Evidence that the inhibition of cartilage proteoglycan breakdown by mannosamine is not mediated via inhibition of glycosylphosphatidylinositol anchor formation

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The effect of mannosamine, an inhibitor of glycosylphosphatidylinositol (GPI) anchor formation, on chondrocyte-mediated cartilage proteoglycan breakdown was investigated using cartilage explant cultures. Mannosamine inhibited interleukin 1 α -, tumour necrosis factor α - and retinoic acid-stimulated proteoglycan release from bovine nasal and articular cartilage, and retinoic acid-stimulated proteoglycan release from human cartilage. Its effects on two GPI-anchored proteins [the urokinase receptor, which binds urokinase-type plasminogen activator (uPA) to cell surfaces, and alkaline phosphatase] were also studied using bovine chondrocytes. Enzyme histochemistry and zymography demonstrated cell-associated uPA-like serine pro-

teinase activity and PA activity respectively which was not reduced by treatment of chondrocytes with mannosamine at concentrations effective at inhibiting cartilage proteoglycan breakdown. Similarly, the activity of cell-associated alkaline phosphatase was not reduced, except at mannosamine concentrations much higher than those used to inhibit proteoglycan breakdown. These results demonstrate that inhibition of proteoglycan breakdown by mannosamine is too potent to be explained by an effect on GPI-anchor formation.

Key words: chondrocyte, interleukin 1 α , retinoic acid, tumour necrosis factor α .

INTRODUCTION

Cartilage breakdown is a major feature of both rheumatoid arthritis and osteoarthritis. However, the mechanisms by which this occurs are not fully elucidated. As a model of cartilage degradation, use has been made of cartilage explant culture systems in which chondrocytes are stimulated to degrade their extracellular matrix by agents such as all-*trans*-retinoic acid (Ret) [1] or the pro-inflammatory cytokines interleukin 1 (IL1) [2] and tumour necrosis factor α (TNF α) [3].

The employment of proteinase inhibitors in this system has implicated the involvement of metalloproteinases [4,5], the cysteine proteinase cathepsin B (EC 3.4.22.1) [4] and serine proteinases [6] in cartilage proteoglycan breakdown. Our studies with the inhibitor of trypsin-like serine proteinases 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin have shown it to be a particularly potent inhibitor of urokinase-type plasminogen activator (uPA) and cytokine-stimulated proteoglycan breakdown. However, a role for other serine proteinases in cartilage proteoglycan breakdown could not be excluded [6].

uPA is predominantly cell-associated, bound to specific receptors, the uPARs (reviewed in [7]). The uPAR is covalently attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor [8]. Binding of uPA to its receptor is advantageous with regard to uPA-mediated catalysis, in that it concentrates uPA at the cell surface and allows more efficient conversion of plasminogen into plasmin [9,10]. In addition, GPI-anchored glycosidases such as hyaluronidase may be involved in cartilage proteoglycan breakdown [11]. It has been suggested that aggrecanase, the enzyme(s) thought to be mainly responsible for cartilage proteoglycan breakdown [4], is GPI-anchored [12].

However, two aggrecanases have recently been cloned, isolated and demonstrated to be secreted proteinases [13,14].

Mannosamine (2-amino-2-deoxy-D-mannose), an inhibitor of GPI anchor formation [15], has been shown previously to block uPAR GPI anchor formation [16]. Mannosamine is thought to inhibit the formation of the α 1,2 mannose linkages present in the conserved core glycan of the GPI anchor, preventing the incorporation of glycans into GPI and its anchoring of proteins [15,17]. The protein component is unaffected, and may be secreted if not GPI-anchored.

We have now investigated the effect of mannosamine on cartilage proteoglycan breakdown *in vitro* in explant cultures, and on the activities of the chondrocyte GPI-anchored proteins alkaline phosphatase (AP) [18] and uPAR.

