Inhibition of bovine nasal cartilage degradation by selective matrix metalloproteinase inhibitors

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N-terminal analysis of aggrecan fragments lost from bovine nasal cartilage cultured in the presence of recombinant human interleukin 1 α revealed a predominant ARGSVIL sequence with an additional ADLEX sequence. Production of the ARGSVIL-containing fragments has been attributed to the action of a putative proteinase, aggrecanase. The minor sequence (ADLEX) corresponds to a new reported cleavage product; comparison of this sequence with the available partial sequence of bovine aggrecan indicates that this is the product of a cleavage occurring towards the C-terminus of the protein. Matrix metalloproteinase (MMP) inhibitors inhibited aggrecan loss from bovine nasal explants incubated in the presence of recombinant human interleukin 1 α . A strong correlation between inhibition of aggrecan metabolism and inhibition of stromelysin 1 (MMP 3)

($r = 0.93$) suggests a role for stromelysin or a stromelysin-like enzyme in cartilage aggrecan metabolism. However, the compounds were approx. 1/1000 as potent in inhibiting aggrecan loss from the cartilage explants as they were in inhibiting stromelysin. There was little or no correlation between inhibition of aggrecan metabolism and inhibition of gelatinase B (MMP 9) or inhibition of collagenase 1 (MMP 1). Studies with collagenase inhibitors with a range of potencies showed a correlation between inhibition of collagenase activity and inhibition of collagen degradation in the cartilage explant assay. This indicates that in interleukin 1 α -driven bovine nasal cartilage destruction, stromelysin (or a closely related enzyme) is involved in aggrecan metabolism, whereas collagenase is principally responsible for collagen degradation.

INTRODUCTION

Cartilage is a fibre-reinforced matrix whose biomechanical properties allow it to resist the compressive, shear and frictional forces after joint motion and load bearing. Pathological remodelling of aggrecan and type II collagen resulting in destruction of articulating cartilage is a feature of both rheumatoid arthritis and osteoarthritis [1]. Aggrecan, the major proteoglycan of articulating cartilage, is a multi-domain glycoprotein, immobilized within a type II collagen network. The hydrophilic characteristics of aggrecan are largely responsible for the biomechanical properties of articulating cartilage. The highly charged keratin and chondroitin sulphate side chains linked to the aggrecan backbone induce a swelling pressure within cartilage, whereas type II collagen, the main collagen component of cartilage, gives the tissue its tensile strength.

Matrix metalloproteinases (MMPs; collagenases, stromelysins, gelatinases and others) [2] are produced by cartilage chondrocytes in response to inflammatory stimuli such as interleukin 1 (IL-1) and tumour necrosis factor α . These enzymes, acting in concert, can degrade all the extracellular matrix components of cartilage. Collagenases are the only enzymes capable of cleaving triple helical collagen within the helical region, whereas stromelysin and other MMPs have been shown to be capable of cleaving aggrecan [3]. Indeed, broad-spectrum inhibitors of these enzymes have been reported to inhibit both aggrecan and collagen degradation in cartilage explant cultures [4–7].

Large aggrecan fragments are lost from cartilage explants

incubated in the presence of IL-1 [8] and also from articular cartilage in inflamed arthritic joints [9–12]. Analysis of these fragments shows that the loss is the result of cleavage in the region between the G1 and G2 globular domains of the aggrecan molecule. Cleavage takes place between Glu³⁷³ and Ala³⁷⁴, resulting in two fragments with a C-terminal NITEGE and an N-terminal ARGSVIL sequence. Specific antibodies to the C-terminal sequence NITEGE recognize this epitope in medium used to culture rat chondrosarcoma cells that synthesize and metabolize aggrecan. Taken together, these observations suggest that cleavage between Glu³⁷³ and Ala³⁷⁴ is the primary event in aggrecan metabolism [12]. The primary cleavage in aggrecan for all MMPs tested so far is within the sequence VDIPENFFGVG, between Asn³⁴¹ and Phe³⁴², which is also located in the G1–G2 interglobular region. This is not the same as the primary cleavage observed in cartilage degradation studies. Therefore the ability of MMP inhibitors to inhibit aggrecan degradation has been explained by the involvement of MMPs in the activation of enzymes that cause aggrecan destruction [9].

