probably reflects the activity of stromelysin within canine, bovine and/or human articular cartilage. On the other hand, indomethacin has been reported to have no effect on the level of neutral MMP activity within human osteoarthritic cartilage [30].

The implications of these findings are complicated, since in most of these studies the levels of MMPs were not measured directly (e.g. using ELISA techniques) but were extrapolated from results obtained by measuring the activity of enzymes. Our present study demonstrates that all NSAIDs as well as glucocorticoids tested have no direct inhibitory potential on the activity of MMP proteoglycanases, suggesting that the findings [17, 21, 30, 49, 50] with naproxen, tiaprofenic acid as well as with glucocorticoids may be the result of an indirect mechanism perhaps one affecting the biosynthesis, release and/or activation of MMPs and/or TIMPs. This assumption is supported by studies performed by DiBattista *et al.* [51] in which the investigators showed that glucocorticoids can reduce the synthesis of MMPs by suppressing transcription, although posttranscriptional effects cannot be ruled out.

In this study, the possible chondroprotective drugs glycosaminoglycan polysulfate and pentosan polysulfate displayed no inhibitory effect on the activity of MMP proteoglycanases *in vitro*. Nethery *et al.* [52], however, reported that glycosaminoglycan polysulfate and sodium pentosan polysulfate produced a concentration-dependent inhibition of the activity of both human fibroblast stromelysin and rat tumour stromelysin using a similar assay to measure the activity of this enzyme. Prophylactic and therapeutic application of glycosaminoglycan polysulfate has also been reported to decrease the level of active and total PG degrading metalloproteinases in different animal models of osteoarthritis [48, 53, 54]. Furthermore, several studies have shown that these possible chondroprotective drugs, as well as chloroquine and hydroxychloroquine, are able to inhibit PG release from rabbit, bovine and canine articular cartilage both *in vivo* and *in situ* [25, 26, 55, 56]. The lack of any inhibitory effect of either drug in our assay system, therefore, might be explained by species differences. The results presented in our study using bovine MMP-proteoglycanases suggest that the reported anticatabolic effects may also be mediated in part by influencing the biosynthesis, release and/or activation of MMPs or their inhibitors.

In this study, none of the other DMARDs proved to possess any inhibitory effects on the activity of MMP proteoglycanases. Limited information is available concerning the effects of DMARDs on these enzymes. Auranofin was reported to reduce the IL-1-mediated PG loss from bovine nasal septum cartilage while methotrexate, D-penicillamin and sulfasalazin were ineffective [57]. Further studies are, however, required to determine by which mechanism auranofin can modulate IL-1-mediated PG loss from cartilage.

The hydroxamic acid-containing peptide U-24522, which was developed as an inhibitor of MMPs, proved to be a potent and broad spectrum inhibitor of MMP proteoglycanases both *in vitro* and *ex vivo*. Our experiments showing that the aggregability of newly synthesized PGs was increased in cultures treated with U-24522 and IL-1 compared to cultures treated with IL-1 alone suggest that this compound inhibited the proteolytic digestion of the core protein and/or link proteins of the PGs, enabling them to form macromolecular aggregates. Demonstration of MMP inhibitor efficacy suggests that cartilage explant cultures provide a useful model for studying the effects of drugs on articular cartilage catabolism *in situ*. Our findings are consistent with data reported by Caputo *et al.* [58] in which a rabbit articular cartilage-derived proteoglycanase was also blocked *in vitro* by this MMP inhibitor. Our determined IC_{50} value of 1.8×10^{-9} M for the *in vitro* inhibition of a mixture of MMP proteoglycanases is at least one order of magnitude lower than the previously reported IC_{50} values of the same agent (6.0×10^{-8} M [58] and 4.2×10^{-6} M [59] respectively), which might reflect differences in species or in the methodologies used. Current theory suggests that the hydroxamic acid functional group chelates the zinc molecule at the active site of the metalloproteinase, thereby inactivating the enzyme [60].

One outstanding example of an inhibitor design for blocking the activity of a metalloproteinase has been the development of ACE inhibitors, which are able to inhibit the metal-dependent exopeptidase angiotensin-converting enzyme. In our study, the tested ACE inhibitors were ineffective in reducing the activity of MMP proteoglycanases *in vitro*, which is probably due to insufficient binding interaction at the active site of these enzymes.

The significance of our results is that the observed *in situ* and *in vivo* effects of some NSAIDs, glucocorticoids, DMARDs and possible chondroprotective drugs on MMP proteoglycanases are not due to a direct inhibition of their activities but rather may be indirect, such as by influencing the biosynthesis, release and/or activation of MMPs or TIMPs. Further studies, some of which are already in progress in our laboratory, are required to determine the molecular mechanisms involved in these pharmacological effects on the level of active MMPs.

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