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Lysosomal cysteine endopeptidases mediate interleukin 1-stimulated cartilage proteoglycan degradation

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The peptidyl diazomethane inactivator of cysteine endopeptidases, benzyloxycarbonyl-Tyr-Ala-CHN_2, was tested as an inhibitor of interleukin 1α-stimulated release of proteoglycan from bovine nasal septum cartilage explants. Like the previously tested epoxidyl peptide proinhibitor *trans*-epoxysuccinyl-leucylamido-(3-methyl)butane ethyl ester, it proved to be an effective inhibitor of proteoglycan release from cartilage, with significant inhibition at a concentration of 1 μM. The inhibition did not seem to be due to a general toxic effect. The rates of inactivation of the bovine cysteine endopeptidases by the peptidyl diazomethane, the epoxidyl peptide proinhibitor and its active form were determined. Benzyloxycarbonyl-Tyr-Ala-CHN_2 proved to be a rapid inactivator of cathepsins L, S and B, but reacted much more slowly with cathepsin H and calpain. Thus it would appear that the latter two enzymes are not implicated in proteoglycan release in our test system. The peptidyl diazomethane and epoxidyl peptide proinhibitor (above) were also tested for their effects on three other interleukin 1-mediated cellular events, namely epidermal growth factor receptor transmodulation, and interleukin 6 and prostaglandin E_2 production. In all cases the inactivators did not interfere with the response to interleukin 1 in human gingival fibroblasts. We conclude that one or more of the lysosomal cysteine endopeptidases cathepsins B, L and S mediate interleukin 1-stimulated cartilage proteoglycan degradation without affecting signal transduction.

INTRODUCTION

The inflammatory cytokine interleukin 1 (IL1) stimulates chondrocytes to degrade the proteoglycan component of their surrounding cartilage matrix. This is one of a number of ways in which IL1 is thought to be involved in the pathogenesis of rheumatoid arthritis (Saklatvala, 1992). The mechanisms by which IL1 alters chondrocyte behaviour are unknown, but it is generally thought that the resulting degradation of cartilage proteoglycan is due to the action of unidentified endopeptidase(s).

The breakdown of cartilage proteoglycan has generally been refractory to inhibition by endopeptidase inhibitors, and this has led to suggestions that the enzymes might be operating in an environment from which the inhibitors are excluded. We recently reported that a hydrophobic proinhibitor, the ethyl ester (referred to here as Ep453) of the fast-acting irreversible inactivator of cysteine endopeptidases, Ep475, was capable of inhibiting IL1-stimulated proteoglycan release, whereas hydrophilic inactivators were ineffective. We therefore concluded that cysteine endopeptidases play a part in cytokine-stimulated cartilage breakdown, and probably act at a site controlled directly by the cell (Buttle *et al.*, 1992).

The active form of the proinhibitor is a rapid inactivator of most mammalian cysteine endopeptidases, including the lysosomal enzymes cathepsins B, H and L (Barrett *et al.*, 1982), and the cytosolic calpains (Parkes *et al.*, 1985). The effectiveness of this reagent therefore does not give any clue as to which cysteine endopeptidase(s) is involved in proteoglycan degradation. Also unknown are the means by which the degradation of cartilage proteoglycan is achieved. The cysteine endopeptidase inactivator could be effective at inhibiting proteoglycan release because of its specific action on proteoglycan-degrading cysteine endopeptidase(s). Although it would seem from earlier work that the inhibitor does not act via a non-specific toxic effect (Buttle *et al.*, 1992), other possibilities still exist, such as an inhibitory effect, through whatever means, on IL1 signal transduction.

We have now addressed these points by the use in our cartilage explant system of a hydrophobic inactivator with some selectivity for individual cysteine endopeptidases, and by investigating the effects of the inactivators on other IL1-dependent cellular events.

