Interleukin 13 blocks the release of collagen from bovine nasal cartilage treated with proinflammatory cytokines

C S Cleaver, A D Rowan, T E Cawston

Abstract

Objective—**To investigate whether interleukin 13 (IL13) could act in a chondroprotective manner and protect cartilage stimulated to resorb with a combination of IL1α and oncostatin M (OSM), in a similar way to the anti-inflammatory cytokine, IL4.**

Methods—**IL13 was added to explant cultures of bovine nasal cartilage stimulated to resorb with IL1α and OSM, and the release of collagen and proteoglycan determined. Collagenolytic and tissue inhibitors of metalloproteinase (TIMP) activities were determined by bioassay. Northern blot analyses were performed to determine the effects of IL13 on the induction of matrix metalloproteinase-1 (MMP-1), MMP-3, MMP-13, and TIMP-1 gene expression.**

Results—**IL13 can prevent the release of collagen from bovine nasal cartilage in a dose dependent manner. This was accompanied by a concomitant decrease in measurable collagenolytic activity in the culture supernates and an increase in TIMP activity. Northern blot analysis showed that IL13 down regulated MMP-3 and MMP-13 levels but up regulated MMP-1 and TIMP-1 gene expression in bovine nasal chondrocytes at 24 hours.**

Conclusion—**This study showed for the first time that IL13 can block collagen release from resorbing cartilage in a similar manner to IL4. This is accompanied by a reduction in detectable collagenolytic activity, a decrease in MMP-3 and MMP-13 mRNA levels, and an up regulation of TIMP-1 expression.**

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Department of Rheumatology, School of Clinical and Medical Sciences, 4th Floor Catherine Cookson Building, The Medical School, Framlington Place, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne NE2 4HH, UK
C S Cleaver
A D Rowan
T E Cawston

Correspondence to:
Dr Cleaver
C.S.Cleaver@ncl.ac.uk

Cartilage is composed of an extensive extracellular matrix of proteoglycans and collagens and relatively isolated numbers of chondrocytes, which maintain tissue integrity. The chondrocytes regulate cartilage metabolism under both normal and pathophysiological conditions. These cells initiate the rapid release of proteoglycan from cartilage in response to proinflammatory cytokines, such as interleukin 1α (IL1α)[1] and tumour necrosis factor α (TNFα),[2] but this component of the matrix is quickly replaced.[3] Collagen is much less readily released from the tissue, and when degradation occurs the structural integrity of the tissue is irreversibly lost.[4] Therefore, collagen degradation is a key point in the control of cartilage turnover.

The matrix metalloproteinases (MMPs) comprise a family of zinc dependent homologous enzymes that collectively can degrade all the components of the extracellular matrix. The three mammalian collagenases, collagenase-1 (MMP-1),[5] neutrophil collagenase (MMP-8),[6] and collagenase-3 (MMP-13),[7] have the ability to cleave the three α chains of types I, II, and III collagens at a single site to give characteristic three quarters and one quarter length products.[8] Gelatinase A (MMP-2)[9] and membrane type 1 MMP (MMP-14)[10] can also cleave collagen in this manner. These potent enzymes are tightly regulated at a number of points, including synthesis and secretion by cytokines and growth factors, the production of pro-enzyme forms requiring proteolytic cleavage for activation, and inhibition of these active forms by naturally occurring inhibitors.[11] The tissue inhibitors of metalloproteinases (TIMPs) are a specific group of inhibitors that form stable 1:1 stoichiometric complexes with active MMPs.[12] This family of inhibitors now consists of at least four members—TIMP-1,[13] TIMP-2,[14] TIMP-3,[15] and TIMP-4.[16]

The involvement of these proteinases in the normal turnover of connective tissue matrix that takes place during growth and development is well established.[17] Furthermore, certain MMPs have been shown to have critical roles in wound healing,[18] tumour growth and metastasis,[19] and the pathological destruction of cartilage and bone in the arthritides.[20 21]

Raised levels of MMP-1 have been seen in arthritic synovial tissue and cartilage[22] and in synovial fluids taken from patients with rheumatoid arthritis (RA).[23 24] Synovial cells and chondrocytes produce MMPs in response to proinflammatory cytokines such as IL1α and TNFα, suggesting their importance in the breakdown of cartilage collagen in arthritic diseases. In a previous study we showed that treatment of bovine nasal cartilage in explant culture, with a combination of IL1α and oncostatin M (OSM) led to the release of proteoglycan and collagen fragments.[25] More recently, we have shown that IL4, a 20 kDa pleiotropic anti-inflammatory cytokine, can act in a chondroprotective manner, blocking the release of collagen fragments from bovine nasal cartilage in explant culture.[26] IL13 is an anti-inflammatory cytokine known to resemble IL4, sharing approximately 30% homology at the protein level and exhibiting many overlapping biological activities.[27] An unglycosylated

protein of 132 amino acids with a molecular size of 10 kDa,[28] IL13 was cloned and sequenced and found to be located in close proximity to the IL4 gene, on chromosome 5q31.[29]

