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Biochemical Pharmacology 64 (2002) 217–227

Biochemical Pharmacology

Effects of polysulfated glycosaminoglycan and triamcinolone acetonid on the production of proteinases and their inhibitors by IL-1α treated articular chondrocytes

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Received 28 January 2002; accepted 26 April 2002

Abstract

In this study we determined the *in vitro* effects of polysulfated glycosaminoglycan (PSGAG) and the glucocorticoid triamcinolone acetonid (TA) on the IL-1 altered expression and activity of matrix metalloproteinases (MMP-1, MMP-3), tissue inhibitor of metalloproteinases-1, the plasminogen activators tPA and uPA and plasminogen activator inhibitor 1 by articular chondrocytes. Bovine chondrocytes were cultured in alginate gel beads. Cells were treated with interleukin-1α (IL-1α) in the presence of vehicle or drugs at various concentrations. After 48 hr mRNA expression of MMP-1, MMP-3, TIMP-1, uPA, tPA and PAI-1 was analyzed by RT-PCR-ELISA. The protein synthesis of TIMP-1 and MMP-3 was determined by immunoprecipitation, PAI-1 protein was quantitated by ELISA. The activity of enzymes and inhibitors was measured by functional assays. Treating chondrocytes with IL-1 induced the expression of MMPs and downregulated TIMP-1 but stimulated both the expression of PAs and PAI-1. Both drugs significantly reduced collagenase and proteoglycanase activities which was accompanied by inhibition of the expression of MMP-1 and MMP-3. The IL-1 decreased expression of TIMP-1 was further reduced by TA, 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 PSGAG. Both drugs inhibited the IL-1-induced mRNA expression of tPA, whereas expression of uPA was only mildly reduced by PSGAG, which also induced PAI-1 above IL-1 stimulated levels. As inhibition of collagenase activities and tPA expression by PSGAG occured at physiological concentrations it might be of clinical relevance, indicating that PSGAG could help reducing cartilage degradation and has a strong anti-fibrinolytic potential. Due to their co-regulation of MMPs and TIMP(s) glucocorticoids should be carefully studied for their overall effect on extracellular matrix proteolysis.

Keywords: Matrix metalloproteinases; Tissue inhibitor of metalloproteinases; Plasminogen activators; Plasminogen activator inhibitor; Glucocorticoids; Polysulfated polysaccharides; Osteoarthritis; Rheumatoid arthritis

1. Introduction

Impairment of cartilage function by depeletion of aggrecan and collagen is a common feature of joint diseases like osteoarthritis (OA) and rheumatoid arthritis (RA). Besides a dysregulated synthesis of matrix components like proteoglycans (PG) and collagens a variety of proteolytic enzymes is involved in this cartilage eroding process. Among the matrix metalloproteinases (MMPs), which are zinc-dependent endopeptidases, the collagenases (MMP-1, -8, -13) are of special relevance due to 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 [1]. The degradation of aggrecan, on the other hand, is mediated by the recently identified aggrecanases (ADAMTS4 and ADAMTS5) which are members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family [2,3] and MMPs. Stromelysin-1 (MMP-3),

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Abbreviations: OA, osteoarthritis; RA, rheumatoid arthritis; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; PA, plasminogen activator; PAI, plasminogen activator inhibitor; PSGAG, polysulfated glycosaminoglycan; IL-1, interleukin-1; TA, triamcinolone acetonid; SD, standard deviation; cAMP, cyclic AMP; PGE_2, prostaglandin E_2.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01073-0

for example, is involved in the degradation of aggrecan by cleavage at the Asn^{341}–Phe^{342} site resulting in the C-terminal neoepitope sequence VDIPEN [4]. VDIPEN neoepitopes were found in synovial fluid, as well as in cartilage of RA and OA patients [5]. Additionally, when experimental RA was induced in MMP-3-deficient mice articular cartilage remained intact whereas in wildtype mice severe erosion was observed [6]. Apart from a pivotal role in cartilage degradation MMP-3 is involved in the activation of proMMP-1 [7].

The PAs are serine proteases, which catalyse the activation of plasminogen to plasmin. Urokinase-type PA (uPA) is secreted as an inactive single-chain protein (pro-uPA) which is then converted into the double-chain active enzyme, whereas both single- and double-chain forms of tissue-type PA (tPA) possess enzymatic activity [8]. PA activity is controlled by two distinct PA inhibitors, PAI-1 and PAI-2 [9,10]. Pro-uPA and uPA interact with cells through a high affinity receptor (uPAR), localizing proteolytic activity to the cell surface which increases the rate of plasminogen activation and modifies inhibition by PAI-1 [11,12]. MMPs are synthesized and released from chondrocytes as inactive proenzymes, which are activated by limited proteolytic cleavage. Plasmin is one candidate involved in the activation process of these enzymes [13]. This enzyme further contributes to cartilage matrix degradation by its direct proteolytic activity on extracellular macromolecules, such as PGs [14].

MMP activity is controlled by tissue inhibitors of metalloproteinases (TIMPs) which form inhibitory complexes with MMPs in a 1:1 stoichiometry. To date four different TIMPs (TIMP-1, -2, -3, -4) are known [15], however, their specific roles are not well understood. TIMP-1 to -3 were found to be expressed by chondrocytes [16,17] and therefore are possibly essential for maintaining an enzyme-inhibitor balance required for physiological ECM turnover.

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 [18]. IL-1 has been found to contribute to the disease process by stimulating the biosynthesis of proteolytic enzymes [19], and also by inhibiting the production of extracellular matrix constituents such as collagen and PGs from articular cartilage [20,21]. The resulting imbalance between the anabolic and catabolic activities leads to the severe degradation of articular cartilage, observed in OA.

