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Inhibition of interleukin 1-stimulated cartilage proteoglycan degradation by a lipophilic inactivator of cysteine endopeptidases

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Inactivators of cysteine endopeptidases were tested as inhibitors of the cytokine-stimulated release of proteoglycan from cartilage. The test system consisted of bovine nasal septum cartilage maintained in organ culture, and the stimulus was provided by recombinant human interleukin 1α. L-3-Carboxy-2,3-*trans*-epoxypropionyl-leucylamido-(4-guanidino)butane (E64) and L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido-(3-methyl)butane (Ep475) showed no inhibition at concentrations up to 100 μM. In contrast, *trans*-epoxysuccinyl-leucylamido-(3-methyl)butane ethyl ester (Ep453), a 'prodrug' of Ep475, was an effective inhibitor. The LL-, LD- and DL-isomers gave significant inhibition at 10 μM, and the DD-isomer was inhibitory at 100 μM. None of the isomers had any detectable effect on protein synthesis or glycolysis, and their inhibitory effects were reversible. Iodoacetate inhibited proteoglycan release by a general toxic effect. Our results suggest that cysteine endopeptidase(s) play a part in cytokine-stimulated cartilage breakdown, but that effective inhibitors must pass through membranes.

INTRODUCTION

As an experimental model of pathophysiological cartilage degradation, chondrocytes can be induced to degrade their surrounding cartilage matrix in tissue culture by application of compounds such as retinol (Hembry *et al.*, 1982) and interleukin 1 (IL1) (Saklatvala *et al.*, 1983, 1984). Analysis of the diffusible products of the degradation indicates that they result from the action of one or more proteolytic enzymes (Tyler, 1985), but these have not been identified, and attempts to block the catabolism with inhibitors of endopeptidases have met with little or no success (Hembry *et al.*, 1982; Saklatvala & Sarsfield, 1988). Caputo *et al.* (1987) reported the inhibition of retinoic acid- and IL1-stimulated proteoglycan release from rabbit articular cartilage explants by two hydroxamic acid-containing peptides previously shown to be inhibitors of matrix metalloendopeptidases, but it remains to be shown whether the response was specific or due to toxicity.

There is now strong evidence for a role for lysosomal cysteine endopeptidases in the catabolism of the organic matrix of bone (Delaissé *et al.*, 1980, 1987), and this prompted us to examine the effects of some inhibitors of such enzymes in the cartilage system. We now report that inhibition has been achieved with low concentrations of a non-polar precursor of a potent inactivator of cysteine endopeptidases, and discuss the implications of this.

EXPERIMENTAL

Materials

Recombinant human IL1α (rhIL1α) was obtained as described previously (Bird & Saklatvala, 1990). The isomers of Ep453 [*trans*-epoxysuccinyl-leucylamido-(3-methyl)butane ethyl ester; also known as EST, E64d and loxistatin] were synthesized as described (Tamai *et al.*, 1987). Papain was Type III from Sigma Chemical Co. L-[^{35}S]Methionine and sodium [^{35}S]sulphate were from Amersham International.

Cartilage cultures

Bovine nasal septum cartilage was dissected from freshly slaughtered animals. The cartilage was sliced, and discs were cut from the slices with the aid of a belt punch. About 200 discs (2 mm diam., 1–3 mm thick) were obtained from each animal. The discs were maintained in 25 ml of Dulbecco's modified Eagle's medium (DMEM) containing gentamicin (25 μg/ml), penicillin G (100 units/ml), streptomycin (0.1 mg/ml), amphotericin B (0.2 μg/ml), heat-inactivated newborn calf serum (5%, v/v) and cortisol (Sigma; 0.1 μg/ml) for 2 days at 37 °C in a humidified atmosphere of 5% CO_2. The discs were washed in serum- and cortisol-free DMEM, then transferred individually into wells of a 96-microwell plate each containing 150 μl of the same medium. Cysteine endopeptidase inactivators (1–100 μM) were added from 100-fold concentrated stock solutions in dimethyl sulphoxide (Me_2SO). Iodoacetate (Sigma) was added from a freshly prepared stock solution in water. After 60 min, rhIL1α was added to 0.3 nM. The cultures were maintained for 30 h, after which the medium and/or discs were taken for analysis.

