Papers

Inhibitors of collagenase but not of gelatinase reduce cartilage explant proteoglycan breakdown despite only low levels of matrix metalloproteinase activity

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Aims—To investigate the level of matrix metalloproteinase activity during the time-course of cartilage explant proteoglycan breakdown; to determine the effects of selective small-molecule inhibitors of matrix metalloproteinases on proteoglycan degradation.

Methods—The levels of matrix metalloproteinase activity in cartilage explant cultures and conditioned media were monitored by use of a quenched fluorescent substrate. The constants for inhibition of certain matrix metalloproteinases by a series of synthetic inhibitors were determined. Bovine and human cartilage explant cultures were treated with interleukin-1, tumour necrosis factor or retinoic acid and the amount of proteoglycan released into the culture medium in the absence and presence of the inhibitors was quantified. Control experiments, examining the inhibition of other proteinases, and investigating possible toxic or non-specific effects of the inhibitors, were carried out.

Results—The profile of inhibition of proteoglycan release suggested the involvement of interstitial collagenase-like, rather than gelatinase- or possibly stromelysin-like, proteinases. No evidence was found for toxic or non-specific mechanisms of inhibition. Very low levels of activity of the known matrix metalloproteinases were present during the time-course of aggrecan breakdown.

Conclusions—A novel collagenase-like proteinase(s) may be involved in cartilage proteoglycan breakdown. Gelatinase-type matrix metalloproteinases do not seem to be involved in this process. Specific collagenase inhibitors may be therapeutically efficacious in the treatment of arthritis.

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Aggregating proteoglycan (aggrecan) and type II collagen are the major extracellular protein components of cartilage. Loss of proteoglycan and its associated water can compromise the structure and function of cartilage by reducing its resilience to compressive loading and increasing friction during joint motion.^{1,2} Thus, depletion of articular cartilage proteoglycan (seen on x ray film as joint space narrowing) may predispose the cartilage to further mechanical damage. Preventing pathological cartilage proteoglycan breakdown may therefore substantially protect the affected joint and help prevent the development of an arthritic lesion. The mechanism by which cartilage proteoglycan is degraded is not fully understood, although recent evidence would suggest the involvement of a number of proteolytic enzymes in a cascade reaction that may result in the activation of an as yet undiscovered enzyme, "aggrecanase" (reviewed elsewhere³).

There is evidence that members of the matrix metalloproteinase (MMP), or matrixin, family of enzymes are involved in cartilage proteoglycan breakdown. The MMPs, like almost all metalloproteinases that have been characterised so far, utilise a zinc atom in the active site.⁴ Based on their substrate specificity the MMPs can be divided into three groups: the collagenase group, including interstitial collagenase (EC 3.4.24.7); neutrophil collagenase (EC 3.4.24.34) and collagenase-3⁵; the stromelysin group, which includes stromelysin 1 (EC 3.4.24.17), stromelysin 2 (EC 3.4.24.22) and matrilysin (EC 3.4.24.23); and the gelatinase group consisting of gelatinases A (EC 3.4.24.24) and B (EC 3.4.24.35).⁶ Stromelysin 3 actually represents a distinct group of the MMPs.^{4,7} In in vitro experiments matrilysin and stromelysin 1 have been shown to be the most efficient members of this subfamily at catalysing aggrecan hydrolysis.⁸

Increased MMP concentrations have been found in both osteoarthritis⁹ and rheumatoid arthritis.¹⁰ Amounts of the MMPs are raised by the action of the proinflammatory cytokines, interleukin-1 (IL-1) and tumour necrosis

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factor (TNF), that mediate catabolic responses in cartilage.¹¹⁻¹⁴

The ability of synthetic inhibitors of the MMPs to suppress cartilage proteoglycan degradation in model culture systems is well documented,¹⁵⁻¹⁸ and it has been proposed that inhibitors of these enzymes might be used for therapeutic intervention in the arthritides.¹⁹ To date, all of the inhibitors found to have a chondroprotective effect are broad spectrum inhibitors that do not discriminate between the various MMPs. For instance, the most potent example to date, the hydroxamate BB94, is a tight binding inhibitor of all the MMPs investigated, with $K_{i(app)}$ and IC_{50} values in the low nanomolar range and varying by only about a factor of 10 between the different MMPs.¹⁵⁻²⁰ Such inhibitors therefore provide us with little information about which members of this large group of enzymes may be involved in cartilage proteoglycan breakdown.

We have now attempted to address the role of MMPs in cartilage proteoglycan breakdown by the use of a series of zinc chelating MMP inhibitors that demonstrate some discrimination for the inhibition of various MMPs, and also by the measurement of levels of MMP activity present in cartilage explant cultures during the time-course of aggrecan depletion.