Polysulfated glycosaminoglycan (PSGAG) is a heparin-like substance with anti-arthritic activities. Therapeutic application of PSGAG, for example, has been reported to decrease the level of active and total PG degrading metalloproteinases in different animal models of OA [22,23]. Furthermore, this drug increased the synthesis of aggrecan by human articular chondrocytes *in vitro* [24] and exhibited anti-inflammatory activities in the rat subcutaneous air pouch model [25]. The potential of polysulfated polysaccharides to act as therapeutic agents in the treatment of OA and RA is also documented by two recent studies dealing with the influence of calcium pentosan polysulfate on aggrecan catabolism and on the production of MMPs and TIMPs [26,27]. Glucocorticoids, on the other hand, have been widely used in the treatment of inflammatory joint diseases. Although they were found to inhibit IL-1 stimulated MMP synthesis in human chondrocytes [28,29], little is known about the effects of steroids on the activating PA/plasmin systems and TIMP(s). However, a comprehensive study not only on the MMP biosynthesis but also on the MMP activating and inhibiting systems appears to be essential to evaluate any drug-mediated anti-proteolytic activity on articular cartilage. Therefore, the present study was designed to compare the effects of PSGAG and triamcinolone acetonid (TA) on collagenase, proteoglycanase, TIMP and PA activities, and on the expression of MMP-1, MMP-3, TIMP-1, uPA, tPA and PAI-1 by bovine articular chondrocytes stimulated with IL-1α.

2. Materials and methods

2.1. Materials

Protein G PLUS/protein A agarose, human urokinase, and anti-TIMP-1 antibody were purchased from Calbiochem, triamcinolone acetonid, ascorbate, penicillin, streptomycin, L-glutamine, α-ketoglutarate, 6-aminocaproic acid, *N*-ethylmaleimide, 4-aminophenylmercuric acetate (APMA), phenylmethylsulfonyl fluoride (PMSF), bovine type I collagen, and mouse IgG were obtained from Sigma. An amount of 72 kDa gelatinase, low range protein molecular weight marker, DIG-labeling mix, biotin labeling mix, and PCR-ELISA were from Boehringer, alginate from Kelco, and trypsin were purchased from Worthington. Ham's F12, fetal bovine serum (FBS), gentamycin, and Trizol® were obtained from Life Technologies, human recombinant IL-1α from R&D Systems, S-2251, plasminogen, and tPA stimulator from Chromogenix, and M-MLV reverse transcriptase as well as Taq polymerase from Promega. CR-ITS^{+TM} was purchased from Collaborative Research (Bedford, UK), gelatine from Bio-Rad and Easy Taq^{TM} express protein labeling mix [^{35}S] from NEN. TintElize® PAI-1 was ordered from Biopool and anti-MMP-3 antibody from Chemicon. PSGAG was a generous gift from Luitpold Pharma.

2.2. Culture of chondrocytes

Isolation and culture of chondrocytes was performed as described previously [30]. Briefly, chondrocytes were isolated from macroscopically healthy metacarpophalangeal joints of 18–24 months old steers and subsequently encapsulated in alginate beads. Twenty alginate beads (10^4 cells

per bead) per well were transferred to a 12 well culture dish containing 2.5 mL per well growth medium (Ham's F12 containing 10% FBS, 25 mM HEPES, 0.3 mg/mL L-glutamine, 0.03 mg/mL α-ketoglutarate, 0.05 mg/mL ascorbic acid, 10 U/mL penicilline and 0.1 mg/mL streptomycine). Media were changed every second day. Cultures were maintained for 8 days at 37°, 5% CO_2 and 95% humidity. During the final 48 hr, culture medium containing 0.5% (v/v) of the serum substitute CR-ITS^{+TM} instead of FBS and IL-1α at a final concentration of 0.5 ng/mL were given to the cells either alone or together with drugs (0.1–50 μM) or the drug vehicle, respectively. After the incubation period, media were collected and stored frozen at −20°. 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 as well as the chondrocytes were stored frozen at −20° until analyzed.

2.3. Determination of TIMP activity

The activity of TIMP(s) was quantitated by its (their) ability to inhibit the 72 kDa gelatinase (MMP-2) in a solid-phase assay employing biotin labeled gelatin adsorbed onto microtiter plate wells as substrate [31]. Briefly, gelatin was biotinylated by the use of a biotin labeling kit according to the manufacturer's instructions. The 96-well microtiterplates were coated with biotin-labeled gelatin and MMP-2 activity in the presence or absence conditioned media and solubilized alginate samples was measured essentially as described elsewhere [32]. 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.

2.4. Determination of collagen and PG degrading activities

Media samples were assayed for collagenolytic activity by their ability to degrade collagen type I coated on microtiter plate wells as previously described [33]. Briefly, proenzymes were activated by treatment with trypsin (0.1 mg/mL) for 20 min at 37°. Soybean trypsin inhibitor was subsequently added to stop the reaction (0.5 mg/mL for 10 min at 37°). The samples were then given in duplicate to the wells of microtiter plates coated with bovine type I collagen. The remaining amount of undigested collagen was detected as previously described [32]. One unit of MMP collagenolytic activity was defined as the digestion of 1% collagen as compared to undigested control. A linear relationship between the amount of enzyme and substrate digestion was confirmed in preliminary experiments (data not shown).

Proteoglycanase activity was determined by degradation of [^{3}H]proteoglycan (PG) monomers entrapped in polyacrylamide gel beads as described [32,34]. Briefly, MMPs in the media were activated by addition of 1 mM APMA for 6 hr at 37°. 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 PG monomers. Release of radioactivity was determined by using a liquid scintillation counter. One unit of proteoglycanolytic activity was defined as one count per minute of tritium released per mg PG beads. A linear relationship between the amount of enzymes 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 metal-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.