Determination of proteoglycan release

The amount of proteoglycan released into the culture medium was determined by use of 1,9-dimethylMethylene Blue (Serva Feinbiochemica), with 0–5 μg of whale chondroitin sulphate A (Sigma) as standard (Farndale *et al.*, 1986).

Protein synthesis

The discs were incubated in groups of five in 1 ml of serum- and cortisol-free DMEM in wells of a 24-well plate, with or without inhibitors (100 μM final concentration) and 0.3 nM-rhIL1α for 30 h. The wells were washed in methionine-free minimal essential medium (MEM) (1 ml, 2 × 30 min), and 1 ml of the same medium (with or without the inhibitors and rhIL1α) containing 3 μCi of [^{35}S]methionine was added. The cultures

Abbreviations used: IL1, interleukin 1; DMEM, Dulbecco's modification of Eagle's medium; E64, L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido-(4-guanidino)butane; Ep453, *trans*-epoxysuccinyl-leucylamido-(3-methyl)butane ethyl ester; Ep475, *trans*-epoxysuccinyl-leucylamido-(3-methyl)butane; MEM, minimal essential medium; rhIL1α, recombinant human IL1α; Me_2SO, dimethyl sulphoxide.

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were maintained for 2 h at 37 °C. The medium was removed and the cartilage discs were washed in 3 % (w/v) trichloroacetic acid (3 × 15 min) and water (3 × 10 min). Each disc was placed in 200 μl of 0.1 M-sodium phosphate/1 mM-EDTA/4 mM-cysteine, pH 6.8, containing 2 mg of papain/ml, for 45 min at 60 °C. This resulted in total dissolution of the tissue. A scintillation cocktail was added and the mixture was counted for radioactivity in a liquid scintillation counter.

Assay for lactate

The lactate concentration in conditioned serum-free DMEM was determined by the lactate oxidase/peroxidase method with a kit supplied by Sigma.

RESULTS

Stimulation of proteoglycan degradation by rhIL1α

The total amount of sulphated glycosaminoglycan per bovine nasal septum cartilage disc was found to be 2.23 mg ± 0.54 mg (mean ± S.E.M., range 1.00–2.78 mg). When a control disc was cultured in serum-free DMEM for 30 h, approx. 70 μg of proteoglycan was released into the medium. The addition of rhIL1α to the culture medium increased the release in a dose-dependent manner. At 0.3 nM-rhIL1α, stimulation was approx. 2–5-fold, and this concentration was chosen for the inhibition experiments. Me_2SO (1 %, v/v) did not affect either control or rhIL1α-stimulated levels of proteoglycan release.

Inhibitory effects of iodoacetate and epoxides

Iodoacetate is a general thiol-alkylating reagent and is thereby a cysteine endopeptidase inactivator. It gave significant inhibition at a concentration of 100 μM (Table 1).

E64 [L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido-(4-guanido)butane] and Ep475 [*trans*-epoxysuccinyl-leucylamido-(3-methyl)butane] are charged compounds, and are rapid, specific inactivators of cysteine endopeptidases (Barrett *et al.*, 1982; Tamai *et al.*, 1987). They did not inhibit proteoglycan release, however.

The uncharged molecule of Ep453 contains two optically active centres: epoxysuccinic acid and the leucine α-carbon atom. As can be seen in Table 1, the LL-, LD- and DL-isomers inhibited the degradation of proteoglycan in a dose-dependent manner, giving statistically significant inhibition at 10 μM and 100 μM. The DD-isomer was also inhibitory, but showed a significant effect only at 100 μM. There was no apparent effect on the control level of proteoglycan release in the absence of rhIL1α (results not shown).