Methods

Human interstitial procollagenase, promatrilysin and prostromelysin 1 were recombinant proteins purified from media conditioned by CHO cells transfected with cDNA coding for the respective enzymes. Progelatinases A and B were isolated from media harvested from the malignant melanoma cell line RPMI 7591, and the monocytic leukaemia cell line THP-1, respectively. The MMP inhibitors used in this study, BB87, BB250, BB2001, BB2014 and BB3003*, were synthesised at British Biotech Pharmaceuticals Ltd., Oxford, UK. Human cathepsin B (EC 3.4.22.1) was purified from liver by affinity chromatography²¹ and human cathepsin D (EC 3.4.23.5) from liver was prepared by the published method.²² Peptidyl dipeptidase A (angiotensin converting enzyme) (EC 3.4.15.1) was purified from rabbit lung.²³ Neprilysin (enkephalinase) (EC 3.4.24.11) was from rabbit kidney, and was obtained in the membrane bound form essentially as described.²⁴ Recombinant human IL-1 α (rhIL-1 α) (specific activity 5×10^7 U/mg) was a gift from Dr K Ray, Glaxo Group Research Ltd, Greenford Road, Greenford, Middlesex, UK. Recombinant human TNF α (rhTNF α) (specific activity 8×10^7 U/mg) was kindly provided by Dr E Amento, Genentech Inc. Boulder, Colorado, USA.¹

The recombinant human IL-1-receptor antagonist protein was a gift from Synergen Inc.,

*The structures of the MMP inhibitors are: BB87, N4-hydroxy-2R-isobutyl-N1-(2-phenyl-1S-[(pyrid-2-ylmethyl)-carbamoyl]-ethyl)-succinamide; BB250, N4-hydroxy-2R-isobutyl-N1-(1S-methyl-carbamoyl-2-phenyl-ethyl)-3S-thiophene-2-sulfonyl-methyl succinamide; BB2001, 3R{1S(3-morpholin-4-yl-propylcarbamoyl)-2-phenyl-ethyl-carbamoyl}-6-phenyl-hexanoic acid; BB2014, 3R-(1S-methylcarbamoyl-2-phenyl-ethylcarbamoyl)-6-phenylhexanoic acid; BB3003, 3R-(1S-methylcarbamoyl-2-phenyl-ethylcarbamoyl)-nonadecanoic acid, dicyclohexylamine salt.

Boulder, Colorado, USA. All-trans-retinoic acid (Ret), gentamicin, cortisol, chondroitin sulfate A, aminophenylmercuric acetate (APMA) and dimethyl sulfoxide were purchased from Sigma Chemical Co., Poole, Dorset, UK. Dulbecco's modification of Eagle's medium (DMEM), minimal essential medium (MEM), newborn-calf serum (NCS), fetal calf serum (FCS), amphotericin B, penicillin, and streptomycin were purchased from Gibco Life Technologies Ltd, Paisley, UK. 1,9-Dimethylmethylene blue was from Aldrich, Gillingham, Dorset, UK. Cetylpyridinium chloride, Triton X-100 and OCT embedding medium were from BDH Chemicals Ltd, Poole, Dorset, UK. 7-Methoxycoumarin-4-ylacetyl (Mca)-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl) (Dpa)-Ala-Arg-NH₂ was provided by Dr C Graham Knight, Strangeways Research Laboratory, Cambridge, UK. Human urokinase-type plasminogen activator (uPa) (EC 3.4.21.73) was obtained from Calbiochem-Novabiochem Ltd, Nottingham, UK. Benzylloxycarbonyl (Z)-Gly-Gly-Arg-aminomethylcoumarylamide (NHMeC) and Z-Arg-Arg-NHMeC were the products of Bachem Feinchemikalien AG, Bubendorf, Switzerland. ³⁵SO₄ and ³⁵S-methionine were products of Amersham International plc, Little Chalfont, Buckinghamshire, UK. All buffers and salts were of analytical grade.

PROTEINASE ASSAYS

The effects of one of the MMP inhibitors, BB250 (10 μ M), on the activities of uPa, cathepsin B and cathepsin D, which have been implicated in cartilage proteoglycan breakdown,³⁻²⁵ were tested. In addition, we examined the capacity of BB250 to inhibit two metallopeptidases that are not MMPs—that is, peptidyl dipeptidase A and neprilysin.

uPa activity was measured by continuous rate assays in 50 mM Tris HCl, 0.1% Triton X-100, pH 7.5 at 37°C, with Z-Gly-Gly-Arg-NHMeC as substrate (100 μ M). The rate of substrate hydrolysis was recorded before and after the addition of the inhibitor to the cuvette. The effect of BB250 on human cathepsin B activity was determined with Z-Arg-Arg-NHMeC (5 μ M) as substrate in 100 mM Na₂H/KH₂PO₄, pH 6.0, containing 1 mM Na₂EDTA and 2 mM dithiothreitol. The activity of cathepsin D in the presence and absence of BB250 was measured at 45°C using 8% (w/v) haemoglobin as substrate in 0.1 M formate buffer, pH 3.3. Following precipitation of undigested protein in 3% (w/v) trichloroacetic acid and filtration the change in A₂₈₀, as compared with a blank without enzyme, was determined. Peptidyl dipeptidase A activity was assayed using N-benzoylglycine-His-Leu, as described previously.²³ Neprilysin was assayed using the quenched fluorescent substrate dansyl-D-Ala-Gly-Phe(pNO₂)-Gly.²⁴