2.5. Determination of PA activity

Conversion of plasminogen to plasmin by PAs in alginate samples and cell lysates was recorded by the hydrolysis of the chromogenic substrate H–D–Val–Leu–Lys–pNA (S-2251), as has been described [35]. No activity could be detected in media samples. Briefly, cells were lysed by repeated freeze/thawing cycles and cell-associated PA activity was then extracted by homogenization in Tris buffer (50 mM Tris and 0.05% Tween® 80, pH 8.0). Samples were mixed with plasminogen (1.3 μM), S-2251 (0.7 mM) and Tris buffer. To maximize the activity of tPA, fibrinogen fragments (125 μg/mL) were added as stimulator. Enzyme activity was calculated by generation of a standard curve with human urokinase and is given in units according to international standards. Control experiments performed without plasminogen showed that the samples contained no intrinsic plasmin activity.

2.6. 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° with a final denaturation step at 95° for 15 min. The cDNA was diluted in sterile water and then stored at −20°. The following primer pairs were chosen from the sequences published in GenBank and used for amplification:

TIMP-1: 5′-ATGGCCTCTGGCATCCTGTTG-3′; 5′-AAAGGTGGGAGTGGAAACACG-3′; MMP-1: 5′-AACTCTGGAGCAATGTCACAC-3′; 5′-CCTCATAATCAGCTTGAAGTC-3′; MMP-3: 5′-GAAATGCAGAAGTTCCT-

TGG-3′; 5′-GTGAAAGAGACCCAGGGAGTG-3′; uPA: 5′-TGCAGGAACCCAGACAATCAG-3′; 5′-GTCAGTGACTTCAGCGCCATA-3′; tPA: 5′-GCACCAACTGGAACAGCAGCG-3′; 5′-ATTTCTGCTCCTCCTCGCCAG-3′; PAI-1: 5′-GATTGAAGAGAAGGGCATGGC-3′; 5′-TGCGACACGTACAGAAACTCT-3′; GAPDH: 5′-GAGATGATGACCCTTTTGG-3′; 5′-GTGAAGGTCGGAGTCAACG-3′.

Depending on the cDNA to be amplified, PCR was performed for 22–28 cycles (denaturing at 95° for 45 s, annealing for 60 s, extension at 72° for 120 s) to keep the reaction in the exponential range of amplification, and thus obtain semiquantitative results (data not shown). Annealing temperatures for the primer pairs were: 55° for TIMP-1, MMP-3, uPA, tPA and PAI-1, 50° for GAPDH, and 48° for MMP-1. PCR products were labeled with digoxigenin (DIG) by addition of a DIG-labeling mix to the reaction mixture. PCR products were commercially sequenced (MWG Biotech, Ebersberg, Germany), which revealed their identity to the predicted sequences (see above).

2.7. 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 microtiterplate 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 anti-digoxigenin 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.

2.8. PAI-1 ELISA

The content of PAI-1 protein in media was determined with the TintElize® PAI-1 ELISA, which detected the active and latent form, as well as complexes of PAI-1 with tPA and uPA. The ability of this assay to detect bovine PAI-1 has previously been shown [36]. The detection limit was 0.5 ng/mL PAI-1. Prior to measurement, the media samples were concentrated 6-fold by lyophilization and subsequently rehydrated. PAI-1 protein levels in cell lysates and solubilized alginate samples were below the detection limit of the assay.

2.9. Determination of TIMP-1 and MMP-3 protein synthesis

TIMP-1 and MMP-3 protein synthesis was analyzed by metabolic labeling of chondrocytes followed by immunoprecipitation. Cells were radiolabeled with 50 μCi/mL [^{35}S]methionine/cysteine during the final 18 hr of the incubation period with IL-1α and drugs. Media and alginate samples were pooled and precleared by incubation with non-immune mouse IgG (TIMP-1) or non-immune rabbit IgG (MMP-3) and protein G plus/protein A agarose suspension for 1 hr. Mouse anti-TIMP-1 antibody (diluted 1:2000), which detected free TIMP-1 as well as TIMP-1 complexed with MMPs, or rabbit anti-MMP-3 antibody (diluted 1:500), which detected the active and latent form of the enzyme, was added to the samples. According to the manufacturers both antibodies reacted with the bovine antigens but not with other TIMP or MMP family members. After overnight incubation at 4°, immune complexes were precipitated by the addition of a 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 27 kDa (TIMP-1) and 58 kDa (proMMP-3) was measured with the Automatic TLC-Linear Analyzer LB284/LB285 (Berthold, Wildbad, Germany). Peaks were analyzed with the Chroma 1D software (Berthold).

The determination of the synthesis of total protein was done as previously described [30].

2.10. Statistical analysis

Data were collected from four to six independent experiments. Samples were determined in duplicate and groups of data were analyzed using a one-way ANOVA with Tukey's multiple comparison test. Significance was set $P \leq 0.05$. Data are presented as mean ± SD.

3. Results

3.1. Effects of drugs on collagenase, proteoglycanase and TIMP activities

In a first set of experiments we were interested whether PSGAG and triamcinolone acetonid could reduce chondrocyte released collagen and PG degrading enzyme activities, which were increased 12.3-fold ($P \leq 0.001$) and 7.5-fold ($P \leq 0.001$), respectively, by IL-1α. As illustrated in Fig. 1 both drugs dose-dependently decreased collagenase activities reaching inhibition by approximately 50% at a low concentration of 0.1 μM. In comparison, proteoglycan degrading activities were inhibited by PSGAG with an IC_{50} value of 9.5 μM whereas the glucocorticoid suppressed enzyme activities by 70–80% at all concentrations tested (Fig. 2).

