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THE PREVENTION OF COLLAGEN BREAKDOWN IN BOVINE NASAL CARTILAGE BY TIMP, TIMP-2 AND A LOW MOLECULAR WEIGHT SYNTHETIC INHIBITOR

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Interleukin-1 stimulated bovine nasal cartilage fragments were cultured in the presence and absence of various metalloproteinase inhibitors. Tissue inhibitor of metalloproteinases (TIMP) and tissue inhibitor of metalloproteinases-2 (TIMP-2) completely blocked the release of collagen from the cartilage but were unable to prevent the release of proteoglycan. Similarly, a low molecular weight synthetic inhibitor (BB87) inhibited collagen release in a dose dependent manner, but was unable to inhibit proteoglycan release at the same concentrations. Significantly greater concentrations of inhibitor than those required to block collagen release did, however, block proteoglycan release. These results indicate that the therapeutic use of naturally occurring or synthetic inhibitors may provide a means of modifying the destruction of connective tissue proteins occurring in the arthritides and other connective tissue pathologies.

The matrix metalloproteinases (MMPs) are a unique family of enzymes that in concert can degrade all the components of the extracellular matrix [1]. These potent enzymes are synthesised in a proenzyme form and activation occurs after leaving the cell. Extracellular activity is also controlled by specific inhibitors, Tissue Inhibitors of Metalloproteinases (TIMPs), that bind to the active forms of the enzyme forming 1:1 complexes and blocking their activity [2].

The inflammatory cytokine interleukin-1 (IL-1) can induce resorption of cartilage in vitro [3] and in vivo when injected into rabbit joints [4] and is also found in joint fluids from patients with rheumatoid arthritis [5,6]. IL-1 is also known to stimulate the production of the MMPs collagenase and stromelysin from human synovial cells and chondrocytes [7]. Diseased cartilage from different rheumatic diseases contains raised levels of both mRNA and enzyme protein [8]. Consequently the MMPs are a valid therapeutic target for inhibition in the rheumatic diseases [9].

Abbreviations: IL-1, Interleukin-1; GAG, glycosaminoglycan; PG, proteoglycan; TIMP, tissue inhibitor of metalloproteinase; MMP, matrix metalloproteinases.

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A number of studies have shown that IL-1 stimulated cartilage model systems can be used to test the effectiveness of proteinase inhibitors. The release of glycosaminoglycan (GAG) fragments (as a measure of proteoglycan degradation) can be prevented by the addition of low molecular weight synthetic inhibitors [10-14] but not by the addition of TIMP [15,16]. However, whilst the release of GAG fragments from cartilage appears to be rapid in vivo in response to IL-1 [17], they are also quickly resynthesised by chondrocytes [18]. In contrast the release of collagen from cartilage is much slower, is less reproducible, and appears to be irreversible as resynthesis is difficult to achieve [19].

In this study we have examined the release of collagen from resorbing cartilage to determine if the naturally occurring MMP inhibitors TIMP and TIMP-2 and low molecular weight synthetic inhibitors can block collagen release from IL-1 stimulated cartilage.

Materials and Methods

Culture medium

Control culture medium was Dulbecco's modification of Eagle's medium containing 25mM HEPES (Gibco) supplemented with glutamine (2mM), streptomycin (100μg/ml), penicillin (100U/ml) and amphotericin (2.5μg/ml).

Cartilage degradation assay

Cartilage slices were dissected from bovine nasal septum cartilage. Discs were cut from the slices (2 x 2mm) and washed twice in HBSS (Gibco). The discs were then incubated at 37°C in groups of three in control medium (600μl) in a 24 well plate for 24 hours for stabilisation. Control medium (600μl) with or without inhibitors and rhIL1α (a generous gift of Roche Products Ltd., UK) was added and the plate incubated at 37°C for 7 days. The supernates were harvested and replaced with fresh medium containing identical test reagents to Day 1. The experiment was continued for a further 7 days and Day 7 and 14 supernates were stored at -20°C until assay. In order to determine the total glycosaminoglycan (GAG) and hydroxyproline (OHPro) content of the cartilage fragments, the remaining cartilage was digested with papain (4.5mg/ml; Sigma) in 0.1M phosphate buffer, pH 6.5, containing 5mM EDTA and 5mM cysteine hydrochloride, incubating at 65°C until digestion was complete (16 h).

