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INTERLEUKIN-1 AND ONCOSTATIN M IN COMBINATION PROMOTE THE RELEASE OF COLLAGEN FRAGMENTS FROM BOVINE NASAL CARTILAGE IN CULTURE

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Interleukin-1 (IL-1) and Oncostatin M (OM) induce a rapid and reproducible release of proteoglycan and collagen fragments from bovine nasal cartilage in culture. Over 90% of the total collagen was released by day 14 compared to a variable release with IL-1 alone. This release was accompanied by the appearance of collagenolytic activity in the medium that cleaved collagen specifically at the one quarter/ three quarter position. Tissue inhibitor of metalloproteinases (TIMP-1) activity was low or absent in media from resorbing tissue. The breakdown of cartilage collagen could be prevented by the addition of BB94, a specific matrix metalloproteinase (MMP) inhibitor. These results suggest that T-cell/macrophage products within inflammed joints can interact with pro-inflammatory cytokines and lead to the rapid destruction of connective tissue collagen.

The matrix metalloproteinases (MMPs) are a family of enzymes that can degrade all the components of the extracellular matrix. These potent enzymes are controlled at a number of key points that include stimulation of synthesis and secretion by cytokines and growth factors, the production of proenzyme forms requiring activation and inhibition by naturally occuring inhibitors (1). One specific family of inhibitors, the tissue inhibitor of metalloproteinases (TIMPs), only inhibit MMPs forming 1:1 stoichiometric complexes with the active forms (2).

Three MMPs, fibroblast collagenase; MMP-1 (3), neutrophil collagenase; MMP-8 (4) and collagenase-3; MMP-13 (5), can all specifically cleave triple helical collagen to give characteristic 3/4 and 1/4 products. MMP-1 consists of two domains linked by an exposed proline-rich peptide (6). Whilst the N-terminal domain contains the active site zinc, the C-terminal domain is important for binding to the substrate. MMP-1 is known to be present within the rheumatoid joint (7), it can be localised to rheumatoid synovial tissue (8) and is upregulated by the proinflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor-α (TNFα) (9) suggesting its importance in the breakdown of cartilage collagen in arthritic diseases.

ABBREVIATIONS:

IL-1 interleukin-1; OM oncostatin M; GAG glycosaminoglycan; MMP matrix metalloproteinase; TIMP tissue inhibitor of metalloproteinases; TNFα tumour necrosis factor α; TGFβ transforming growth factor β; IFNγ interferon γ.

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Cartilage is composed of collagen fibres within which are trapped proteoglycan molecules that pull water into the tissue and allow cartilage to resist compression. Relatively low numbers of chondrocytes maintain the integrity of the tissue. The chondrocytes initiate the rapid release of proteoglycan from the tissue in response to the cytokines IL-1 and TNFα (10) but this matrix component can be replaced relatively quickly (11). In contrast collagen is much less readily released, but when degradation of collagen does occur, the structural integrity of the tissue is irreversibly lost (12). This makes collagen degradation a key control point in cartilage turnover.

Many growth factors and cytokines affect cartilage proteoglycan turnover. IL-1 and TNFα both induce the rapid release of proteoglycan (10). Little is known about collagen destruction in cartilage; although IL-1 can cause the release of collagen fragments this is slow and irreproducible (13). We have recently shown that combinations of growth factors synergise with retinoic acid and IL-1 to profoundly affect the level of MMPs and TIMP produced by fibroblasts in culture (14,15). Growth factors and cytokines act together in vivo and in previous studies we have shown that when transforming growth factor β (TGFβ) is added to cartilage in culture it reduces proteoglycan release (16), probably by stimulating TIMP production (25). Additionally interferon γ (IFNγ) in the presence of IL-1 also reduced proteoglycan release but this effect was acheived by down regulating the release of MMPs (18).

We decided to investigate the effect of combinations of agents that were known to markedly increase TIMP production to see if the release of both proteoglycan and collagen fragments in bovine cartilage could be altered. Interleukin-6 (IL-6) and other members of this family of growth factors are known to increase the production of TIMP in a variety of cell types (19-21). We have examined the effect of combinations of cytokines and growth factors on the release of proteoglycan and collagen fragments from cartilage discs in culture. In this study we report that Oncostatin M in combination with IL-1 causes a massive release of proteoglycan and collagen fragments from bovine nasal cartilage and this could indicate a new mechanism of collagen destruction that is relevant to joint diseases.