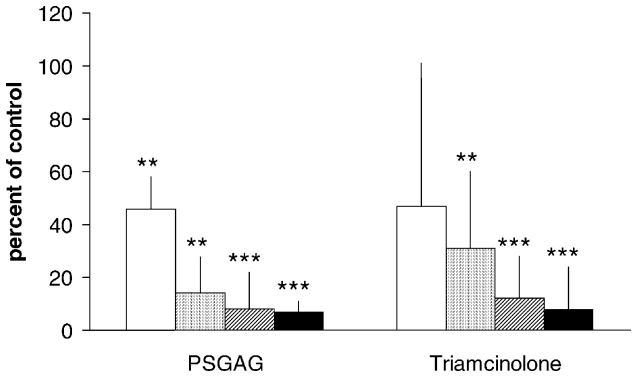

Fig. 1. PSGAG and TA dose-dependently decrease collagen type I degrading activities. Cells were incubated with drugs in the presence of 0.5 ng/mL IL-1α for 48 hr at the following concentrations: 0.1 μM (open bars), 1 μM (dotted bars), 10 μM (hatched bars), 50 μM (dark bars). MMPs in media samples were activated with trypsin and analyzed for enzymatic activities against collagen type I immobilized on microtiterplates. Values are expressed in mean ± SD (N = 4–6). Data were analyzed using ANOVA with Tukey's multiple comparison test. Statistically significant different from control values (IL-1 + vehicle = 100%): (**) $0.001 < P \leq 0.01$; (***) $P \leq 0.001$.

TIMP(s) plays a pivotal role in the control of metalloproteolytic activities. An increase or decrease of the production of inhibitor(s) would ultimately lead to an altered net proteolysis of cartilage components. Therefore, we next examined the influence of PSGAG and triamcinolone acetonid on TIMP activity in the presence of IL-1α. Neither the addition of cytokine alone or in combination with PSGAG to culture media significantly altered inhibitor activities. Table 1, however, demonstrates that triamcinolone acetonid inhibited TIMP activities by 20–60% at the concentrations tested.

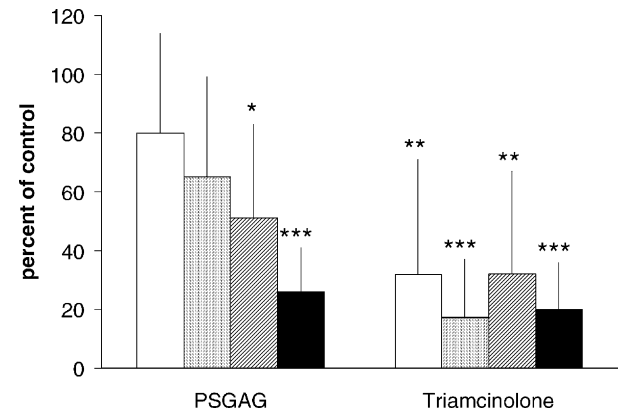

Fig. 2. PSGAG and TA dose-dependently reduce proteoglycan degrading activities. Cells were incubated with drugs in the presence of 0.5 ng/mL IL-1α for 48 hr at the following concentrations: 0.1 μM (open bars), 1 μM (dotted bars), 10 μM (hatched bars), 50 μM (dark bars). MMPs in media samples were activated with APMA and analyzed for enzymatic activities against PG monomers Values are expressed in mean ± SD (N = 4–6). Data were analyzed using ANOVA with Tukey's multiple comparison test. Statistically significant different from control values (IL-1 + vehicle = 100%): (*) $0.01 < P \leq 0.05$; (**) $0.001 < P \leq 0.01$; (***) $P \leq 0.001$.

Table 1
TIMP activity and biosynthesis of TIMP-1 after treatment of chondrocytes with 0.5 ng/mL IL-1α and TA for 48 hr

Treatment	Activity	Protein synthesis	mRNA expression
No cytokine	103 ± 32	207.6 ± 60.9[a]	191.9 ± 77.3[a]
IL-1 + vehicle	100	100	100
IL-1 + TA			
0.1 μM	34.8 ± 14.0[c]	32.9 ± 34.9[c]	58.2 ± 20.4[b]
1 μM	64.4 ± 17.7[b]	49.4 ± 36.7[b]	49.6 ± 15.3[c]
10 μM	82.5 ± 13.8	36.7 ± 14.3[c]	57.6 ± 24.5[b]
50 μM	51.2 ± 21.5[c]	38.7 ± 11.1[b]	54.1 ± 20.6[b]

Experiments were performed between culture day 6 and 8 in the presence of the serum substitute CR-ITS^{+TM}. Media and solubilized alginate samples were harvested and analyzed for TIMP activity and TIMP-1 protein. For determination of the mRNA expression, total RNA was isolated and transcript levels were quantitated by RT-PCR-ELISA. All analysis were performed as described in Section 2. Values are mean ± SD (N = 4–6). Statistical significant different from control values (IL-1 + vehicle = 100%): (a) $0.01 < P \leq 0.05$; (b) $0.001 < P \leq 0.01$; (c) $P \leq 0.001$.

3.2. Effects of drugs on the expression of MMP-1, MMP-3 and TIMP-1

Having determined that PSGAG and TA could inhibit MMP and TIMP activities we next examined whether these drugs elicited their effects by direct inhibition of enzymes and inhibitors or by downregulation of the expression of MMPs and TIMPs. For this we looked at the mRNA expression of MMP-1 and MMP-3 which was increased 5.8-fold ($P \leq 0.001$) and 4.8-fold ($P \leq 0.001$), respectively, by IL-1α. Furthermore, stimulation with this cytokine resulted in a 50-fold ($P \leq 0.001$) induction of MMP-3 protein synthesis. In contrast, IL-1α-decreased levels of TIMP-1 mRNA and protein by approximately 50% (Table 1).

