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Inhibition of bone resorption in culture by inhibitors of thiol proteinases

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Leupeptin, antipain, tosyl-lysylchloromethane (Tos-Lys-CH_2Cl) and benzyloxycarbonylphenylalanylalanyldiazomethane (Z-Phe-Ala-CHN_2) inhibit reversibly the resorption induced by parathyroid hormone or heparin in cultured mouse bones. Leupeptin and antipain do not affect collagenase production and activity or the enhanced secretion of β-glucuronidase induced by the bone-resorbing agents. They might thus act by a direct (extracellular?) inhibition of lysosomal thiol proteinases.

The osteoclasts are the main effector cells of bone resorption, but their biochemical mode of action is still conjectural (Raisz, 1976). In our previous work, we found no correlation between the resorption of cultured mouse bones and their secretion of collagenase (Lenaers-Claeys & Vaes, 1979). On the contrary, good correlations exist between bone resorption and the release of lysosomal hydrolases, suggesting that these enzymes, acting in the segregated subosteroclastic resorption zones, may contribute to the digestion of bone (Vaes, 1968, 1980). As two lysosomal thiol proteinases, cathepsin B (Burleigh *et al.*, 1974) and cathepsin N (Ducastaing & Etherington, 1978), have been reported to degrade native collagen, the major organic constituent of bone, we investigated the action of inhibitors of thiol proteinases, leupeptin, antipain (Umezawa & Aoyagi, 1977), Tos-Lys-CH_2Cl (Barrett, 1977) and Z-Phe-Ala-CHN_2 (Shaw & Dean, 1980) on the resorption of bone explants in culture.

Materials and methods

Cultures

Calvaria from 19-day-old NMRI mouse embryos were cultured as described by Lenaers-Claeys & Vaes (1979) under an atmosphere of air in medium 199 supplemented or not with heparin (300μg/ml), purified parathyroid hormone (parathyrin; 'PTH') (0.3 U.S. Pharmacopeia unit/ml) or proteinase inhibitors. The bones (two calvaria/2ml of medium) were cultured either for 2–4 days with renewal of medium every day (scheme A) or for 4 days without renewal of medium (scheme B). The media were then collected, buffered at pH 7.5 with 0.04M-Tris/HCl, filtered on 0.45μm Millipore filters and assayed immediately or after storage at −20°C. All the data reported were obtained in several (usually at least three) repeated experiments.

Abbreviations used: Tos-Lys-CH_2Cl, tosyl-lysylchloromethane (7-amino-1-chloro-3-L-tosylamidoheptan-2-one; 'TLCK'); Z-Phe-Ala-CHN_2, benzyloxycarbonylphenylalanylalanyldiazomethane.

Analytical

Bone resorption was evaluated (Lenaers-Claeys & Vaes, 1979) by measuring the loss of hydroxyproline and of calcium from the explants and by scoring 0–5 the extent of the resorption lacunae (Gaillard, 1959; Vaes, 1965) observed in the tissues under a dissecting microscope. Loss of hydroxyproline from the calvaria correlates well ($r = 0.87$) with the development of the lacunae and represents a valid index of collagen degradation in this model, where synthesis of new collagen is minimal, as evidenced by the lack of significant hydroxyproline gain by calcitonin-treated, non-resorbing control explants (Lenaers-Claeys & Vaes, 1979). β-Glucuronidase was assayed as described by Robins *et al.* (1968); one unit of activity refers to the decomposition of 1pmol of substrate/min. Collagenase was assayed after complete activation of its latent form with trypsin, as described by Vaes (1972).

Materials

Leupeptin and antipain were provided by the U.S.–Japan Co-operative Cancer Research Program through the courtesy of Dr. W. Troll, New York University Medical Center. Tos-Lys-Ch_2Cl was from Sigma Chemical Co., St. Louis, MO, U.S.A.; Z-Phe-Ala-CHN_2 was from Bachem Feinchemikalien A.G., Bubendorf, Switzerland; soya-bean trypsin inhibitor was from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; and aprotinin [Trasylol, 5400 KI (kallikrein-inhibitory) units/mg] was from Bayer A.G., Wuppertal, Germany. Other chemicals were from suppliers

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previously mentioned (Lenaers-Claeys & Vaes, 1979).