The principal structural component of cartilage is type II collagen. At physiological pH, mammalian collagenases hydrolyse triple helical collagen at a specific locus three-quarters of the way along the molecule. Non-specific MMP inhibitors have been shown to inhibit collagen metabolism in cartilage explant assay [4,7].

In the studies reported here we have used potent MMP inhibitors with differing selectivities for the human MMPs, in conjunction with a bovine nasal explant system, to explore the

Abbreviations used: Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; IL-1, interleukin 1; Mca, (7-methoxycoumarin-4-yl)acetyl; MMP, matrix metalloproteinase; rHu-IL-1 α , recombinant human IL-1 α .

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Figure 1 Time course of the loss of aggrecan from cultured bovine nasal cartilage explants

Explants were cultured alone (●) or in the presence of 0.1 ng/ml rHu-IL-1α (■), 1.0 ng/ml rHu-IL-1α (▲) or 11 ng/ml rHu-IL-1α (▼). Each point represents the mean $\pm$ S.E.M for 10 determinations.

roles of these enzymes in the degradation of the two major components of cartilage. These inhibitors bind exclusively to the catalytic domains of MMPs. Inhibitors with selectivities determined for human enzymes were used to study the degradation of bovine cartilage. There is sequence identity between the catalytic domains of human stromelysin 1 and bovine stromelysin 1 [13]. Human and bovine collagenases 1 have 91 % similarity in their catalytic domains (SwissProt Database, release 34.0, October 1996) and we have shown similar potencies for a range of inhibitors against these two enzymes (results not shown). There is 92 % similarity between the catalytic domains of bovine and human gelatinase B (SwissProt Database, release 34.0, October 1996). Therefore we would expect that the potencies of these inhibitors against the human MMPs would be similar to those obtained against the corresponding bovine enzymes.

MATERIALS AND METHODS

Materials

All the MMP inhibitors described and the novel substrate (Mca-Pro-Lys-Pro-Leu-Gly-Leu-Dpa-NH₂) [where Mca is (7-methoxycoumarin-4-yl)acetyl and Dpa is *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl] were synthesized within the Chemistry Department at Roche (Welwyn Garden City, Herts., U.K.). Recombinant human IL-1α (rHu-IL-1α) was prepared and supplied by Roche (Nutley, NJ, U.S.A.) [14]. All tissue culture materials were supplied by Gibco. GlucNH[®] test kit and equine serum were supplied by Roche Diagnostics (Welwyn Garden City). Chondroitinase ABC lyase (protease-free) was obtained from ICN. The monoclonal antibody 3-B-3, which principally binds to 6-sulphated 'stubs' of proteoglycan cores, was supplied by the Seikagaku Corporation; keratinase, gelatin-agarose, trypsin, trypsin inhibitor and hydroxyproline were obtained from Sigma. Horseradish peroxidase-linked rat anti-mouse IgM was obtained from Amersham.

Determination of MMP inhibitor potencies

Medium from human dermal fibroblasts (CCD45) cultured in the presence of rHu-IL-1α (25 ng/ml) was used as the source of collagenase. Collagenase activity was determined by measuring the degradation of ¹⁴C-labelled type I collagen fibrils [15]. In

these assays the IC₅₀ was approximately twice the K_i. Human prostromelysin 1 was antibody-affinity purified from conditioned human fibroblast culture medium [16]. Progelatinase B was purified by gelatin-agarose affinity chromatography from human neutrophils [17]. Both prostromelysin and progelatinase B were activated by treatment with trypsin. Stromelysin and gelatinase B activities were determined by measuring the cleavage of the fluorogenic substrate Mca-Pro-Lys-Pro-Leu-Gly-Leu-Dpa-NH₂; assays were performed in 50 mM borate buffer/1 mM CaCl₂ containing 0.05 % Brij-35, at a substrate concentration of 2 μM. Assays were started by the addition of enzyme to a mixture of substrate and inhibitor, and incubated for 4 h at 37 °C; the assay was stopped by the addition of acetic acid to a final concentration of 0.17 M. The fluorescence of the product Mca-Pro-Lys-Pro-Leu-Gly (λ_{ex} 325 nm; λ_{em} 395 nm) was measured with a Hitachi F-4500 fluorescence spectrophotometer. Assay conditions were such that IC₅₀ values approximate to K_i values, and slow and tight binding effects of inhibitors were minimized.