We determined an IC_{50} value of 2.2 μM for the inhibition of MMP-1 mRNA expression by PSGAG whereas TA nearly completely abolished transcription of MMP-1 (Fig. 3). These results are somewhat in contrast to the observed inhibition of collagenase activities with IC_{50} values of 0.1 μM, indicating that MMP-1 was not alone responsible for the measured degradation of collagen. On the other hand, a good correlation was observed between the inhibition of proteoglycanase activities and downregulation of MMP-3 expression by the two drugs. Thus, the transcript level and protein synthesis of MMP-3 was lowered by PSGAG with IC_{50} values of 13.9 and 7.5 μM, respectively, whereas in the presence of TA inhibition ranged from 50 to 95% (Figs. 4 and 5).

Inhibition of the biosynthesis of TIMP-1 by IL-1α was not modified by PSGAG but was further lowered by TA (Table 1). This inhibition was not dependent on the concentrations used in our experiments. Protein and mRNA expression of TIMP-1 was always diminished by ~60 and ~40%, respectively.

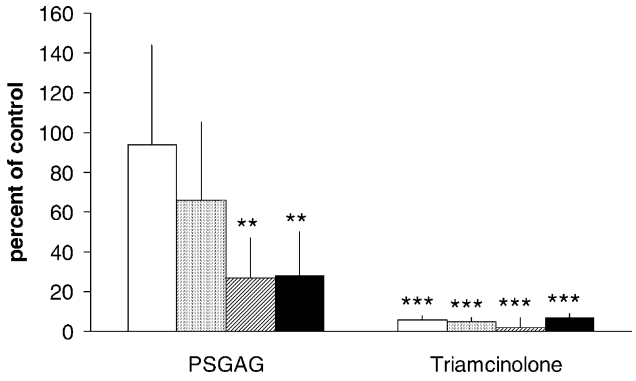

Fig. 3. PSGAG and TA inhibit transcript levels of MMP-1. Cells were incubated with drugs in the presence of 0.5 ng/mL IL-1α for 48 hr at the following concentrations: 0.1 μM (open bars), 1 μM (dotted bars), 10 μM (hatched bars), 50 μM (dark bars). Total RNA was isolated and MMP-1 mRNA was analyzed by RT-PCR-ELISA. All analysis were performed as described in Section 2. Values are mean ± SD (N = 4–6). Statistical significant from control values (IL-1 + vehicle = 100%): (**) $0.001 < P \leq 0.01$; (***) $P \leq 0.001$.

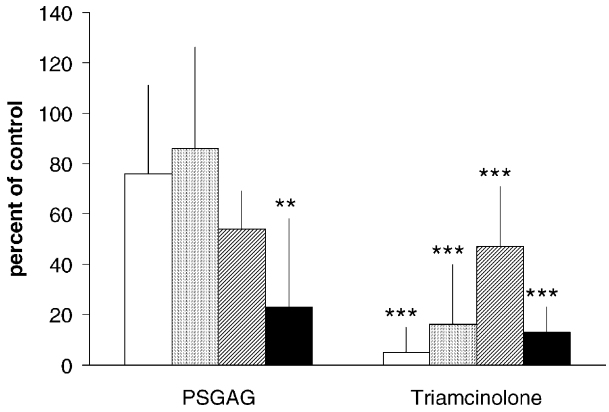

Fig. 5. PSGAG and TA inhibit transcript levels of MMP-3. Cells were incubated with drugs in the presence of 0.5 ng/mL IL-1α for 48 hr at the following concentrations: 0.1 μM (open bars), 1 μM (dotted bars), 10 μM (hatched bars), 50 μM (dark bars). Total RNA was isolated and MMP-3 mRNA was analyzed by RT-PCR-ELISA. All analysis were performed as described in Section 2. Values are mean ± SD (N = 4–6). Statistical significant from control values (IL-1 + vehicle = 100%): (**) $0.001 < P \leq 0.01$; (***) $P \leq 0.001$.

3.3. Effects of drugs on the expression of uPA, tPA and PAI-1

Untreated chondrocytes released significant levels of PA activity which was increased 1.3-fold ($P \leq 0.005$) by IL-1α. Evaluation of the mRNA expression of the two enzymes revealed, that uPA was enhanced 1.8-fold ($P \leq 0.001$), whereas the transcript level of tPA was induced 3.2-fold ($P \leq 0.001$) by this cytokine. No effects could be observed on the protein content of PAI-1, although transcription of this inhibitor was significantly increased (2.6-fold, $P \leq 0.001$).

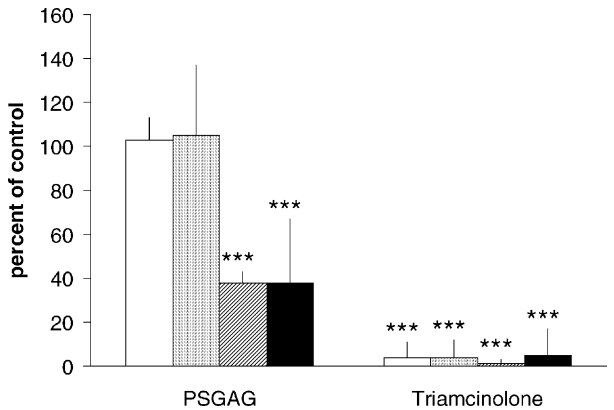

Fig. 4. PSGAG and TA reduce protein synthesis of MMP-3. Cells were incubated with drugs in the presence of 0.5 ng/mL IL-1α for 48 h at the following concentrations: 0.1 μM (open bars), 1 μM (dotted bars), 10 μM (hatched bars), 50 μM (dark bars). MMP-3 was immunoprecipitated from culture supernatants after labeling chondrocytes with 50 μCi/mL [^{35}S]methionine/cysteine during the final 18 hr of the incubation period with IL-1α and drugs. All analysis were performed as described in Section 2. Values are mean ± SD (N = 4–6). Statistical significant from control values (IL-1 + vehicle = 100%): (***) $P \leq 0.001$.