EXPERIMENTAL

Materials

Recombinant human (rh) IL1 α (specific activity 3×10^8 units/mg) was a gift from The National Cancer Institute (Frederick Research and Development Centre, MD, U.S.A.). rhTNF α (specific activity 8×10^7 units/mg) was a gift from Genentec Inc. (San Francisco, CA, U.S.A.). Papain (EC 3.4.22.2) (type III), collagenase from *Clostridium histolyticum* (EC 3.4.24.3) (type I), Ret, bovine tracheal chondroitin sulphate A, D-mannosamine hydrochloride, D(+)-glucosamine hydrochloride, Sigma Fast *p*-nitrophenyl phosphate tablets and *p*-nitrophenol were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). 1,9-Dimethylmethylene Blue was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Z-Gly-Gly-Arg-4M β NA (where Z- is benzyl-

Abbreviations used: AP, alkaline phosphatase (EC 3.1.3.1); DMEM, Dulbecco's modified Eagle's medium; GPI, glycosylphosphatidylinositol; IL1 α , interleukin 1 α ; 4M β NA, 4-methoxy- β -naphthylamide; PA, plasminogen activator; PI-PLC, phosphatidylinositol-specific phospholipase C (EC 3.1.4.10); Ret, all-*trans*-retinoic acid; rhIL1 α , recombinant human IL1 α ; TNF α , tumour necrosis factor α ; rhTNF α , recombinant human TNF α ; sGAG, sulphated glycosaminoglycan; tPA, tissue-type PA (EC 3.4.21.68); uPA, urokinase-type PA (EC 3.4.21.73); uPAR, uPA receptor; Z-, benzyloxycarbonyl-.

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oxycarbonyl- and 4M β NA is 4-methoxy- β -naphthylamide) was from Bachem Feinchemikalien AG (Bubendorf, Switzerland), and L-[35 S]methionine was the product of NEN Life Science Products UK (Hounslow, Middx., U.K.). Trypsin (EC 3.4.21.4) (0.25%) and reagents for tissue culture were obtained from Gibco Life Technologies Ltd. (Paisley, Scotland, U.K.). Phosphatidylinositol-specific phospholipase C (PI-PLC), from *Bacillus thuringiensis* (EC 3.1.4.10), was obtained from Oxford Glycosciences (U.K.) Ltd. (Abingdon, Oxon., U.K.)

Cartilage explant cultures

Explants of bovine nasal septum cartilage and articular cartilage from the metacarpophalangeal joint were prepared and cultured in serum-free or serum- and glucose-free Dulbecco's modified Eagle's medium (DMEM) as previously described [4,19]. Mannosamine (10 nM–10 mM) and glucosamine (0.01–1 mM) were added to the cultures from 100-fold stock solutions in water.

Nasal and articular cartilage explant cultures, without or with rhIL1 α (0.3 nM and 2 nM respectively), were maintained for 30 h. All other cultures (nasal and articular cartilage with or without 1 μ M Ret; nasal cartilage with or without 3 nM rhTNF α ; articular cartilage with or without 6 nM rhTNF α) were sustained for 5 days, with a medium change on day 2; at the end of the 5 days the medium and explants were taken for analysis. These conditions had been found previously to produce on average a 50% loss of total proteoglycan by day 2 (IL1 α) or day 5 (TNF α and Ret) [6]. Only experiments in which the addition of cytokines or Ret led to at least a doubling of the amount of proteoglycan released, compared with basal levels, were included in the analyses of the effects of mannosamine or glucosamine on stimulated release.

Macroscopically normal human femoral head cartilage from seven females (median age 80 years; range 77–84 years) was sliced and maintained in culture for 6 days in serum-free DMEM, with or without Ret (1 μ M) and with or without mannosamine (0.1–10 mM). Medium was changed on day 3.

Quantification of proteoglycan release

Proteoglycan released into culture media was determined as sulphated glycosaminoglycan (sGAG) using the Dimethylmethylene Blue assay, with bovine tracheal chondroitin sulphate A as the standard [20]. Proteoglycan within explants was quantified by the same method following digestion with papain [21].

Protein synthesis determination

Following a 2-day culture period with or without rhIL1 α and various concentrations of mannosamine, bovine nasal septum cartilage explants were given a 6-h pulse with [35 S]methionine, and its incorporation was determined as described previously [19].