Bovine nasal cartilage explant assay: measurement of aggrecan metabolism

Bovine nasal cartilage explants (25–30 mg) were cultured at 37 °C in Dulbecco's modified Eagle's medium containing penicillin (50 i.u./ml), streptomycin (50 μg/ml) and fungizone (250 μg/ml) [4]. Degradation of aggrecan was induced by the addition of 11 ng/ml of rHu-IL-1α to the culture medium. After 5 days the medium around each explant was aspirated and retained for the determination of aggrecan concentration. The remaining cartilage was digested with papain (21 units/mg of cartilage) to remove non-metabolized aggrecan. Aggrecan concentrations in the medium and the digest from each explant were determined with a dimethylMethylene Blue-dye-binding colorimetric assay [18] adapted to a 96-well microtitre plate format. The concentration of aggrecan present in each sample was determined by comparison with a bovine aggrecan standard. The amount of aggrecan lost from each cartilage explant (aggrecan in medium) was expressed as a percentage of the total amount of aggrecan present in that explant at the start of the experiment (aggrecan present in medium plus explant digest).

Bovine nasal cartilage explant assay: measurement of collagen metabolism

The culture conditions used to measure bovine nasal cartilage collagen degradation were similar to the conditions used for the measurement of aggrecan degradation, the differences being that the level of rHu-IL-1α used was 113 ng/ml and the cultures were incubated for 14–21 days. During this period culture media containing rHu-IL-1α and inhibitors were renewed every 7 days. When a considerable portion of each cartilage piece had disappeared, the assay was stopped by removing the remaining cartilage explant, which was then analysed for hydroxyproline content [19]. This was measured as a marker of cartilage collagen content.

N-terminal amino acid sequence analysis of aggrecan fragments

Medium that had been used to culture cartilage explants in the presence of rHu-IL-1α was pooled and concentrated, then aggrecan glycosaminoglycan and keratin sulphate side chains were removed by treatment with chondroitinase ABC lyase (0.12 units/ml) and keratinase (0.14 units/ml). The deglycosylated proteins were dialysed against SDS/PAGE running buffer [0.24 M Tris (pH 8.3)/1.92 M glycine/1 % (w/v) SDS]. The

Table 1 Potency of inhibitors against human collagenase 1 (MMP 1), stromelysin 1 (MMP 3), gelatinase B (MMP 9) and aggrecan metabolism

Compounds **i–xii** represent a series of hydroxamic acid analogue inhibitors of matrix metalloproteinases. Each IC_{50} is the mean of 2–20 determinations. The S.D. for each mean was within 0–30% of the mean (results not shown).

No.	Structure	IC_{50} , stromelysin (nM)	IC_{50} , gelatinase (nM)	IC_{50} , collagenase (nM)	IC_{50} , inhibition of aggrecan metabolism (μ M)
i		160	10	5	300
ii		38	8	9	50
iii		7.3	17.5	>10000	40
iv		53	2.35	2.5	30
v		5	250	8200	10
vi		5	1	3	3
vii		2.7	0.7	1.6	2
viii		8	2.6	1.5	1.1
ix		0.46	1.1	24	0.5
x		0.9	1	6	0.34
xi		0.4	0.1	30	0.3
xii		0.08	0.1	24.5	0.16

resulting dialysed protein sample was then split into two and duplicate SDS/PAGE analysis performed with 8 % (w/v) polyacrylamide precast gels (Novex). Separated proteins were transferred to an Immobilon P (PVDF) membrane. Aggrecan proteins from the first blot were identified by using 3-B-3, a monoclonal antibody that recognizes terminal chondroitin 6-sulphate residues [9]. Immunolocalized proteins were detected with a horseradish peroxidase-linked rat anti-(mouse IgM). The second blot was stained with Ponceau S. Stained bands on the second blot corresponding to the immunolocalized bands on the first blot were excised and N-terminally sequenced [20].

Assessment of the cytotoxic actions of inhibitors

The cytotoxic actions of inhibitors on cartilage chondrocytes were assessed by measuring the decrease in glucose utilization by cartilage explants over the course of the experiment. Glucose utilization was determined by measuring the glucose concentration in the culture medium around the explants at the end of the experiment. The samples were analysed with a Cobas Bio (Roche Diagnostics) using a GlucNH[®]-ultimate 5 test kit.