EXPERIMENTAL

Materials

The LL-isomers of Ep453 (also known as EST, E64d and loxistatin) and Ep475 (known also as E64c) were gifts from Dr. M. Tamai, Research Centre, Taisho Pharmaceuticals Co. Ltd., Saitama 330, Japan. Benzyloxycarbonyl (Z)-Tyr-Ala-CHN_2 was a gift from Dr. E. Shaw, Friedrich Miescher Institut, CH-4002 Basel, Switzerland. Recombinant human IL1α (rhIL1α) was obtained as described previously (Bird & Saklatvala, 1990). L-[^{35}S]Methionine was from Amersham International. Papain (type III) and reactive-red 120-agarose were from Sigma Chemical Co. 1,9-Dimethylmethylene Blue was obtained from Serva Feinbiochemica, Heidelberg, Germany. Hydroxyapatite HT was the product of Bio-Rad Laboratories, Hemel Hempstead HP2 7TD, U.K. All other chromatographic media and columns were from Pharmacia LKB Biotechnology, Milton Keynes MK9 3HP, U.K. Peptidyl-4-methyl-7-coumarylamide (NHMec) substrates were from Bachem Feinchemikalien AG, Bubendorf CH-4416, Switzerland.

Abbreviations used: Bz, benzoyl; EGF, epidermal growth factor; Ep475, *trans*-epoxysuccinyl-leucylamido-(3-methyl)butane; Ep453, *trans*-epoxysuccinyl-leucylamido-(3-methyl)butane ethyl ester; IL1, interleukin 1; IL6, interleukin 6; k_2, second-order rate constant for inactivation; $k_{2(app.)}$, apparent second-order rate constant in the presence of substrate; $k_{obs.}$, apparent pseudo-first-order rate constant for inactivation; NHMec, 4-methyl-7-coumarylamide; PGE_2, prostaglandin E_2; rhIL1α, recombinant human IL1α; Suc, 3-carboxypropionyl; Z, benzyloxycarbonyl.

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Protein determination

Protein concentrations were determined by the Bio-Rad dye binding assay, with BSA as standard.

Enzyme assays

Cathepsin B was assayed at 40 °C with Z-Arg-Arg-NHMec (5 μM) in 50 mM-Mes/1 mM-EDTA/0.005% Brij 35/4 mM-cysteine, pH 5.5. Cathepsin L was assayed at 30 °C with Z-Phe-Arg-NHMec (5 μM) in the same buffer. Cathepsin H was assayed at 40 °C with Arg-NHMec (5 μM) in 50 mM-sodium phosphate/1 mM-EDTA/0.005% Brij 35/4 mM-cysteine, pH 6.0. Cathepsin S was assayed with benzoyl (Bz)-Phe-Val-Arg-NHMec (5 μM) at 40 °C in 100 mM-sodium phosphate/1 mM-EDTA/0.005% Brij 35/4 mM-cysteine, pH 7.5. Calpain was assayed at 30 °C with 0.6% azocasein in 100 mM-Tris/acetate, pH 7.5, containing 100 mM-KCl, 5 mM-$CaCl_2$ and 0.02% (v/v) 2-mercaptoethanol. The reaction was stopped by the addition of a 10% (w/v) final concentration of trichloroacetic acid. Following removal of the precipitate by centrifugation, the A_{366} of the supernatant was recorded.

Enzyme purification

Cathepsin L (EC 3.4.22.15) was purified from bovine liver by a method based on that for the purification of the human enzyme (Mason *et al.*, 1985), with the use of the S-Sepharose High Performance 35/100 prepacked column run on the f.p.l.c. system at 10 ml/min, in place of chromatography on CM-Sephadex C-50.

Cathepsin B (EC 3.4.22.1) was purified from the peak of proteins that did not bind to the S-Sepharose 35/100 chromatogram (above), by active site-directed affinity chromatography (Rich *et al.*, 1986).

Cathepsin H (EC 3.4.22.16) was purified from the peak of activity against Arg-NHMec that was eluted early from chromatography on the S-Sepharose 35/100 column (above), by chromatography on Sephacryl S-200, hydroxyapatite and concanavalin A–Sepharose (Schwartz & Barrett, 1980).

Cathepsin S (EC 3.4.22.27) was purified from bovine spleen as described (Kirschke *et al.*, 1989), with a linear gradient of NaCl on the S-Sepharose 35/100 column replacing stepwise chromatography on CM-Sephadex C-50.

m-Calpain (EC 3.4.22.17) was purified from bovine skeletal muscle (Goll *et al.*, 1986).