IL13 has been shown to inhibit a range of proinflammatory cytokines, including IL1β, TNFα, IL6, and IL8 in monocytes, macrophages, B cells, natural killer cells, and endothelial cells in a similar way to IL4.[28–31] Addition of exogenous IL13 to cultures has been shown to reduce significantly the production of IL1β and TNFα by synovial fibroblasts and mononuclear cells.[32] Studies have also shown that in vitro IL13 can inhibit bone resorption.[33] Furthermore, subcutaneous inoculation of vector cells, engineered to secrete IL13 into mice, ameliorated collagen induced arthritis, supporting the notion that IL13 has anti-inflammatory potential in RA.[34] In view of these apparent structural and functional similarities, we investigated whether IL13 can protect cartilage by preventing collagen release in the same manner as we demonstrated for IL4.[26]

In this study we show for the first time that IL13 acts in a chondroprotective manner by specifically blocking collagen release from cartilage stimulated to resorb with IL1α + OSM by reducing the levels of active collagenase(s).

Materials and methods
CULTURE MEDIUM AND TEST REAGENTS
Human recombinant IL1α and IL4 were generous gifts from Glaxo Group Research Ltd (Greenford, UK). IL1α was used at a final concentration of 1 ng/ml. Human recombinant OSM and IL13 were obtained from R&D Systems Ltd (Oxon, UK). OSM was used at a final concentration of 10 ng/ml in all experiments. IL1α, OSM, and IL13 were stored at −80°C and diluted from stock solutions into culture medium. IL13 was used in a range of concentrations from 1 to 50 ng/ml. Pro-MMP-3 was a generous gift from Professor Hideaki Nagase (Kennedy Institute, London, UK). Control culture medium was Dulbecco's modification of Eagle's medium (DMEM) containing 25 mM HEPES (Gibco, Paisley, UK) supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), glutamine (2 mM), and gentamycin (2.5 μg/ml). All other chemicals and biochemicals were commercially available analytical grade reagents available from Sigma (Poole, UK) or BDH (Poole, UK) or have been previously described.[25]

CELL CULTURE
Bovine nasal cartilage was cut into 2 mm pieces and chondrocytes isolated by sequential enzymatic digestion of the tissue to produce a primary culture. Briefly, the cartilage was incubated at 37°C for 15 minutes with hyaluronidase at 1 mg/ml in phosphate buffered saline (PBS) (Sigma). After three washes with PBS, 0.25% (w/v) trypsin was added for 30 minutes at 37°C. Finally, 3 mg/ml bacterial collagenase was added to the cartilage and left at 37°C overnight. Chondrocytes were collected by centrifugation at 1100 rpm for five minutes and incubated in DMEM supplemented with 10%

fetal calf serum, 100 U/ml of penicillin, 100 μg/ml streptomycin, 40 U/ml nystatin, and 100 μg/ml ascorbate. Cells were grown to 70–80% confluence, then starved for 24 hours in serum-free DMEM before adding cytokines.

CARTILAGE DEGRADATION ASSAY
Bovine nasal septum cartilage was held at 4°C overnight after slaughter. Discs were cut from 2 mm slices to give pieces of 2 mm diameter and washed twice in PBS. Three discs per well of a 24 well plate were incubated for 24 hours at 37°C in 600 μl control medium. Fresh control medium (600 μl), with or without test reagents (four wells for each condition), was then added and the plate incubated at 37°C for seven days. Supernates were collected and replaced with fresh medium containing identical test reagents to day 1. The experiment was continued for a further seven days and day 7 and 14 supernates were stored at −20°C until assay. Where MMP-3 was added at day 7 of culture, pro-MMP-3 was activated by addition of trypsin (Sigma) for 15 minutes at room temperature to a final concentration of 1 mg/ml. Soybean trypsin inhibitor (Sigma) was then added in excess for one hour at room temperature to inhibit the trypsin present. MMP-3 was diluted into culture medium immediately before use at a concentration of either 0.83 μg/ml or 3.33 μg/ml to give 0.5 μg/well or 2 μg/well respectively and fresh cytokines added. Control plates had no MMP-3 added. The experiment was continued for a further seven days and day 7 and 14 supernatants were stored at −20°C until assay.

CYTOTOXICITY ASSAY
Lactate dehydrogenase assays were performed on day 7 and day 14 media to assess viability of explanted tissue using the CytoTox 96 cytotoxicity assay (Promega, Southampton, UK). No increase in lactate dehydrogenase levels with any of the cytokine combinations was found. Serum was excluded from cartilage explants because it can increase cartilage metabolism in the absence of exogenous cytokines.[35] The absence of serum was also shown not to affect viability of the tissue (data not shown).

PROTEOGLYCAN AND COLLAGEN DEGRADATION
To determine the total glycosaminoglycan (GAG) and hydroxyproline (OHPro) content of the cartilage samples, the remaining cartilage was digested with papain (4.5 mg/ml; Sigma) in 0.1 M phosphate buffer, pH 6.5, containing 5 mM EDTA and 5 mM cysteine hydrochloride, with incubation at 65°C until digestion was complete (16 hours).

