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ARTICLES

Retinoic Acid Combines With Interleukin-1 to Promote the Degradation of Collagen From Bovine Nasal Cartilage: Matrix Metalloproteinases-1 and -13 are Involved in Cartilage Collagen Breakdown

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Abstract Retinoic acid (RetA) and interleukin-1α (IL-1) together can induce a reproducible release of proteoglycan fragments from bovine nasal cartilage in culture. However, release of collagen fragments with either agent alone is often variable. In this study over 70% of the total collagen was released from bovine nasal cartilage in culture by day 14 when RetA and IL-1 were combined. This release was accompanied by the appearance of collagenolytic activity in the culture medium that cleaved collagen specifically at the ¼/¾ position. Tissue inhibitor of metalloproteinases (TIMP) activity was present at day 7 but low or absent in media from resorbing tissue at day 14. The breakdown of cartilage collagen could be prevented by the addition of BB-94, a specific metalloproteinase inhibitor. These results suggest that RetA promotes the early release of TIMP from the tissue and that IL-1 stimulates pro-collagenase secretion which, when activated, exceeds the local concentration of TIMP. Thus in the later stages of culture collagen destruction occurs. Both MMP-1 and MMP-13 were detected and appear to be involved in IL-1 + RetA induced bovine cartilage destruction. However, for the first time, we also present evidence to suggest that MMP-13 is the predominant collagenase in this system. J. Cell. Biochem. 79:519–531, 2000.

Key words: rheumatoid arthritis; collagenase; matrix metalloproteinase; MMP; TIMP; cytokine; retinoid

The matrix metalloproteinases (MMPs) can degrade all the components of the extracellular matrix. This potent family of enzymes are controlled at a number of key points that include the stimulation of synthesis and secretion by cytokines and growth factors, the production of pro-enzyme forms requiring activation and inhibition by naturally occurring inhibitors [Woessner, 1991]. One specific family of inhibitors, the tissue inhibitor of metalloproteinases (TIMPs), inhibit MMPs forming 1:1 stoichiometric complexes with the active forms of all MMPs [Cawston, 1986].

Three mammalian MMPs, MMP-1; fibroblast collagenase [Stricklin et al., 1977], MMP-8; neutrophil collagenase [Hasty et al., 1990], MMP-13; collagenase-3 [Freije et al., 1994], can all specifically cleave triple helical collagen to give characteristic ¾ and ¼ products. MMP-2 (gelatinase A) and MT1-MMP (membrane type 1-MMP; MMP-14) have also been shown to cleave fibrillar collagen at this specific site [Aimes and Quigley, 1995; Ohuchi et al., 1997]. MMP-1 consists of two domains linked by an exposed proline-rich polypeptide [Li et al., 1995]. Whilst the N-terminal domain contains the active site, the C-terminal domain is important for binding to the substrate.

MMP-1 is known to be present within the rheumatoid synovial fluid [Clark et al., 1993] and has been localised to the rheumatoid joint [Brinckerhoff, 1991; Firestein et al., 1991; Okada et al., 1990; Tetlow and Woolley, 1998]. MMP-1 is upregulated by the proinflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor-α (TNFα) [Saklatvala et al., 1984; Mitchell and Cheung, 1991] suggesting its importance in the breakdown of cartilage collagen in arthritic diseases. MMP-13 is expressed by normal and osteoarthritic chondrocytes [Mitch-

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ell et al., 1996] and has been localised to both rheumatoid and osteoarthritic cartilage [Mitchell et al., 1996; Moldovan et al., 1996; Fernandes et al., 1998; Tetlow and Woolley, 1998]. MMP-13 is also the most efficient collagenase against type II collagen [Knäuper et al., 1996; Mitchell et al., 1996] suggesting an important role in cartilage collagen turnover.

Cartilage is composed of type II 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. These cells initiate the rapid release of proteoglycan from the tissue in response to cytokines such as IL-1 [Dingle et al., 1987] and TNFα [Saklatvala, 1986] but this matrix component can be replaced relatively quickly [Page-Thomas et al., 1991]. In contrast collagen is much less readily released, but when degradation of collagen does occur, the structural integrity of the tissue is irreversibly lost [Jubb and Fell, 1980]. This makes collagen degradation a key point in the control of cartilage turnover [Shingleton et al., 1996].

All-*trans*-retinoic acid (RetA) is a naturally occurring, biologically active retinoid. It is derived from retinol and is a member of the vitamin A family of compounds. RetA is known to be important in cartilage development and profoundly affects many aspects of cartilage and bone metabolism. For example, it can regulate the mineralisation of cartilage [Iwamoto et al., 1994] and increase the production of Type X collagen from chick chondrocytes [Pacifici et al., 1991]. It can interact with other growth factors such as transforming growth factor-β (TGFβ) [Morales et al., 1992; Von den Hoff et al., 1994], and can inhibit the IL-1-induced production of IL-6 by human lung fibroblasts [Zitnik et al., 1994]. It is also known to upregulate the production of plasminogen activators [Brown et al., 1992; Medh et al., 1992; Vitti and Hamilton, 1988].

Early studies showed that RetA could initiate the release of both proteoglycan and collagen from chick cartilage in explant culture [Dingle et al., 1961]. Whilst the release of proteoglycan was rapid, collagen release was slower and often dependent on the presence of other bone derived cells being present in the explant [Dingle et al., 1975]. These results suggested that factors produced by additional cell types in the explants might interact with RetA to promote collagen destruction. It is known that RetA alone can interact with connective tissue cells in culture and we and others have shown that RetA increased the synthesis and secretion of TIMP-1 from connective tissue cells, while blocking synthesis and secretion of collagenase [Brinckerhoff et al., 1980; Wright et al., 1991]. In addition, we have recently shown that combinations of growth factors synergise with RetA to profoundly affect the level of MMPs and TIMP-1 produced by fibroblasts in culture [Bigg and Cawston, 1995; 1996].