MMP activity in bovine nasal cartilage explant cultures and conditioned media was assayed by use of the quenched fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂. This substrate is cleaved efficiently by all of the MMPs

tested so far, including interstitial collagenase, stromelysin 1, matrilysin, gelatinase A,²⁶ gelatinase B,¹⁵ neutrophil collagenase, and collagenase-3,²⁷ and can therefore be used to assess the combined levels of activity of these enzymes. The MMP activity in conditioned media from explants cultured with or without rhIL-1 α (0.3 nM), rhTNF α (3.5 nM) or Ret (1 μ M) for up to five days, with and without APMA (0.1 mM; one hour) activation of pro-MMPs, was assessed by continuous-rate assays at 37°C. The buffer used was 0.1 M Tris HCl, pH 7.5, containing 0.1 M NaCl, 10 mM CaCl₂, 0.2% Triton X-100, and Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (5 μ M) (ex. 328 nm, em. 393 nm). The fluorimeter was standardised with a known concentration of Mca-Pro-Leu.

In order to obviate potential problems of fluorescence quenching, the substrate (10 μ M) was added to explant cultures (two explants of approximate dimensions 2 $\times$ 2 $\times$ 2 mm/culture) in 0.4 ml phosphate buffered isotonic saline (PBS), pH 7.2, containing 4.5 mg/ml D-glucose and antibiotics (see later), for periods of up to 24 hours. We have previously found that cartilage explants can be successfully cultured for at least two days under these conditions, during which time they behave in a similar manner to explants cultured in DMEM, and resorb the proteoglycan component of the extracellular matrix upon treatment with catabolic stimuli. BB94¹⁵ (10 μ M) was also added to some cultures; 0.3 ml of each culture was diluted with an equal part of Tris buffer (earlier), and the quantity of product formed was determined by comparing the amount of fluorescence with that of a known concentration of standard.

DETERMINATION OF KINETIC PARAMETERS

Gelatinase A was activated overnight at 4°C in 1mM APMA. Gelatinase B was activated by the same means at ambient temperature for two hours. Interstitial collagenase was activated with 0.66 mM APMA for 16 hours at 37°C, prior to purification. Purified stromelysin 1 was activated with trypsin (30 minutes, 37°C), and matrilysin was autoactivated by freeze-thawing prior to purification.

For the determination of IC₅₀ values, all assays were performed at 37°C for 16 hours in 25 mM Hepes, pH 7.5, containing 5 mM CaCl₂, 0.05% (w/v) Brij 35 and 0.02% NaN₃. Collagenase activity was determined in an assay²⁸ utilising ¹⁴C-collagen as substrate.²⁹ Matrilysin and stromelysin 1 activities were measured in assays using ¹⁴C-casein as the substrate.³⁰ Gelatinase A and B activities were assayed by a modification of the previously described procedure,³¹ with the addition of 50 μ g/ml gelatin. IC₅₀ values were found by comparing enzyme activity in the presence of varying concentrations of the inhibitors, and fitting a curve to the data thus obtained. The inhibitor concentration that gave 50% inhibition was then acquired by interpolation.

CARTILAGE CULTURES

Most of the work reported here has been done on normal bovine cartilage, which has been

shown previously to behave catabolically in a manner similar to the human tissue.³ However, a limited number of experiments were done using two samples of non-arthritis human cartilage. One sample was from the femoral condyle of an 81 year old man with no history of arthritis disease. The second sample was from the interphalangeal joint of the left big toe of a 14 year old boy who underwent fusion of the joint in response to a non-arthritis foot deformity. Cartilage was sliced, to give an explant size of approximately 3 $\times$ 3 $\times$ 2 mm. The slices were pre-incubated in MEM containing 10% (v/v) FCS, penicillin (100 u/ml) and streptomycin (100 μ g/ml) for two days. They were then transferred individually to wells of a 96-well plate containing 195 μ l MEM containing 1% (v/v) FCS to which the inhibitors were added from 100-fold concentrated stock solutions in methanol. After one hour Ret was added to a final concentration of 1 μ M. The cultures were maintained for five days after which the media and explants were stored frozen pending analysis.

Bovine nasal septum cartilage explants were prepared and preincubated as described previously.³² The cartilage discs were transferred individually to wells of a 96-well plate, with 195 μ l of serum-free DMEM to which the metalloproteinase inhibitors were added from 100-fold stock solutions in methanol. A 1% (v/v) concentration of methanol did not affect cartilage catabolism (data not shown). After one hour, rhIL-1 α , rhTNF α , or Ret was added to final concentrations of 250 U (0.3 nM), 5000 U (3.5 nM), or 10⁻⁶ M, respectively. The cultures were maintained for up to five days with a medium change after two days, the fresh medium containing exactly the same constituents as the original. Conditioned media and discs were stored frozen pending analysis. In studies examining the effects of the MMP inhibitors on stimulated proteoglycan release from all types of cartilage examined, only experiments in which the addition of the stimulus resulted in at least a doubling of the amount of proteoglycan release were included in the analyses. This helped to ensure that the detected inhibition was of stimulated, as well as basal, release.