As a measure of proteoglycan release, media samples and digests were assayed for sulphated GAG, using a modification of the 1,9-dimethylmethylene blue dye binding assay.[36] Sample or standard (40 μl) was mixed with dye reagent in the well of a microtitre plate, and the absorbance at 525 nm determined immediately. Bovine chondroitin sulphate (5–40 μg/ml) was used as a standard. As a measure of collagen release, OHPro released to the culture medium was assayed by a microtitre modifica-

tion of the assay described by Bergman and Loxley.[37] Fresh chloramine T (7% (w/v) solution) was diluted 1:4 in acetate citrate (57 g sodium acetate, 37.5 g trisodium citrate, 5.5 g citrate acid, 385 ml propan-2-ol/l water). *p*-Dimethylaminobenzaldehyde (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 hours 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 40 µl of sample or standard (OHPro; 5–30 µg/ml) added to microtitre plates together with chloramine T reagent (25 µl) and then dimethylaminobenzaldehyde reagent (150 µl) after four minutes. The plate was heated at 60°C for 35 minutes, cooled, and the absorbance at 560 nm determined. Results were expressed as a percentage of the total.

ENZYME AND INHIBITOR ASSAYS

[3]H acetylated collagen was used to measure collagenolytic activity by the diffuse fibril assay.[38] A trypsin control was included to confirm the labelled collagen had not become denatured. Latent enzyme was activated with aminophenyl mercuric acetate (APMA; final concentration 0.7 mmol/l). Inhibitory activity was measured by addition of samples to a known amount of active rabbit collagenase in the diffuse fibril assay. The percentage inhibi-

tion was then calculated. One unit of collagenase degrades 1 µg of collagen per minute at 37°C. One unit of inhibitory activity inhibits two units of collagenase by 50%.

RNA ISOLATION AND NORTHERN BLOTTING

Total cellular RNA from chondrocytes was extracted and purified using the RNeasy kit (Qiagen, Crawley, UK) under RNAse-free conditions according to the manufacturer's instructions. The extracted RNA was quantified spectrophotometrically. Equal amounts (20 µg) of total RNA were resolved on 1% agarose formaldehyde gels, and transferred to GeneScreen Plus membrane (NEN, Boston, USA) by capillary transfer and ultraviolet cross linked. RNA was stained with ethidium bromide and visualised under ultraviolet light to check for integrity. Membranes were prehybridised for two hours in 10 ml of hybridisation solution (50% formamide, 1% sodium dodecyl sulphate, 1 M NaCl, 1 × Denhardt's solution (Sigma), 100 µg/ml denatured salmon sperm DNA) and warmed to 42°C. Blots were probed for 18 hours at 42°C with cDNA probes (human MMP-1, MMP-3, MMP-13, TIMP-1) labelled with α-[32P]dCTP using random priming.

The membrane was then washed twice in 2 × saline-sodium citrate (SSC) for 15 minutes at room temperature, followed by two washes in 2
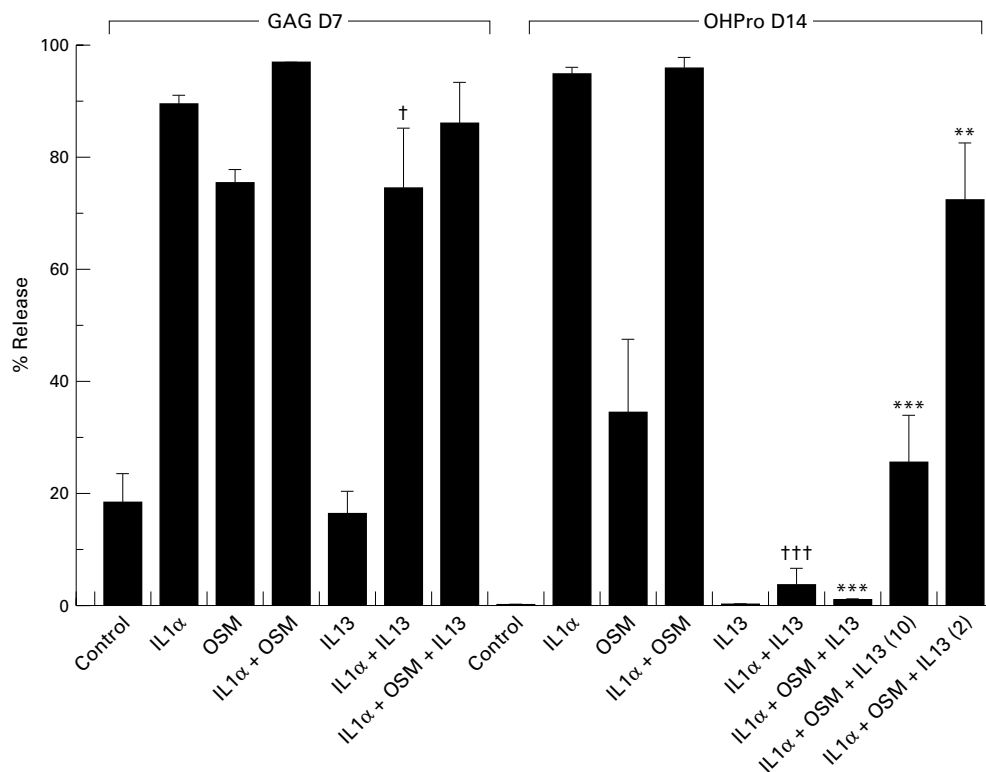

