Esculetin inhibits cartilage resorption induced by interleukin 1 α in combination with oncostatin M

S Elliott, A D Rowan, S Carrère, P Koshy, J B Catterall, T E Cawston

Abstract

Objective—To determine if a new inhibitor, esculetin (EST), can block resorption of cartilage.

Methods—Interleukin 1 α (IL1 α , 0.04–5 ng/ml) and oncostatin M (OSM, 0.4–50 ng/ml) were used to stimulate the release of proteoglycan and collagen from bovine nasal cartilage and human articular cartilage in explant culture. Proteoglycan and collagen loss were assessed by dimethyl-methylene blue and hydroxyproline assays, respectively. Collagenase levels were measured by assay of bioactivity and by enzyme linked immunosorbent assay (ELISA). The effects of EST on the expression of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the transformed human chondrocyte cell line T/C28a4 were assessed by northern blot analysis. TIMP-1 protein levels were assayed by ELISA. The effect of EST on the MMP-1 promoter was assessed using a promoter-luciferase construct in transient transfection studies.

Results—EST inhibited proteoglycan and collagen resorption in a dose dependent manner with significant decreases seen at 66 μ M and 100 μ M EST, respectively. Collagenolytic activity was significantly decreased in bovine nasal cartilage cultures. In human articular cartilage, EST also inhibited IL1 α + OSM stimulated resorption and decreased MMP-1 levels. TIMP-1 levels were not altered compared with controls. In T/C28a4 chondrocytes the IL1 α + OSM induced expression of MMP-1, MMP-3, and MMP-13 mRNA was reduced to control levels by 250 μ M EST. TIMP-1 mRNA levels were unaffected by EST treatment. All cytokine stimulation of an MMP-1 luciferase-promoter construct was lost in the presence of the inhibitor.

Conclusion—EST inhibits degradation of bovine nasal cartilage and human articular cartilage stimulated to resorb with IL1 α + OSM.

(Ann Rheum Dis 2001;60:158–165)

In rheumatoid arthritis (RA) and osteoarthritis (OA), progressive loss of articular cartilage occurs, in particular, degradation of collagen and proteoglycan.¹ The integrity of these macromolecules is vital to cartilage and joint function. In response to various stimuli, degradation of proteoglycan is easily achieved relative to collagen, which is lost much less

readily.² However, proteoglycan can be resynthesised, whereas collagen, once degraded, is not replaced.^{3–4}

Matrix metalloproteinases (MMPs) are key enzymes in RA and OA pathogenesis. Collectively they can degrade all components of the extracellular matrix.^{5–7} MMP-1, MMP-8, and MMP-13 specifically cleave fibrillar collagen to produce characteristic one quarter and three quarter length products.^{8–10} These MMPs and the collagen cleavage products are found in stimulated explant cultures, in the synovium and serum of rheumatoid patients, and in animal models, providing evidence of MMP involvement in collagen breakdown in vivo.^{11–15} In addition, a number of MMPs are reported to activate other members of the MMP family—for example, it has been proposed that MMP-3 plays a part in the activation cascade of MMP-1, leading to collagen degradation.^{16–17} Aggrecan, the principal proteoglycan in cartilage, can be cleaved at two key sites: Asn³⁴¹-Phe³⁴² and Glu³⁷³-Ala³⁷⁴.^{18–19} Degradation at the Asn³⁴¹ site can be effected by a number of MMPs, including MMP-1, MMP-3, and MMP-8.^{19–20} However, a new metalloproteinase family, termed ADAM-TS (a disintegrin and metalloproteinase with thrombospondin motifs), may have a more significant effect than MMPs on aggrecan degradation because they cleave at the Glu³⁷³ site that is typical of aggrecan cleavage in vivo.^{21–24} These enzymes are produced by cartilage in culture but it is not clear if they are identical to the recently described membrane associated aggrecanase.²⁵ In addition, synthesis of proteoglycan may be inhibited by the presence of cytokines, such as interleukin 1 α (IL1 α), with the overall effect of reducing the levels of proteoglycan present in the tissue.²⁶

The natural inhibitors of MMPs are α_2 macroglobulin and tissue inhibitors of metalloproteinases (TIMPs).^{27–28} The former is a general endoproteinase inhibitor, whereas the latter interact with active MMPs, forming 1:1 complexes. The in vivo inhibitors of the aggrecan-degrading ADAM-TS proteins have not yet been identified. Normally, active MMP and TIMP levels are in equilibrium such that controlled turnover of the extracellular matrix occurs. However, in rheumatoid cartilage, this equilibrium moves in favour of the active MMPs and tissue destruction occurs, ultimately resulting in the loss of cartilage.^{14–29–30} Connective tissue turnover can be prevented by reducing levels of active MMPs or increasing levels of TIMPs, and treatments that act in this way are therefore of therapeutic interest.^{31–33}

Department of Rheumatology, Cookson Building, Medical School, Newcastle-upon-Tyne NE2 4HH, UK
S Elliott
A D Rowan
S Carrère
P Koshy
J B Catterall
T E Cawston

Correspondence to: Dr Elliott
s.f.elliott@ncl.ac.uk

Accepted 31 July 2000

Figure 1 Esculetin (6,7-dihydroxycoumarin).

Current treatments for RA and OA reduce pain and swelling of the joint but generally do little to block cartilage destruction.³⁴ Evidence indicates that MMPs and ADAM-TS proteins degrade cartilage and are therefore potential targets for inhibition. Of the drugs currently in use, there have been conflicting reports of the effects of steroidal and non-steroidal treatments, when tested at therapeutic concentrations, on enzyme profiles.^{35–36} In addition, synthetic molecules have been developed that bind to the catalytic site of MMPs, inhibiting the enzymes and therefore potentially preventing cartilage degradation.^{32–36–42} A number of such inhibitors have been investigated in animal models and some are currently being examined in clinical trials. However, to date, these inhibitors are not used in routine clinical practice.