Inhibitors

TIMP-1 and TIMP-2 were prepared from WI-38 culture medium. TIMP-1 was purified by a monoclonal-based affinity column [20] and TIMP-2 as previously described [21].

BB87 (British Biotechnology, Oxford, UK) was dissolved in ethanol and diluted in control medium to appropriate concentrations.

Proteoglycan degradation

Media samples and papain digests were assayed for sulphated glycosaminoglycans (as a measure of proteoglycan release) using a modification of the 1,9-dimethylmethylene blue dye binding assay [22]. Sample or standard (40μl) was mixed with dye reagent (250μl), prepared as described [22], in the well of a microtitre plate, and the absorbance at 525nm determined immediately.

Chondroitin sulphate from shark fin (5-40μg/ml) was used as a standard. The complex formed with 1,9-dimethylmethylene blue results in a decrease in absorbance at 525nm. Hyaluronate causes no absorbance change [23].

Collagen degradation

Hydroxyproline release was assayed (as a measure of collagen degradation) using a microtitre plate modification of the method of Bergman and Loxley [24]. Chloramine T (7% (w/v)) was diluted 1:4 in acetate-citrate buffer (57g sodium acetate, 37.5g tri-sodium citrate, 5.5g citrate acid, 385 ml propan-2-ol per litre water). P-dimethylaminobenzaldehyde (DAB; 20g in 30ml 60%

perchloric acid) was diluted 1:3 in propan-2-ol. Specimens were hydrolysed in 6M HCl for 20h at 105°C and the hydrolysate neutralised by drying over NaOH in vacuo. The residue was dissolved in water and 40µl sample or standard (hydroxyproline; 5-30µg/ml) added to microtitre plate together with chloramine-T reagent (25µl) and then DAB reagent (150µl) after 4 min. The plate was covered and heated to 60°C for 35 min, cooled for 5 min to room temperature and the absorbance at 560nm determined.

Results

A high dose of human recombinant IL-1α (1000u/ml) was used to stimulate the release of matrix components from bovine nasal cartilage. Over 90% of the GAG was released by day 7. However, no collagen release was detected until Day 10 and up to 80% was released by day 15 (Figure 1). Addition of TIMP and TIMP-2 (100 u/ml) had no effect on the release of GAG fragments into the medium at day 7 (data not shown), as found previously for a pig articular cartilage model system [16]. However, the addition of the same concentration of TIMP or TIMP-2 on Day 7 completely blocked the release of collagen fragments from the tissue (Figure 2).

Additional experiments were completed with this same model system but with the addition of a low molecular weight synthetic inhibitor of matrix metalloproteinases. BB87 is a substrate analogue of the cleavage site in collagen hydrolysed by the action of collagenase attached to a hydroxamic acid chelating group. Addition of this inhibitor at a range of concentrations (10^{-5}M to 10^{-8}M) was able to prevent release of collagen components in a dose dependent manner. Figure 3 illustrates that approximately 70% of the cartilage collagen is released into the medium after the addition of IL-1 and subsequent culture for 14 days. In the presence of 10^{-5}M and 10^{-6}M BB87 complete inhibition of collagen release was observed with increasing release at 10^{-7}M