*Figure 1 The effect of interleukin 1α (IL1α) in combination with oncostatin M (OSM), with or without IL13, on the release of proteoglycan and collagen from bovine nasal cartilage in explant culture. Three discs of cartilage per well in quadruplicate were cultured in 600 µl control medium alone, IL1α (1 ng/ml), OSM (10 ng/ml), IL1α + OSM, with and without IL13 (2–50 ng/ml), for 0–7 days and the media removed. Each well was replenished under identical conditions and left for a further seven days. At day 14 media were removed and the remaining cartilage digested with papain. The levels of glycosaminoglycan (GAG) and hydroxyproline (OHPro) released into the medium on days 7 and 14 were determined and the results expressed as a percentage of the total. Results are expressed as mean (SD). The data shown are representative of three independent experiments. Student's unpaired two tailed t test was used to compare IL1α and IL1α + IL13, where †††p<0.001 and †p<0.05. The same test was used to compare IL1α + OSM with IL1α + OSM + IL13, where ***p<0.001 and **p<0.01.*
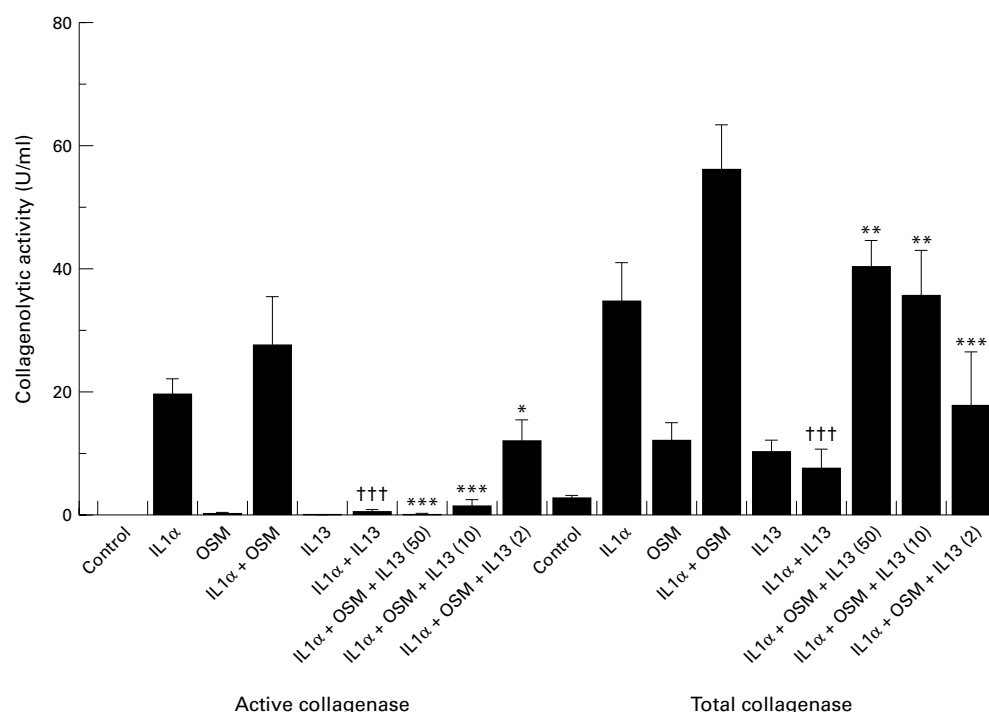
*Figure 2 Levels of active and total collagenase activity in media samples removed from cartilage cultures at day 14 after stimulation with interleukin 1α (IL1α), oncostatin M (OSM), IL1α + OSM with and without IL13 (50 ng/ml). Cartilage was incubated and treated as described in fig 1. The levels of active and total collagenase released into the medium on day 14 were measured and the results expressed as U/ml (mean (SD)). Active and total collagenase were measured as described in "Materials and methods". Student's unpaired two tailed t test was used to compare IL1α and IL1α + IL13, where ††† p<0.001. The same test was used to compare IL1α + OSM with IL1α + OSM + IL13, where ***p<0.001, **p<0.01, and *p<0.05.*

× SSC + 0.5% sodium dodecyl sulphate for 30 minutes at 65°C. Images were visualised using a STORM 860 phosphorimager (Molecular Dynamics, Chesham, UK). The mRNA levels were measured by scanning densitometry of the bands using ImageMaster 1D software (Amersham Pharmacia Biotech, Little Chalfont, UK). Equal loading of RNA was assessed from the ethidium bromide stained membrane.

STATISTICAL ANALYSIS
The statistical significance between two groups was determined by Student's unpaired two tailed t test. Values of p<0.05 were considered significant.

Results

EFFECT OF IL13 ON THE RELEASE OF PROTEOGLYCAN AND COLLAGEN FROM BOVINE NASAL CARTILAGE TREATED WITH IL1α, OSM, AND IL1α + OSM
Previous studies have shown that IL1α and OSM together can reproducibly stimulate the release of collagen from bovine nasal cartilage in explant culture by day 14.[25][39] These conditions were used in the present study as the cytokine stimulus to promote cartilage degradation. This culture system has been designed and validated for the specific purpose of studying collagen release from cartilage. In this study IL1α and IL1α + OSM both release proteoglycan from the cartilage. IL13 partially inhibited the release of proteoglycan by IL1α alone, by day 7, but was unable to prevent the release induced by IL1α + OSM. In the presence of IL13 at 50 ng/ml the effect on col-

lagen release in response to IL1α alone or IL1α + OSM could be blocked. A dose dependent effect was seen on collagen release by day 14, with complete inhibition seen at concentrations of IL13 of 50 ng/ml, and a small but significant inhibition still seen at 2 ng/ml (fig 1). IL13 alone (50 ng/ml) had no effect on either GAG or OHPro release compared with control values, and both were found to be viable at day 14.