Effect of cysteine endopeptidase inactivators on the metabolic activity of cartilage explants

rhIL1α-mediated proteoglycan release is dependent upon the metabolic activity of the tissue (Saklatvala & Sarsfield, 1988), so that non-specific inhibition of proteoglycan release would be seen with toxic compounds. We therefore investigated the cysteine endopeptidase inactivators for toxicity by looking at the reversibility of inhibition, and the rates of glycolysis and protein synthesis.

The ability of the tissue to recover from the presence of the inactivators was assessed by allowing the explants to equilibrate in inactivator-free DMEM containing serum and cortisol for 24 h following the 30 h period with the cytokine and cysteine endopeptidase inactivators, and then presenting rhIL1α for a second 30 h period, in the absence of inactivators. In all cases except that of iodoacetate, the cartilage discs responded to the second exposure to cytokine (Table 2).

The effects of 100 μM concentrations of iodoacetate and the isomers of Ep453 on glycolysis were tested by measuring the amount of lactate produced by the cartilage explants. Control discs produced 64.9 ± 3.8 μg of lactate/disc, and dead tissue produced 4.1 ± 0.9 μg. In contrast to what was found with a fibroblast line grown in cell culture (Bird *et al.*, 1987), rhIL1α

Table 1. Inhibition of rhIL1α-stimulated proteoglycan release from bovine nasal septum cartilage by cysteine endopeptidase inactivators

The experiments were carried out on cartilage explants stimulated with rhIL1α as described in the Experimental section. Control levels of proteoglycan release were subtracted, and the effect of the presence of the inactivators was compared with the rhIL1α-stimulated response in the absence of inactivator, by the unpaired Student's *t* test. The results are given as percentage inhibition of proteoglycan release (means ± S.E.M.) compared with the effect of rhIL1α alone. Significance of differences: $*P < 0.05$, $\dagger P < 0.005$, $\ddagger P < 0.0005$, $\S P < 0.00005$.

Inactivator	Concentration (μM)	Inhibition (% of control)
Iodoacetate	1	42 ± 15
	10	36 ± 18
	100	81 ± 4†
E64	10	−20 ± 12
	100	−8 ± 15
Ep475	1	23 ± 13
	10	13 ± 19
	100	0 ± 18
Ep453 (LL)	1	37 ± 18
	10	47 ± 10*
	100	76 ± 6‡
Ep453 (LD)	1	22 ± 20
	10	59 ± 10†
	100	91 ± 6§
Ep453 (DL)	1	−39 ± 19
	10	59 ± 10*
	100	84 ± 8‡
Ep453 (DD)	1	0 ± 26
	10	30 ± 9
	100	56 ± 3‡

Table 2. Reversibility of the inhibition of proteoglycan release by cysteine endopeptidase inactivators

The experiment was carried out as described in the Experimental section. After incubation of explants in serum-free medium containing various combinations of cytokines and inactivators for 30 h as indicated (Treatment 1), and a 24 h culture period in the absence of both cytokine and inactivator, discs were incubated for 30 h in medium containing only rhIL1α (Treatment 2). Values for the release of proteoglycan are expressed as means ± S.E.M.

Treatment 1 additions		Proteoglycan release (μg/disc)	
rhIL1α (nM)	Inactivator	Treatment 1	Treatment 2
–	–	58 ± 5	134 ± 27
0.3	–	157 ± 30	184 ± 24
0.3	LL-Ep453 (100 μM)	62 ± 7	126 ± 27
0.3	LD-Ep453 (100 μM)	96 ± 13	222 ± 55
0.3	DL-Ep453 (100 μM)	91 ± 12	168 ± 30
0.3	DD-Ep453 (100 μM)	96 ± 9	147 ± 35
0.3	Iodoacetate (100 μM)	92 ± 5	42 ± 2

had only a minimal effect on lactate production by cartilage explants, increasing lactate production to 84.2 ± 11.2 μg/disc. Lactate production was decreased to 11.3 ± 3.4 and 20.1 ± 7.3 μg by iodoacetate in the presence and absence respectively of rhIL1α, but none of the isomers had any significant effect on lactate production with or without rhIL1α (range 62.8–81.3 μg/disc).