Assay for lactate

Lactate concentrations in culture media of bovine cartilage explants were determined by the lactate oxidase/peroxidase method using a kit supplied by Sigma Chemical Co.

Enzyme histochemical analysis of the effects of mannosamine and PI-PLC on chondrocyte uPA-like serine proteinase activity

Chondrocytes were obtained from bovine nasal and metacarpophalangeal joint cartilage as previously described [22]. The

cells were plated on to wells of 2-well chamber slides at sufficient density to form a confluent monolayer ($1.6 \times 10^5/\text{cm}^2$ for nasal chondrocytes and $2.0 \times 10^5/\text{cm}^2$ for articular chondrocytes). The adherent cells were incubated in DMEM containing 10% (v/v) fetal calf serum for at least 2 days. Then the medium was removed, and nasal and articular chondrocytes were incubated with or without rhIL1 α (0.3 nM and 2 nM respectively), rhTNF α (3 nM and 6 nM respectively) or Ret (1 μ M), with or without mannosamine (1 mM in unstimulated cultures and 0.01 mM in all other cultures) for 2 days in serum- and glucose-free DMEM. In order to visualize chondrocyte uPA-like activity, enzyme histochemical detection using Z-Gly-Gly-Arg-4M β NA as a fluorimetric substrate was carried out as described previously [6].

The effect of PI-PLC, which cleaves GPI-anchored proteins from the cell surface [15], on cell-associated uPA-like activity was also investigated. Bovine nasal chondrocytes were incubated with or without PI-PLC (1 unit/ml) for 2 days prior to enzyme histochemistry.

Zymographic analyses of the effect of mannosamine on PA activity

Bovine nasal and articular chondrocytes were isolated as described above. The adherent cells, in 24-well plates, were then incubated with or without Ret (1 μ M), rhIL1 α (0.3 nM and 2 nM for nasal and articular chondrocytes respectively) or rhTNF α (3 nM and 6 nM respectively), and with or without mannosamine (0.01 mM for stimulated cells; 0.1 mM for unstimulated cells), in serum- and glucose-free DMEM (300 μ l/well) for 24 h. Zymographic analysis for PA activity was carried out as described previously [6].

Determination of chondrocyte AP activity

Bovine articular chondrocytes were isolated and cultured, as described above, in 12-well plates. The cells were incubated with or without mannosamine (100 nM–10 mM) in the presence or absence of rhIL1 α (2 nM) or rhTNF α (6 nM) for 2 days in Phenol Red-, serum- and glucose-free DMEM. The culture media were removed and centrifuged to remove any cells or cell debris, and the supernatants were stored at -20°C prior to assay. Cell-associated AP activity was determined by incubating the cells with 400 μ l of *p*-nitrophenyl phosphate substrate for 10–30 min and measuring the change in absorbance at 405 nm. Soluble AP activity in culture media was measured by incubating 50 μ l of medium with 150 μ l of AP substrate for 10–30 min and measuring the change in absorbance as above. One unit of AP activity was defined as that which produces 1 μ mol of *p*-nitrophenol per min at 25°C and pH 8.0. AP activities, as units of activity, were expressed relative to the activities in untreated samples from the same experiment.

Statistical analyses

Except where stated otherwise, each experiment was repeated with material from at least two animals, and the combined data gave ≥ 24 replicates for each treatment group. Results of treatments were compared using the unpaired two-tailed Mann-Whitney test.

RESULTS

sGAG release from cartilage explants

Bovine nasal cartilage discs contained $1407 \pm 147 \mu\text{g}$ of sGAG. On stimulation by cytokines or Ret, between 50 and 75% was

Table 1 Inhibition by mannosamine and glucosamine of proteoglycan release from bovine nasal cartilage, cultured in serum- and glucose-free medium

Cartilage explants were cultured in serum- and glucose-free medium with or without mannosamine or glucosamine for 30 h or for 5 days with one medium change, as described in the Experimental section. Culture media and tissue were analysed for the amount of proteoglycan. In the analyses of stimulated proteoglycan release, basal levels were subtracted. The results are shown as percentage inhibition of proteoglycan release, expressed as means $\pm$ S.E.M., with $n \geq 24$ for each treatment group. The effect of the presence of the amino sugar was analysed statistically; significant differences are indicated by: * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$. The value of greater than 100% inhibition reflects total inhibition of stimulated release plus some inhibition of the basal level.