ANALYSIS OF MEDIUM FROM BOVINE NASAL CARTILAGE CULTURES FOR COLLAGENOLYTIC AND TIMP INHIBITORY ACTIVITIES
Bovine nasal cartilage stimulated with IL1α, OSM, or IL1α + OSM, with and without IL13, was assayed for collagenolytic activity (fig 2) and MMP inhibitory activity (fig 3). Collagenolytic activity was present in IL1α and IL1α + OSM media removed at day 14. Conversely, little activity could be detected in the IL1α or IL1α + OSM in the presence of IL13 (50 ng/ml) treated samples, although as the concentration of IL13 decreased, the levels of active collagenase increased in a dose dependent manner. The appearance of active collagenolytic activity correlated with the release of collagen. TIMP levels were reduced in IL1α and IL1α + OSM treated cartilage to below control levels. The presence of IL13 (50 ng/ml) with IL1α or IL1α + OSM increased TIMP levels significantly (fig 3). APMA activation of any pro-enzyme present resulted in a significant increase in detectable collagenolytic activity in the IL13 treated samples. Interestingly, the level of total collagenase activity was
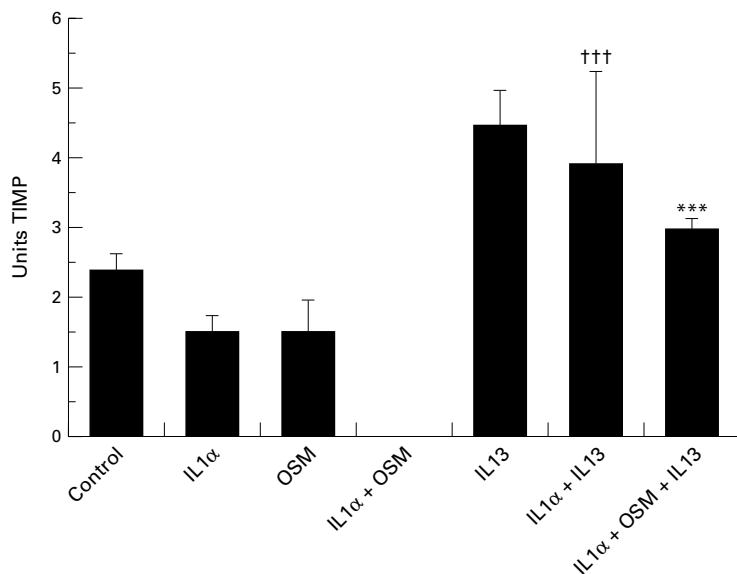
*Figure 3 Levels of tissue inhibitors of metalloproteinase (TIMP) inhibitory activity in media samples removed from cartilage cultures at day 14 after stimulation with interleukin 1α (IL1α), oncostatin M (OSM), IL1α + OSM, with and without IL13. Cartilage was incubated and treated as described in fig 1. The levels of TIMP released into the medium on day 14 were measured and the results expressed as U/ml (mean (SD)). Student's unpaired two tailed t test was used to compare IL1α and IL1α + IL13, where †††p<0.001. The same test was used to compare IL1α + OSM with IL1α + OSM + IL13, where ***p<0.001.*

slightly reduced in the IL1α + OSM + IL13 (2 ng/ml) sample, below the levels seen for the higher concentrations of IL13. However, this reduction was not significant (p=0.1193).

CARTILAGE COLLAGEN RELEASE INHIBITED BY IL13 AND IL4 CAN BE RECOVERED BY ADDITION OF AN EXOGENOUS ACTIVATOR OF PROCOLLAGENASES

MMP-3 has been shown to activate procollagenases and to be important in the initiation of collagen damage.[40][41] Therefore, to investigate further whether IL13 was preventing activation, a known activator of pro-MMPs, MMP-3, was exogenously added to bovine nasal cartilage stimulated with IL1α + OSM, with and without IL13 (50 ng/ml). To compare potency both IL4 and IL13 were used in this experiment. Figure 4A indicates the reproducible chondroprotective effects of IL4 and IL13. In the presence of exogenous MMP-3 at 0.83 µg/ml, the percentage release of collagen was significantly increased in a dose dependent manner (fig 4B). The addition of MMP-3 at 3.33 µg/ml resulted in a dramatic release of collagen with levels close to those seen for IL1α + OSM (fig 4C).