Purified cysteine endopeptidases were standardized by E64 titration (Barrett *et al.*, 1982) and then further characterized by their kinetic behaviour with substrates and inactivators. Whenever comparison was possible, the values obtained were close to published figures (see below).

Determination of kinetic parameters

All kinetic parameters were determined at 30 °C by the use of continuous rate assays in a Perkin–Elmer fluorimeter (excitation 360 nm, emission 460 nm) linked to a personal computer running the FLUSYS (Rawlings & Barrett, 1990) and Enzfitter (Elsevier Biosoft, Cambridge, U.K.) software packages. Total substrate hydrolysis never exceeded 2%. Cathepsins B and L were assayed in 100 mM-sodium acetate/1 mM-EDTA/0.01% Brij 35/4 mM-cysteine, pH 5.5, using Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec respectively as substrates. For cathepsin S, the buffer was 50 mM-potassium phosphate containing 0.01% Triton X-100/1 mM-EDTA/4 mM-cysteine, pH 6.5, with Bz-Phe-Val-Arg-NHMec as substrate. Cathepsin H was assayed in 50 mM-sodium phosphate/0.01% Brij 35/4 mM-cysteine, pH 6.0, with Arg-NHMec as substrate. m-Calpain was studied using 50 mM-Tris/HCl/5 mM-$CaCl_2$/10 mM-dithiothreitol/0.1% Triton X-100, pH 7.5, containing Suc-Leu-Tyr-NHMec as substrate.

K_m values were calculated by non-linear regression analysis of at least six individual experiments at substrate concentrations varying by at least one order of magnitude. The values found were 158 ± 12 μM (mean ± S.E.M.) for cathepsin B with Z-Arg-Arg-NHMec, 54 ± 9 μM for cathepsin H with Arg-NHMec, and 890 ± 260 μM for m-calpain with 3-carboxypropionyl (Suc)-Leu-Tyr-NHMec. These values are not dissimilar to those for human cathepsins B and H (180 μM and 150 μM respectively; Kirschke & Barrett, 1987; Schwartz & Barrett, 1980) and chicken calpain (400 μM; Crawford *et al.*, 1988).

k_2 values were derived as $k_{obs.}$ by non-linear regression. $k_{2(app.)}$ was calculated as $k_{obs.}/[I]$ (where [I] is inhibitor concentration). In most cases, at least six different experiments were carried out with inactivator concentrations varying over at least one order of magnitude. In all cases, $k_{obs.}/[I]$ did not vary with varying [I]. k_2 was then found by the standard correction for the effect of competition by substrate, using the K_m values for cathepsins B and H and m-calpain (above), and the published values of 2.2 μM for bovine cathepsin L with Z-Phe-Arg-NHMec (Mason, 1986) and 8.1 μM for bovine cathepsin S with Bz-Phe-Val-Arg-NHMec (Brömme *et al.*, 1989). m-Calpain is known to lose activity due to autolysis. In order to allow for the loss of enzyme activity, $k_{obs.}$ was measured for the loss of activity in the absence of inactivator. This value (-0.003 s^{-1}) was subtracted from the value of $k_{obs.}$ found in the presence of inactivator, and the corrected $k_{obs.}$ was then entered into the calculations.

Cartilage cultures

Bovine nasal septum cartilage was prepared and cultured as previously described (Buttle *et al.*, 1992). Z-Tyr-Ala-CHN_2 was added from a 100-fold concentrated stock solution in dimethyl sulphoxide 60 min prior to the addition of rhIL1α (0.3 nM).

Determination of proteoglycan release

After 30 h in culture the amount of proteoglycan released into the culture medium was determined by use of 1,9-Dimethylmethylene Blue (Farndale *et al.*, 1986). In most instances a response to the cytokine (as measured by at least a doubling of the amount of released proteoglycan) was easily detectable at this time, but occasionally the response was slower. These latter experiments were left in culture for a total of 48 h. The small number of experiments in which proteoglycan release had not at least doubled by this time in response to treatment with IL1 were not analysed further.