MATERIALS AND METHODS

Materials

Chemicals were obtained from the following suppliers: Oncostatin M from Genzyme, Kent, UK. IL-1α was a generous gift from Glaxo Group Research Ltd, Greenford, UK. BB94 was a generous gift of British Biotechnology, Oxford, UK All other chemicals and biochemicals were commercially available analytical grade reagents obtained from Fisons, Loughborough, UK or BDH, Poole, UK or have been previously described (13,14,16).

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

Bovine nasal septum cartilage was held at 4°C overnight after slaughter. Discs were cut from 2mm slices to give pieces 2mm in diameter and washed twice in HBSS (Gibco). Three discs per well of a 24 well plate were incubated at 37°C in control medium (600μl) for 24 hours. Control medium (600μl) with or without test reagents (4 wells for each condition) 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. 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).

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) 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.

Collagen degradation

Hydroxyproline release was assayed (as a measure of collagen degradation) using a microtitre plate modification of the method in (23). 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. using a Savant speed vac. The residue was dissolved in water and 40µl sample or standard (hydroxyproline; 5-30µg/ml) added to microtitre plates together with chloramine-T reagent (25µl) and then DAB reagent (150µl) after 4 minutes. The plate was heated to 60°C for 35 min, cooled and the absorbance at 560nm determined.

Enzyme and inhibitor assays

^{3}H-acetylated collagen was used to measure collagenolytic activity by the diffuse fibril assay (26). Aminophenyl mercuric acetate was added at a concentration of 0.7mM to activate procollagenase. Inhibitory activity was assayed by the addition of samples to a known amount of active collagenase in the diffuse fibril assay and the % inhibition calculated. One unit of collagenase activity degrades 1µg of collagen per minute at 37°C and one unit of inhibitory activity inhibits 2 units of collagenase by 50%. SDS PAGE was as previously described (24).

Growth factors and cytokines

Interleukin-1 was stored at -70°C and diluted from stock solutions into culture medium and used at a final concentration of 5ng ml^{-1} in all experiments. Oncostatin M was stored at -70°C and diluted in culture medium to a final concentration of 50ng ml^{-1}. All dilutions were prepared immediately prior to use and added to the plates on day 0 or day 7.

RESULTS

Treatment of bovine cartilage with IL-1 in the presence of OM.

A significant increase in proteoglycan release was noted on day 7 after treatment with IL-1, OM or IL-1+OM. As IL-1 treatment alone in this experiment released most of the GAG it was not possible to determine if a greater release of GAG occurred when IL-1 and OM were added together. In other experiments (data not shown), where the release of GAG in response to IL-1 alone was lower, OM+IL-1 released higher levels of GAG suggesting that the effect of these reagents was additive. The combination of IL-1 + OM stimulated the release of collagen such that >80% of the total collagen was released by day 14 (Fig 1) whilst only 25% was released by IL-1 alone and 5% by OM alone.. In some experiments (data not shown) no increase of collagen

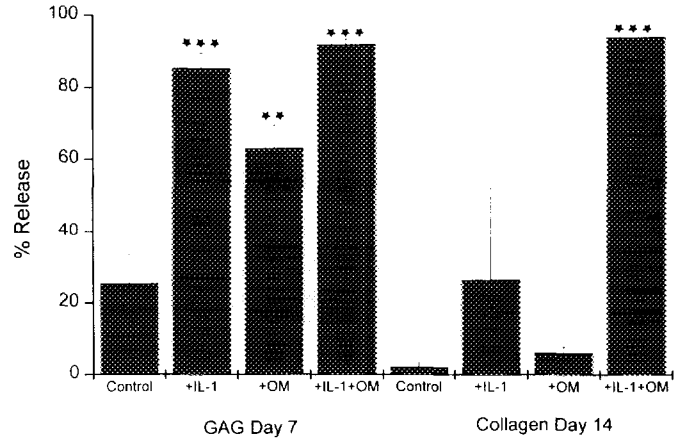


Fig.1.

The effect of IL-1 in combination with OM on the release of GAG and collagen fragments from bovine nasal cartilage.