Amino sugar	Inhibition (%)			
	Basal	+ rhIL1 α	+ rhTNF α	+ Ret
[Mannosamine] (mM)				
0.00001		4 $\pm$ 8*	-8 $\pm$ 12	11 $\pm$ 8
0.0001		33 $\pm$ 6***	34 $\pm$ 10*	18 $\pm$ 7*
0.001		41 $\pm$ 7***	31 $\pm$ 10**	42 $\pm$ 8***
0.01		34 $\pm$ 5***	39 $\pm$ 11*	57 $\pm$ 6***
0.1		58 $\pm$ 5***	83 $\pm$ 4***	91 $\pm$ 6***
1	-8 $\pm$ 7*	74 $\pm$ 4***	96 $\pm$ 4***	114 $\pm$ 6***
[Glucosamine] (mM)				
0.01		-22 $\pm$ 18	1 $\pm$ 10	-20 $\pm$ 9*
0.1		15 $\pm$ 13	-19 $\pm$ 13	-9 $\pm$ 8
1	-5 $\pm$ 10	-47 $\pm$ 17*	-5 $\pm$ 11	-22 $\pm$ 8**

Table 2 Inhibition by mannosamine and glucosamine of proteoglycan release from bovine articular cartilage, cultured in serum- and glucose-free medium

Cartilage explants were cultured in serum- and glucose-free medium with or without mannosamine for 30 h or for 5 days with one medium change, as described in the Experimental section. The culture medium was analysed for the amount of proteoglycan released from the explants, and the results are presented as described in the legend to Table 1. The effect of the presence of the amino sugar was analysed statistically; significant differences are indicated by: * $P < 0.05$; ** $P < 0.001$; *** $P < 0.0001$ ($n \geq 24$ for each treatment group).

Amino sugar	Inhibition (%)			
	Basal	+ rhIL1 α	+ rhTNF α	+ Ret
[Mannosamine] (mM)				
0.01	10 $\pm$ 16	63 $\pm$ 11***	59 $\pm$ 9***	40 $\pm$ 10**
0.1	16 $\pm$ 6*	89 $\pm$ 7***	84 $\pm$ 8***	76 $\pm$ 10***
1	22 $\pm$ 6**	79 $\pm$ 8***	86 $\pm$ 8***	73 $\pm$ 10***
[Glucosamine] (mM)				
0.01	-30 $\pm$ 13	-49 $\pm$ 15*	-24 $\pm$ 15	-17 $\pm$ 12
0.1	-42 $\pm$ 14*	-18 $\pm$ 9	-7 $\pm$ 13	-16 $\pm$ 13
1	-50 $\pm$ 12*	-37 $\pm$ 18	-39 $\pm$ 15	-37 $\pm$ 12*

Table 3 Inhibition by mannosamine of proteoglycan release from human femoral head cartilage

Cartilage explants were cultured in serum-free medium with or without mannosamine for 6 days with one medium change, as described in the Experimental section. The culture medium and tissue remaining at the end of culture were analysed for the amount of proteoglycan. The effect of the presence of mannosamine was analysed statistically; significant differences are indicated by: * $P < 0.05$; *** $P < 0.0001$ compared with controls ($n \geq 24$ for each treatment group).

[Mannosamine] (mM)	Presence of Ret	Inhibition (%)
0.1	—	-42 $\pm$ 13*
1	—	-48 $\pm$ 8***
5	—	-28 $\pm$ 18
10	—	-7 $\pm$ 10
0.1	+	43 $\pm$ 9***
1	+	54 $\pm$ 6***
5	+	74 $\pm$ 9***
10	+	62 $\pm$ 7***

Table 4 Effect of mannosamine on lactate production by bovine nasal septum cartilage explants cultured in serum- and glucose-free medium

Explants were cultured for 30 h with or without rhIL1 α (0.3 nM) and mannosamine. Values are means $\pm$ S.E.M. Lactate levels in the culture medium, produced in the presence of mannosamine, were compared statistically with those in its absence; significant differences are indicated by: * $P < 0.05$ ($n \geq 24$ for each treatment group). Explants that had been frozen and thawed three times were included as controls (†).