Protein synthesis

A 2 h pulse with [^{35}S]methionine, following the 30 h culture with and without cytokine and inactivator, was used to assess the rate of protein synthesis, as previously described (Buttle *et al.*, 1992).

Assay for lactate

The lactate concentrations in the culture media were determined by the lactate oxidase/peroxidase method with a kit supplied by Sigma.

IL1-dependent epidermal growth factor (EGF) receptor transmodulation

The down-regulation of EGF binding to human gingival fibroblasts in response to IL1 was measured as described previously (Bird & Saklatvala, 1989).

IL1-stimulated IL6 synthesis

IL6 production by human gingival fibroblasts in response to

Table 1. Second-order rate constants for the inactivation of bovine cysteine endopeptidases by Ep475, its proinhibitor Ep453, and Z-Tyr-Ala-CHN_2

See the Experimental section for details. The values are means ± S.E.M.

	k_2 ($M^{-1} \cdot s^{-1}$)				
Inhibitor	Cathepsin B	Cathepsin L	Cathepsin S	Cathepsin H	Calpain
Ep475	64200 ± 2500	113000 ± 10000	168000 ± 5000	1900 ± 100	4870 ± 780
Ep453	104 ± 14	530 ± 40	1140 ± 90	138 ± 19	12.6 ± 2.3
Z-Tyr-Ala-CHN_2	1800 ± 200	120000 ± 3000	1740 ± 220	< 10	< 10

rhIL1α (1 nM) was determined by culturing cells in the presence and absence of IL1 and inactivators for 18 h, then measuring IL6 in the culture medium by e.l.i.s.a. (Taktak *et al.*, 1991).

Prostaglandin E_2 (PGE_2)

This was measured by radioimmunoassay.

RESULTS AND DISCUSSION

Rates of inactivation of bovine cysteine endopeptidases by some specific inactivators

The second-order rate constants for the inactivation of the bovine cysteine endopeptidases cathepsin B, cathepsin H, cathepsin L, cathepsin S and m-calpain by the epoxidyl peptide Ep475, its proinhibitor Ep453 and the peptidyl diazomethane Z-Tyr-Ala-CHN_2, were determined, and are shown in Table 1.

Ep475 was found to be a fast-acting inactivator of all the bovine enzymes tested, although the rate constants for the inactivation of cathepsins B and L were lower than those reported for the human enzymes (Barrett *et al.*, 1982), and the rate for the inactivation of bovine m-calpain was about half that reported for chicken calpain (Parkes *et al.*, 1985).

Compared with Ep475, the ethyl ester Ep453 was a slower inactivator by between one and three orders of magnitude (Table 1), supporting the suggestion that it is a proinhibitor, and highlighting the importance of the carboxy group of Ep475 in the rapid inactivation of cysteine endopeptidases. The crystal structure of the papain–Ep475 complex has been reported, and shows the interaction of the carboxy group of Ep475 with His-159, Gln-19 and Cys-25 of papain (Yamamoto *et al.*, 1991).

Z-Tyr-Ala-CHN_2 was found to be a very rapid inactivator of bovine cathepsin L (Table 1). The rates for the inactivation of bovine cathepsins B and S, although lower by nearly two orders of magnitude, were still quite high. In contrast, the inactivation of bovine cathepsin H and m-calpain was too slow to measure. Our results for the inactivation of bovine cathepsins B and L and m-calpain by this reagent are very similar to those previously published for the human cathepsins and chicken calpain (Crawford *et al.*, 1988).

The inactivation of cathepsin S by epoxysuccinyl peptides has not been previously reported. The inactivation of this enzyme by Ep475 is rapid, the rate being slightly greater than that for cathepsin L. In contrast, the rate of inactivation of cathepsin S by the peptidyl diazomethane is relatively slow, and more closely resembles that for cathepsin B (Table 1).