In this study we report that RetA in combination with IL-1α, an agent known to stimulate cartilage turnover, caused a rapid release of proteoglycan and collagen fragments from bovine nasal cartilage in explant culture. The collagen release was blocked by the addition of a metalloproteinase inhibitor, BB-94, and our data suggest that both MMP-1 and -13 are involved in collagen destruction mediated by IL-1 in combination with RetA.

MATERIALS AND METHODS

Materials

Chemicals were obtained from the following suppliers: All-*trans*-retinoic acid from Sigma Chemical Company (Poole, UK). IL-1α was a generous gift from GlaxoWellcome Ltd. (Greenford, UK). BB-94 and cDNAs for human MMP-1 and TIMP-1 were kind gifts from British Biotech Pharmaceuticals Ltd. (Oxford, UK). cDNA for MMP-13 was from Dr. V. Knäuper (UEA, Norwich, UK) and cDNA for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was as described [Fort et al., 1985]. All other chemicals and biochemicals were commercially available analytical grade reagents obtained from Fisher (Loughborough, UK), BDH (Poole, UK), Sigma, or have been previously described [Andrews et al., 1989; Ellis et al., 1994; Bigg and Cawston, 1995]. Control culture medium was Dulbecco's modification of Eagle's medium (DMEM) containing 25 mM HEPES (Gibco, Paisley, UK) supplemented with glutamine (2 mM), streptomycin (100 μg/ml), penicillin (100 U/ml), and nystatin (40 U/ml).

Cartilage Degradation Assay

Bovine nasal septum cartilage was held at 4°C overnight after slaughter. The connective tissue sheath was removed from the cartilage

that was cut into 2 mm slices. Discs were cut from the slices to give pieces 2 mm in diameter and washed twice in phosphate buffered saline (PBS). Three discs per well of a 24-well plate were incubated at 37°C in control medium (600 μl) for 24 h. Control medium (600 μl) with or without test reagents (four wells for each condition) was added and the plate incubated at 37°C for 3 days. The supernates were harvested and replaced with fresh medium containing test reagents. This was repeated at day 7 and the experiment was continued for a further 7 days and day 3, 7, and 14 supernates were stored at −20°C until assayed. To determine the total glycosaminoglycan (GAG) and hydroxyproline (OHPro) content of the cartilage fragments, the remaining cartilage was digested with papain (4.5 mg/ml) in 0.1 M phosphate buffer, pH 6.5, containing EDTA (5 mM) and cysteine hydrochloride (5 mM), incubating at 65°C until digestion was complete (16 h).

Cytotoxicity Assay

Viability of cartilage explants was assessed by screening for the production of lactate dehydrogenase (LDH) using the Cytotox 96 assay (Promega, Southampton, UK). No increase in LDH levels with any of the cytokine or inhibitor combinations was found (data not shown). Serum is excluded from cartilage explants since it can increase cartilage metabolism in the absence of exogenous cytokine(s) [Sah et al., 1994]. The aim of this study was to create a model of cartilage breakdown; therefore the presence of serum was avoided as it contains chondroprotective agents such as insulin-like growth factor-1 [Luyten et al., 1988; Tyler, 1989]. The absence of serum was also shown not to affect the viability of the tissue (data not shown) and previous studies have shown that cartilage in serum-free culture for 8–9 days can respond to serum and other growth factors [Hascall et al., 1983]. In the present study cytokines/growth factors were added after 1 day of serum-free culture.

Proteoglycan Degradation

Media samples and papain digests were assayed for sulphated GAGs (as a measure of proteoglycan release) using a microtitre plate modification of the 1,9-dimethylmethylene blue dye binding assay [Farndale et al., 1986]. Briefly, sample or standard (40 μl) was mixed with dye reagent (250 μl) in the well of a microtitre plate, and the absorbance at 525 nm determined immediately. Chondroitin sulphate from bovine trachea (5–40 μg/ml) was used as a standard. The complex formed with 1,9-dimethylmethylene blue results in a decrease in absorbance at 525 nm.

Collagen Degradation

As a measure of collagen degradation, OHPro release was assayed using a microtitre plate modification of the method in [Bergman and Loxley, 1963]. Chloramine T (7% [w/v]) was diluted 1:4 in acetate-citrate buffer (57 g sodium acetate, 37.5 g tri-sodium citrate, 5.5 g citrate acid, 385 ml propan-2-ol per litre of water). p-dimethylaminobenzaldehyde (DAB; 20 g in 30 ml 60% perchloric acid) was diluted 1:3 in propan-2-ol. Samples were hydrolysed in 6 M HCl for 20 h at 105°C and the hydrolysate neutralised by drying over NaOH in vacuo using a Savant speed vac. The residue was dissolved in water (200 μl) and 40 μl of sample or standard (OHPro; 5–30 μg/ml) was added to microtitre plates together with chloramine-T reagent (25 μl) and then DAB reagent (150 μl) after 4 min. The plate was heated to 65°C for 35 min, cooled, and the absorbance at 560 nm determined.

Enzyme and Inhibitor Assays

^{3}H-acetylated calf skin collagen was used to measure collagenolytic activity by the modified diffuse fibril assay [Koshy et al., 1999]. Trypsin was used as a control to ensure that the collagen was not denatured. If trypsin released significant amounts of collagen then the substrate was discarded and assays repeated. If inhibition of metalloproteinases was required, 1,10-phenanthroline was added to a final concentration of 2 mM. Aminophenylmercuric acetate (APMA) was added at a final concentration of 0.7 mM to activate pro-collagenases. Inhibitory activity was assayed by the addition of samples to a known amount of active collagenase in the diffuse fibril assay and the percent inhibition calculated. One unit of collagenase activity degrades 1 μg of collagen per minute at 37°C and one unit of inhibitory activity inhibits two units of collagenase by 50%.

Electrophoresis and Western Blotting

Supernatants from cartilage explants treated with IL-1 + RetA for 14 days were

incubated with soluble calf skin type I collagen at 22°C overnight. Samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with reduction [Laemmli and Farvre, 1973]. Gels were stained with Coomassie Brilliant Blue G250 to visualise the degradation products.