We investigated a new inhibitor of cartilage degradation, esculetin (EST) (fig 1). EST is a coumarin derivative which has been found to protect rabbit articular cartilage from proteoglycan loss in response to IL1 α .⁴³ We used established cartilage explant models inducing resorption with the proinflammatory cytokines IL1 α and oncostatin M (OSM).^{44–45} We found that EST was chondroprotective for both collagen and proteoglycan loss, and decreased cytokine stimulated increases in collagenolytic activity and MMP-1 levels. In addition, using a transformed human chondrocyte cell line (T/C28a4), we found that EST decreased the expression of MMP-1, MMP-3, and MMP-13 mRNA, and inhibited MMP-1 promoter activity. These results indicate that EST can protect cartilage from degradation by acting pre-translationally.

Materials and methods

EST was supplied by Kureha Chemical Industry Co Ltd, Tokyo, Japan. It was solubilised in dimethyl sulphoxide (DMSO), stored at -20°C , and subsequently diluted into culture media with a final DMSO concentration less than 0.06% (v/v). Bovine nasal septa were obtained at the time of slaughter. Fresh human articular cartilage was obtained from a local orthopaedic unit. Both tissues were stored at 4°C and used within 24 hours. The transformed human chondrocyte cell line T/C28a4 was a gift from Professor Mary Goldring (Harvard Institute for Medicine, Boston, MA, USA⁴⁶). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco BRL (Paisley, UK). DMEM was supplemented with penicillin-streptomycin (penicillin 50 U/ml, streptomycin 50 $\mu\text{g}/\text{ml}$), nystatin (20 U/ml), gentamycin (5 $\mu\text{g}/\text{ml}$), and glutamine (2 mmol/l). L-Ascorbic

acid was purchased from Wako Pure Chemical Industries (Alpha Laboratories, Eastleigh, UK). ITS+ Premix was from Becton Dickinson (Bedford, MA, USA). Human recombinant IL1 α and OSM were gifts from Glaxo-Wellcome (Stevenage, UK) and Professor J Heath (Department of Biochemistry, University of Birmingham, UK), respectively. α [^{32}P]dCTP was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). All other chemicals were reagent grade. The MMP-1 promoter-luciferase construct (-517/+63 base pairs (bp))⁴⁷ was supplied by Professor C Brinckerhoff (Dartmouth Medical School, NH, USA).

BOVINE TISSUE EXPERIMENTS

Discs (1 mm diameter) were cut from 1 mm thick slices of bovine nasal cartilage. Three discs per well were placed in 600 μl supplemented DMEM overnight at 37°C , 5% CO_2 , 95% air. Medium was removed and replaced with DMEM, with or without test reagents, thus: IL1 α (0.04–1 ng/ml), OSM (0.4–10 ng/ml), and EST (0–330 $\mu\text{mol}/\text{l}$) to a final volume of 600 μl . Discs were incubated for 14 days. Media were harvested and replenished at day 7 with identical test reagents. At day 14, media were harvested, and the remaining cartilage papain digested overnight at 65°C as described previously.⁴⁴ Media and digested cartilage were stored at -20°C until assayed.

HUMAN TISSUE EXPERIMENTS

Macroscopically normal human articular cartilage from excised knee joints was cut into 2 mm³ pieces. Articular cartilage from different patients was used in separate experiments. Three pieces were placed in each well in 600 μl supplemented DMEM, and incubated overnight as above. Media were removed and replaced with supplemented DMEM containing IL1 α (5 ng/ml), OSM (50 ng/ml), and EST (0–500 $\mu\text{mol}/\text{l}$) to a final volume of 600 μl . At days 7 and 14, media were harvested and replenished as above. At day 21, media were harvested and the remaining cartilage papain digested. Media and digested cartilage were stored at -20°C until assayed.

CULTURE CONDITIONS

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 because it can increase cartilage metabolism in the absence of exogenous cytokine(s).⁴⁸ This study aimed at creating a model of cartilage breakdown; therefore the presence of serum was avoided as it contains chondroprotective agents, such as insulin-like growth factor 1.^{49–50} 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 eight to nine days can respond to serum and other growth

Figure 2 Esculetin (EST) inhibits collagen loss from bovine nasal cartilage stimulated to resorb with interleukin 1α (IL1α) and oncostatin M (OSM). Bovine nasal cartilage discs were cultured for 14 days with IL1α (1 ng/ml) in combination with OSM (10 ng/ml) ± EST (10–330 μmol/l). Media were harvested and replenished at day 7. Collagen release is expressed as a percentage of the total present in the explant. The cumulative collagen loss over 14 days of culture is presented. Results are expressed as mean (SD) (n=4). Statistical analyses compared collagen loss seen with IL1α + OSM alone to that seen with IL1α + OSM + EST. ***p<0.001.

factors.⁵¹ In this study, cytokines were added after one day of serum-free culture.