Table 2. Inhibition of IL1-stimulated proteoglycan release from bovine nasal septum cartilage by Z-Tyr-Ala-CHN_2

Cartilage discs (approx. 2 mm × 2 mm) were cultured in serum-free medium with or without IL1 (0.3 nM) and Z-Tyr-Ala-CHN_2 for 30 or 48 h. The culture medium was then analysed for the amount of proteoglycan released from the explants. Background levels of proteoglycan release were subtracted. The results are presented as percentage inhibition of proteoglycan release (means ± S.E.M.). The effect of the presence of Z-Tyr-Ala-CHN_2 was compared with the IL1 response in the absence of inactivator using Student's unpaired *t* test: $*P < 0.05$; $**P < 0.0005$ compared with controls.

[Z-Tyr-Ala-CHN_2] (μM)	Inhibition (% of control)
0.1	1 ± 9
1	29 ± 6*
10	36 ± 6**
100	78 ± 8**

Table 3. Lactate production by bovine nasal septum cartilage explants in the presence of Z-Tyr-Ala-CHN_2

Explants were cultured for 30 h with or without IL1 and Z-Tyr-Ala-CHN_2. Cartilage was killed by freezing and thawing prior to culture. Lactate levels are expressed as percentages of controls, i.e. with IL1 only (means ± S.E.M.). The effect of the presence of Z-Tyr-Ala-CHN_2 was compared with the control (rhIL1α alone) by use of Student's unpaired *t* test: $*P < 0.05$; $**P < 0.005$.

[Z-Tyr-Ala CHN_2] (μM)	Presence of IL1	Lactate (% of control)
0	–	83 ± 4*
0	+	100 ± 13
0.1	+	90 ± 3
1	+	90 ± 3
10	+	86 ± 4*
100	+	51 ± 4**
Dead tissue	+	5 + 2

Inhibition of IL1-stimulated proteoglycan degradation by Z-Tyr-Ala-CHN_2

We tested the lipophilic peptidyl diazomethane for its effect on the rhIL1α-stimulated release of proteoglycan from bovine nasal septum cartilage explants. Statistically significant inhibition was obtained at a reagent concentration of 1 μM, and inhibition was dose-dependent up to 100 μM (Table 2).

We have previously reported a similar inhibitory effect of the lipophilic proinhibitor Ep453, and a lack of effect in this system of the active, but hydrophilic, form Ep475 and of another hydrophilic epoxidyl peptide, E64 (Buttle *et al.*, 1992). Our results with Z-Tyr-Ala-CHN_2 therefore support the hypothesis that cysteine endopeptidases are involved in IL1-stimulated proteoglycan release from bovine nasal septum cartilage explants, but that inhibitors must be able to traverse membranes in order to inhibit this response. The radioiodinated form of Z-Tyr-Ala-CHN_2 has been shown to specifically label cathepsins B and L in intact mouse fibroblasts within 3 h of its addition to the medium at a concentration of 0.1 μM (Mason *et al.*, 1989), and to label these enzymes in intact purified lysosomes by 10 min (Wilcox &

Mason, 1989). The lack of inhibition of bovine cathepsin H and calpain by Z-Tyr-Ala-CHN_2 (Table 1) strongly suggests that these two cysteine endopeptidases are not involved in the degradative response to IL1.

Effect of Z-Tyr-Ala-CHN_2 on the metabolic activity of cartilage explants

Non-specific inhibition of proteoglycan release is seen with toxic compounds, such as iodoacetate (Buttle *et al.*, 1992). We therefore tested the peptidyl diazomethane for non-specific effects by investigating the reversibility of its inhibitory action, and its effect on rates of glycolysis and protein synthesis.

The ability of the explants to recover from the presence of the inactivator was investigated by subjecting the tissue to a second presentation of cytokine following a 24 h recovery period, as previously described (Buttle *et al.*, 1992). Explants cultured in the presence of rhIL1α (0.3 nM) and Z-Tyr-Ala-CHN_2 (100 μM) released 49 ± 17 μg of glycosaminoglycan/disc. The second treatment with the cytokine alone resulted in the release of 161 ± 42 μg of glycosaminoglycan/disc, demonstrating that the explants were capable of responding to a second exposure to the cytokine.