Bovine nasal cartilage was cultured in quadruplicate in 600uL of medium alone, medium + 5ng ml^{-1} IL-1, medium + 50 $ngml^{-1}$ OM and medium + 5ng ml^{-1} IL-1 + 50 ng ml^{-1} OM. Medium was removed on day 7 and replaced as described above for a further 7 days. At fourteen days the medium was removed and the remaining cartilage digested with papain. The levels of GAG and OH-proline released into the medium on day 7 and 14 was measured and the results are expressed as % of total released. Results are expressed as mean +/- SD. *** $p = < 0.001$; ** $p = < 0.005$.

release was seen with IL-1 alone but in all experiments the combination of OM + IL-1 markedly increased collagen release over that observed with any other treatment.

Time course of release of proteoglycan and collagen fragments from bovine cartilage.

Cartilage was incubated as described above but the media in all wells was changed on day 3 and day 10 in addition to day 7. Treatment with IL-1 and OM caused 70% of the proteoglycan present to be released by day 3 whilst with IL-1 alone 25% was released by day 7 and a further 40% by day 10. The combination of IL-1 and OM also released collagen earlier than other treatments with 25% and a further 66% being released by day 7 and 10 respectively (Fig 2).

Prevention of proteoglycan and collagen fragments release with MMP inhibitors.

To determine if the release of proteoglycan and collagen fragments was mediated by MMPs bovine cartilage was incubated with the reagents described above both with and without the broad spectrum metalloproteinase inhibitor BB94 at 10^{-4}M as previously described (22). Almost complete inhibition of GAG release and complete inhibition of the release of collagen fragments was seen in the wells treated with BB94 as shown in Fig 3.

Analysis of medium from OM/IL-1 treated cartilage for proteinases and inhibitors.

Up to 90% of the total collagen could be released after treatment for 14 days with OM and IL-1 (Fig 1). The inhibition by BB94 suggests that MMPs were responsible for this release. Therefore the medium was analysed for both pro and active collagenolytic activity Some procollagenase was released at day 7 when cartilage was treated with OM + IL-1 with high levels present at day 14 (Fig 3). Some release of procollagenase occurred at day 14 with IL-1 alone but none with OM alone. The levels of active collagenase in medium from cartilage treated with IL-1 or OM

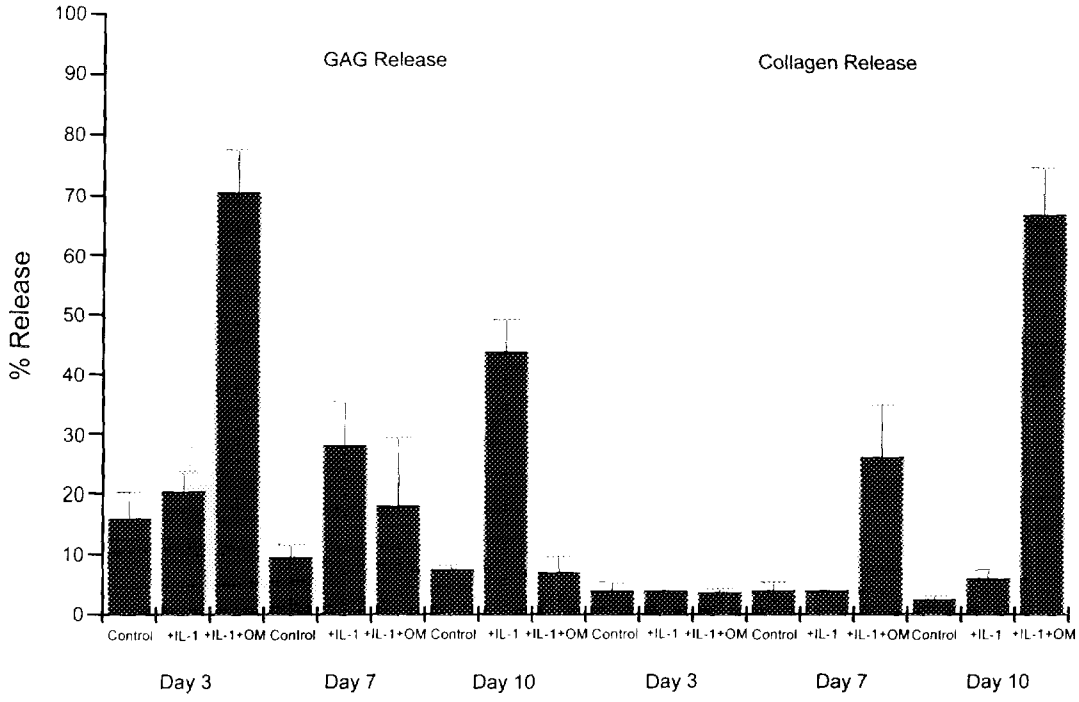


Fig.2. Time course of the release of GAG and collagen in response to IL-1 and OM.
Cartilage was treated as described for Fig 1 but medium was removed on day 3, 7, and 10 and assayed for GAG and collagen and the results are expressed as % of total released. Mean +/- SD.