[Mannosamine] (mM)	Presence of rhIL1 α	Lactate (μ g/disc)
0†	—	17 $\pm$ 1
0	—	86 $\pm$ 5
5	—	89 $\pm$ 5
10	—	84 $\pm$ 7
0†	+	12 $\pm$ 1
0	+	95 $\pm$ 4
1	+	100 $\pm$ 5
5	+	75 $\pm$ 4*

released, as previously observed [6]. sGAG release occurring in the absence of stimulation accounted for between 20 and 25% of the total. Bovine articular cartilage explants contained 265.4 $\pm$ 26.7 μ g of sGAG. As with nasal cartilage, > 50% of this was released following addition of rhIL1 α , rhTNF α or Ret, with basal release not usually exceeding 25%.

Mannosamine inhibits proteoglycan release from bovine cartilage cultures

Mannosamine potently inhibited proteoglycan release from IL1 α -, TNF α - and Ret-stimulated bovine nasal cartilage explants cultured in serum- and glucose-free DMEM (Table 1). Significant effects were observed at concentrations as low as 0.1 μ M. By contrast, mannosamine at 1 mM had no inhibitory effect on basal proteoglycan release.

Glucose-free medium was used for most experiments, since glucose and mannosamine may compete for the same transporter [23]. When glucose was present (25 mM), higher concentrations of mannosamine were required to produce inhibition of IL1-

Figure 1 Enzyme histochemical demonstration of the effects of mannosamine and PI-PLC on chondrocyte Z-Gly-Gly-Arg-4M β NA-hydrolysing activity

Bovine metacarpophalangeal or nasal chondrocytes were isolated, cultured and processed for enzyme histochemical analysis of uPA-like activity as described in the Experimental section. A period of 30 min at ambient temperature was allowed for the generation of the yellow fluorescent product. Bovine metacarpophalangeal chondrocytes were cultured for 2 days in: (A) serum- and glucose-free DMEM; (B) serum- and glucose-free DMEM containing mannosamine (1 mM); (C) serum- and glucose-free DMEM containing rhTNF α (6 nM); (D) serum- and glucose-free DMEM containing rhTNF α (6 nM) and mannosamine (0.01 mM). Bovine nasal chondrocytes were cultured for 2 days in: (E) serum-free DMEM; (F) serum-free DMEM containing PI-PLC (1 unit/ml). Original magnification $\times 50$.

stimulated proteoglycan release, with 10 mM mannosamine producing $55 \pm 6\%$ inhibition.

In experiments with bovine articular cartilage explants in glucose- and serum-free-medium, mannosamine inhibited, with high potency, rhIL1 α -, rhTNF α - and Ret-stimulated proteoglycan breakdown (Table 2).

The effects of mannosamine may be non-specific, or common to other amino sugars. Therefore the effects of glucosamine, a structurally similar amino sugar, on proteoglycan release were also studied. In these experiments nasal and articular cartilage explants were cultured using glucose-free DMEM to allow a direct comparison of the results with those observed when using mannosamine. At the concentrations of glucosamine used in our experiments (0.01–1 mM), no inhibition of cartilage proteoglycan

release occurred (Tables 1 and 2). In fact glucosamine appeared to enhance proteoglycan release, an effect that reached significance in Ret- and IL1 α -stimulated nasal and articular cultures and basal articular cartilage cultures, although there was no obvious dose effect. Other workers have observed inhibition of Ret-stimulated aggrecan breakdown by glucosamine, but at concentrations greater than 2 mM [12].