The effect of Z-Tyr-Ala-CHN_2 on protein synthesis was assessed by measuring [^{35}S]methionine incorporation into trichloroacetic acid-precipitable material during a 2 h pulse in the presence of cytokine and inactivator, immediately after the 30 h incubation in the presence of the inactivator and rhIL1α. At a concentration of 100 μM, Z-Tyr-Ala-CHN_2 had no significant effect on protein synthesis, with levels of incorporation in the presence of the inactivator and cytokine being 96 ± 2% of those in the presence of the cytokine alone, whereas dead (frozen-and-thawed) tissue only incorporated 5 ± 1% of control levels.

The effect of the peptidyl diazomethane on glycolysis was tested by measuring the amount of lactate produced by the cartilage explants. The presence of rhIL1α led to a statistically significant increase in lactate production. When Z-Tyr-Ala-CHN_2 was also added to the culture medium to a concentration of 100 μM, it was found to suppress lactate excretion by about 50% (Table 3). At 10 μM, suppression of lactate production was still significantly decreased to 86% of the control level, roughly the same as the cultures without either the cytokine or the inactivator. Below a 10 μM concentration of inactivator, lactate levels were not significantly reduced (Table 3).

It is clear that Z-Tyr-Ala-CHN_2 has an inhibitory effect on glycolysis. However, the concentration required to significantly reduce lactate levels (to those produced by explants cultured in the absence of IL1) is 10-fold higher than the concentration (1 μM) at which significant inhibition of proteoglycan degradation is seen (Table 2). This observation, along with the finding that the suppression of glycolysis, even by 100 μM-Z-Tyr-Ala-CHN_2, is not sufficient to significantly alter levels of protein synthesis by the explants, and that the inhibition by 100 μM-Z-Tyr-Ala-CHN_2 is reversible, leads us to conclude that it is unlikely that the inhibition of IL1-stimulated proteoglycan degradation in the presence of Z-Tyr-Ala-CHN_2 is due to non-specific effects of the inactivator.

Effects of Z-Tyr-Ala-CHN_2 and Ep453 on other cellular responses to rhIL1α

From our results so far, we conclude that the lysosomal cysteine endopeptidases cathepsins L, S and B are implicated in the IL1-stimulated loss of proteoglycan from bovine nasal septum cartilage. One way in which the inactivators could affect the response of the cartilage is by interfering with the transduction of the signal generated by the cytokine binding to its receptor, which passes to the nucleus and induces subsequent changes in gene expression. IL1 signal transduction almost certainly involves activation of protein kinases (Mizel, 1990; O'Neill *et al.*, 1990), and leads to the up-regulation of a number of genes (Muegge & Durum, 1990). Although the exact nature of the protein kinases stimulated by IL1 is uncertain, an early indication of their activation is rapid phosphorylation of the EGF receptor, which causes a loss of affinity of the receptor for its ligand: so-called transmodulation (Bird & Saklatvala, 1989). We therefore investigated the effects of Z-Tyr-Ala-CHN_2 and of the other hydrophobic cysteine endopeptidase inactivator previously shown to be effective in our model system, Ep453 (Buttle *et al.*, 1992), upon EGF receptor transmodulation caused by IL1. We also examined their possible effects on the stimulation of production of IL6 and PGE_2 in connective tissue cells by IL1. IL6 is an early-response gene (Elias & Lentz, 1990), whereas induction of PGE_2 synthesis is slow, taking 6–12 h to develop.

Table 4. Effect of hydrophobic cysteine endopeptidase inactivators on IL1-dependent EGF receptor transmodulation

Confluent human gingival fibroblasts were treated with Ep453 or Z-Tyr-Ala-CHN_2 (100 μM) for 2 h at 37 °C, then incubated in the presence of rhIL1α for 10 min. The cell layers were washed and incubated at 4 °C for 10 min before the addition of ^{125}I-EGF for 3 h at 4 °C. After washing, the cells were lysed and counted for radioactivity on a γ-radiation counter. Non-specific binding was estimated by incubating the cells with ^{125}I-EGF in a 100-fold excess of unlabelled ligand. The values for EGF bound (c.p.m.) are the means of triplicate cultures.