ASSAY OF BIOCHEMICAL MARKERS OF DEGRADATION

Proteoglycan and collagen in media and cartilage samples were assayed as previously described.⁴⁴ Briefly, glycosaminoglycan (GAG) levels were determined by the amount of polyanionic material reacting with 1,9-dimethylmethylene blue, using shark chondroitin sulphate as standard. Levels of

Figure 3 Esculetin (EST) inhibits proteoglycan loss from bovine nasal cartilage. Bovine nasal cartilage discs were incubated for 14 days with interleukin 1α (IL1α) and oncostatin M (OSM) at the concentrations indicated ± EST (100 μmol/l). Media were harvested and replenished at day 7; media and cartilage were harvested at day 14. Proteoglycan loss is expressed as a percentage of the total present in each explant; the data illustrated are for proteoglycan loss by day 7 of culture. Results are mean (SD) (n=4). Statistical analyses compared proteoglycan loss seen with IL1α + OSM treatment to that seen with IL1α + OSM + EST for each set of cytokine concentrations as indicated by the bars. ***p<0.001, *p<0.05.

hydroxyproline in samples were assayed as a measure of collagen, using hydroxyproline as standard.⁴⁴ The proteoglycan and collagen content of media and digested cartilage was expressed as a percentage of the total GAG or total hydroxyproline present in each explant. Collagenolytic activity present in media was assayed by the ³H diffuse collagen fibril assay,⁵² with modifications according to Koshy *et al.*⁵³ When appropriate, *p*-aminophenylmercuric acetate (APMA) was added to 0.7 mmol/l to activate procollagenases. Levels of total MMP-1 (pro-form, active or TIMP-bound) and total TIMP-1 (free and bound to MMPs) in human media samples were assayed using specific enzyme linked immunosorbent assays (ELISAs).^{11 54}

NORTHERN BLOT ANALYSIS

T/C28a4 cells were grown in T25 flasks in supplemented DMEM with 10% FCS, L-ascorbic acid (100 μg/ml). When cells reached 60% confluence the media were replaced with serum-free supplemented DMEM, with ITS+. After overnight culture, cells were washed with phosphate buffered saline and then stimulated with IL1α (1 ng/ml) and OSM (10 ng/ml) ± EST (50–500 μmol/l) in serum-free supplemented DMEM with ascorbate. Total cellular RNA was harvested 24 hours after stimulation using the RNeasy system (Qiagen, Crawley, UK) according to the manufacturer's instructions. Northern blot analysis was carried out essentially as previously described.⁴⁵ In brief, for each treatment, 15–20 μg of RNA was fractionated on a 1% agarose gel and blotted onto a nitrocellulose membrane. Expression of mRNA was detected using complementary human DNA probes to MMP-1 and TIMP-1,⁴⁵ MMP-3 (from A Galloway, British BioTechnology, Oxford, UK), MMP-13 (from Dr V Knäuper, University of East Anglia, Norwich, UK), c-fos, c-jun (from Dr I Clark, University of East Anglia, Norwich, UK), and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH).⁵⁵ Autoradiographs of blots were scanned and radioactive bands quantified with Alphavision (Flowgen, Lichfield, UK). GAPDH was used to normalise the data.

TRANSIENT TRANSFECTION AND REPORTER GENE ASSAY

T/C28a4 cells at 50–80% confluence in 10 cm diameter tissue culture dishes were transfected with 8 μg of plasmid DNA for the MMP-1 promoter-luciferase construct (-517/+63 bp)⁴⁷ and 32 μl of lipofectamine reagent (Gibco, BRL) per well according to the manufacturer's instructions. The following day, each dish was passaged into 2 × 6 well plates and incubated overnight in DMEM/1% AT-FCS at 37°C. After which, cells were incubated with IL1α ± OSM ± EST for 24 hours. Lysis buffer (400 μl/well, Promega) was applied at -20°C for two hours to release cytosolic proteins. Supernates were removed and used in the luciferase assay (below). The remaining nuclear pellets were immediately stored at -20°C until used in the Hirt's assay⁵⁶ to quantify the amount of

Figure 4 Esculetin (EST) inhibits interleukin 1α (IL1α) + oncostatin M (OSM) induced collagenolytic activity in bovine nasal cartilage. Bovine nasal cartilage discs were incubated for 14 days with IL1α (1 ng/ml) in combination with OSM (10 ng/ml) ± EST (10–330 μmol/l), with media changes at day 7. Media were harvested at day 14 and assessed by using the diffuse collagen fibril assay for active collagenolytic activity (A) and total collagenolytic activity by the inclusion of p-aminophenylmercuric acetate (0.7 mmol/l) in the assay (B). Statistical analyses compared the activity of IL1α + OSM treatment with that of IL1α + OSM + EST treatment (n=4). *p<0.05.

transfected plasmid. Supernates (10 μl) were mixed with 100 μl of luciferase substrate (bee-tle luciferin, Promega) in a white walled, 96 well plate (Wallac, Milton Keynes, UK) and read on a TriLux MicroBeta 1450 luminescence and scintillation counter (Wallac). Triplicate samples were performed in three experiments. Results were normalised to the amount of plasmid per well, as determined by Hirt's assay.

STATISTICS

In experiments requiring statistical analysis, each treatment was performed in quadruplicate. Student's *t* test was performed, assuming two tailed distributions and equal variance. Significance is denoted thus: *0.05>p>0.01; **0.01>p>0.001; ***p<0.001.