Treatment of chondrocytes with PSGAG in the presence of IL-1α resulted in a dose-dependent inhibition of PA activity. In contrast, TA reduced enzyme activities to approximately 70% of control values at all concentrations tested (Fig. 6). To determine whether this inhibition was reflected in changes of the expression of uPA and tPA we measured the steady-state levels of PA mRNA. As could be seen from Figs. 7 and 8 uPA and tPA significantly differed in their regulation by the two drugs. Thus, PSGAG ($IC_{50} = 1.3$ μM) and TA selectively inhibited tPA expression, whereas the expression of uPA remained unchanged.

Finally, we examined the influence of PSGAG and TA on PAI-1 protein and transcript levels in the presence of IL-1α. As summarized in Table 2, a significant 2-fold increase in secreted amounts of PAI-1 could be observed in the

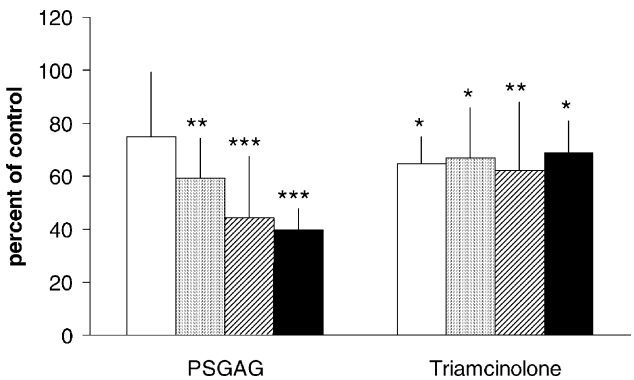

Fig. 6. PSGAG and TA reduce PA activities. Cells were incubated with drugs in the presence of 0.5 ng/mL IL-1α for 48 hr at the following concentrations: 0.1 μM (open bars), 1 μM (dotted bars), 10 μM (hatched bars), 50 μM (dark bars). PA activity represents the sum of activities determined in solubilized alginate samples and cell lysates. All analysis were performed as described in Section 2. Values are mean ± SD (N = 4–6). Statistical significant from control values (IL-1 + vehicle = 100%): (*) $0.01 < P \leq 0.05$; (**) $0.001 < P \leq 0.01$; (***) $P \leq 0.001$.

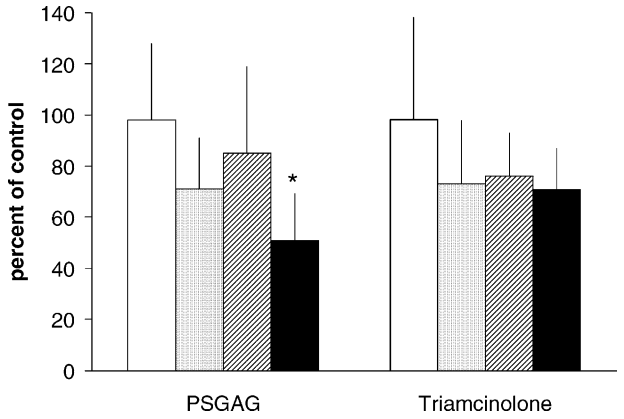

Fig. 7. mRNA expression of uPA after treating chondrocytes with PSGAG and TA. Cells were incubated with drugs in the presence of 0.5 ng/mL IL-1α for 48 hr at the following concentrations: 0.1 μM (open bars), 1 μM (dotted bars), 10 μM (hatched bars), 50 μM (dark bars) Total RNA was isolated and analyzed by RT-PCR as described in Section 2. DIG-labeled PCR products were quantitated by the use of a PCR-ELISA. Results are expressed as mean ± SD (N = 4–6). Statistical significant from control values (IL-1 + vehicle = 100%): (*) $0.01 < P \leq 0.05$.

presence of 50 μM PSGAG. Examining the mRNA expression of PAI-1 revealed that this drug only moderately induced the inhibitor's expression above IL-1α stimulated levels (Table 2). No effects could be observed for TA (data not shown).

3.4. Drug effects on the viability and protein synthesis of IL-1α treated articular chondrocytes

The viability of chondrocytes after treatment with IL-1α in the absence or presence of drugs 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 PSGAG and TA to IL-1α treated chondrocytes did not alter the synthesis of total protein and expression of GAPDH.

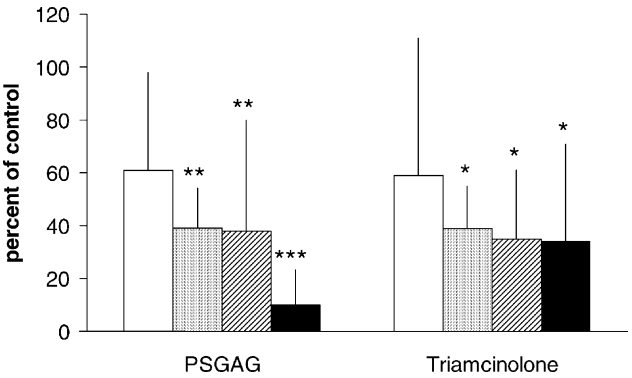

Fig. 8. mRNA expression of tPA after treating chondrocytes with PSGAG and TA. Cells were incubated with drugs in the presence of 0.5 ng/mL IL-1α for 48 hr at the following concentrations: 0.1 μM (open bars), 1 μM (dotted bars), 10 μM (hatched bars), 50 μM (dark bars) Total RNA was isolated and analyzed by RT-PCR as described in Section 2. DIG-labeled PCR products were quantitated by the use of a PCR-ELISA. Results are expressed as mean ± SD (N = 4–6). Statistical significant from control values (1 + vehicle = 100%): (*) $0.01 < P \leq 0.05$; (**) $0.001 < P \leq 0.01$; (***) $P \leq 0.001$.