Supernatants from explant and monolayer culture were separated by SDS-PAGE with reduction and transfered to nitrocellulose and then probed with sheep anti-porcine MMP-1 IgG (1 μg/ml; as described in Cawston et al., 1998), rabbit anti-human MMP-2 IgG (1:1000; TCS Biologicals Ltd., Botolph Claydon, UK) or a rabbit anti-human MMP-13 IgG (1 μg/ml) prepared in house using recombinant human pro-MMP-13 antigen [Knäuper et al., 1996]. Horseradish peroxidase-conjugated secondary antibodies were either rabbit anti-sheep IgG or swine anti-rabbit IgG (both from Dako, Ely, UK). Colour was developed using metal enhanced diaminobenzidine tetrahydrochloride (Pierce & Warriner, Chester, UK) as substrate.

Chondrocyte Isolation and Culture

Bovine nasal septa were cut into slices, and chopped into small pieces, approximately 2 mm^3, washed three times in PBS containing penicillin (100 U/ml), streptomycin (100 μg/ml), and nystatin (40 U/ml). The cartilage pieces were then subjected to a sequential digest by hyaluronidase (1 mg/ml, 15 min, 37°C), trypsin (2.5 mg/ml, 30 min, 37°C), and bacterial collagenase (3 mg/ml, 16 h, 37°C). Tissue debris was allowed to settle and the chondrocyte suspension removed. Cells were pelleted by centrifugation at 400*g* for 5 min, washed in PBS, and resuspended in DMEM supplemented with 10% fetal calf serum (FCS; Gibco). Cells were seeded into 25 cm^2 tissue culture flasks (Corning/Costar, High Wycombe, UK) at 1×10^6 cells per flask. Once cells had reached confluence, medium was removed, the cells washed with PBS, and medium replaced with serum-free DMEM (control medium). After 24 h serum starvation medium was replaced with serum-free DMEM with or without IL-1α (1 ng/ml) or RetA (10^{-6} M) or combinations thereof. Media and cells were harvested at 1 to 7 days post-stimulation. Media were stored at −20°C until assayed. At the time of harvest an aliquot of each culture medium sample was treated with the following cocktail of protease inhibitors to prevent autolysis of any MMPs that may be present: EDTA (10 mM), N-ethylmalemide (10 mM), benzamidine hydrochloride (10 mM), 6-aminocaproic acid (100 mM). Cells were harvested into RNeasy lysis buffer (Qiagen, Crawley, UK), and total RNA isolated using RNeasy spin columns (Qiagen) according to the manufacturer's instructions.

RNA Electrophoresis and Northern Blotting

Total RNA (20 μg/lane) was separated on 1% agarose formaldehyde gels, transfered to GeneScreen plus membrane (NEN, Boston, MA, USA), and cross-linked by UV irradiation. Blots were probed in turn with ^{32}P-labelled cDNA probes to human MMP-1, -13, and TIMP-1. GAPDH was used as the housekeeping gene. The probes and hybridisation conditions used were as described previously [Cawston et al., 1998]. Northern blots were exposed to a Storage Phosphor Screen (Molecular Dynamics, Chesham, UK), Bands were detected by PhosphorImager (Storm 860, Molecular Dynamics) and quantified using 1D Prime software (Amersham Pharmacia Biotech, Little Chalfont, UK). Results were expressed as integrated density values (IDV).

Statistics

To determine any significance between data obtained from material treated with RetA or IL-1 alone and the material treated with IL-1 + RetA, two-tailed, unpaired Student's *t*-test was applied to the data.

RESULTS

Treatment of Bovine Cartilage With RetA in the Presence of IL-1

The effect of either RetA or IL-1 alone on collagen release was variable (compare Fig. 1A and B). IL-1 alone always induced some collagen release and RetA alone induced collagen release in seven of ten experiments. In some experiments the release stimulated by RetA alone was greater than IL-1 alone (Fig. 1B). However, the combination of IL-1 + RetA throughout the 14 days of culture significantly increased the release of collagen (Fig. 1A; IL-1 + RetA[i]). The effect of IL-1 + RetA was more reproducible than either agent alone as indicated by the smaller error bars (Fig. 1A; IL-1 + RetA[i]). Significant increase in collagen release, compared to control, was also seen when these two agents were only present dur-