alone were low but high levels of collagenolytic activity were present in medium from cartilage treated with IL-1 in combination with OM at day 14. This shows that in addition to the increased production of procollagenase, activation of the enzyme had also occurred (Fig 4). The levels of collagenolytic activity were extremely high when compared to the levels normally observed in cell culture medium. Levels of TIMP were also measured and are shown in Fig 5; TIMP levels were higher at day 7 than at day 14 and an increase in TIMP was seen at day 7 after treatment with OM alone. The lower levels of TIMP in OM + IL-1 treated medium accompanied by high levels of collagenolytic activity, some of which is active, indicates that these conditions would obviously favour collagen release. When medium from cartilage treated with IL-1 + OM was added to collagen the enzyme(s) present was able to effect the three quarter one quarter cleavage of fibrillar collagen showing that a true collagenase was present (Fig 6).

DISCUSSION

The proinflammatory cytokines IL-1 and TNFα are known to affect the release of proteoglycan from cartilage tissue in vivo and in vitro and block the synthesis of new proteoglycan molecules (10,11,16). Whilst the proteoglycans are obviously important to the structural integrity of cartilage the chondrocytes in culture can rapidly replace proteoglycan once the cytokine stimulus

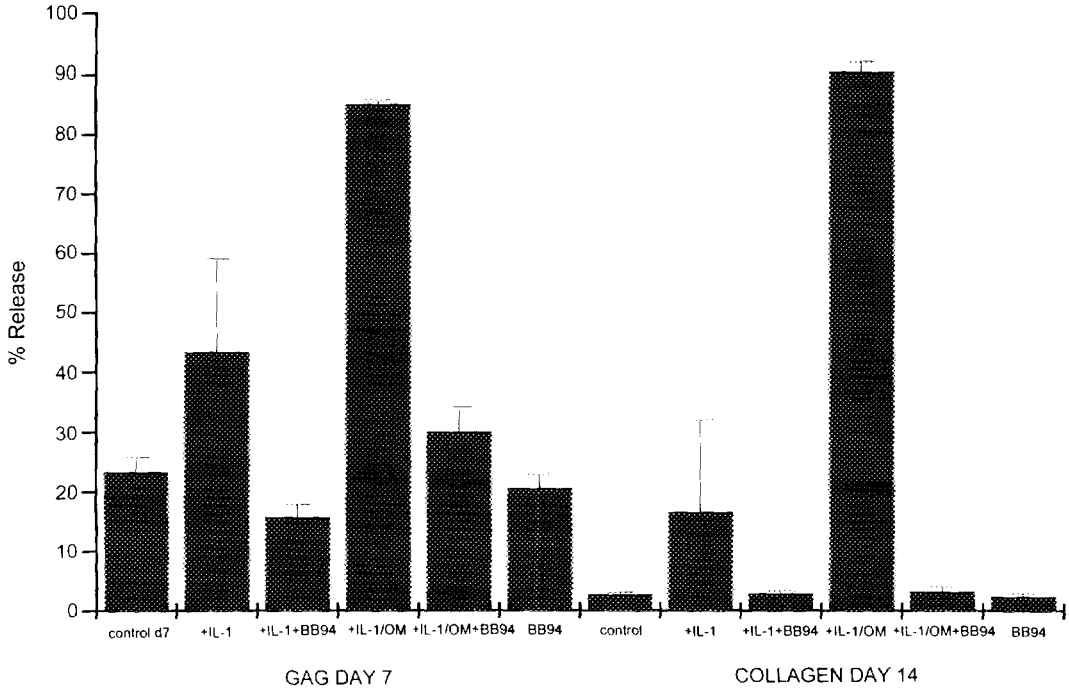


Fig.3. The effect of the specific MMP inhibitor BB94 on the release of GAG and collagen fragments from bovine nasal cartilage.