RESULTS

Time course of aggrecan metabolism in bovine nasal cartilage explants

Figure 1 shows a time course of aggrecan loss from bovine nasal explants cultured alone and in the presence of different concentrations of rHu-IL-1 α . rHu-IL-1 α accelerated aggrecan loss from the explants in a concentration-dependent manner. At day 5 the loss of aggrecan was approx. 70 % in the presence of 11 ng/ml rHu-IL-1 α ; this time point and concentration of rHu-IL-1 α were used for subsequent studies with MMP inhibitors. By day 9 in the presence of 11 ng/ml rHu-IL-1 α , more than 95 % of the cartilage aggrecans had been released into the culture medium; after this time the medium was removed for N-terminal sequence analysis. The use of these higher levels of rHu-IL-1 α than in published data [4] shortened the assay duration, making the system more useful for measuring the effects of a range of MMP inhibitors. The shortened assay duration did not affect the inhibitory potency of a standard MMP inhibitor when compared with the previously published assay conditions (results not shown).

N-terminal analysis of aggrecan metabolism products derived from bovine nasal cartilage explants

Aggrecan fragments in the medium from cartilage explants were separated by SDS/PAGE and were identified on Western blots with 3-B-3 [9]. Eight fragments were observed, four of which were successfully N-terminal sequenced. Three major protein bands had molecular masses of approx. 200 kDa and an ARGSVIL N-terminal sequence; the fourth protein of molecular mass 116 kDa had an ADLEXTXG N-terminal sequence. Aggrecan fragments recovered from the medium of stimulated and non-stimulated cultures showed similar molecular masses and the same N-terminal sequences, which suggests that the same enzymes are involved in cleaving aggrecan in both non-stimulated and stimulated explant cultures.

Inhibitors of aggrecan metabolism

A comparison of the activities of 12 inhibitors against the human MMPs collagenase 1 (MMP 1), gelatinase B (MMP 9) and stromelysin 1 (MMP 2) with their ability to inhibit aggrecan

Figure 2 Inhibition of cartilage explant aggrecan metabolism and inhibition of stromelysin 1, collagenase 1 and gelatinase B

Double logarithmic transformed IC_{50} values from Table 1, showing inhibition of aggrecan metabolism in the bovine nasal explant assay against transformed IC_{50} values against stromelysin 1 (a), collagenase 1 (b) and gelatinase B (c). Least-square linear regressions are plotted and the correlation coefficients (r) between the log IC_{50} values are shown.

metabolism is shown in Table 1 and Figure 2. There is a strong correlation ($r = 0.93$) between inhibition of stromelysin 1 and inhibition of aggrecan metabolism (Figure 2a); however, the potency for inhibition of aggrecan breakdown is approx. 1/1000 that for inhibition of stromelysin. In contrast, there is little relationship ($r = 0.18$) between inhibition of collagenase 1 and inhibition of aggrecan metabolism (Figure 2b). Although there is a loose correlation ($r = 0.71$) between inhibition of gelatinase B and inhibition of aggrecan breakdown (Figure 2c), there are anomalies, for example compound v (see Table 1 and Figure 2). Additionally, compounds vi, ix and x were equipotent inhibitors

Table 2 Inhibition of cartilage explant collagen breakdown and inhibition of stromelysin 1 (MMP 3), gelatinase B (MMP 9) and collagenase 1 (MMP 1)

Each IC₅₀ is the mean of 2–20 determinations. The S.D. for each mean was within 0–30% of the mean (results not shown).

No.	Structure	IC ₅₀ (nM)			
		Stromelysin	Gelatinase	Collagenase	Collagen protection
i		160	10	5	76
iii		7.3	17.5	>10000	>10000
xiii		2175	237	10.1	30–100
xiv		527	59	7	60

of gelatinase B but showed large variations in inhibitory potency in the aggrecan assay.

Inhibition of collagen metabolism

A comparison of the activities of four inhibitors of MMPs with their ability to inhibit collagen degradation is shown in Table 2. There is a strong agreement between inhibition of collagenase 1 and inhibition of cartilage collagen metabolism. There is no correlation between inhibition of stromelysin 1 or gelatinase B and collagen metabolism.

Assessment of cytotoxic action of inhibitors

At concentrations of compounds that greatly inhibited collagen or aggrecan breakdown, glucose consumption remained similar to that observed in IL-1-stimulated controls. Therefore there was no evidence to suggest that inhibition of aggrecan or collagen breakdown was due to a cytotoxic action by the inhibitors on the cartilage chondrocytes (results not shown).