Bovine articular cartilage slices were dissected from metacarpophalangeal joints and preincubated as reported previously,¹⁵ after which the explants were treated as described earlier for the nasal cartilage discs. As shown elsewhere¹⁵ articular cartilage was less responsive than nasal cartilage to the presence of human cytokines. The concentrations of rhIL-1 α and rhTNF α were therefore increased to 1800 U (2 nM) and 10 000 U (7 nM), respectively. The cultures were maintained for five days with a medium change on day 2, after which the media and cartilage pieces were stored frozen.

DETERMINATION OF PROTEOGLYCAN DEGRADATION

The amount of proteoglycan in the culture medium was determined as sulfated glycosaminoglycan by use of dimethylmethylen

Table 1 Constants for the inhibition of some members of the MMP subfamily by a series of MMP inhibitors

	IC ₅₀ values (nM)				
	BB250	BB87	BB2001	BB2014	BB3003
Interstitial collagenase	5	30	12 000	15 000	>100 000
Stromelysin 1	20	600	7000	1000	>100 000
Gelatinase A	20	30	100	30	30
Gelatinase B	2	10	2000	150	3000
Matrilysin	10	80	12 000	>100 000	>100 000

blue by adaptation of the method³³ for use with a plate reader.³⁴ The binding of sulfated glycosaminoglycan to the dye was accompanied by a metachromatic shift, measured as a decrease in absorbance at 570 nm. Determination of residual sulfated glycosaminoglycan in the cartilage explants was by the same method following digestion with papain.³⁵

HISTOLOGY

Cartilage explants cultured with or without a catabolic stimulant or MMP inhibitor were embedded in OCT, snap frozen in liquid N₂ cooled isopentane and stored at -70°C until immediately before use. Sections (7 µm) were cut on a cryostat, placed on slides and left to dry in air for 30 minutes. The sections were then fixed in 4% paraformaldehyde in phosphate buffer, pH 7.2, for 15 minutes and stained with 1% (w/v) toluidine blue in 3% (v/v) acetic acid, pH 2.5, for 15 seconds, destained in 3% (v/v) acetic acid for 30 seconds and mounted using glycerine.

LACTATE DETERMINATION

The concentration of lactate in the medium was determined with a kit supplied by Sigma Chemical Co. which uses the lactate oxidase/peroxidase method.

PROTEIN SYNTHESIS

Bovine nasal cartilage explants were cultured with or without rhIL-1α and MMP inhibitor for two days. The explants were washed in DMEM and given a two hour pulse with ³⁵S-methionine and then treated as described previously.³²

PROTEOGLYCAN SYNTHESIS

The amount of incorporation of ³⁵SO₄ into cetylpyridinium chloride precipitates was assessed by the previously described method³⁶ with the modification that the explants were cultured in DMEM rather than SO₄-free Iscove's MEM.

STATISTICAL ANALYSES

The results were analysed using the two-tailed Mann-Whitney U test for non-parametric data.

Results

CONSTANTS FOR MMP INHIBITION

IC₅₀ values for the inhibition of the MMPs are given in table 1. These results demonstrate that BB250 (like BB94, already shown to be a potent inhibitor of cartilage proteoglycan breakdown¹⁵) is a tight binding inhibitor of all the MMPs tested. BB87 is a tight binding

inhibitor of interstitial collagenase, gelatinases A and B, but inhibits stromelysin 1 relatively poorly. BB2001, BB2014 and BB3003 are selective inhibitors of gelatinase A. We also examined the inhibition of two non-matrix metallopeptidases, peptidyl dipeptidase A and neprilysin, by the MMP inhibitors. In all cases, IC₅₀ values for the inhibition of these two enzymes were greater than 10 000 nM (results not shown). We could detect no inhibition of human cathepsin B, human cathepsin D or human uPa by BB250 (10 µM) (results not shown).

STIMULATION OF PROTEOGLYCAN BREAKDOWN FROM BOVINE CARTILAGE

Although there was some variability between the behaviour of bovine nasal cartilage from different animals, greater than 50% release of total cartilage proteoglycans was usually achieved by two days of culture with 0.3 nM rhIL-1α. The same level was often not reached until five days in the presence of 1 µM Ret. rhTNFα elicited a catabolic response in bovine nasal cartilage, leading to a notable increase in release of proteoglycan, as reported previously.³⁷ The tissue was less responsive to rhTNFα than to rhIL-1α, however, such that more than a 10-fold higher concentration (3.5 nM instead of 0.3 nM) was required to cause, on average, a 50% release of proteoglycan by day 5 (data not shown). The IL-1-receptor antagonist protein, when present in a 100-fold molar excess, completely inhibited rhIL-1α stimulated proteoglycan breakdown from nasal explants. TNF stimulated release, however, was not inhibited by this concentration of the receptor antagonist, demonstrating that TNF stimulated proteoglycan release did not occur as a secondary response to TNF stimulation of IL-1 synthesis by the chondrocytes (results not shown).

Similar results were found using bovine metacarpophalangeal cartilage. In terms of stimulation of proteoglycan breakdown this tissue was generally less responsive to the cytokines, such that the concentrations of rhIL-1α and rhTNFα had to be increased (see the Methods section) in order to achieve a 50% release of total proteoglycan by day 5. In contrast, the response to Ret was not very different between the two bovine cartilages.