Cartilage was incubated as described in Fig 1 both with and without the metalloproteinase inhibitor BB94 at 10 $^{-4}$M. Medium was removed on day 7 and replaced as described above for a further 7 days. At fourteen days the medium was removed and the remaining cartilage digested with papain. The levels of GAG and OH-proline released into the medium on day 7 and 14 was measured and the results are expressed as % of total released. Mean +/- SD.

is removed (11). However, loss of the collagen fibrillar network has a profound effect on the tissue and represents the irreversible phase of cartilage destruction (12).

Our recent studies have attempted to investigate the mechanisms of collagen release from cartilage (13, 27). However these studies have been hampered by the relatively erratic release of collagen from cartilage in culture. Bovine nasal cartilage was found in previous studies to release collagen more reproducibly than porcine cartilage (13).

OM is a cytokine produced by T-cells (28) and monocytic cells (29) which can regulate growth in a number of different cell lines. It upregulates the production of acute phase proteins by liver cells and shares this property with the IL-6 family of cytokines (30). This family of cytokines signal through a common receptor gp130 but differ in the second receptor component that binds with gp130 to effect binding and subsequent signalling (31). A variety of cell types including fibroblasts, synovial cells and chondrocytes have been shown to possess receptors (32) Recently OM (19) and other members of this family (20,21,33,) were shown to upregulate TIMP-1 production. Therefore in this study we investigated the effect of adding OM in the presence of IL-1 to bovine nasal cartilage in culture to determine if upregulation of TIMP was able to prevent the release of either proteoglycan or collagen fragments.

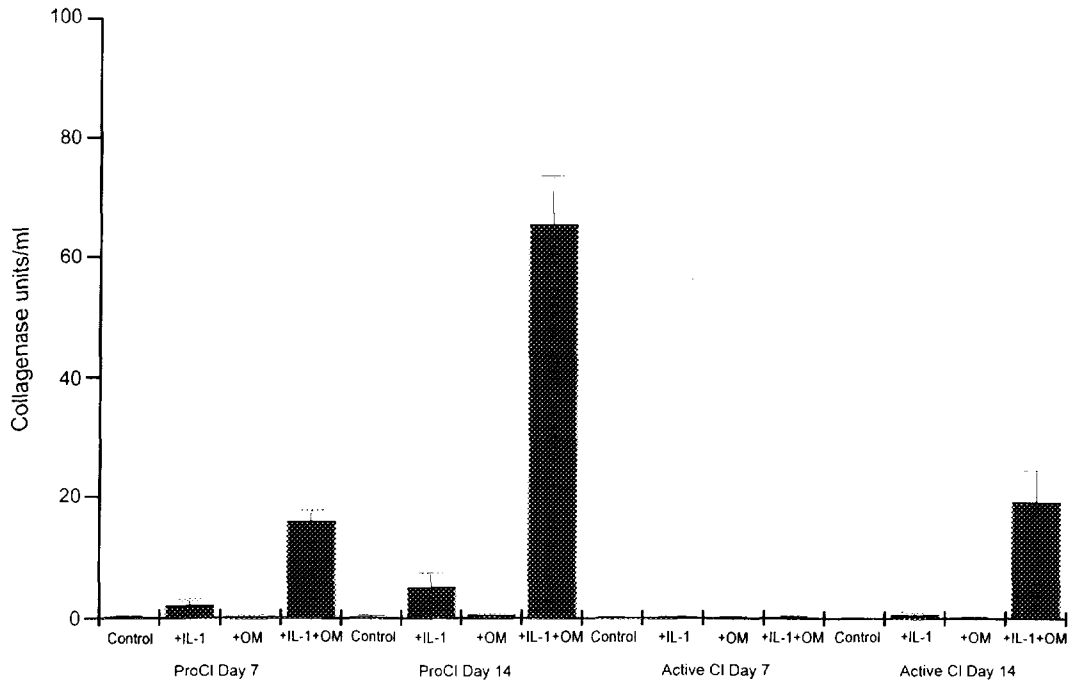
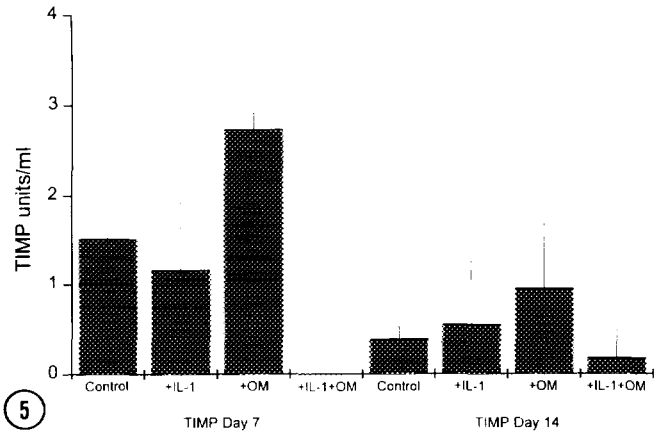