EFFECTS OF IL13 ON MMP-1, MMP-3, MMP-13, AND TIMP-1 MRNA PRODUCTION BY BOVINE NASAL CHONDROCYTES

Northern blot analysis showed that at 24 hours IL1α and IL1α + OSM significantly up regulated expression of MMP-1, MMP-3, and MMP-13 (fig 5). In the presence of IL13, expression of both MMP-3 and MMP-13 induced by both IL1α and IL1α + OSM was significantly reduced. The observed effect was more pronounced for MMP-3 (figs 5B and C).
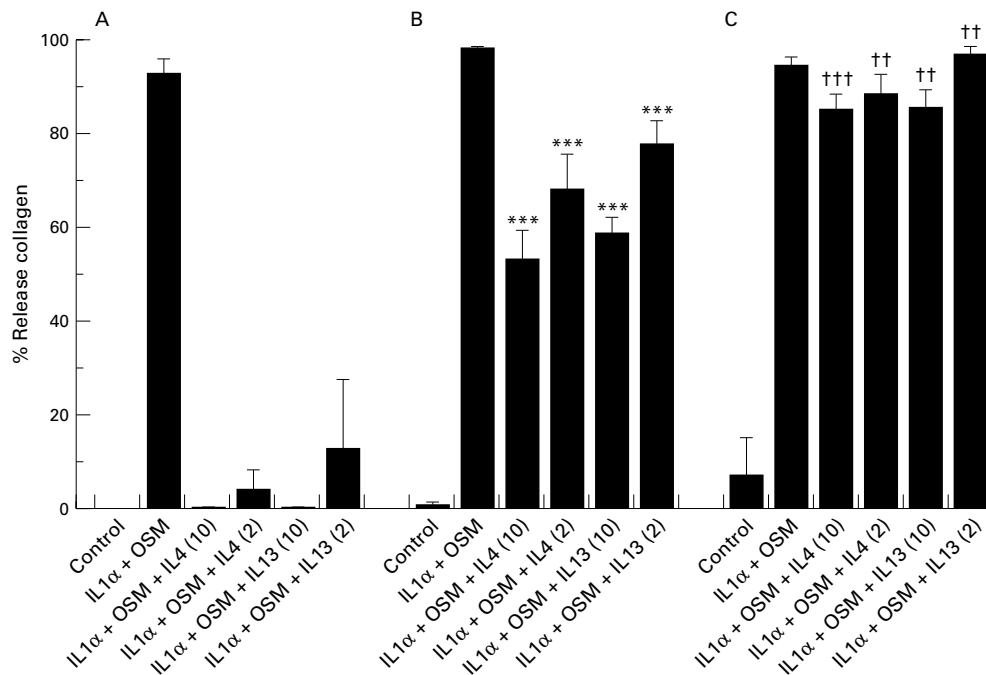

*Figure 4 Cartilage collagen release inhibited by interleukin 13 (IL13) and IL4 can be recovered by addition of an exogenous activator of procollagenases. Three discs of cartilage per well in quadruplicate were cultured in 600 µl control medium alone, IL1α (1 ng/ml) + oncostatin M (OSM) (10 ng/ml), with and without IL4/13 (2–50 ng/ml), for 0–7 days, and the media removed. Media containing identical cytokine combinations to day 1 were replenished. One set received no further additions (A), whereas others had exogenous active matrix metalloproteinase-3 (MMP-3) added at 0.83 µg/ml (B), or 3.33 µg/ml (C). All cultures were left for a further seven days. At day 14 media were removed and the remaining cartilage digested with papain. The levels of hydroxyproline (OHPro) released into the medium on days 7 and 14 were determined and the results expressed as a percentage of the total released. Results are expressed as mean (SD). The data shown are representative of three independent experiments. Student's unpaired two tailed t test was used to analyse data, firstly, where the addition of MMP-3 at 0.83 µg/ml (B) was compared with its identical treatment in the absence of exogenous MMP-3 (A), where ***p<0.001, and, secondly, where the addition of MMP-3 at 3.33 µg/ml (C) was compared with its identical treatment in the presence of MMP-3 at 0.83 µg/ml (B), where †††p<0.001 and ††p<0.01.*

A

MMP-1

B

MMP-3

C

MMP-13

D

TIMP-1

28 S

IL1α (1 ng/ml)	−	+	−	+	+	+	+	+	+	+	+
OSM (10 ng/ml)	−	−	+	+	−	−	−	−	+	+	+
IL13 (ng/ml)	−	−	−	−	50	10	5	1	50	10	5	1