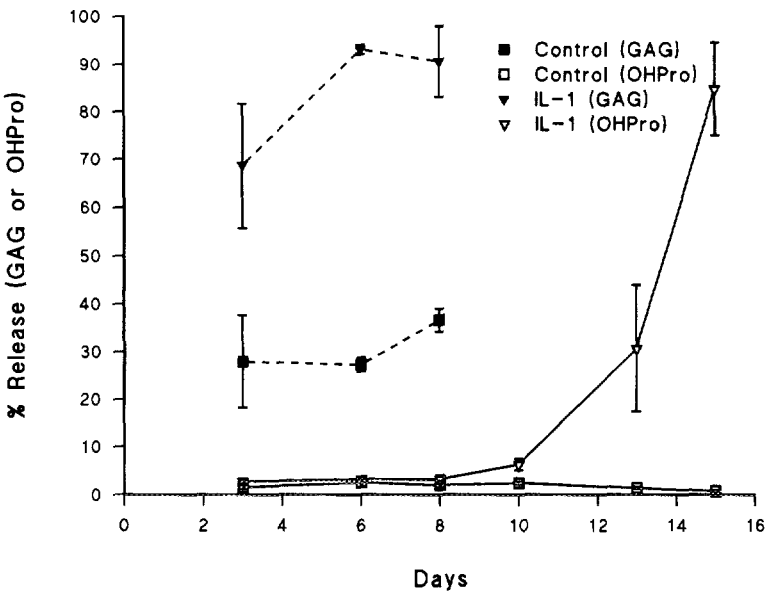

Figure 1. Time course of the release of GAG and OHPro fragments from IL-1 treated bovine nasal cartilage.

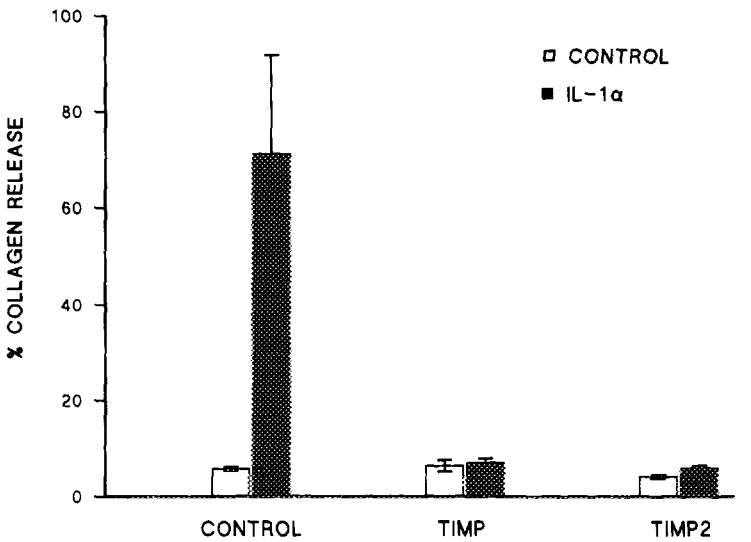

Figure 2. The effect of TIMP and TIMP-2 on the release of collagen from IL-1-stimulated bovine nasal cartilage.
Cartilage was treated with IL-1 (1000u/ml) at day 1 and day 7. TIMP and TIMP-2 (100u/ml) were added to the cartilage at day 7, after the removal of GAG-containing medium. Collagen release was measured at day 14 using the hydroxyproline assay. No release of collagen occurred from day 1 to day 7. Both TIMP and TIMP-2 significantly inhibited collagen release at day 14 compared to the control cultures.

and 10^{-8}M BB87. The same concentrations of BB87 were unable to prevent the release of GAG fragments from the cartilage, but we have previously show that higher concentrations of this inhibitor (10^{-3}M and 10^{-4}M) are able to inhibit GAG release in a pig articular system (data not shown).

Discussion

Much previous work has been published on the prevention of cartilage damage as a rational therapeutic target in the rheumatic diseases [25]. Numerous animal models and in vitro cartilage models have been used [26,27]. Many of these have focussed almost exclusively on monitoring the release of GAG fragments from the tissue as a measure of damage to the articular cartilage. However recent results would suggest that cartilage can tolerate relatively high levels of GAG turnover before permanent damage ensues . Whilst the release of GAG is rapid, and consequently easy to measure, it can also be rapidly replaced by the chondrocytes [18]. Perhaps a more appropriate measure of cartilage degradation is the release of collagen fragments, as such release is followed by irreversible damage [19]. However, release of collagen from cartilage is not always reproducible after treatment with cytokines, and is always delayed. In the present study treatment with high concentrations of IL-1α only ensured release of collagen after day 7 but before day 14. Consequently many studies have elected to follow GAG release as a rapid and reproducible measure of cartilage destruction.