EFFECTS OF THE MMP INHIBITORS ON BASAL AND STIMULATED PROTEOGLYCAN RELEASE FROM CARTILAGE EXPLANTS

We first examined the effect of a fixed concentration (10 µM) of the MMP inhibitors on basal and stimulated proteoglycan release from bovine nasal septum cartilage explants. Only two of the five inhibitors tested produced statistically significant inhibition of stimulated release, BB250 and BB87 (table 2). These compounds, both of which are tight binding inhibitors of interstitial collagenase (table 1), were potent inhibitors of proteoglycan release stimulated by rhIL-1α, rhTNFα and Ret. All of the inhibitors produced some inhibition of basal (unstimulated) proteoglycan breakdown

Table 2 Percentage inhibition of proteoglycan release from bovine cartilage explants

Inhibitor (10 μM)	Basal	Stimulant		
		rhIL-1α	rhTNFα	Ret
<i>Nasal</i>				
BB250	48 (3)†	105 (5)†	28 (7)*	78 (4)†
BB87	61 (4)†	108 (2)†	97 (4)†	85 (5)†
BB2001	12 (7)	5 (18)	-13 (10)	-4 (4)
BB3003	22 (9)	-15 (14)	-14 (3)	-6 (6)
BB2014	32 (4)†	-10 (17)	-68 (12)*	-19 (7)
<i>Articular</i>				
BB87	56 (6)†	137 (10)*	146 (6)†	104 (9)*
BB250	76 (3)†	110 (13)†	163 (4)†	91 (6)*

In the analyses of stimulated release basal levels of proteoglycan release were subtracted. The results are shown as percentage inhibition of proteoglycan release, expressed for purposes of clarity as mean (SEM). *p < 0.005; †p < 0.0005 compared with controls. Values of greater than 100% inhibition of stimulated release reflect total inhibition of stimulated release plus some inhibition of the basal level.

Table 3 Dose response for the inhibition of rhIL-1α stimulated proteoglycan release from bovine nasal cartilage explants by BB87 and BB250

Inhibitor concentration (µM)	Per cent inhibition	
	BB250	BB87
0.001	27 (20)	
0.01	43 (17)	
0.1	26 (7)*	7 (6)
1	77 (5)†	35 (6)†
10	105 (5)†	108 (2)†

Cartilage explants were cultured for two days with or without rhIL-1α and varying concentrations of the MMP inhibitors before the culture media and explants were analysed for sulfated glycosaminoglycan content. Results are expressed as mean (SEM). *p < 0.005; †p < 0.0005 compared with controls.

(table 2), though again BB250 and BB87 were the most potent. Inhibition of basal release of proteoglycan by the gelatinase inhibitor BB2014 was also statistically significant. BB2014 produced a statistically significant stimulation of proteoglycan release in the presence of rhTNFα, however. Cells responsive to TNF are capable of shedding their TNF receptors, a process dependent on a proteolytic cleavage.³⁸ The soluble receptors then compete with signalling receptors for the ligand. Our result could thus be explained by the inhibition by BB2014 of a proteinase involved in TNF receptor shedding, or indeed of an enzyme that is capable of degrading TNFα. These possibilities were not explored further.

We next determined whether BB250 and BB87 were capable of inhibiting proteoglycan breakdown from bovine metacarpophalangeal cartilage. As can be seen in table 2, a 10 µM concentration of either inhibitor produced virtually complete inhibition of rhIL-1α, rhTNFα and Ret stimulated release, as well as a substantial inhibition of basal proteoglycan breakdown.

In cultures of rhIL-1α stimulated nasal cartilage explants a dose dependent inhibition of proteoglycan release was seen in the presence of both BB87 and BB250 (table 3). At a concentration of 1 µM both inhibitors produced statistically significant reductions in proteoglycan release from the tissue, though BB250 seemed to be the more potent. This was confirmed at the lower concentration of 0.1 µM, where BB250, but not BB87, produced statistically significant inhibition.

A small number of experiments were carried out to investigate the inhibitory effects of BB87

and BB250 on human articular cartilage proteoglycan breakdown. As described in the Methods section, we obtained two samples of non-arthritic cartilage. Both of these responded catabolically to the presence of 1 µM Ret, releasing about 40% of total glycosaminoglycan by day 5 in the presence of the vitamin, and about 10% in its absence. BB250 and BB87 at a dose of 10 µM both strongly inhibited the release of proteoglycan from human cartilage. In one sample, basal levels of release were inhibited by (mean (SEM)) 32% (12%) (p < 0.001) by BB250 and 41% (6%) (p < 0.0001) by BB87. Inhibition of Ret stimulated release was even more notable, the two inhibitors giving 93% (5%) (p < 0.001) and 99% (4%) (p < 0.001), respectively. In a second experiment with cartilage from a different patient, BB250 at a concentration of 10 µM produced 38% (16%) (NS) inhibition of basal release and 102% (5%) (p < 0.001) inhibition of Ret stimulated proteoglycan release.