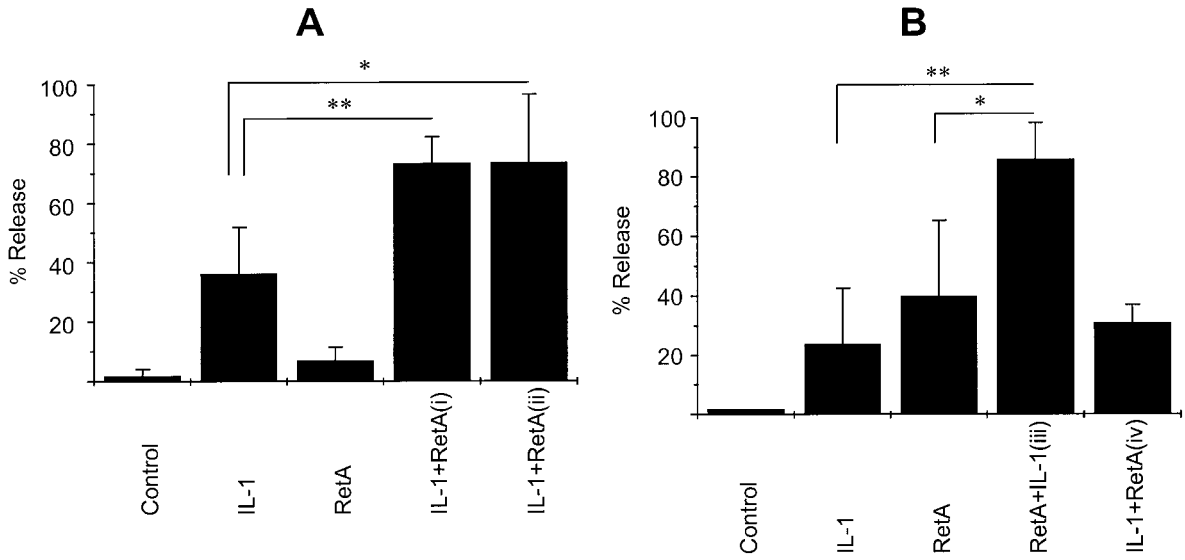

Fig. 1. The effect of IL-1 and RetA on the release of collagen. IL-1α (1 ng/ml) and RetA (10^{-6} M) were used to stimulate bovine nasal cartilage in explant culture. **A**: IL-1 + RetA(i), both factors present for the full 14 days of culture, with significant increase in collagen release compared to IL-1α alone (**$P < 0.01$); IL-1 + RetA(ii); both factors present for the first 7 days of culture only, followed by control medium only for days 7–14, with significant increase in collagen release compared to IL-1α alone (*$P < 0.05$). **B**: RetA + IL-1(iii), RetA for the first 7 days of culture, followed by IL-1α for days 7–14, with significant increase in collagen release compared to either IL-1α alone or RetA alone for both weeks of culture (**$P < 0.01$, *$P < 0.05$); IL-1 + RetA(iv), IL-1α for the first 7 days of culture, followed by RetA for days 7–14. The data presented in A are derived from a different experiment to that presented in B, and are representative of 10 separate experiments.

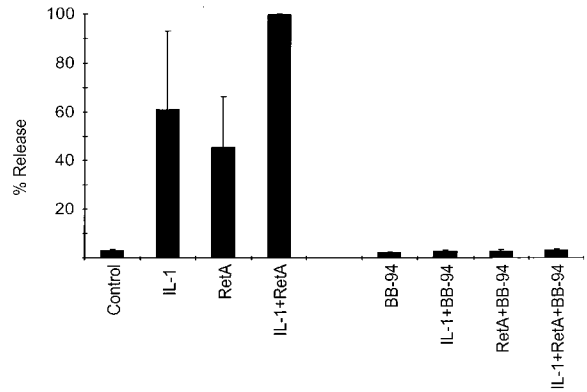

Fig. 2. Collagen release from bovine nasal cartilage explants stimulated with IL-1 and RetA in the presence of BB-94. Bovine nasal cartilage was stimulated in the presence or absence of IL-1α (1 ng/ml), RetA (10^{-6} M), and BB-94 (10^{-5} M) for the full 14 days of culture. Collagen release was determined by measuring OHPro and expressed as a percentage of the total. The cumulative release of collagen by day 14 is presented.

ing the first 7 days of culture (Fig. 1A; IL-1 + RetA[ii]). When RetA was added during the first 7 days followed by IL-1 for the final 7 days, then a significant increase in collagen release was observed when compared to either agent alone (Fig. 1B; RetA+IL-1[iii]). Incubation with IL-1 first followed by RetA induced collagen release only comparable to either agent alone (Fig.1B; IL-1+RetA[iv]).

Bovine nasal cartilage released approximately 30% of GAG by day 3 when treated with either RetA or IL-1 (data not shown). The effect of combining these two agents increased the amount of GAG released by day 3, but this increase was not statistically significant compared to either agent alone (data not shown). By day 7 all treatments had induced 80–90% release of the total GAG (data not shown).

Incubation of the cartilage with IL-1+RetA in the presence of the metalloproteinase inhibitor BB-94 at 10^{-5} M completely blocked IL-1+RetA induced collagen degradation (Fig. 2).

Analysis of Medium From IL-1/RetA Treated Cartilage and Chondrocytes for Proteinases and Inhibitors

The media removed on day 7 and 14 from the cartilage explant cultures were assayed for total collagenase, active collagenase, and TIMP using the activity assays described in Materials and Methods. These assays measure total activity against fibrillar collagen or total inhib-

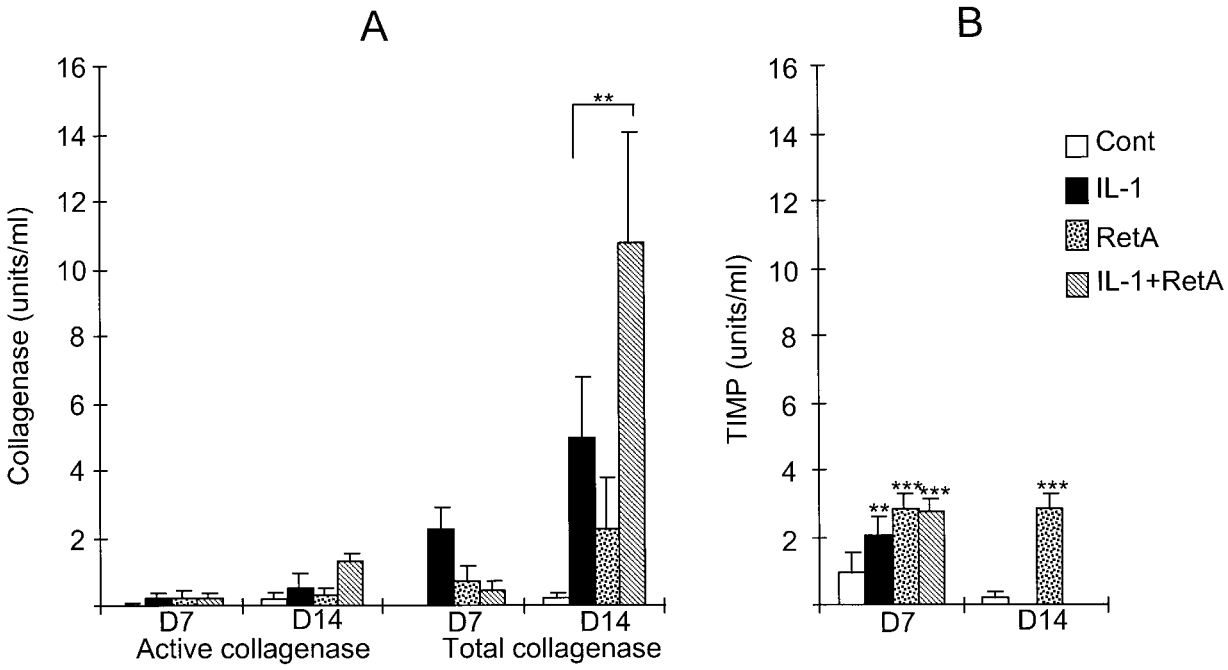

Fig. 3. The effect of IL-1 and RetA on the production of collagenolytic activity and TIMP activity from bovine nasal cartilage in explant culture. Bovine nasal cartilage was cultured for 14 days with IL-1α (1 ng/ml) and RetA (10^{-6} M), and collagenolytic and TIMP activities in the culture supernatants at days 7 and 14 determined. **A**: Collagenolytic activity; **B**: TIMP inhibitory activity (***$P < 0.001$, **$P < 0.01$).