Results

EFFECT OF EST ON THE RESORPTION OF BOVINE NASAL CARTILAGE

EST was found to block the release of collagen and proteoglycan loss from bovine nasal cartilage in culture (figs 2 and 3). EST inhibited IL1α + OSM induced collagen resorption in a dose dependent manner, with 66 μM EST sig-

Figure 5 Esculetin (EST) inhibits collagen loss and collagenolytic activity up to 72 hours after cytokine stimulation of bovine nasal cartilage. Bovine nasal cartilage discs were cultured for 14 days with interleukin 1α (IL1α; 1 ng/ml) and oncostatin M (OSM; 10 ng/ml). EST (100 μmol/l) was added simultaneously with the cytokines or up to 120 hours thereafter. Media were harvested and replenished at day 7; media and cartilage were harvested at day 14. (A) Cumulative collagen loss over 14 days of culture. Collagenolytic activity was measured in day 7 media in the presence (B) or absence (C) of the metalloproteinase activator, p-aminophenylmercuric acetate (0.7 mmol/l). Statistical analyses of collagen loss and collagenolytic activity levels compared IL1α + OSM + EST (0 hours) with IL1α + OSM + EST added at time points thereafter (n=4). ***p<0.001, *p<0.05.

nificantly inhibiting collagen depletion (p<0.001; fig 2). Similar results were obtained when bovine nasal cartilage was cultured with IL1α alone (1 ng/ml); there was a significant reduction in the stimulated collagen release at 66 μM EST (p<0.05; data not shown).

Although the major emphasis of this study was collagen breakdown, proteoglycan resorption stimulated with IL1α (1 ng/ml) in combination with OSM (10 ng/ml) was also significantly reduced when EST was present at 330 μmol/l (p<0.05) but not at lower concentrations (data not shown). However, when the IL1α concentration was reduced to 0.2 or 0.04 ng/ml, with OSM maintained at 10 ng/ml, EST (100 μmol/l) could significantly inhibit the loss of proteoglycan (fig 3; p<0.05). Similar results

Figure 6 Esculetin (EST) inhibits interleukin 1 α (IL1 α) + oncostatin M (OSM) stimulated resorption of human articular cartilage. Pieces of human articular cartilage (2 mm²) were cultured with IL1 α (5 ng/ml) in combination with OSM (50 ng/ml) $\pm$ EST (50–500 μ mol/l). Media were harvested and replenished at days 7 and 14; media and cartilage were harvested at day 21. Proteoglycan and collagen release are presented as a percentage of the total. (A) Cumulative proteoglycan loss over 21 days of culture. (B) Cumulative MMP-1 levels over 21 days of culture. (C) Cumulative collagen loss over 21 days of culture. Statistical analyses compared the data for IL1 α + OSM with that for the IL1 α + OSM + EST treatments ($n=4$). *** $p<0.001$, ** $p<0.01$.

were observed when OSM was decreased to 2 or 0.4 ng/ml, with IL1 α at 1 ng/ml (fig 3; $p<0.05$).

PROCOLLAGENOLYTIC AND ACTIVE COLLAGENOLYTIC ACTIVITY IN THE PRESENCE OF EST

Having established that EST can inhibit the resorption of collagen, we investigated its

effects on collagenolytic activity in the media harvested from the cultured bovine nasal cartilage (fig 4A). Addition of a metalloproteinase activator, APMA, activated procollagenases present, allowing the measurement of total collagenase levels (fig 4B). It is evident that the levels of collagenolytic activity mirrored those seen for collagen loss under the same conditions (figs 2, 4A and B). By day 14 of culture, 66 μ M EST significantly inhibited IL1 α + OSM stimulated increases in active and total collagenolytic activity (figs 4A and B). IL1 α stimulated effects were also inhibited by EST (data not shown).

TIME COURSE OF EST EFFICACY IN BOVINE NASAL CARTILAGE

To determine if EST can prevent collagen release at various times after the addition of cytokines, the inhibitor was added to bovine nasal cultures up to 72 hours after the addition of IL1 α + OSM. EST inhibited the release of collagen when added up to 72 hours after stimulation (fig 5A). If added thereafter, collagen loss was not inhibited ($p<0.001$). Similarly, levels of total and active collagenolytic activity were suppressed by EST added up to 72 hours after stimulation (figs 5B ($p<0.05$) and C).

EST EFFECTS ON HUMAN ARTICULAR CARTILAGE RESORPTION

To confirm the results from the bovine studies we examined the effects of EST on resorption of human articular cartilage. Previous studies have shown that the response of human articular cartilage to cytokines is less dramatic than that for animals, but that all human cartilages respond to IL1 α + OSM by resorbing proteoglycan, with approximately 50% of these also releasing low levels of collagen.⁴⁵ In this study with human articular cartilage, proteoglycan loss stimulated by IL1 α + OSM, and MMP-1 levels were inhibited by EST (figs 6A and B): 50 μ mol/l inhibited control levels ($p<0.001$ and $p<0.01$, respectively). Collagen loss was also significantly inhibited by EST at 50 μ mol/l in the same explant culture (fig 6C; $p<0.01$). EST at 50 μ mol/l effectively blocked cartilage resorption in four of seven experiments, but in the remaining experiments the effect was seen at higher concentrations of the inhibitor. In contrast with these results, no consistent effect of EST on TIMP-1 production was seen when assayed by ELISA (data not shown).

EFFECT OF EST ON MMP EXPRESSION IN CHONDROCYTES

We then used a human chondrocyte cell line to dissect the mechanism of action of EST. At 24 hours of stimulation, IL1 α + OSM up regulated the expression of MMP-1, MMP-3, MMP-13, and TIMP-1 mRNA in the T/C28a4 cell line (fig 7). EST (100–500 μ mol/l) inhibited the increased MMP expression in a dose dependent manner. EST at 250 μ mol/l was sufficient to decrease mRNA to levels similar to those seen under control conditions (that is, media alone), while a partial effect was seen at 100 μ mol/l and 50 μ mol/l. Similar effects were seen at 48 and 72 hours of

Figure 7 Esculetin (EST) reduces the interleukin 1α (IL1α) + oncostatin M (OSM) induced expression of MMP-1, MMP-3, and MMP-13 mRNA in T/C28a4 chondrocytes. T/C28a4 chondrocytes were stimulated for 24 hours with IL1α (1 ng/ml) in combination with OSM (10 ng/ml) ± EST (100–500 μmol/l). Total RNA was harvested, northern blotted, and the membranes probed for MMP-1, MMP-3, MMP-13, and TIMP-1 mRNA. Glyceraldehyde-3-phosphate dehydrogenase was used to correct for RNA loading. The data are representative of three separate experiments.

incubation (data not shown). Expression of TIMP-1 was unaffected by EST when normalised to GAPDH levels. GAPDH expression was also unaffected by the concentrations of EST used. No MMP mRNA was expressed in the presence of EST alone (data not shown).