Table 2
Secreted amounts of PAI-1 protein and PAI-1 transcript levels after treatment of chondrocytes with 0.5 ng/mL IL-1α and PSGAG for 48 hr

Treatment	PAI-1 protein	PAI-1 mRNA
No cytokine	91.9 ± 24.5	35.6 ± 12.7[b]
IL-1 + vehicle	100	100
IL-1 + PSGAG		
0.1 μM	158.2 ± 25.6[a]	111.7 ± 36.7
1 μM	188.2 ± 63.0	122.5 ± 32.8
10 μM	176.8 ± 66.2	148.1 ± 42.8
50 μM	203.4 ± 70.1	132.9 ± 33.8

Experiments were performed between culture day 6 and 8 in the presence of the serum substitute CR-ITS^{+TM}. Media were harvested and analyzed for PAI-1 content as described in Section 2. Total RNA was isolated and analyzed by RT-PCR as described in Section 2. DIG-labeled PCR products were quantitated by the use of a PCR-ELISA. Results are expressed as mean ± SD (N = 4–6). Statistical significant from control values (IL-1 + vehicle = 100%): (a) $0.01 < P \leq 0.05$; (b) $P \leq 0.001$.

4. Discussion

In this work we compared the effects of two drugs belonging to different pharmacologic classes on proteolytic activities released by chondrocytes under the influence of IL-1α. On the one hand we treated chondrocytes with a glucocorticoid (GC), which belong to the most often used substances in treating inflammatory joint diseases and act through binding to intracellular GC receptors. PSGAG on the other hand is a heparin-like substance. Polysulfated polysaccharides have attracted interest because of their potential to favorably modify the pathogenesis of OA.

Our results indicate that TA and PSGAG could significantly reduce IL-1 increased collagen and proteoglycan degrading activities which are mainly responsible for cartilage erosion during OA and RA. However, whereas collagenase activities were suppressed at nearly equal potencies, TA was much more efficient in reducing proteoglycanase activities than PSGAG. The determination of transcript levels of MMP-1 and MMP-3 which could be considered a prototypic collagenase and proteoglycanase, respectively, revealed further differences in the effects of the two drugs studied. Thus, the concentration of PSGAG needed for half-maximal inhibition of IL-1-induced MMP-1 mRNA expression was more than 10-fold higher than for inhibition of collagenase activity. In contrast, TA nearly completely abolished expression of MMP-1, although collagenase activities were determined in the presence of this drug. From previous studies we can exclude the possibility that these drugs directly interfered with collagenase activities [37]. However, due to the expression of MMP-8 and MMP-13 by chondrocytes, the measured

collagen degrading activity is only a rough estimate of MMP-1 production. Therefore, it might follow that both drugs possibly have also some influence on the expression of the two other mammalian collagenases, MMP-8 and MMP-13, as well.

The same might hold true for the modulation of proteoglycanase activities by TA and PSGAG, as proteoglycanase activities have been described for a number of MMPs (MMP-1, -2, -3, -7, -8, -9, -13 -14) *in vitro* and therefore do not only reflect the activity of MMP-3. Although in our experiments a good correlation between the inhibition of proteoglycanase activities and downregulation of MMP-3 by both drugs was observed. Additionally, a major component of PG degradation measured by our assay was possibly mediated by the recently identified aggrecanases ADMATS4 and ADMATS5 [2,3]. In this respect the weak inhibition of proteoglycan degrading activities by PSGAG is of interest, as in a recent study it has been shown that calcium pentosan polysulfate (CaPPS) which also belongs to the group of polysulfated polysaccharides inhibited IL-1-induced aggrecan loss from cartilage explant cultures at a concentration which was 10-fold lower than our determined IC_{50} value for the inhibition of proteoglycanase activities [26]. Furthermore, in the study by Munteanu and *et al.* [26] aggrecan degrading activities in conditioned media could be directly inhibited by CaPPS, whereas in a similar assay PSGAG had no significant influence on enzyme activities [38]. Thus, it appears that members of the group of polysulfated polysaccharides are quite heterogenous in their functions. This is further underscored by yet another study in which no effects of CaPPS on the IL-1-induced production of various MMPs including MMP-1 and MMP-3 could be measured [27].

Taken together our results suggest that the glucocorticoid TA is more effective in suppressing MMP-mediated cartilage degradation than PSGAG, as TA decreased proteoglycan degradation much stronger than did PSGAG. However, enhanced net proteolysis of cartilage components during OA and RA is likely to be the result of an excess of MMPs in comparison to TIMPs. Increasing the biosynthesis of TIMPs could therefore be an alternative pharmacological approach to reduce the articular cartilage matrix destruction during OA and RA. In our study, IL-1 inhibited the production of TIMP-1 by approximately 50%, which agrees with findings by Häuselmann *et al.*, who recently reported an IL-1-mediated downregulation of TIMP-1 protein in human chondrocytes [39]. In contrast, a coordinate upregulation of MMPs and TIMP-1 in response to IL-1 has been demonstrated for synovial fibroblasts [40]. Thus, it seems that articular cartilage is more vulnerable to cytokine action than the synovium, as in the former tissue, an imbalance between MMP and TIMP activities is evoked, which could form the base for cartilage proteolysis.