The effects of rhIL-1α and BB87 on bovine nasal cartilage explants were assessed histologically, by use of toluidine blue, which binds to sulfated glycosaminoglycans. Cartilage cultured for 24 hours in the absence of catabolic stimulus retained most of its metachromasia and hence its proteoglycan content (fig 1A), the sections staining darker in the territorial than in the interterritorial matrix. This was also the case with cartilage taken from freshly slaughtered cattle and processed for histology without the culture period (not shown). Explants cultured in the presence of rhIL-1α for 24 hours stained less intensely (fig 1B). The presence of BB250 in rhIL-1α treated 24 hour cultures was chondroprotective, the histological appearance (fig 1C) being very similar to that of the unstimulated controls (fig 1A).

EFFECTS OF BB87 AND BB250 ON THE METABOLIC ACTIVITY OF NASAL SEPTUM CARTILAGE EXPLANTS

Proteoglycan release from cartilage explants can be inhibited non-specifically by toxic compounds such as iodoacetate.³² We therefore evaluated the effects of the inhibitory compounds on some metabolic activities of chondrocytes, and determined whether the inhibitory effect on proteoglycan degradation was reversible.

The ability of nasal cartilage explants to recover from the effects of BB87 and BB250 was investigated. The explants were cultured for two days with rhIL-1α (0.3 nM) and either of the inhibitors (10 µM) after which the explants were allowed to recover in cytokine-free DMEM for 24 hours. The explants were then placed in DMEM containing 0.3 nM rhIL-1α. During the first incubation with inhibitor, BB87 and BB250 inhibited rhIL-1α stimulated release by 78% and 59%, respectively. Because of the inhibition of proteoglycan breakdown during the first incubation with the inhibitors, these explants retained a larger amount of glycosaminoglycan that could be subsequently lost from the tissue. Indeed, when these explants were thereafter stimulated with rhIL-1α in the absence of the inhibitors they

Figure 1 Toluidine blue staining of sections from bovine nasal cartilage explants cultured in the absence and presence of rhIL-1 α and BB87. Sections were stained with 1% (w/v) toluidine blue. (A) Section from a cartilage explant cultured for 24 hours in DMEM alone. (B) Section from a cartilage explant cultured in the presence of rhIL-1 α for 24 hours. (C) Section from a cartilage explant cultured in the presence of rhIL-1 α and BB87 (10 μ M) for 24 hours. Original magnification $\times 200$.

released even more sulfated glycosaminoglycan (a mean of 1728 μ g/disc following exposure to BB250 and 1548 μ g/disc after BB87 treatment) than discs treated with rhIL-1 α and no inhibitor for each incubation (404 μ g/disc), showing that the effects of the inhibitors were reversible.

Chondrocytes are known to respire largely anaerobically.³⁹ IL-1 causes increased lactate excretion by fibroblasts,⁴⁰ rheumatoid synovial cells⁴¹ and cartilage cultures.⁴² In two separate experiments we again found that rhIL-1 α increased the concentrations of lactate in the culture media. However, BB87 and BB250 (10 μ M) had no significant effect on lactate production either in the presence or absence of this cytokine (results not shown).

BB87 and BB250 were also tested for their effects on protein synthesis, as assessed by incorporation of ³⁵S-methionine into material that was insoluble in trichloroacetic acid. BB250 had no effect on ³⁵S-methionine incorporation, either in the presence or absence of rhIL-1 α . BB87 slightly decreased ³⁵S-methionine incorporation (19.7% at a concentration of 10 μ M) in the absence of IL-1, but had no effect in its presence.

It is possible that proteinase inhibitors affect signal transduction pathways and subsequent gene expression rather than the activities of proteolytic enzymes involved specifically in the breakdown of cartilage proteoglycan. IL-1, TNF and Ret inhibit the synthesis of cartilage proteoglycan by chondrocytes.^{37 43 44} We therefore examined the effect of BB87 on rates of proteoglycan synthesis by nasal cartilage discs in the presence of rhIL-1 α , in the knowledge that interference with signal transduction pathways by the inhibitor would be manifested by an increase in the rate of synthesis when compared with the effect of the cytokine on its own. The MMP inhibitor at a concentration of 10 μ M did not seem to have any effect on rates of proteoglycan synthesis. The control level of incorporation of 7900 (700) (mean (SEM)) counts per minute (cpm)/disc was not significantly altered by the presence of BB87 (7200 (700) cpm/disc). As expected, rhIL-1 α significantly depressed ³⁵SO₄ incorporation, to 3000 (200) cpm/disc ($p < 0.0001$). Again, this value was not significantly changed by the presence of BB87 (2900 (200) cpm/disc).

We conclude from these studies on the metabolic activity of cartilage explants that the inhibitory action of the MMP inhibitors on proteoglycan breakdown is likely to be due to a specific inhibition of the activity of a metallo-proteinase, rather than to any toxic or non-specific effect of the compounds.