EST INHIBITS CYTOKINE STIMULATED MMP-1 PROMOTER ACTIVITY

We have clearly shown that EST inhibits IL1α + OSM mediated MMP-1 gene expression, and therefore we studied its effects at the transcriptional level. To this end, we used the -517/+63 bp sequence of the human MMP-1 promoter cloned upstream of a luciferase reporter gene.⁴⁷ The effect of cytokines and EST was assessed after transient transfection

Figure 8 Esculetin (EST) inhibits matrix metalloproteinase-1 (MMP-1) promoter activity. T/C28a4 cells were transiently transfected with an MMP-1/luciferase promoter construct. Two days after transfection, cells were incubated with interleukin 1α (IL1α; 1 ng/ml) and/or OSM (10 ng/ml) ± EST (100 μmol/l) for 24 hours. Cells were then harvested and luciferase activities measured, and normalised to the amount of DNA plasmid in each well. The results represent the mean (SD) (n=3) of a representative experiment performed three times.

into T/C28a4 cells. Treatment of these cells with OSM (10 ng/ml) showed stimulation, whereas IL1α + OSM (1 and 10 ng/ml, respectively) synergistically stimulated the MMP-1 promoter construct (fig 8). IL1α alone was unable to stimulate this construct, as previously described.⁴⁷ When 100 μM EST was added simultaneously with the cytokines to the transiently transfected cells, all stimulation of the promoter construct by OSM and IL1α + OSM was lost. Following on from this study, we investigated members of the activator protein 1 family that we believe plays a part in MMP transcription: we found that EST does not alter the up regulation of c-fos and c-jun expression induced by IL1α and/or OSM (data not shown). We are currently investigating the mechanism of action of EST further.

Discussion

We have shown that the coumarin derivative, EST, can inhibit cartilage resorption induced by IL1α + OSM in bovine nasal cartilage and human articular cartilage explant cultures by significantly preventing collagen and proteoglycan degradation in a dose dependent manner. In bovine nasal cartilage, EST decreased the induced total and active collagenolytic activity. These findings corroborate the human articular cartilage data, which showed that total MMP-1 levels were reduced by EST. Further evidence indicates that EST prevents the IL1α + OSM stimulated expression of MMP-1, MMP-3, and MMP-13 mRNA, and thus the production of secreted proenzymes, whereas it has no effect on TIMP-1. In addition, in T/C28a4 cells EST (100 μmol/l) can abolish OSM ± IL1α stimulation of an MMP-1 promoter construct, indicating that EST can inhibit the cytokine stimulated activation of this promoter. Overall, these findings suggest that EST inhibits MMP production, and that it does not act by regulating TIMP-1 or by preventing activation of pro-MMPs. This work also confirms the earlier findings of Watanabe *et al* that EST does not interact directly with collagenases to inhibit their activity.⁴³

It has been found that IL1α can inhibit proteoglycan synthesis, in addition to the stimulation of metalloproteinase production.²⁶ This results in an overall loss of proteoglycan. Watanabe *et al* reported that EST does not alter the effects of IL1 on proteoglycan synthesis,⁴³ and in this study we examined the inhibition of proteoglycan resorption by EST. To obtain significant inhibition of proteoglycan release from bovine nasal cartilage stimulated to resorb with IL1α and OSM (1 and 10 ng/ml respectively) required EST at 330 μmol/l. However, we have previously found that IL1α and OSM at these concentrations promote a maximal and rapid loss of proteoglycan by day 7 of culture of bovine nasal cartilage.⁴⁵ To determine if lower EST concentrations could inhibit proteoglycan resorption, we decreased the concentration of these cytokines, thus decreasing the rate of resorption. Using cytokines at concentrations 5- to 20-fold lower than those typically used, we found that significant inhibition did occur with EST at 100 μmol/l. We conclude that EST

can inhibit proteoglycan loss, but where explants are exposed to high levels of cytokines over prolonged periods then this inhibition can be overcome. In addition, it was apparent that the standard deviations increased. We have noted this on a number of occasions when cytokine concentrations are reduced, and propose that not all of the explants respond to the reduced levels of cytokine, resulting in an increased standard deviation.

In response to IL1 α + OSM, it is typical for human articular cartilage to respond with rapid proteoglycan loss but low collagen degradation.⁵⁷ The experiment illustrated had a higher collagen loss relative to many similar experiments. However, the potential inhibitory effect of EST in the human system was also determined by a specific ELISA, which showed that MMP-1 levels were always significantly reduced to control levels in the presence of the inhibitor, corroborating the collagen results in human articular cartilage.