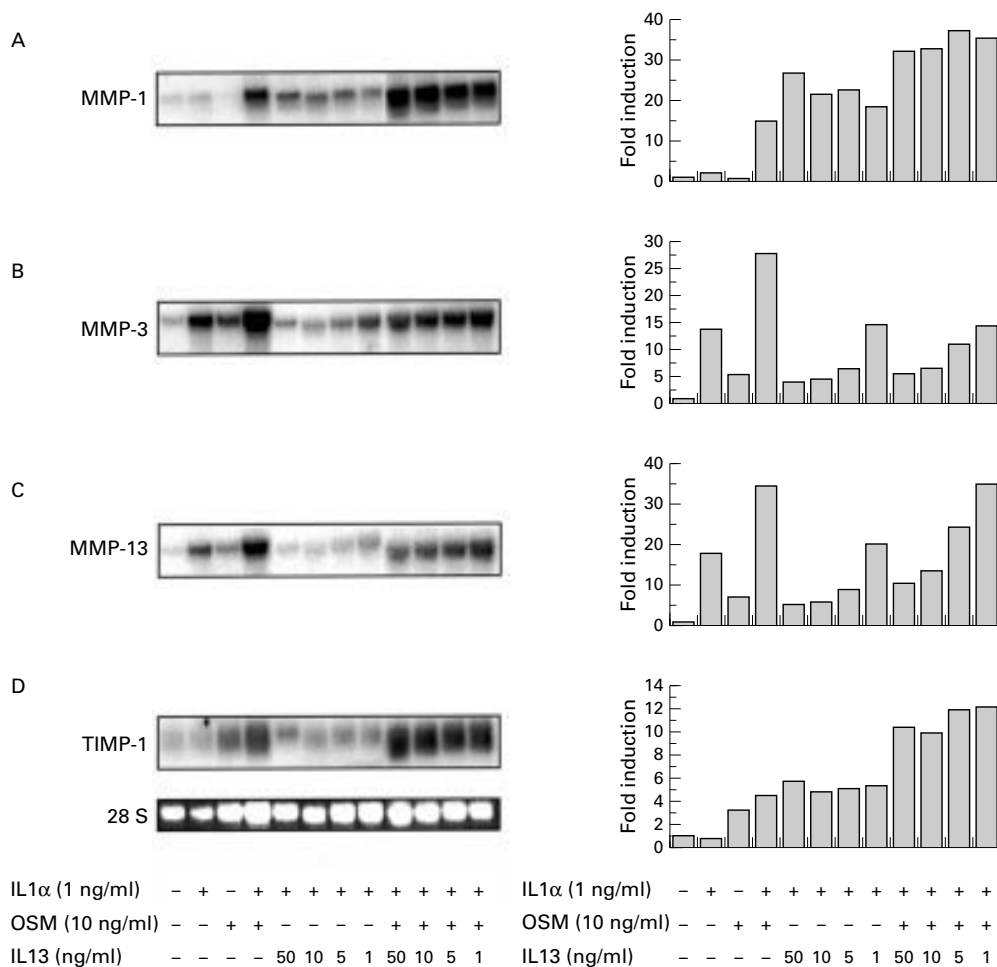| IL1α (1 ng/ml) | − | + | − | + | + | + | + | + | + | + | + |
| OSM (10 ng/ml) | − | − | + | + | − | − | − | − | + | + | + |
| IL13 (ng/ml) | − | − | − | − | 50 | 10 | 5 | 1 | 50 | 10 | 5 | 1 |

Figure 5 Interleukin 13 (IL13) inhibits IL1α and IL1α + oncostatin M (OSM) induction of metalloproteinase-3 (MMP-3) and MMP-13 mRNA but not MMP-1 or tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) mRNA in bovine nasal chondrocytes at 24 hours. Total RNA was isolated at 24 hours after the addition of IL1α (1 ng/ml), OSM (10 ng/ml), IL1α + OSM, alone or in combination with IL13 in cultures of bovine nasal chondrocytes. Northern blots were hybridised to cDNA probes corresponding to MMP-1 (A), MMP-3 (B), MMP-13 (C), and TIMP-1 (D). Ethidium bromide stained 28 S rRNA was used for normalisation of RNA loading. The northern blots are shown on the left hand side of the figure and were measured by scanning densitometry. The results are expressed as fold induction over control on the right hand side. The data shown are representative of three independent experiments.

In contrast with both MMP-3 and MMP-13, IL13 had a pronounced stimulatory effect on the expression of MMP-1, strongly up regulating levels when in the presence of both IL1α and IL1α + OSM (fig 5A). IL13 up regulated TIMP-1 gene expression when in combination with IL1α and IL1α + OSM (fig 5D).

Discussion
The loss of the collagen fibrillar network has a profound effect on the fundamental integrity of cartilage and represents the irreversible phase of cartilage destruction. We investigated the effect of adding IL13 to cartilage induced to resorb with IL1 + OSM, to examine whether IL13, like IL4, could confer protection to cartilage from collagen loss. We have shown, for the first time, that IL13 can decrease collagen loss in a bovine nasal cartilage model as previously described for IL4.[26]

T cells have a prominent role in the initiation and maintenance of the rheumatoid joint.[42 43] Two main groups of T cells exist which can be distinguished by the cytokines they produce. T helper 1 (Th1) cells produce cytokines and growth factors such as IL2 and interferon γ, and these in turn can promote the activation of proinflammatory cytokines such as IL1 and TNFα. The proinflammatory cytokines then initiate the production of degradative enzymes, including collagenases, which in turn leads to collagen loss and cartilage destruction in the joint. Th1 cells have been shown to predominate in the joints of patients with RA.[44] Th2 cells generally secrete high levels of inhibitory or anti-inflammatory cytokines like, IL4, IL5, IL10, and IL13. Recently, anti-inflammatory cytokines have been implicated in the resolution of inflammatory reactions, presumably in part through their effect of decreasing production of proinflammatory cytokines.[45] It has been shown that these anti-inflammatory cytokines may also contribute to the down regulation of the inflammatory response through production of other receptor antagonists, such as IL1 receptor antagonist (IL1ra).[46 47] IL13, like IL4, has been shown to inhibit production of a range of proinflammatory cytokines, including IL1, TNFα, IL6, and IL8 and also stimulate the production of IL1ra

in monocytes, macrophages and B cells.[27] More recently, IL13 has been shown to inhibit production of IL1β, TNFα, and MMP-3 and also to stimulate the production of IL1ra in human osteoarthritic synovial membranes in ex vivo culture.[48]