^{125}I-EGF	IL1	Inhibitor	EGF bound (c.p.m.)
Non-specific	–	–	552
+	–	–	1728
+	–	Ep453	1456
+	–	Z-Tyr-Ala-CHN_2	1266
+	+	–	1024
+	+	Ep453	1067
+	+	Z-Tyr-Ala-CHN_2	825

The experiments investigating the effects of the inactivators of EGF receptor transmodulation were carried out using confluent human gingival fibroblasts. As can be seen (Table 4), treatment with rhIL1α roughly halved the amount of EGF binding specifically to the cells, confirming earlier results (Bird & Saklatvala, 1989). Although there may have been a slight reduction in the amount of EGF bound to the cells in the presence of 100 μM of the inactivators whether or not IL1 was present, it is clear that in each case the inactivators did not interfere with the reduction in binding of EGF due to the presence of the cytokine.

We initially attempted to investigate IL6 and PGE_2 production by bovine nasal septum cartilage explants in the presence of the inactivators. However, the amounts of IL6 and PGE_2 produced by the explants, even upon stimulation with rhIL1α, were barely above the limit of detection. Instead, as with EGF receptor transmodulation (above), we conducted the experiments using a monolayer culture of human gingival fibroblasts. These cells produced easily detectable levels of IL6 and PGE_2, even in the absence of the cytokine. The presence of 1 nM-rhIL1α increased IL6 production by about two orders of magnitude, whether or not the inactivators were present (Table 5). Similarly, the 50-fold increase in PGE_2 synthesis caused by IL1 was unaffected by the inactivators.

Concluding remarks

Our results suggest a role for the lysosomal cysteine endopeptidases cathepsins L, S and/or B in the loss of

Table 5. IL1-stimulated IL6 and PGE_2 production by human gingival fibroblasts in the presence and absence of lipophilic cysteine endopeptidase inactivators

Human gingival fibroblasts were treated with Ep453 or Z-Tyr-Ala-CHN_2 (100 μM) in the presence and absence of IL1 (1 nM) for 18 h (for measurement of IL6) or 24 h (for measurement of PGE_2). The culture medium was then assayed by e.l.i.s.a. for the presence of IL6 or by radioimmunoassay for PGE_2. Values (ng/ml) are the means ± S.E.M. of quintuplicate cultures.

IL1	Inhibitor	IL6 (ng/ml)	PGE_2 (ng/ml)
−	−	0.258 ± 0.096	0.39 ± 0.05
−	Ep453	0.138 ± 0.010	0.33 ± 0.03
−	Z-Tyr-Ala-CHN_2	0.080 ± 0.004	0.08 ± 0.01
+	−	19.7 ± 1.2	24 ± 1
+	Ep453	17.0 ± 0.5	27 ± 2
+	Z-Tyr-Ala-CHN_2	17.0 ± 1.1	20 ± 1

proteoglycan from bovine nasal septum cartilage treated with IL1. We could find no evidence to suggest that the enzyme inactivators were affecting IL1 signal transduction or an early change in gene expression. Nor did they affect the stimulation of PGE_2 synthesis, which is a late response. Other mechanisms therefore appear more likely. The endopeptidases could be having a direct effect on the proteoglycan aggregates in cartilage, releasing the bulk of the molecule from the hyaluronate-binding region. Both cathepsins B and L have recently been shown to have this capacity *in vitro* (Nguyen *et al.*, 1990). Alternatively, the cysteine endopeptidases could be rate-limiting in a cascade. For instance, cathepsin B is capable of activating pro-urokinase-type plasminogen activator (Kobayashi *et al.*, 1991) and prostromelysin (Murphy *et al.*, 1992). Plasminogen activation might lead to a direct hydrolytic action of plasmin on cartilage proteoglycan (Lack & Rogers, 1958) and to its participation, along with cathepsin B, in the activation of prostromelysin (Okada *et al.*, 1988). The metalloendopeptidase could itself then contribute to proteoglycan release. Direct evidence for such a cascade is generally lacking, however. Further work is also necessary to clarify the means by which the IL1 signal affects lysosomal physiology, and to determine whether cartilage proteoglycan degradation occurs in or outside chondrocyte plasma membranes.

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