Despite the inhibition of TIMP-1 production by IL-1, we could observe no reduction of activity. This might be due to the presence of other TIMPs in the culture samples, which are not susceptible to cytokine action, as has been proposed for TIMP-2 [41]. Taking into account the high affinity of TIMP-2 for MMP-2, the enzyme used in our activity assay, it might be possible that the sustained level of inhibitor activity was caused by TIMP-2.

PSGAG did not reverse the inhibitory effect of IL-1 on the expression of TIMP-1 or increased TIMP activity. On the other hand, TA enhanced downregulation of protein and mRNA expression of TIMP-1 by IL-1, which resulted in a significant loss of TIMP activity. The decrease of TIMP activity, however, was weaker than the TA inhibition of mRNA expression and protein synthesis of TIMP-1. As chondrocytes also express other members of the TIMP family, the TIMP activity measured by our assay might not only reflect TIMP-1 activity. We can therefore not exclude that the expression of other TIMPs was not affected by TA resulting in a weaker inhibition of total TIMP activity. We have previously reported that dexamethasone inhibits TIMP-1 expression and TIMP activity by chondrocytes [32]. In addition, other studies have already shown that expression of TIMP-3 by chondrocytes is reduced by dexamethasone [42,17]. Notably, the activities of membrane-type MMPs and the aggrecanases are preferentially inhibited by TIMP-3 [43–46], making this inhibitor important for the maintenance of articular cartilage. Thus, simultaneous suppression of TIMPs and MMPs might question the cartilage preserving function which has been proposed for glucocorticoids [47]. On the other hand, CaPPS has been shown to augment production of TIMP-3 [27]. It would therefore be of special interest to determine whether PSGAG has similar properties.

MMPs are secreted as inactive zymogens which have to be extracellularly processed to gain full activity. In articular cartilage, plasmin, the conversion product of plasminogen, is thought to be involved in the activation of MMPs [13]. We found a preferential stimulation of tPA mRNA expression by IL-1 as compared to uPA, corroborating an early study by Bunning *et al.* [48]. Furthermore, TA and PSGAG had comparable inhibitory activities on IL-1-induced transcript levels of tPA. It has been shown that cultured bovine chondrocytes express a functional prostaglandin E_2 (PGE_2) receptor [49]. PGE_2 synthesis is induced by IL-1 and the subsequent binding of PGE_2 to this receptor could increase intracellular cAMP levels, which is involved in the IL-1 induction of PAs. As both drugs can suppress PGE_2 production [50], this might be a mechanism by which inhibition of tPA expression was achieved.

Interestingly, uPA expression was not affected by TA and only weakly inhibited by PSGAG, indicating different gene-regulatory mechanisms of the related but genetically distinct forms of PA. Indeed, species and cell-type specific responsiveness to cAMP of tPA and uPA is well documented. For instance, cAMP or cAMP-enhancing agents cannot directly induce the expression of tPA in several human and mouse cells [51,52], whereas uPA expression in

murine macrophages is actually reduced by cAMP agonists [53]. A, thus, uncoordinated regulation of their genetic expression is also evidenced by several studies in which expression of one PA form was found to be dominant over expression of the other form. Although some variation exists, in most studies dealing with RA-affected joints the predominant enzyme has been identified as uPA [54,55]. Thus, it remains questionable whether reduction of PA activity by PSGAG and TA is of therapeutic relevance as inhibition occured by mainly targeting expression of tPA. At least *in vitro*, however, some evidence exists that TA can reduce proteoglycan degradation by inhibition of the PA/plasmin system [56]. To our knowledge, downregulation of tPA expression by PSGAG has not been previously reported. However, a similar selective inhibition of tPA in arterial smooth muscle cells has been demonstrated for heparin [57]. The possibility that the two drugs tested here have direct effects on PA activities can be excluded from our previous studies [58].

PAI-1 is the major physiological inhibitor of PAs in connective tissue. In this study the transcript levels of PAI-1 were significantly elevated by IL-1 without an accompanying increase in secreted PAI-1 protein. We have at present no explanation for this phenomenon but similar effects have already been reported by Treadwell *et al.* for IL-1 treated human cartilage [59] and by DiBattista *et al.* for phorbol ester treated synovial fibroblasts [60].

PSGAG led to elevated inhibitor levels in culture media, which was accompanied by a mild induction of PAI-1 mRNA expression above IL-1 stimulated levels. On the other hand, TA failed to have any influence on the production of PAI-1. Regarding the PA/plasmin system, it thus seems that PSGAG has more favourable properties than TA, as it not only inhibited PA activity but also elevated PAI-1.

Although the effective concentrations observed for PSGAG in this study are above drug levels that are reached within synovial fluid after a single therapeutical dose (0.05 μM after 125 mg i.m.) one has to consider that due to the high affinity of PSGAG for cartilage drug concentrations in this tissue can rise by 10-fold [61]. Therefore, inhibition of collagenase activities and tPA expression might be of clinical relevance, confirming previous studies on the pharmacological usefulness of this drug in reducing or at least slowing the progression of OA. Our data on TA once more suggest, that glucocorticoids even at low doses are potent inhibitors of MMP synthesis and might therefore reduce cartilage degradation. However, due to their co-regulation of MMPs and TIMP(s) glucocorticoids should be carefully studied for their overall effect on ECM proteolysis.

Acknowledgments

This work was supported in part by BONFOR. The authors would like to thank I. Pelzer for technical assistance.

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