LEVELS OF MMP ACTIVITY DURING THE TIME-COURSE OF PROTEOGLYCAN BREAKDOWN

As described in the Methods section, we used a quenched fluorescent substrate that is cleaved by all the MMPs to monitor the appearance of MMP activity during the time-course of proteoglycan breakdown in bovine nasal septum cartilage cultures. Very low levels of activity were found in media conditioned by the explants, even after five days of culture, irrespective of whether the explants were cultured in the presence or absence of agents that stimulate proteoglycan breakdown (table 4), even though the amount of glycosaminoglycan released into the medium was increased by at least threefold in the presence of the catabolic stimuli (results not shown). Slightly

Table 4 Quenched fluorescent substrate hydrolysing activity in media conditioned by bovine nasal cartilage explants

Stimulant	Days 0–2		Days 3–5	
	APMA			
	–	+	–	+
None	0.39 ± 0.12	0.75 ± 0.72	0.48 ± 0.30	2.61 ± 1.50
rhIL-1 α	0.21 ± 0.21	1.14 ± 0.81	—	—
rhTNF α	0.57 ± 0.33	1.29 ± 0.78	0.51 ± 0.33	2.04 ± 1.33
Ret	0.60 ± 0.21	2.28 ± 1.05	0.36 ± 0.27	2.46 ± 1.74

Explants were cultured in serum-free DMEM with or without a catabolic stimulant for up to five days with one medium change on day 2. The conditioned media were assayed for Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂-hydrolysing activity in continuous rate assays as described in Methods. The results are expressed as pmol/min of product per explant.

Table 5 Quenched fluorescent substrate hydrolysing activity in bovine nasal explant cultures

Stimulant	BB94	Time (hours)				
		1	2	4	6	24
None	–	0.78 ± 0.04	0.66 ± 0.05	0.84 ± 0.06	0.78 ± 0.07	0.54 ± 0.03
None	+	0.66 ± 0.05	0.84 ± 0.08	0.60 ± 0.04	0.66 ± 0.05	0.54 ± 0.03
rhIL-1 α	–	0.78 ± 0.07	0.72 ± 0.06	0.84 ± 0.07	0.60 ± 0.04	0.48 ± 0.04
rhIL-1 α	+	0.72 ± 0.05	0.54 ± 0.06	0.66 ± 0.05	0.60 ± 0.04	0.48 ± 0.01

Explants were cultured in PBS and glucose, as described in Methods, in the presence of Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (10 μ M), with or without BB94 (10 μ M). The increase in fluorescence, as compared with the amount of fluorescence in unconditioned PBS, was then determined. The results are expressed as pmol/min of product per explant.

more activity was present following treatment with APMA, suggesting the presence of low amounts of proMMPs. The low levels of activity generated during the time-course of proteoglycan breakdown (≤ 0.6 pmol/min substrate hydrolysed/explant, without APMA treatment) can be contrasted with the level of over 15 000 pmol/min/explant found in week 3 medium from explants cultured in the presence of IL-1 α , when collagen breakdown is occurring.⁴⁵ There is thus a difference in MMP activity of over four orders of magnitude, between the times of proteoglycan and collagen breakdown.

It is possible that the bulk of MMP activity in our cultures remained associated with the explants rather than being secreted into the surrounding medium. It has been reported that active stromelysin 1⁴⁶ and other MMPs bind to components of the extracellular matrix, and indeed the enzymes can be extracted from solid tissues.⁴⁷ In order to examine this possibility we determined the levels of MMP activity in the cultures themselves, as opposed to the activity present in isolated conditioned media (earlier). Once again, the levels of active enzyme were very small, and there were no differences in the amounts of activity detected in cultures with and without rhIL-1 α , or any increase of enzyme activity with time (table 5). Most important was the finding that the activity was not inhibited by BB94, a broad spectrum MMP inhibitor that also inhibits proteoglycan breakdown.¹⁵ We conclude from these experiments that MMP activity is very low during the first few days in cartilage explants cultured in the presence of agents, such as IL-1 and TNF, that have been reported to promote the expression of these enzymes^{11–14} as well as proteoglycan breakdown. Instead, we found that levels of active MMPs in cartilage explant cultures correlated with collagen breakdown.⁴⁵

Discussion

The results presented here are in agreement with earlier work demonstrating the efficacy of MMP inhibitors at limiting the breakdown of cartilage proteoglycan in experimental systems.^{15–18} Our findings also suggest that at least one metalloproteinase may be involved irrespective of the catabolic stimulus to cartilage breakdown (the cytokines IL-1 and TNF, or the vitamin Ret) in both bovine and human systems. As far as we are aware, this study is the first to investigate the effect of MMP inhibitors on TNF stimulated breakdown and the first to demonstrate inhibition of proteoglycan release from human cartilage by MMP inhibitors.

Our results clearly show the lack of inhibition of stimulated proteoglycan breakdown by compounds, BB2001, BB2014 and BB3003, that show a considerable degree of selectivity for the inhibition of gelatinase activity. It therefore seems unlikely that gelatinase(s) is involved in proteoglycan breakdown stimulated by IL-1, TNF or Ret in our model systems.