We have previously shown that MMP-1 is up regulated when cartilage is cultured in the presence of IL1 + OSM, but this enzyme must be activated before collagen degradation will proceed.[25 39] However, the proteinases responsible for the activation of procollagenases in cartilage tissue are unidentified. IL13 has a dose dependent, chondroprotective effect on the release of collagen from bovine nasal cartilage. The effect of IL13 on proteoglycan release was less dramatic. IL1 + OSM has been shown previously to be more effective than IL1 alone in promoting proteoglycan release from bovine nasal cartilage,[25] presumably by inducing aggrecan degrading activity.[49] It is not known if this membrane associated activity is the same as that recently described as ADAMTS-5.[50 51] Possibly, IL13 effects are specific to collagenases and not aggrecanases. Alternatively, it might be that where proteoglycan release is driven by high concentrations of cytokines then IL13 is unable to reverse this process. Recent studies have shown that both IL4 and transforming growth factor β can decrease proteoglycan loss in a dose dependent manner when concentrations of IL1α and OSM are reduced to 0.2 ng/ml and 2 ng/ml, respectively (Hui W, personal communication).

Measurement of collagenolytic activity showed that where active collagenase was present, a concomitant increase in collagen release was seen. In the presence of high concentrations of IL13, active collagenase was not detectable. We can extrapolate, therefore, that there is no collagenolytic activity in the tissue because no collagen release is seen. However, this does not exclude any sequestered TIMP/collagenase complex. Activation of procollagenases with APMA significantly increased the levels of detectable collagenase in IL13 treated samples. Interestingly, when IL13 (2 ng/ml) was in the presence of IL1α + OSM the total collagenolytic activity appeared to be reduced to levels below those seen when IL13 concentrations were increased. This result was unexpected and surprising. Possibly, total collagenase activity might be reduced if the enzyme is sequestered by matrix components or inhibitors or possibly destroyed by other proteinases. However, the results indicate that there is sufficient procollagenase present to promote extensive collagen loss if activated and supports the notion that down regulation of collagenolytic activity is an important mechanism in cartilage protection. We suggest that IL13 can prevent activation of procollagenases, by down regulation of an enzyme(s) present in the activation cascade, and/or by promoting local levels of TIMP, thus preventing matrix turnover. Possibly, IL13 may down regulate plasminogen activators, known to play an important part in the activation of procollagenases.[52] Addition of an exogenous activator

(MMP-3) increased collagen release in the presence of IL13 and IL4 in a dose dependent manner to levels close to those for IL1α + OSM alone. In the MMP-3 treated controls there was no increase in collagen release. This was expected because MMP-3 cannot cleave native fibrillar collagen. If IL13 and IL4 were simply preventing activation of procollagenases by down regulating an activating enzyme(s), it might be speculated that addition of MMP-3 at its lowest concentration should be sufficient to cause total collagen release. However, IL13 and IL4 may be preventing activation through production of an inhibitor. Therefore, addition of MMP-3 at low concentrations is not sufficient to overcome the local levels of inhibitor present, thus resulting in partial collagen release. By increasing the MMP-3 concentration, the local levels of inhibitor are exceeded and hence the dramatic release of collagen is recovered.

We have investigated the transcriptional regulation of various genes of importance in cartilage breakdown and shown, for the first time, that IL13 can down regulate the MMP-13 gene expression in a dose dependent manner. Interestingly, IL13 appeared to increase MMP-1 gene expression. Possibly, IL13 can differentially regulate MMP-1 and MMP-13 expression in bovine chondrocytes. Recently, it has been reported that transforming growth factor β can differentially regulate MMP-1 and MMP-13 expression in human fibroblasts.[53] A similar finding was also described in normal and osteoarthritic chondrocytes.[54] The effect of IL13 on MMP-3 levels was similar to the pattern of expression seen with MMP-13. Recently, it was reported that in human synovial fibroblasts, IL4 down regulated production of MMP-3 induced by IL1 at the mRNA level.[55] IL13 increased TIMP-1 gene expression at the mRNA level and also in the bioassay. However, because the TIMP bioassay measures all TIMPs and not just TIMP-1, we cannot attribute this increase solely to TIMP-1. The results indicate that levels of TIMP detected are unlikely to counteract the IL1 + (OSM) induced MMP activities on collagen. Therefore, further investigations are under way to identify whether other members of the TIMP family are indeed up regulated in response to IL13.

In summary, we have shown that IL13 can act in a chondroprotective manner by (*a*) specifically preventing loss of collagen from bovine nasal cartilage, (*b*) reducing the amount of active collagenases that are released from the cartilage, and (*c*) act in a dose dependent manner and significantly reduce IL1 and IL1 + OSM induced production of both MMP-13 and MMP-3 mRNA. Further studies will enable us to elucidate the mechanism by which IL13 prevents breakdown of cartilage collagen.

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