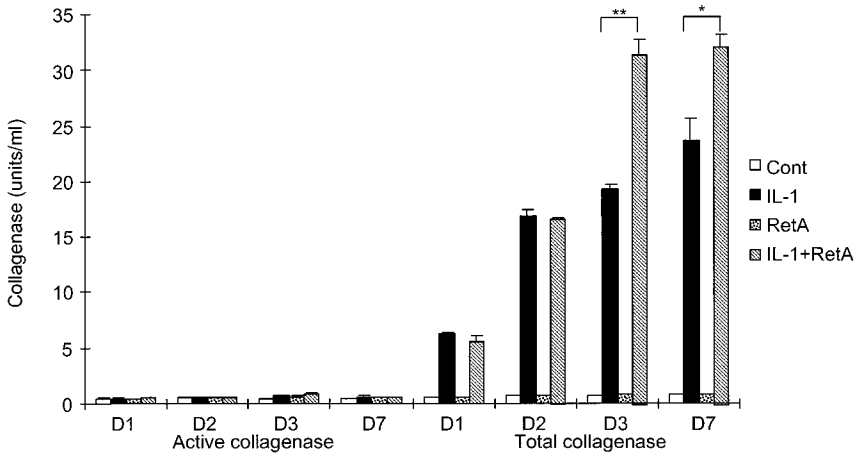

Fig. 4. The effect of IL-1 and RetA on the production of collagenolytic activity from bovine nasal chondrocytes in monolayer culture. Bovine nasal chondrocytes were stimulated with IL-1α (1 ng/ml) and RetA (10^{-6} M), and culture supernatants harvested and assayed for collagenolytic activity. A significant increase in pro-collagenase at days 3 and 7 was induced by IL-1 + RetA compared to IL-1α alone (**$P < 0.01$, *$P < 0.05$).

itory activity, but do not distinguish between the different collagenases or TIMPs. Low levels of collagenolytic activity were present at day 7, with the highest levels present in the IL-1 treated cultures although very little active collagenase was present (Fig. 3A). At day 14 total collagenase levels were greatest in the medium from cartilage treated with IL-1 + RetA, and this induction was synergistic (Fig. 3A). The highest level of active collagenase was also seen in the medium from cartilage treated with IL-1 + RetA at day 14 (Fig. 3A). This coincides with the release of collagen fragments into the medium (Fig. 1). In contrast, significant induction of TIMP activity was detected for all treatments at day 7 (Fig. 3B). By day 14, only medium from cartilage treated with RetA alone contained any detectable TIMP activity.

No active collagenase could be detected in the culture media supernatants from chondrocytes grown in monolayer culture. However, substantial levels of pro-collagenase were de-

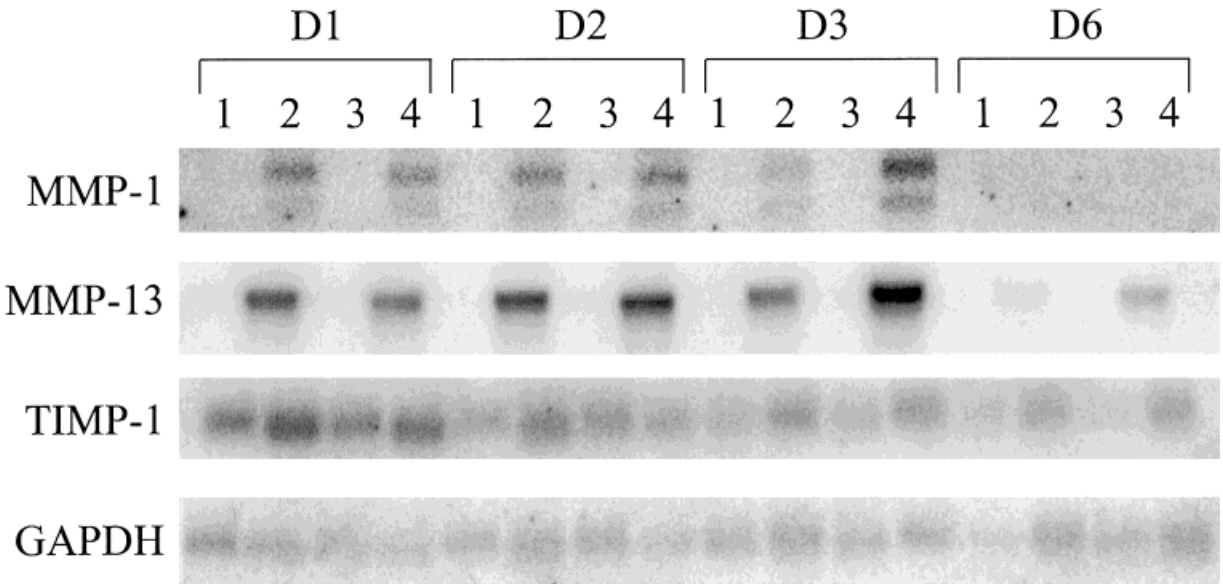

Fig. 5. Northern blot analysis of total RNA from IL-1 and RetA stimulated bovine nasal chondrocytes. Bovine nasal chondrocytes were stimulated in monolayer culture and total cellular RNA harvested at day 1 (D1) through to day 6 (D6) and Northern blotted for MMP-1, MMP-13, and TIMP-1. GAPDH was used for normalisation. Lane 1: Control; Lane 2: IL-1α (1 ng/ml); Lane 3: RetA (10^{-6} M); Lane 4: IL-1α + RetA (1 ng/ml + 10^{-6} M).

tected in the media from cells treated with IL-1 or IL-1 + RetA (Fig. 4). IL-1 alone induced a steady increase in the induction of collagenase throughout the seven day culture period. IL-1 and RetA in combination induced a significant increase in pro-collagenase compared to IL-1 alone. Maximal stimulation with IL-1 + RetA was achieved by day 3, which was then maintained through to day 7. RetA alone did not induce any collagenase secretion. Unlike the cartilage explant experiments, no induction of TIMP activity was detected in chondrocyte conditioned medium by RetA or IL-1 or combinations thereof (not shown).