The effect of mannosamine on proteoglycan release from human femoral head cartilage was also analysed (Table 3). Cartilage pieces were cultured in glucose-containing serum-free DMEM with and without Ret. In line with the results from the experiments using bovine tissue, mannosamine significantly inhibited Ret-stimulated proteoglycan release, but did not inhibit basal proteoglycan breakdown. In fact, in the absence of Ret,

Figure 2 Zymographic demonstration of PA activity in bovine nasal chondrocytes

Cell culture and zymography were carried out as described in the Experimental section. Staining of gels was with 0.1% Coomassie Blue R250. The cells were cultured with rhIL1 α (0.3 nM), rhTNF α (3 nM) or Ret (1 μ M), or without any of these reagents, and with or without mannosamine (0.01 mM and 1 mM in stimulated and unstimulated cultures respectively). (A) and (C) are conditioned media; (B) and (D) are cell lysates. Treatments for (A) and (B): lane 1, control; lane 2, mannosamine; lane 3, rhIL1 α ; lane 4, rhIL1 α + mannosamine; lane 5, high- M_r human uPA (positive control); lane 6, human tPA (positive control). Treatments for (C) and (D): lane 1, control; lane 2, mannosamine; lane 3, rhTNF α ; lane 4, rhTNF α + mannosamine; lane 5, Ret; lane 6, Ret + mannosamine; lane 7, high- M_r human uPA (positive control); lane 8, human tPA (positive control).

low doses of the amino sugar significantly stimulated proteoglycan release from human cartilage.

Effects of mannosamine on the metabolic activity of bovine nasal cartilage explants

In the presence of compounds that are toxic to cells, non-specific inhibition of proteoglycan release is observed [19], and the release of cartilage proteoglycan is dependent upon the metabolic activity of the tissue [24]. The effects of mannosamine on various activities of the chondrocytes were therefore investigated, as was the reversibility of its inhibitory effect on proteoglycan release.

All experiments were carried out in serum- and glucose-free DMEM.

An investigation into the ability of explants to recover from exposure to mannosamine was carried out by subjecting explants, initially cultured in the presence of rhIL1 α (0.3 nM) and mannosamine (1, 10 and 100 mM), to a second presentation of IL1 following a 24 h recovery period, as previously described for cysteine proteinase inactivators [19]. When exposed to a second presentation of cytokine in the absence of mannosamine, explants initially exposed to 1, 10 and 100 mM mannosamine in the presence of IL1 released 422 ± 36 μ g, 693 ± 37 μ g and 150 ± 7 μ g of sGAG per disc respectively. Explants exposed to IL1 only throughout the experiment (no mannosamine) released 420 ± 34 μ g. Clearly the explants were capable of responding to a second treatment with the cytokine following exposure to mannosamine, although it seems likely that the recovery was incomplete after exposure to 100 mM mannosamine.

Chondrocytes are known to respire largely anaerobically [25]. We analysed the influence of mannosamine on lactate production by nasal cartilage explants cultured with and without stimulation by IL1. The presence of IL1 caused a slight increase in lactate production, although this was not found to be statistically significant (Table 4). In the absence of IL1, lactate levels remained stable at all concentrations of mannosamine tested. In the presence of IL1 a statistically significant decrease in lactate production was seen at a concentration of 5 mM mannosamine. From this experiment we can assume that, at concentrations below 5 mM, mannosamine is not having a general suppressive effect on anaerobic respiration by our cartilage cultures. There was little lactate production by cartilage explants killed by freezing and thawing.

The incorporation of [35 S]methionine into trichloroacetic acid-precipitable material was used as a measure of protein synthesis in bovine nasal cartilage explants cultured with or without rhIL1 α (0.3 nM) and mannosamine. In the absence of rhIL1 α , protein synthesis was significantly suppressed at a concentration of mannosamine of 1 mM (7200 ± 400 and 5000 ± 500 d.p.m. of [35 S]methionine incorporated for untreated and mannosamine-treated explants respectively; means $\pm$ S.E.M.; $P < 0.05$). However, in the presence of rhIL1 α , no significant suppression of protein synthesis by mannosamine at either 0.1 or 1 mM was observed (5000 ± 400 , 5000 ± 500 and 4700 ± 300 d.p.m. of [35 S]methionine incorporated for explants treated with IL1 alone, IL1 + 0.1 mM mannosamine and IL1 + 1 mM mannosamine respectively). Mannosamine thus exerts its chondroprotective effect at inhibitor concentrations that are not detrimental to protein synthesis.