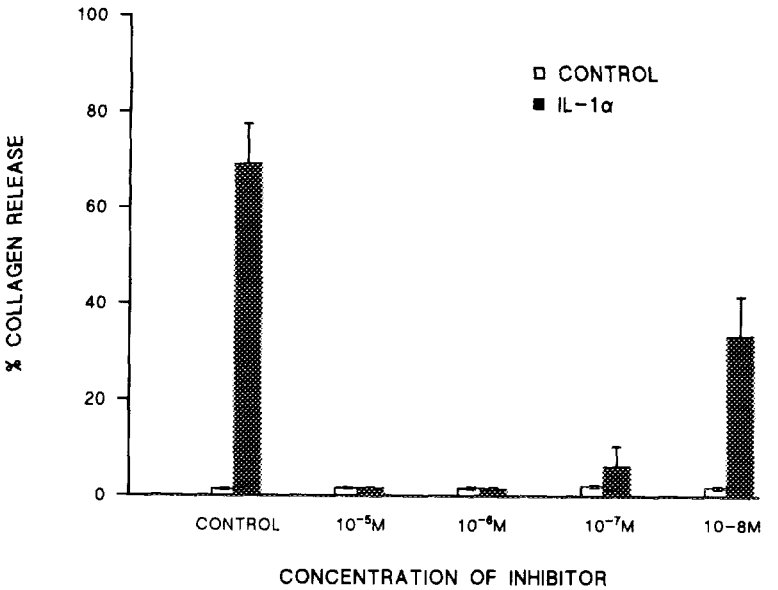

Figure 3. The effect of a low molecular weight synthetic inhibitor (BB87) on collagen release from IL-1 stimulated bovine nasal cartilage.
IL-1 (1000u/ml) and BB87 were added to the cartilage on day 1 and day 7. Collagen release was measured on day 7 and day 14 using the hydroxyproline assay. BB87 gave almost total inhibition of collagen release at the higher concentrations of 10^{-5}M and 10^{-6}M.

In this study TIMP and TIMP-2 had no effect on GAG release from bovine nasal cartilage confirming our previously published results using pig articular cartilage [16]. However, both inhibitors completely prevented the release of collagen fragments. Whilst it is possible that the release of GAG fragments is initiated by a proteinase not inhibited by TIMP, this seems unlikely as low molecular weight inhibitors at higher concentrations are able to block release as shown in previous studies [10-16]. All members of the MMP family purified to date are inhibited by the TIMPs. It is more likely that both TIMPs are unable to gain access to the cartilage matrix in the presence of high concentrations of the highly charged GAG fragments. In our previous study with pig articular cartilage [16] we were able to show that whilst TIMP was bound to the cartilage it did not penetrate the matrix to any extent and so was unable to block the release of GAG. Removal of GAG fragments from the cartilage on day 7 in this study allows the inhibitors to penetrate the cartilage as over 75% of the GAG has been released at this time. TIMP and TIMP-2 are thus able to reach the sites of cartilage collagen destruction surrounding the cells and prevent the action of collagenase on the collagen matrix.

The results with the low molecular weight inhibitors are also interesting. There is a marked difference in concentration required to block GAG release compared to the lower concentrations that block collagen release. This could be explained in the same way as the results obtained with the addition of the TIMPs. The highly charged GAGs at high concentration possibly perturb the interaction of proteinase with inhibitor by competing as the substrate and so a

much higher concentration of inhibitor is required than would be expected from the inhibition constants obtained with isolated enzymes. However high concentrations of collagen are also present as substrate and the same effect would also be expected with the concentration of inhibitor required to inhibit collagenase. Alternatively, the inhibitors are targeted to known members of the MMP family, particularly collagenase and stromelysin. If release of the GAG fragments is effected by a proteinase other than stromelysin or collagenase then the currently available inhibitors would not necessarily be expected to inhibit GAG release at the same range of concentrations as found effective against stromelysin.

Buttle et al [28] have recently proposed a model for cartilage PG breakdown involving a lysosomal proteinase cathepsin B and a MMP in a cascade mechanism with two pathways converging at the point of activation of an "aggrecanase" MMP. Studies by Sandy et al [29] have proposed that stromelysin is not the proteinase responsible for GAG release as the fragments released after cartilage resorption in vitro and in vivo are produced by cleavage of the aggrecan molecule at a position not cleaved by stromelysin. However this alternative "aggrecanase" enzyme has not yet been identified.

In conclusion, our results indicate that the use of metalloproteinase inhibitors will be of significant importance in the future to aid in the prevention of cartilage collagen destruction occurring in conditions such as rheumatoid arthritis.

Acknowledgments

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