Analysis of mRNA Expression by Northern Analysis

Following stimulation of bovine nasal chondrocytes, grown in monolayer culture, with IL-1 alone, an increase in the level of MMP-1 mRNA expression was observed (Figs. 5 and 6A). RetA alone did not appear to induce MMP-1 gene expression. The combination of IL-1 + RetA induced a further induction of MMP-1 mRNA expression over that observed with IL-1 alone (Fig. 6A). This induction was transient, not appearing until day 3 post-stimulation, and returned to control levels by day 6. Reduced MMP-1 mRNA expression, compared to maximal stimulation, was detected at day 5 post-stimulation (not shown), but no MMP-1 mRNA was detected at day 6 post-stimulation with any treatment (Figs. 5 and 6A).

MMP-13 mRNA was induced by IL-1 alone (Figs. 5 and 6B). This induction persisted above control levels through to day 6 post-stimulation. RetA alone did not induce MMP-13 mRNA expression. The combination of IL-1 + RetA did not increase MMP-13 mRNA expression at days 1 and 2, when compared to IL-1 alone. However, a dramatic induction was observed by day 3 (Figs. 5 and 6B). This induction was maximal at day 3 and still evident at day 6 (Figs. 5 and 6B), but had returned to control levels by day 7 (not shown).

TIMP-1 mRNA was detected throughout the culture period, but levels decreased with time (Figs. 5 and 6C). In some cell culture experiments TIMP-1 mRNA was not detected beyond day 2 (not shown).

Confirmation of Specific Cleavage of Collagen by Collagenolytic Activity

Media (day 7–14) removed from cartilage treated with IL-1 + RetA for 14 days were incubated at 22°C with soluble type I collagen overnight. After separation by SDS-PAGE and

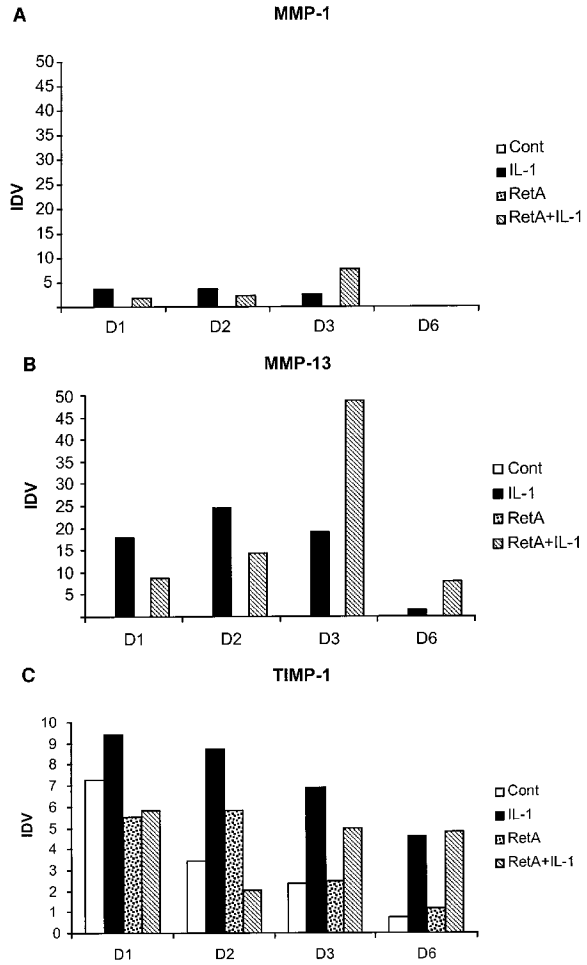

Fig. 6. Representation of Northern blot data after normalisation to GAPDH. The Northern blots shown in Figure 5 were scanned, quantified, and normalised to the intensity of GAPDH detected. **A**: MMP-1; **B**: MMP-13; **C**: TIMP-1.