Enzyme histochemical detection of uPA-like activity

The uPAR is a GPI-anchored protein [8] which binds uPA to cell surfaces. uPA or similar enzymes have been shown to be implicated in our models of chondrocyte-mediated proteoglycan breakdown [6]. We therefore analysed chondrocytes for the presence of uPA-like activity with and without prior treatment with mannosamine. As described previously, bovine nasal and articular chondrocytes in monolayer culture expressed Z-Gly-Gly-Arg-4M β NA-hydrolysing activity [6]. Yellow fluorescence was barely detectable upon addition of the incubation mixture to articular chondrocytes (results not shown), but became visible after a period of 30 min in unstimulated as well as rhIL1 α -, rhTNF α - and Ret-stimulated cells. Mannosamine (1 mM for unstimulated cells; 0.01 mM for stimulated cells) had little effect on the level of fluorescence observed. Figures 1(A)–1(D) show unstimulated and rhTNF α -stimulated articular cartilage

Figure 3 Bovine chondrocyte cell-associated and soluble AP activity

Bovine articular chondrocytes were isolated and cultured as described in the Experimental section. They were treated with or without mannosamine (0–10 mM) in the presence or absence of IL1 α (2 nM) or TNF α (6 nM) for 2 days. Cell-associated (A) and soluble (B) AP activity was measured using *p*-nitrophenyl phosphate as substrate. AP activities (means $\pm$ S.E.M.; $n \geq 4$) are expressed relative to the activities of untreated samples (100%). Significance of differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with unstimulated cells; † $P < 0.05$ compared with IL1 α alone; ‡ $P < 0.05$, §§ $P < 0.01$ compared with TNF α alone.

chondrocytes cultured in the presence or absence of mannosamine. Similar results were seen when using bovine nasal chondrocytes (not shown). In contrast, treatment of bovine nasal chondrocytes with PI-PLC, which cleaves GPI anchors, caused a reduction in fluorescence (Figures 1E and 1F).

Zymographic detection of PA activity

Zymography of bovine nasal chondrocyte cell lysates clearly demonstrated the presence of PA activity in all samples (Figures 2B and 2D). As analysed by scanning densitometry (results not shown), mannosamine treatment (0.01 mM and 1 mM) did not decrease the uPA activity of cell lysates, as would have been expected following the inhibition of uPAR GPI anchor formation [16]. Tissue-type PA (tPA)-like activity was increased in bovine nasal chondrocytes treated with IL1 α and mannosamine (Figure 2B).

Culture media from bovine nasal chondrocytes contained little or no detectable PA activity, irrespective of treatment (Figures 2A and 2C). The levels of soluble uPA activity would be expected to be increased following inhibition of GPI anchor synthesis, but this was not observed. Similar results were obtained for cell lysates and conditioned media from bovine articular chondrocytes (results not shown).

Effect of mannosamine on chondrocyte AP activity

AP is a GPI-anchored protein present on the surface of chondrocytes ([18]; V. Everts, personal communication). The effect of mannosamine on cell-associated and soluble AP activity was

therefore studied as a more direct means of investigating its effects on GPI anchors over a range of concentrations. Concentrations of 1 and 10 mM mannosamine were required to produce a significant reduction in cell surface AP activity in cytokine-stimulated cells. A coincident significant increase in AP activity in the culture medium was observed at 10 mM mannosamine for unstimulated cells or cells stimulated with IL1 α or TNF α (Figures 3A and 3B). The concentrations of mannosamine that affect GPI anchor formation are therefore much higher than those required to inhibit cytokine-stimulated breakdown of cartilage proteoglycan.

DISCUSSION

Mannosamine has been shown to block the synthesis of GPI, preventing the attachment of GPI-anchored proteins to cell membranes [15,17]. The present study has shown that it also potentially inhibits cytokine- and Ret-stimulated proteoglycan release from bovine nasal and metacarpophalangeal joint cartilage explants in culture, in a non-toxic manner. There was little effect of mannosamine on basal proteoglycan release, suggesting a different pathway for basal proteoglycan release compared with Ret- or cytokine-stimulated release. Ret-stimulated proteoglycan release from human femoral head cartilage was also inhibited by mannosamine.