EST can inhibit IL1 α + OSM stimulated collagen degradation when added up to 72 hours after the cytokines. Furthermore, both the total and active levels of collagenase are decreased by addition of EST over the same time period. As mentioned above, it has been shown elsewhere that EST does not inhibit collagenases by direct interaction with the enzymes.⁴³ Therefore, these results suggest that in disc culture, three days are required for IL1 α + OSM to induce the production and secretion of collagenolytic proenzymes by chondrocytes. EST, which at 325 (mol wt) is relatively small, may be able to diffuse through the cartilage matrix to inhibit the IL1 α + OSM effects. It may be that the cytokines' actions are exerted for up to three days of culture and are not required thereafter. In support of this, we have evidence to suggest that collagenolytic proenzymes are present in the matrix by day 3 of culture (data not shown). Furthermore, in culture of T/C28a4 chondrocytes, cytokines encounter no restrictions in reaching the cells and MMP mRNAs are produced within 24 hours of stimulation, with levels of MMP-1 protein rising by 24 hours of stimulation and peaking at 72 hours.⁴⁵

There are a number of points at which EST could act: it may interfere with transcription factors that bind the promoters for MMP-1, MMP-3, or MMP-13 and so prevent transcription. We investigated members of the activator protein 1 family that are believed to be involved in MMP transcription: we found that EST does not alter the up regulation of c-fos and c-jun expression induced by IL1 α and/or OSM,⁵⁸ suggesting that EST does not interfere with cytokine receptor components. These results for EST therefore seem to differ from a study reported by Martel-Pelletier *et al.*,⁵⁹ which showed decreased IL1 receptor expression on OA and RA synovial fibroblasts in the presence of the antirheumatic drug, Tenidap. EST might bind directly to upstream promoter sites of MMPs or to other associated transcription factor genes. It might also bind to and prevent the binding of known transcription factors to relevant genes. Alternatively, EST may inter-

fere with cytokine signalling pathways, or with the cytokine receptor components.

The concentrations of EST required to block cartilage resorption were not due to cytotoxic effects. If cytotoxicity was the mode of action of EST, one would expect to see a decrease in the mRNA expressed for all proteins examined by northern blot. However, expression of TIMP-1 and GAPDH mRNAs was not affected by the EST concentrations used (0–500 μ mol/l), indicating that transcription had not been universally altered, and suggesting that the inhibition by EST was not based on cytotoxic activity. We appreciate that the concentrations of inhibitor required are in the micromolar range and, although relatively high compared with other metalloproteinase inhibitors, the mode of action of EST is different from those inhibitors that directly block the active site of the enzymes.⁴² This is a lead compound representing a new class of molecule in this field. It is currently undergoing further development and more potent derivatives will be available in the future. The bioavailability of compounds in vivo may also alter their efficacy, such that lower concentrations of more potent agents may be sufficient to block cartilage resorption in patients.

Thus we have shown that EST is an effective inhibitor of cartilage resorption. It significantly reduces proteoglycan and collagen loss, and collagenolytic activity in resorbing cartilage. These effects are not due to changes in the levels of TIMP-1, but can be seen at the mRNA level for MMP-1, MMP-3, and MMP-13. It therefore appears that EST exerts its effects at a pre-transcriptional stage, thereby reducing enzyme levels. The mechanism is new, in that most MMP inhibitors function by direct inhibition of active MMPs or by increasing TIMP levels,^{33 35 39 42} whereas EST does neither of these. We are currently investigating the precise mechanisms of action of EST.

SE was funded by Kureha Chemical Industry Co Ltd, Tokyo, Japan. ADR, SC, PK, and JBC were funded by the Arthritis Research Campaign, UK. We are indebted to John Matthews, professor of statistics, Newcastle University, UK, for assistance in the statistical analysis of data. We are grateful to Professor Constance Brinckerhoff and Joni Rutter, Dartmouth College Medical School, Hanover, New Hampshire, USA for the MMP-1 promoter construct.

- Kuettner KE. Biochemistry of articular cartilage in health and disease. *Clin Biochem* 1992;25:155–63.
- Dingle JT, Page-Thomas DP, King B, Bard DR. In vivo studies of articular tissue damage mediated by catabolin/interleukin 1. *Ann Rheum Dis* 1987;46:527–33.
- Page-Thomas DP, King B, Stephens T, Dingle JT. In vivo studies of cartilage regeneration after damage induced by catabolin/interleukin-1. *Ann Rheum Dis* 1991;50:75–80.
- Jubb RW, Fell HB. The breakdown of collagen by chondrocytes. *J Pathol* 1980;130:159–67.
- Krane SM. Clinical importance of metalloproteinases and their inhibitors. *Ann N Y Acad Sci* 1994;732:1–10.
- Woessner JF Jr. The family of matrix metalloproteinases. *Ann N Y Acad Sci* 1994;732:11–21.
- Nagase H, Woessner JF. Matrix metalloproteinases. *J Biol Chem* 1999;274:21491–4.
- Stricklin GP, Bauer EA, Jeffrey JJ, Eisen AZ. Human skin collagenase: isolation of precursor and active forms from both fibroblast and organ cultures. *Biochemistry* 1977;16:1607–15.
- Hasty KA, Jeffrey JJ, Hibbs MS, Welgus HG. The collagen substrate specificity of human neutrophil collagenase. *J Biol Chem* 1987;262:10048–52.
- Freije JM, Diez-Itza I, Balbin M, Sanchez LM, Blasco R, Tolivia J, *et al.* Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. *J Biol Chem* 1994;269:16766–73.