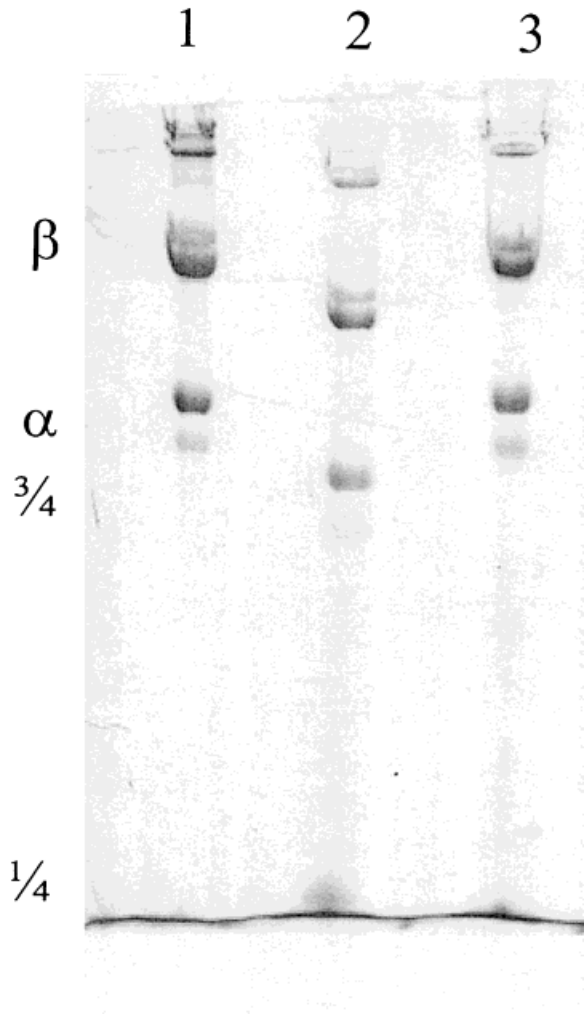

Fig. 7. Confimation of collagenase-specific activity in the culture medium from IL-1 + RetA stimulated bovine nasal cartilage. Culture medium (day 7–14) from IL-1α (1 ng/ml) and RetA (10^{-6} M) stimulated bovine nasal cartilage were incubated overnight with native/soluble type I collagen prior to SDS-PAGE. Lane 1: collagen incubated alone; Lane 2: incubated collagen + medium; Lane 3: collagen + medium + 1,10-phenanthroline (2 mM).

staining, the specific cleavage of the collagen into ¾ and ¼ fragments could be seen (Fig. 7, lane 2). Incubation with 1,10-phenanthroline prevented cleavage of the collagen (Fig. 7, lane 3).

Identification of the Collagenase Responsible for Collagen Cleavage

To determine which enzyme or enzymes were responsible for collagen cleavage we separated day 14 media from cartilage treated with both IL-1/RetA combinations by SDS-PAGE and transfered the proteins electrophoretically onto nitrocellulose. These membranes were Western blotted with antibodies to MMP-1, MMP-2, and MMP-13. A band was recognised by the antibody to MMP-1, however no reaction was seen when blots were probed with polyclonal antibodies to human MMP-2 or MMP-13 (data not shown). This could mean no MMP-13 was produced or that MMP-13 was not stable in culture medium for 7 days.

To test this, bovine nasal chondrocytes were grown in monolayer culture and supernatants were treated with a cocktail of proteinase inhibitors at the point of harvest (see Materials and Methods). These supernatants were then subjected to Western blot analysis. MMP-13 was detected at day 3 post-stimulation in these supernatants (Fig. 8). Low levels were detected

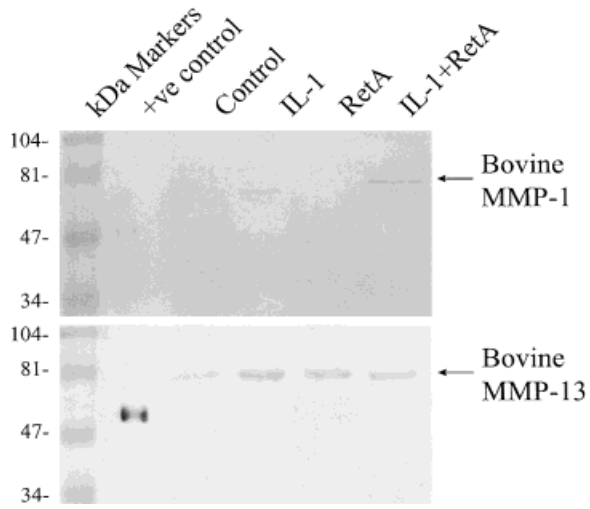

Fig. 8. Identification of the collagenases present in the culture medium from IL-1+RetA stimulated bovine nasal chondrocytes. Bovine nasal chondrocytes were grown in monolayer culture and stimulated with IL-1α (1 ng/ml) and RetA (10^{-6} M) for 3 days. A cocktail of proteinase inhibitors was added to culture supernatants at the time of harvest. Supernatants were then Western blotted for MMP-1 and MMP-13.

in control medium, but were clearly detected in supernatants from IL-1, RetA, and IL-1 + RetA stimulated cells. The presence of a band migrating below the main immunoreactive band (pro-MMP-13) was just detectable in the IL-1 + RetA medium, indicating the presence of active MMP-13 in this sample. When media from chondrocytes grown in the presence of cytokines for 6 or 7 days were analysed by Western blot, little or no immunoreactive protein was detected with the MMP-13 antibody (not shown). Bovine MMP-13 appears larger than the recombinant human positive control. The size differences may be due to variations in amnio acid sequence and/or glycosylation between the two species. MMP-1 was also detected at day 3 post-stimulation (Fig. 8) in supernatants from IL-1 and IL-1 + RetA treated chondrocytes. MMP-1 was still detected in supernatants from IL-1 and IL-1 + RetA stimulated chondrocytes at day 6 (not shown).

DISCUSSION

In this study we report the novel observation that IL-1 + RetA can combine to induce the rapid release of collagen fragments from bovine nasal cartilage. This collagen degradation involved both MMP-1 and MMP-13. We also show, for the first time, that MMP-13 is likely to be the predominant collagenase in this system.

The pro-inflammatory cytokines IL-1 and TNFα are known to effect the release of proteoglycan from cartilage tissue in vivo and in vitro, and block the synthesis of new proteoglycan molecules [Saklatvala, 1986; Page-Thomas et al., 1991]. Our results confirm previous studies where IL-1 and RetA have been shown to promote GAG release from cartilage [Bonassar et al., 1997; Hughes et al., 1998]. Whilst the proteoglycans are obviously important to the structural integrity of cartilage, the chondrocytes within cartilage explant cultures can rapidly replace proteoglycan once the cytokine stimulus is removed [Page-Thomas et al., 1991]. However, loss of the collagen fibrillar network has a profound effect on the tissue and represents the irreversible phase of cartilage destruction [Jubb and Fell, 1980].