Results and discussion

Effects on bone resorption

Parathyroid hormone, alone or together with heparin, induced a typical bone resorption in the explants, with extension of lacunae, loss of hydroxyproline and loss of calcium (Lenaers-Claeys & Vaes, 1979). Leupeptin, antipain, Tos-Lys-CH_2Cl and Z-Phe-Ala-CHN_2 (but not aprotinin or soya-bean trypsin inhibitor) inhibited these processes (Table 1). A 50% decrease in the loss of hydroxyproline caused by parathyroid hormone was achieved by 0.5 μg of leupeptin/ml (about 1.2 μM) (Fig. 1), by about 1 μg of Z-Phe-Ala-CHN_2/ml (2.6 μM) or by 5–10 μg of antipain/ml (8.8–17.6 μM) (results not shown). In several experiments, the loss of calcium appeared less inhibited than that of hydroxyproline, suggesting that the inhibitors act primarily on mechanisms leading to the resorption of organic matrix and that calcium losses were only secondarily affected.

Under some culture conditions (scheme B; see the Materials and methods section), heparin alone induced bone resorption. Leupeptin (Table 1, expt. A) or Tos-Lys-CH_2Cl (expt. C) also inhibited this effect; their inhibitory action was thus not restricted to the interaction between parathyroid hormone and bone cells. Occasional 'spontaneous' resorption in control explants was also inhibited.

Bones cultured with parathyroid hormone, whose resorption was inhibited by leupeptin, antipain, Tos-Lys-CH_2Cl (Fig. 2) or Z-Phe-Ala-CHN_2 (results not shown) subsequently developed normal resorp-

Table 1. *Effects of proteinase inhibitors on bone resorption and on β-glucuronidase release*

In expts A, B and C, calvaria were cultured (scheme B; see the Materials and methods section) in media without additive or with heparin (300 μg/ml), parathyroid hormone (PTH, 0.3 unit/ml), leupeptin (10 μg/ml), antipain [(*a*) 10 μg; (*b*) 25 μg/ml] and/or Tos-Lys-CH_2Cl (TLCK, 100 μM). After 4 days, β-glucuronidase was assayed in the media, and bone resorption was evaluated by the extension of the lacunae and by the amounts of hydroxyproline or calcium left in the explants. In expt. D, bone resorption was evaluated in calvaria cultured for 2 days (scheme A) with or without parathyrin (0.3 unit/ml), Z-Phe-Ala-CHN_2 (6 μg/ml), aprotinin (50 μg/ml) or soya-bean trypsin inhibitor (SBTI, 50 μg/ml). The results are presented as means ± S.D. of three (expt. B) or four (expts. A, C and D) cultures. Statistical significances (*t* test) are calculated within each experiment either (for the subgroups cultured with heparin and/or parathyroid hormone only) relative to the corresponding 'no additive' subgroup or (for the subgroups cultured with inhibitors with or without other additives) relative to the corresponding subgroups cultured with the same additives but without inhibitors. Only the significant differences are indicated: §$P<0.05$; ‡$P<0.02$; †$P<0.01$; *$P<0.001$.