Glucosamine, an amino sugar that is structurally very similar to mannosamine, did not inhibit proteoglycan release at such low concentrations, although it has recently been shown to do so at higher concentrations via inhibition (either direct or indirect) of aggrecanase activity [12] and is, like mannosamine, an inhibitor of GPI-anchor formation [17]. Glucosamine, at the low concentrations used, showed some stimulation of proteoglycan release. It is used by osteoarthritis patients, in whom it has been reported to play a role in the conservation and repair of cartilage [26]. Its reported stimulatory effects on glycosaminoglycan synthesis [27,28] may be more important at low concentrations than its inhibition of cartilage breakdown via effects on aggrecanase activity.

The potency of the inhibitory effect of mannosamine on bovine cartilage proteoglycan release was increased when cartilage was cultured in the absence of glucose. This is consistent with previous work in which effects of mannosamine on GPI anchor formation were found to be reversible upon addition of high concentrations of glucose to culture media [23], suggesting that mannosamine is taken up into cells via a glucose transporter system [29]. However, using human cartilage, we found potent effects of mannosamine even in the presence of glucose.

We have recently demonstrated that an inhibitor of uPA is a potent inhibitor of cytokine-stimulated proteoglycan release from cartilage explants in culture [6]; furthermore, in model systems of tumour invasion, binding of uPA to its receptor has been shown to enhance extracellular matrix breakdown *in vitro* [30]. The uPAR is anchored to the cell membrane via a GPI anchor [8], and the possibility existed that the inhibitory effect of mannosamine was mediated by a reduction in the amount of cell-bound uPAR and a concomitant increase in levels of uPAR in the culture medium, leading to a decrease in cell-associated plasminogen activation [9,10,31].

Zymographic determination of uPA activity did not, however, demonstrate either a decreased amount of cell-associated activity or increased levels of activity in culture media in the presence of mannosamine. This would have been expected if binding of uPA to the cell surface were prevented by a decrease in cell surface uPAR. Lisanti et al. [15] have observed reduced cell association and increased secretion of the GPI-anchored fusion protein gD-

1-DAF on treatment with mannosamine at a concentration of 10 mM.

Other studies [15–17], including one on uPAR, have used mannosamine concentrations in the range 2–10 mM, usually in low-glucose or glucose-free medium, to inhibit GPI anchor formation. Our study has shown that concentrations of mannosamine over four orders of magnitude lower than this significantly inhibited Ret- or cytokine-stimulated proteoglycan release, suggesting that concentrations of mannosamine effective at inhibiting proteoglycan release may not be high enough to inhibit GPI anchor formation. This was confirmed by studies of the effect of mannosamine on levels of chondrocyte cell-associated and soluble AP activity, where significant effects on the GPI anchoring of AP (as indicated by a decrease in cell-associated AP and an increase in AP activity in the culture medium) were observed only at 1 mM and 10 mM mannosamine for cytokine-stimulated cells.

Results from enzyme histochemistry of uPA-like activity also supported this interpretation, since little difference in fluorescence was observed in cells treated or not with mannosamine. However, when cells were treated with PI-PLC a reduction in fluorescence was seen, suggesting that some of the fluorescence was due to GPI-anchored uPA-like enzyme(s).

The effects of mannosamine, at low concentrations, on proteoglycan release may therefore be independent of its effects on GPI anchor formation. At higher concentrations, effects on GPI-anchored proteins cannot be excluded. Sandy et al. [32] have recently demonstrated complete inhibition of the aggrecanase response to IL1 and Ret by mannosamine at 0.45 mM in bovine cartilage explants, which appears to be dependent on the inhibition of synthesis of GPI anchors.

The mechanism(s) by which mannosamine exerts its inhibition of proteoglycan breakdown remains unknown. Its inhibitory action does not appear to be mediated by effects on metabolic activity, since even in glucose-free medium the inhibition by mannosamine of lactate production occurred only at concentrations much higher than those needed to stimulate proteoglycan release. Further work is required to elucidate the mechanism(s) involved.

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