Fig. 4. The levels of procollagenase and active collagenase activity in media samples removed from cartilage cultures at Day 7 and 14 after stimulation with IL-1 and OM.

Cartilage was incubated and treated as described for Fig 1. The levels of procollagenase and active collagenase released into the medium on day 7 and 14 was measured and the results expressed as units ml^{-1}. Mean +/- SD.

The dramatic release of collagen fragments we observed correlated with the appearance in the medium of procollagenase with approximately one third of the enzyme converted to the active form. At the end of the assay period most of the cartilage treated with OM + IL-1 contained less than 10% of the total collagen; 90% had been released into the medium. This collagenolytic activity was inhibited by the chelating agent 1,10-phenanthroline showing that it was a metalloproteinase. As there are three enzymes that break down the interstitial collagens, MMP-1, MMP-8 and MMP-13, it is not clear if a mixture of collagenolytic activities are present. What is clear is that one or more of these enzymes are present as the three quarter/one quarter clips were effected in the Type I collagen molecule. Further work is in progress to establish which of these enzymes, along with other members of the MMP family, are present.

Although the rapid release of proteoglycan fragments reported in this study in response to OM and IL-1 is of interest the subsequent reproducible release of collagen fragments is of greater significance. In vivo the loss of the collagen fibrillar network heralds the irreversible phase of collagen destruction (12,34). It is not known if OM is present within the rheumatoid joint where cartilage is known to be destroyed although IL-6 and LIF, also members of this family, are known to be present (35). OM is produced by T-cells and macrophages (also present within the inflammed joint). The appearance of proinflammatory cytokines with OM within the joint could



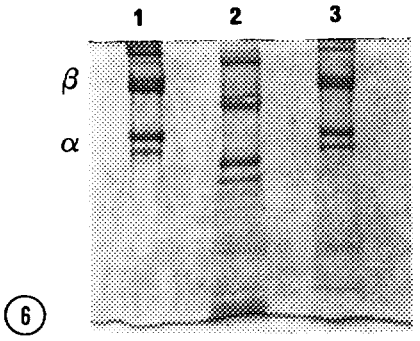


Fig.5. The levels of TIMP activity in media samples removed from cartilage cultures at Day 7 and 14 after stimulation with IL-1 and OM.

Cartilage was incubated and treated as described for Fig 1. The levels of TIMP released into the medium on day 7 and 14 was measured and the results expressed as units ml^{-1}. Mean +/- SD.

Fig.6. SDS gel electrophoresis of IL-1 and OM stimulated bovine nasal cartilage media after incubation with soluble Type I collagen at 22°C.

Media samples from cartilage treated for 14 days with IL-1 5ng ml^{-1} + OM 50 ng ml^{-1} were incubated with and without 1,10 phenanthroline (OP) at 2mM with an equal volume of bovine Type I collagen at 1 mg/ml in Tris HCl buffer, pH 7.6, containing 1M glucose (to prevent fibril formation) overnight. Samples were then treated with SDS and boiled for 3 minutes and loaded onto a SDS/PAGE gel. After electrophoresis the gel was fixed and stained. Lane 1 = incubated collagen; Lane 2 = incubated collagen + day 14 media; Lane 3 = incubated collagen + day 14 media + 1,10-phenanthroline at 2mM.

initiate cartilage destruction seen in the rheumatic diseases with the serious loss of collagen and subsequent irreversible damage to the tissue. Further work will determine if other members of this family of growth factors that signal through gp 130 also combine with IL-1 to upregulate collagenolytic enzymes and so initiate cartilage destruction.

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