Additives to the cultures	Resorption lacunae	Hydroxyproline (μg/calvarium)	Calcium (μmol/calvarium)	β-Glucuronidase released (units/4 days per calvarium)
Expt. A				
None	0.1 ± 0.2	24.4 ± 2.2	3.02 ± 0.39	26.4 ± 3.3
+Heparin	2.1 ± 0.8*	21.2 ± 2.5	2.75 ± 0.18	37.5 ± 7.7§
+Heparin + leupeptin	0.1 ± 0.3‡	24.8 ± 2.2	3.2 ± 0.08	39.4 ± 10.4
+Heparin + PTH	4.9 ± 0.3*	15.6 ± 2.2†	2.45 ± 0.31	56.4 ± 7.6*
+Heparin + PTH + leupeptin	0.6 ± 0.5*	22.4 ± 2.3†	2.69 ± 0.26	51 ± 12.2
Expt. B				
None	1.5 ± 1.9	22.1 ± 0.9	2.57 ± 0.29	27.2 ± 7.1
+Antipain (*b*)	0*	23.2 ± 3.4	2.87 ± 0.67	25.8 ± 2.5
+Heparin + PTH	4.7 ± 0.5‡	12.4 ± 1.3†	1.67 ± 0.09§	57.5 ± 6.5§
+Heparin + PTH + antipain (*a*)	2.5 ± 0.5†	16.7 ± 0.5†	—	54 ± 3.8
+Heparin + PTH + antipain (*b*)	0.5 ± 0.6*	19.5 ± 4.7	2.6 ± 1.06	54 ± 4.1
Expt. C				
None	0	18.3 ± 4	2.48 ± 0.53	25.3 ± 7.6
+TLCK	0	21.6 ± 0.5	2.45 ± 0.25	17 ± 0.9
+Heparin	3.1 ± 1.1*	12.7 ± 1.8§	1.81 ± 0.32	43.5 ± 10.2‡
+Heparin + TLCK	0*	21.4 ± 4.5‡	2.29 ± 0.72	17.8 ± 2.7†
+Heparin + PTH	4 ± 0*	9.8 ± 1.8†	1.36 ± 0.34†	61 ± 7.5*
+Heparin + PTH + TLCK	0.5 ± 0.7*	18.2 ± 0.5*	1.69 ± 0.14	22 ± 4*
Expt. D				
None	0	21.8 ± 1.2	2.51 ± 0.23	
+PTH	3.7 ± 0.5*	14.7 ± 1.8*	1.47 ± 0.20‡	
+PTH + Z-Phe-Ala-CHN_2	0*	18.0 ± 0.9‡	2.11 ± 0.20†	
+PTH + aprotinin	4 ± 0	12.9 ± 2	1.33 ± 0.27	
+PTH + SBTI	3.3 ± 0.5	14.2 ± 2.1	1.57 ± 0.16	

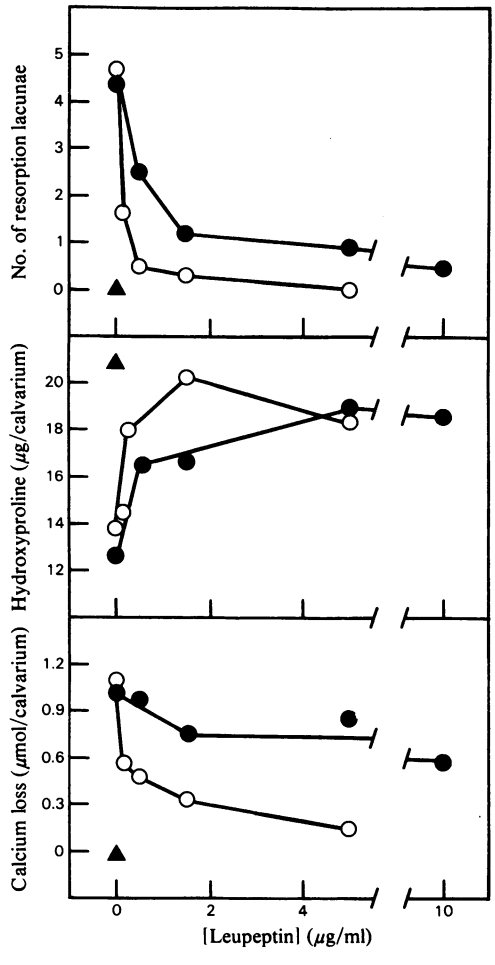

Fig. 1. *Inhibition of bone resorption by leupeptin*
Calvaria were cultured (scheme A; see the Materials and methods section) in the absence of any additive (▲) or in media containing parathyroid hormone alone (●) or together with heparin (○) and supplemented as indicated with increasing amounts of leupeptin. After 3 days, bone resorption was evaluated by the extension of the lacunae, by the amount of hydroxyproline left in the explants and by the amount of calcium released in the medium. Each point is the mean result for four cultures.