We found that inhibitors of collagenase-type MMPs were effective at inhibiting cartilage proteoglycan breakdown, implicating the activity of a collagenase-like MMP in proteoglycan loss. We must interpret this result with some caution, because inhibitor concentrations well in excess of the IC₅₀ values determined in *in vitro* experiments must be used to obtain inhibition of proteoglycan breakdown in tissue systems. An example of this phenomenon was our earlier work with BB94,¹⁵ an inhibitor with low nanomolar inhibition constants with all the MMPs tested so far. Even so, in terms of the inhibition of proteoglycan breakdown, concentrations of about 0.1 μ M were required to effect 50% inhibition. The reasons for this apparent discrepancy are not yet clear, but may be related to problems of accessibility to the target enzyme in cartilage, competition between the inhibitor and very high concentrations of substrate within cartilage or, alternatively, may suggest that the physiologically important enzyme has yet to be identified. Our finding that very low levels of activity of the known MMPs were present in cultures during the time-course of proteoglycan breakdown strengthens the latter possibility. It is worth noting that compounds designed to inhibit the MMPs are not necessarily entirely specific for this family of enzymes. Some other metalloproteinases share sufficient structural similarity, particularly around the active site, to be grouped together with the MMPs as the metzincins⁴⁸ or proteinase clan MB.⁴ Thus, the structural determinants that govern interactions with small molecule inhibitors may be shared features between these families of metalloproteinases. An example is the binding with similar affinity of a small molecule inhibitor to gelatinase A and to atrolysin C (EC 3.4.24.42), a snake venom proteinase of the repolysin family.⁴⁹ Mammalian representatives of the repolysin family have been identified.⁴

Stromelysin 1 has long been considered to be the most likely MMP to be involved in cartilage breakdown, either by a direct mechanism⁹ or via activation of other proMMPs, such as

procollagenase.⁵⁰ However our data might argue against a role for stromelysin 1 in that BB87, a potent inhibitor of cartilage proteoglycan breakdown (table 2), is a 20-fold tighter binding inhibitor of interstitial collagenase than it is of stromelysin 1 (table 1). Although a recent independent study⁵¹ has also provided evidence against a role for stromelysin in chondrocyte mediated proteoglycan breakdown, confirmation will await the results of experiments using probes that are more selective for stromelysins.

The loss of aggrecan from human cartilage, both in various disease states and in tissue culture models, predominantly occurs coincident with a specific proteolytic cleavage produced by the action of an as yet unidentified proteinase referred to as "aggrecanase", at a Glu-Ala bond within the interglobular region towards the N-terminus of aggrecan. This results in the loss from the matrix of almost the complete aggrecan molecule.^{15 52-55} The Glu-Ala bond is not cleaved in vitro by any known MMP other than neutrophil collagenase (EC 3.4.24.34),⁵⁶ perhaps suggesting that neutrophil collagenase is "aggrecanase" (reviewed elsewhere³). Although data presented here, and the fact that neutrophil collagenase has been identified recently in chondrocytes,⁵⁷ would seem to support this hypothesis, there are observations that lend credence to the view that the enzyme responsible for aggrecan cleavage is not neutrophil collagenase. Neutrophil collagenase cleaves another bond in the interglobular region of aggrecan, a Asn-Phe bond that is the favoured cleavage site of most MMPs,⁵⁸⁻⁶⁰ more efficiently than it cleaves the Glu-Ala bond.⁵⁶ Although the product of cleavage of the Asn-Phe bond can be found in human cartilage,⁶¹ the available evidence suggests that this is only a minor product and that cleavage at the Glu-Ala bond prevails in the human arthritic diseases⁵⁴ and in cartilage culture systems.⁵⁵

Similarly, there is evidence that interstitial collagenase and collagenase-3 are not responsible for aggrecan breakdown. Although interstitial collagenase is capable of slowly cleaving aggrecan in the test tube,⁶² and cleaves the interglobular region at two distinct sites,⁵⁹ neither of these correspond to the "aggrecanase" cleavage site. Most importantly we have demonstrated that virtually no active collagenase is produced by bovine cartilage explants during the first week of culture.⁴⁵ It is also worth noting that the natural protein inhibitor of the MMPs, Timp1, does not inhibit cartilage explant proteoglycan release.¹⁶ This observation, together with the very low levels of MMP activity present in our cultures, virtually precludes a role for any of the known MMPs in aggrecan degradation. Our results do indicate, however, that a collagenase-like metalloproteinase may be important in aggrecan breakdown, and the previous demonstration of "aggrecanase"-like activity by neutrophil collagenase⁵⁶ may suggest that "aggrecanase" is a metalloproteinase with some active site features similar to those of the neutrophil enzyme.

The cascade nature of the events leading up to aggrecan breakdown³ unfortunately do not

allow us to discriminate between the inhibition of an enzyme acting directly on aggrecan as opposed to an enzyme involved in "proaggrecanase" activation. The involvement of members of the gelatinase group of MMPs in the events leading to aggrecan breakdown in our model system does now seem unlikely, however.

In any event, the demonstration of potent inhibition of chondrocyte mediated aggrecan breakdown by inhibitors of collagenase-like MMPs may provide a lead for the development of selective inhibitors as therapeutic agents for the treatment of arthritic diseases. The same inhibitors also inhibit type II collagen breakdown in cartilage explants⁴⁵ and discriminating inhibitors are less likely to produce unwanted side effects than those with a broader inhibition profile.

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