The main aim of this investigation was to study mechanisms of collagen breakdown. Our previous studies [Ellis et al., 1994; Cawston and Ellis, 1996], however, have been hampered by the relatively irreproducible release of collagen from cartilage in culture. Dramatic and reproducible collagen release was observed when IL-1 and oncostatin M (OSM) were added together to cartilage in culture [Cawston et al., 1995; 1998]. This was a surprising result since OSM induces expression of TIMP-1 in a variety of cell types [Richards et al., 1993], and some consider that this agent is chondroprotective [Malik et al., 1995; Nemoto et al., 1996]. Since RetA has been proposed as an agent that could be used to prevent tissue degradation [Bauer et al., 1983], is known to upregulate TIMP-1 [Wright et al., 1991; Bigg and Cawston, 1995; 1996] and can downregulate MMP-1 [Brinckerhoff et al., 1980] we examined the effect of adding RetA in the presence of IL-1 to bovine nasal cartilage in culture to determine the effect on the release of collagen fragments.

The dramatic release of collagen fragments observed with IL-1 + RetA correlated with the appearance in the medium of pro-collagenase with approximately one seventh of the enzyme converted to the active form. This is indicative of the destructive nature of the collagenases without the presence of sufficient TIMPs to control their activity.

The increased collagen degradation observed with IL-1 + RetA were combined was dependant on RetA being present during the first 7

days of culture. RetA may induce the production of activators of the collagenases. Indeed, RetA has been demonstrated to induce plasminogen activators [Brown et al., 1992; Medh et al., 1992; Vitti and Hamilton, 1988]. The absence of RetA from the first 7 days of culture may mean insufficient activators can be produced in time to activate the pro-collagenases present. However, Northern data suggests that de novo protein synthesis may be required to facilitate the synergisic induction of both MMP-1 and -13 mRNAs. Maximal induction with IL-1 alone was seen by day 1 and did not increase further, whereas IL-1 + RetA reached a maximum at day 3. It may be that RetA increased the expression of stimulatory cytokines, increased IL-1 receptor expression or increased the expression of intracellular signalling components. Ongoing studies hope to elucidate which of these mechanisms is occurring.

Five members of the MMP family are known to be able to specifically cleave collagen to produce ¾ and ¼ fragments. MT1-MMP and MMP-2 have been shown to cleave fibrillar collagen but much less efficiently than the three collagenases (MMP-1, -8, and -13). MT1-MMP and MMP-8, were not investigated in this study. MT1-MMP is membrane bound and unlikely to have been detected in culture media supernatants. MMP-8 is predominantly expressed by neutrophils, a cell type not present in our culture system. However, low levels of MMP-8 have been demonstrated to be produced by chondrocytes [Cole et al., 1994] and its potential presence in cartilage should not be discounted.

Two members of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin repeats) family, ADAMTS-4 [Arner et al., 1999] and ADAMTS-5 (formerly known as ADAMTS-11) [Abbaszade et al., 1999] have now been shown to have aggrecanase activity. A previous study in our laboratory showed that an aggrecanase activity was inhibited by BB-94 [Billington et al., 1998] and BB-94 partially inhibited GAG release in this study (data not shown), suggesting adamolysins may be inhibited by BB-94. However, to date no member of the adamolysin family of proteinases has been shown to cleave triple helical collagen.

MMP-1 was shown to be present by Western blotting. However, MMP-13 could not be detected in the media from cartilage explant experiments and was only detected in the medium from chondrocyte monolayers after the addition of protease inhibitors. The inability to detect MMP-13 from the culture medium of cartilage explants could be due to proteolytic degradation of MMP-13 [Knäuper et al., 1996]. It has also been proposed that MMP-13 may be internalised via a specific cell surface receptor complex [Barmina et al., 1999], which may represent a further point of control for MMP-13. Thus, these factors need to be taken into account when establishing the relative roles of MMP-1 and MMP-13 in cartilage collagen breakdown.

RetA alone can induce collagen degradation and pro-MMP-13 can be detected in chondrocyte monolayer supernatants. However, no MMP-13 mRNA was detected by Northern blot. The levels of message may be too low to be detected, but sufficient to allow accumulation of pro-MMP-13 in the medium. However, collagen release in response to RetA alone was variable. We have found it difficult to detect MMP-13 protein and it may be that in some experiments no MMP-13 is produced. The data presented in Figures 4–6 derive from the same experiment, while the Western blots in Figure 8 were from one of three repeat experiments. This could explain why RetA alone does not always induce collagen release. No induction of MMP-1 was ever observed, at mRNA or protein levels, in chondrocytes stimulated with RetA alone. It cannot be discounted that RetA could induce collagen degradation by an MMP-independent mechanism [Kozaci et al., 1998; Price et al., 1999]. However, our data suggest that IL-1 + RetA induced collagen degradation is most probably MMP driven since it can be inhibited by BB-94 and the initial collagen cleavage is at the ¾/¼ site.

The early introduction of RetA rapidly stimulates the production of TIMP whilst IL-1 promotes the production of MMP-1 and MMP-13. Levels of TIMP then fall whilst collagenase levels remain high or increased later in the culture period. Data from the Northern blot analysis confirmed these observations. When activation occurs there is insufficient local TIMP to prevent collagen destruction.

Both MMP-1 and MMP-13 are upregulated in response to IL-1 + RetA, however, there is evidence of differential regulation. The response of MMP-13 to this stimulus appears stronger and longer lived than that of MMP-1. The only quan-

titative method of determining collagenase activity available to us was the bioassay. This assay does not distinguish between the enzymes known to cleave fibrillar collagen but does give a measure of total activity. Available enzyme-linked immunoabsorbent assay techniques do not cross-react with bovine material. However, the semi-quantitative Northern analysis suggests that MMP-13 rather than MMP-1 is the major collagenase involved in bovine cartilage destruction when stimulated with this combination of reagents. These data, combined with the fact that MMP-13 cleaves type II collagen five to 10 times more efficiently that MMP-1 [Knäuper et al., 1996; Mitchell et al., 1996], suggests that MMP-13 plays a major role in bovine cartilage collagen degradation when stimulated with RetA.

This study gives further support to the hypothesis that irreversible cartilage damage is caused when collagenases are activated and exceed local TIMP concentrations they destroy the collagen component of the matrix. MMP-1 and MMP-13 appear to have a role in IL-1 + RetA induced cartilage collagen degradation.

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