DISCUSSION

In this study N-terminal sequence analysis of metabolized aggrecan from bovine cartilage indicated that the principal cleavage occurred between Glu³⁷³ and Ala³⁷⁴ in the G1–G2 interglobular domain; this agrees with previously reported studies with human and bovine cartilage [9–11,21]. We have also identified a novel cleavage product that contains the N-terminal amino acid sequence ADLEXTXG and differs from additional reported aggrecan cleavage products [9,22]. The full sequence of bovine aggrecan has not been published. However, comparison of the ADLEXTXG N-terminal sequence with available sequence segments of bovine aggrecan (SwissProt Database, release 34.0,

October 1996) indicates that the fragment (N-terminal ADLEXTXG) is produced as a result of a cleavage in the sequence FHEADLE, which occurs towards the C-terminus of the bovine aggrecan molecule.

All the reported proteolysis products of bovine aggrecan from cartilage explant studies [9,21] reveal that cleavage occurs between a glutamic residue and a small aliphatic residue, normally alanine or leucine, which suggests that the same enzyme is responsible for all these cleavages. Our results show that there is a strong correlation between inhibition of human stromelysin 1 (MMP 3) and inhibition of aggrecan metabolism in our explant assay. Human stromelysin 1 cleaves isolated aggrecan primarily between Asn³⁴¹ and Phe³⁴² in the G1–G2 interglobular domain [23,24]. This cleavage is N-terminal to the cleavage between Glu³⁷³ and Ala³⁷⁴ that is observed in this and other cartilage explant studies [9–11] and with a rat chondrosarcoma cell line [12]; there was no evidence for cleavage between Asn³⁴¹ and Phe³⁴². Only collagenase 2 (MMP 8) has been shown, at high concentrations, to cleave between Glu³⁷³ and Ala³⁷⁴ [25]; however, this follows an initial cleavage between Asn³⁴¹ and Phe³⁴².

The strong correlation between stromelysin inhibition and inhibition of aggrecan degradation argues for the involvement of this enzyme in aggrecan metabolism. However, there was an approx. 1000-fold difference in potencies between inhibition of stromelysin and inhibition of aggrecan degradation. Furthermore there is no evidence for the production of aggrecan with an N-terminal ³⁴²FFG sequence, the expected proteolysis product of stromelysin action on isolated aggrecan [23]. There are several explanations that reconcile these apparently conflicting observations. The enzyme responsible for the cleavage of aggrecan in the explant studies is similar to, but not identical with, stromelysin 1. Alternatively, stromelysin 1 is operating indirectly as a part of a cascade of enzymes responsible for aggrecan metabolism. The decrease in potency might arise because these inhibitors cannot

easily penetrate the cartilage surrounding the chondrocytes or because the inhibited enzyme is not rate-limiting in the degradative process.

Our studies have additionally shown that potent collagenase 1 (MMP 1) inhibitors are effective in preventing cartilage collagen degradation in bovine nasal cartilage explants. Furthermore there is no correlation between inhibition of gelatinase or stromelysin and inhibition of cartilage collagen metabolism. Consequently, inhibition of collagenase alone is sufficient to prevent cartilage collagen breakdown. Three collagenases (1, 2 and 3) have been identified and all are reported to be present in articular cartilage [27,28]. However, currently the relative contribution of each to the destruction of cartilage in either model cartilage degradation systems or osteoarthritis and rheumatoid arthritis is unknown.

Potent MMP inhibitors can prevent cartilage aggrecan and type II collagen degradation. Therefore, what are the prospects that these inhibitors might prove to be effective cartilage protective agents, and would they be useful in the treatment of arthritis? We have shown that extremely potent stromelysin inhibitors are only modest inhibitors of aggrecan metabolism. This may make it difficult to achieve sufficiently high tissue concentrations of a stromelysin inhibitor to prevent this process *in vivo*. The loss of aggrecan that contributes to articular cartilage destruction in arthritis is a rapidly reversible process [29]. Provided that an intact type II collagen network is maintained and chondrocytes within the cartilage remain functional, these cells have the ability to replace proteoglycans lost by proteolysis, thereby restoring full cartilage function. In contrast, destruction of the type II collagen network is essentially the irreversible step in the destruction of cartilage [30]. Therefore inhibitors of collagenase, by preventing collagen II destruction, offer greater potential as an effective cartilage protective therapy.

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