The cysteine endopeptidase inactivators were also tested for their effects on protein synthesis. A 2 h pulse of [^{35}S]methionine followed the 30 h culture in the presence of cytokine and inactivators. Iodoacetate decreased protein synthesis almost to the level found in dead tissue, but the isomers of Ep453 had no effect (range 98.6–115.3 % of control).

DISCUSSION

E64 and Ep475 (also known as E64c) are very specific, potent, irreversible inactivators of cysteine endopeptidases (Barrett *et al.*, 1982; Tamai *et al.*, 1986). Nevertheless, these compounds did not inhibit IL1-mediated proteoglycan degradation, even at high concentrations. This finding was consistent with the negative results reported previously for 2 mM-Ep475 and -E64, and for leupeptin (1 mM), the reversible inhibitor of cysteine endopeptidases (Saklatvala & Sarsfield, 1988).

E64, Ep475 and leupeptin are all polar compounds with very low membrane permeability. In view of this, the non-polar ethyl ester of Ep475 has been synthesized as a prodrug form for use in biological systems, in which it is converted into the highly reactive Ep475 (Tamai *et al.*, 1986). Ep453 has recently been shown to react with cysteine endopeptidases in intact cells and lysosomes (Wilcox, 1990). Neither Ep475 nor its ethyl ester inactivates non-cysteine endopeptidases or other enzymes with essential thiol groups, such as glyceraldehyde-3-phosphate dehydrogenase and hexokinase (Tamai *et al.*, 1987).

Iodoacetate at the concentration that inhibited the degradation of proteoglycan (100 μM) showed clear evidence of toxicity. Thus the tissue did not respond to IL1 after treatment with iodoacetate, and iodoacetate severely inhibited both lactate production and protein synthesis. We therefore attribute the inhibition of proteoglycan degradation by iodoacetate to non-specific toxicity of this thiol-blocking reagent.

In contrast, we take the potent inhibition of IL1-mediated degradation of proteoglycan by the isomers of Ep453 as *prima facie* evidence for a role of cysteine endopeptidases in this process. These compounds did not compromise the ability of the tissue to respond to a second treatment with IL1, and did not depress glycolysis or protein synthesis, and were therefore apparently acting directly on the interleukin-mediated proteoglycan degradation.

The reason for the effectiveness of the lipophilic compounds but not the related hydrophilic compounds is not explained. We tested the lipophilic membrane-permeant compounds because there was a theoretical possibility that cartilage matrix degradation might be taking place in a membrane-limited compartment, like osteoclast-mediated resorption of bone matrix (Delaissé *et al.*, 1980, 1987; Baron *et al.*, 1985). We are not aware of any ultrastructural evidence for such a compartment in cartilage matrix catabolism, but working with a similar test system, Saklatvala & Sarsfield (1988) obtained strong inhibition with cytochalasins B and D (2 μM). This could be taken as evidence that cell membrane movement and perhaps endocytosis are involved in the degradative response.

Our present results do not show whether the cysteine endopeptidases contribute to proteoglycan degradation by acting directly on proteoglycan, or by some indirect effect, such as the activation of prostromelysin (Murphy *et al.*, 1991), or by interfering with IL1 signal transduction. We also cannot say which cysteine endopeptidases may be active. Candidates include the lysosomal cysteine endopeptidases, especially cathepsins B, L and S, and perhaps the predominantly cytosolic calpain.

In conclusion, we consider that the potent inhibition of IL1-mediated proteoglycan degradation in the culture system indicates that cysteine endopeptidases contribute to this process. It is reasonable to suppose that they are also involved in the response to IL1 *in vivo*, and they may well contribute to the degradation of cartilage matrix in response to other agents. The determination of which cysteine endopeptidase(s) are involved in the matrix degradation, and exactly how they act, must clearly await further work.

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