- 11 Clark IM, Powell LK, Wright JK, Cawston TE, Hazleman BL. Monoclonal antibodies against human fibroblast collagenase and the design of an enzyme-linked immunosorbent assay to measure total collagenase. *Matrix* 1992;12:475-80.
- 12 Mort JS, Dodge GR, Roughley PJ, Liu J, Finch SJ, DiPasquale G, *et al.* Direct evidence for active metalloproteinases mediating matrix degradation in interleukin 1-stimulated human articular cartilage. *Matrix* 1993;13:95-102.
- 13 Dodge GR, Pidoux I, Poole AR. The degradation of type II collagen in rheumatoid arthritis: an immunoelectron microscopic study. *Matrix* 1991;11:330-8.
- 14 McCachren SS. Expression of metalloproteinases and metalloproteinase inhibitor in human arthritic synovium. *Arthritis Rheum* 1991;34:1085-93.
- 15 Brinckerhoff CE. Joint destruction in arthritis: metalloproteinases in the spotlight. *Arthritis Rheum* 1991;34:1073-5.
- 16 Murphy G, Cockett MI, Stephens PE, Smith BJ, Docherty AJ, Stromelysin is an activator of procollagenase. A study with natural and recombinant enzymes. *Biochem J* 1987;248:265-8.
- 17 Suzuki K, Englund JJ, Morodomi T, Salvesen G, Nagase H. Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin). *Biochemistry* 1990;29:10261-70.
- 18 Sandy JD, Boynton RE, Flannery CR. Analysis of the catabolism of aggrecan in cartilage explants by quantitation of peptides from the three globular domains. *J Biol Chem* 1991;266:8198-205.
- 19 Flannery CR, Lark MW, Sandy JD. Identification of a stromelysin cleavage site within the interglobular domain of human aggrecan. Evidence for proteolysis at this site in vivo in human articular cartilage. *J Biol Chem* 1992;267:1008-14.
- 20 Fosang AJ, Last K, Knäuper V, Neame PJ, Murphy G, Hardingham TE, *et al.* Fibroblast and neutrophil collagenases cleave at two sites in the cartilage aggrecan interglobular domain. *Biochem J* 1993;295:273-6.
- 21 Lark MW, Gordy JT, Weidner JR, Ayala J, Kimura JH, Williams HR, *et al.* Cell-mediated catabolism of aggrecan. Evidence that cleavage at the "aggrecanase" site (Glu³⁷³-Ala³⁷⁴) is a primary event in proteolysis of the interglobular domain. *J Biol Chem* 1995;270:2550-6.
- 22 Arner EC, Hughes CE, Decicco CP, Caterson B, Tortorella MD. Cytokine induced cartilage proteoglycan degradation is mediated by aggrecanase. *Osteoarthritis Cartilage* 1998;6:214-28.
- 23 Arner EC, Pratta MA, Trzaskos JM, Decicco CP, Tortorella MD. Generation and characterization of aggrecanase. A soluble, cartilage-derived aggrecan-degrading activity. *J Biol Chem* 1999;274:6594-601.
- 24 Arner EC, Pratta MA, Decicco CP, Xue C-B, Newton RC, Trzaskos JM, *et al.* Aggrecanase. A target for the design of inhibitors of cartilage. *Ann N Y Acad Sci* 1999;878:92-107.
- 25 Billington CJ, Clark IM, Cawston TE. An aggrecan-degrading activity associated with chondrocyte membranes. *Biochem J* 1998;336:207-12.
- 26 Tyler JA. Articular cartilage cultured with catabolin (pig interleukin 1) synthesizes a decreased number of normal proteoglycan molecules. *Biochem J* 1985;227:869-78.
- 27 Barrett AJ. Alpha-2 macroglobulin. *Methods Enzymol* 1981;80:737-54.
- 28 Henriot P, Blavier L, DeClerck YA. Tissue inhibitors of metalloproteinases (TIMP) in invasion and proliferation. *APMIS* 1999;107:111-19.
- 29 Pelletier JP, Mineau F, Faure MP, Martel-Pelletier J. Imbalance between the mechanisms of activation and inhibition of metalloproteinases in the early lesions of experimental osteoarthritis. *Arthritis Rheum* 1990;33:1466-76.
- 30 Martel-Pelletier J, McCollum R, Fujimoto N, Obata K, Cloutier JM, Pelletier JP. Excess of metalloproteinases over tissue inhibitor of metalloproteinase may contribute to cartilage degradation in osteoarthritis and rheumatoid arthritis. *Lab Invest* 1994;70:807-15.
- 31 Vincenti MP, Clark IM, Brinckerhoff CE. Using inhibitors of metalloproteinases to treat arthritis. Easier said than done? *Arthritis Rheum* 1994;37:1115-26.
- 32 Cawston TE. Metalloproteinase inhibitors and the prevention of connective tissue breakdown. *Pharmacol Ther* 1996;70:163-82.
- 33 DiGiulio A, Barrachini A, Cellai L, Martuccio C, Amicosante G, Oratore A, *et al.* Rifamycins as inhibitors of collagenase activity: their possible pharmacological role in collagen degradative diseases. *International Journal of Pharmaceutics* 1996;144:27-35.
- 34 Conaghan PG, Brooks P. Disease-modifying antirheumatic drugs, including methotrexate, gold, antimalarials, and D-penicillamine. *Curr Opin Rheumatol* 1995;7:167-73.
- 35 Yamada H, Kikuchi T, Nemoto O, Obata K, Sato H, Seiki M, *et al.* Effects of indomethacin on the production of matrix metalloproteinase-3 and tissue inhibitor of metalloproteinases-1 by human articular chondrocytes. *J Rheumatol* 1996;23:1739-43.