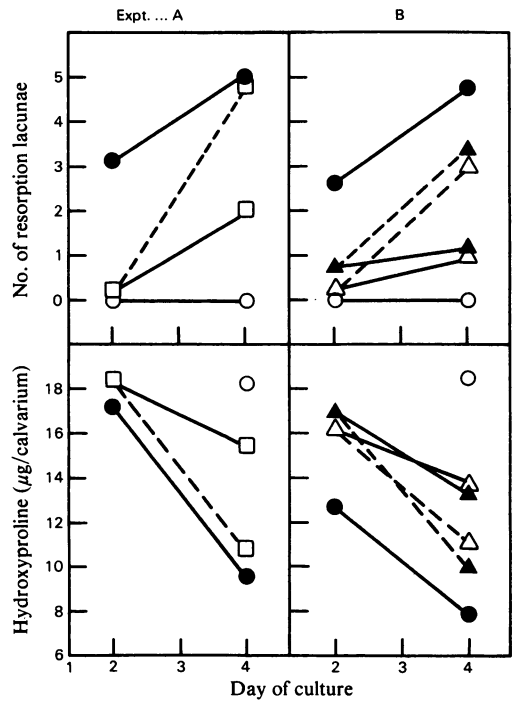

Fig. 2. *Reversibility of the inhibition of bone resorption exerted by leupeptin, antipain or Tos-Lys-CH_2Cl*
Bone explants were cultured for 4 days (scheme A; see the Materials and methods section) in the absence of any additive (○) or together with parathyroid hormone and heparin without (●) or with the following proteinase inhibitors: in expt. A (left), leupeptin, 10μg/ml (□); in expt. B (right), antipain, 10μg/ml (▲) or Tos-Lys-CH_2Cl, 100μM (△). The inhibitors were present in the media during the whole 4-day period of culture (——) or only during the first 2 days (– – – –). Resorption was evaluated by the extension of the lacunae and by the loss of hydroxyproline from the explants. Each point is the mean result for four cultures (for eight cultures for the measurements made after 2 days culture with the inhibitors).

tion after the removal of the inhibitors from the culture media. Thus the action of these agents cannot be attributed to irreversible cytotoxic effects.

Secretion of β-glucuronidase and collagenase

β-Glucuronidase was assayed in the culture media as a marker for the secretion of lysosomal enzymes that parallels bone resorption in culture (Vaes, 1968, 1980). The increased release of β-glucuronidase by the resorbing explants was not modified by concentrations of leupeptin or antipain that inhibited considerably bone resorption (Table 1). This indicates that the release of lysosomal hydrolases that accompanies bone resorption cannot be considered as a mere consequence of bone resorption. Moreover, it suggests that these inhibitors do not act by preventing the secretion of lysosomal enzymes from bone cells but may exert their effect by a direct extracellular inhibition of bone-resorbing proteinases. On the contrary, the inhibition of bone resorption by Tos-Lys-CH_2Cl may be due to another mechanism, as this inhibitor prevented the enhanced release of β-glucuronidase induced by heparin and parathyroid hormone (Table

1, expt. C). At the concentrations used, leupeptin, antipain and Tos-Lys-CH_2Cl had no direct effect on the activity of β-glucuronidase (results not shown).

As expected (Vaes, 1972; Lenaers-Claeys & Vaes, 1979), the secretion of collagenase by the explants was induced by heparin, and the enzyme was mainly recovered in a trypsin-activatable latent form. Its accumulation in the media as well as its activity was not significantly affected by leupeptin, antipain or Tos-Lys-CH_2Cl (results not shown). Thus these agents inhibited bone resorption independently of any effect on collagenase secretion and activity.

General discussion

Leupeptin, antipain and Tos-Lys-CH_2Cl inhibit not only thiol proteinases but also several serine proteinases. However, bone resorption was also inhibited by Z-Phe-Ala-CHN_2, an inhibitor of thiol proteinases that has no action on representative serine proteinases such as chymotrypsin (Shaw & Dean, 1980) or trypsin (our own controls; results not shown). Moreover it was unaffected by aprotinin and soya-bean trypsin inhibitor, both inhibitors of several serine proteinases (Barrett, 1977). Thus our data strongly suggest that thiol proteinases control a critical step in the cellular actions leading to bone resorption. The nature of that step remains to be determined, particularly as proteinase inhibitors may have profound effects on the metabolism of cultured cells (Roblin *et al.*, 1975). However, in our experiments, leupeptin and antipain did not affect the secretion of β-glucuronidase and collagenase by the bones, and the inhibitory effect of all inhibitors on bone resorption was fully reversible.

Thus our observations, taken together with the other evidence reviewed in the introduction to this paper (lack of correlation between collagenase production and bone resorption, release of lysosomal enzymes in the subosteoclastic resorption zones, collagenolytic activity of lysosomal thiol proteinases), allow us to propose, as a working hypothesis, that the collagenolytic action of lysosomal thiol proteinases might be a limiting step in the resorption of the organic matrix of bone, possibly by allowing the extracellular solubilization (and the subsequent endocytosis) of collagen monomers out of collagen fibres (Burleigh *et al.*, 1974) or by inhibiting a critical step in the intracellular digestion of endocytosed collagen fragments.

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