- 36 Steinmeyer J, Daufeldt S. Pharmacological influence of antirheumatic drugs on proteoglycans from interleukin-1 treated articular cartilage. *Biochem Pharmacol* 1997;53:1627-35.
- 37 Lewis EJ, Bishop J, Bottomley KM, Bradshaw D, Brewster M, Broadhurst MJ, *et al.* Ro 32-3555, an orally active collagenase inhibitor, prevents cartilage breakdown in vitro and in vivo. *Br J Pharmacol* 1997;121:540-6.
- 38 Brewster M, Lewis EJ, Wilson KL, Greenham AK, Bottomley KM. Ro 32-3555, an orally active collagenase selective inhibitor, prevents structural damage in the STR/ORT mouse model of osteoarthritis. *Arthritis Rheum* 1998;41:1639-44.
- 39 DiMartino M, Wolff C, High W, Stroup G, Hoffman S, Laydon J, *et al.* Anti-arthritis activity of hydroxamic acid-based pseudopeptide inhibitors of matrix metalloproteinases and TNF alpha processing. *Inflamm Res* 1997;46:211-15.
- 40 Ellis AJ, Curry VA, Powell EK, Cawston TE. The prevention of collagen breakdown in bovine nasal cartilage by TIMP, TIMP-2 and a low molecular weight synthetic inhibitor. *Biochem Biophys Res Commun* 1994;201:94-101.
- 41 Greenwald RA, Golub LM, Ramamurthy NS, Chowdhury M, Moak SA, Sorsa T. In vitro sensitivity of the three mammalian collagenases to tetracycline inhibition: relationship to bone and cartilage degradation. *Bone* 1998;22:33-8.
- 42 Bottomley KM, Borkakoti N, Bradshaw D, Brown PA, Broadhurst MJ, Budd JM, *et al.* Inhibition of bovine nasal cartilage degradation by selective matrix metalloproteinase inhibitors. *Biochem J* 1997;323:483-8.
- 43 Watanabe K, Ito A, Sato T, Hayashi H, Niitani Y. Esculetin suppresses proteoglycan metabolism by inhibiting the production of matrix metalloproteinases in rabbit chondrocytes. *Eur J Pharmacol* 1999;370:297-305.
- 44 Cawston TE, Ellis AJ, Humm G, Lean E, Ward D, Curry V. Interleukin-1 and oncostatin M in combination promote the release of collagen fragments from bovine nasal cartilage in culture. *Biochem Biophys Res Commun* 1995;215:377-85.
- 45 Cawston TE, Curry VA, Summers CA, Clark IM, Riley GP, Life PF, *et al.* The role of oncostatin M in animal and human connective tissue collagen turnover and its localisation within the rheumatoid joint. *Arthritis Rheum* 1998;41:1760-71.
- 46 Goldring MB, Birkhead JR, Suen L, Yamin R, Mizuno S, Glowacki J, *et al.* Interleukin-1 B-modulated gene expression in immortalized human chondrocytes. *J Clin Invest* 1994;94:2307-16.
- 47 Rutter J, Benbow U, Coon C, Brinckerhoff CE. Cell-type specific regulation of human interstitial collagenase-1 gene expression by interleukin-1 β (IL-1 β) in human fibroblasts and BC-8701 breast cancer cells. *J Cell Biochem* 1997;66:322-36.
- 48 Sah RL, Chen AC, Grodzinsky AJ, Trippel SB. Differential effects of bFGF and IGF-I on matrix metabolism in calf and adult bovine cartilage explants. *Arch Biochem Biophys* 1994;308:137-47.
- 49 Luyten FP, Hascall VC, Nissley SP, Morales TI, Reddi AH. Insulin-like growth factors maintain steady-state metabolism of proteoglycans in bovine articular cartilage explants. *Arch Biochem Biophys* 1988;267:416-25.
- 50 Tyler JA. Insulin-like growth factor 1 can decrease degradation and promote synthesis of proteoglycan in cartilage exposed to cytokines. *Biochem J* 1989;260:543-8.
- 51 Hascall VC, Handley CJ, McQuillan DJ, Hascall GK, Robinson HC, Lowther DA. The effect of serum on biosynthesis of proteoglycans by bovine articular cartilage in culture. *Arch Biochem Biophys* 1983;224:206-23.
- 52 Cawston TE, Barrett AJ. A rapid and reproducible assay for collagenase using [¹⁴C] acetylated collagen. *Anal Biochem* 1979;99:340-5.
- 53 Koshy PJT, Rowan AD, Life PF, Cawston TE. 96-well plate assays for measuring collagenase activity using [³H] acetylated collagen. *Anal Biochem* 1999;275:202-7.
- 54 Plumpton TA, Clark IM, Plumpton C, Calvin J, Cawston TE. Development of an enzyme-linked immunosorbent assay to measure total TIMP-1 (free TIMP-1 and TIMP-1 in combination with matrix-metalloproteinases) and measurement of TIMP-1 and CRP in serum. *Clin Chim Acta* 1995;240:137-54.
- 55 Fort Ph, Marty L, Piechacyk M, El Sabrouy S, Dani C, Jeanteur Ph, *et al.* Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate dehydrogenase multigenic family. *Nucl Acid Res* 1985;13:1431-42.
- 56 Hirt B. Selective extraction of polyoma DNA from infected mouse cell cultures. *J Mol Biol* 1967;26:365-9.
- 57 Cawston TE, Billington C, Cleaver C, Elliott S, Hui W, Koshy P, *et al.* The regulation of MMPs and TIMPs in cartilage turnover. *Ann N Y Acad Sci* 1999;878:120-9.
- 58 Benbow U, Brinckerhoff CE. The AP-1 site and MMP gene regulation: What is all the fuss about? *Matrix Biol* 1997;15:519-26.
- 59 Martel-Pelletier J, Mineau F, Tardif G, Fernandes JC, Ranger P, Loose L, *et al.* Tenipad reduces the level of interleukin 1 receptors and collagenase expression in human arthritic synovial fibroblasts. *J Rheumatol* 1996;23:24-31.