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CATHEPSIN B AND L ACTIVITIES IN ISOLATED OSTEOCLASTS

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SUMMARY: Cathepsin B and L activities were examined with chicken osteoclasts isolated by sequential filtration and inhibitors were added to disaggregated rat osteoclasts on cortical bovine bone. Z-Phe-Phe-CHN_2, a selective inhibitor of cathepsin L, at 1, 5, and 10 μM, inhibited bone resorption by rat osteoclasts 50, 85, and 100 per cent and, in chicken osteoclasts, cathepsin L activity was comparably inhibited. Cathepsin L in avian osteoclasts was also 25-fold higher than cathepsin B. Chicken osteoclasts treated with Z-Phe-Ala-CHN_2, a generalized cysteine proteinase inhibitor, had both cathepsins inhibited to the same extent. Cathepsin L may play a key role in resorption.

Bone resorption occurs mainly in an acidic extracellular compartment covered by the ruffled border of osteoclasts, the subosteoclastic resorption zone (1,2). The acidic pH optima of almost all of the lysosomal hydrolases (3) strongly suggested that this environment must be provided for enzyme action. Thus, demineralization and the degradation of calcified collagen may occur in this zone through the action of proton pumps (4). It has been shown that inhibitors of lysosomal cysteine proteinases blocked bone resorption both in vivo (5) and in vitro (6-8). Vaes (9) earlier proposed a collagenase-independent metabolic pathway for the removal of the collagenous matrix in mineralized tissues. This idea has since received support from studies on isolated osteoclasts which have shown a collagen-degrading acid-protease (10). In fact, degradation of bone and dentin collagens occurred most rapidly near or below pH 4 (11). The cysteine proteinases B, L, N, and S are effective at an acidic pH in degrading fibrillar collagen

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Abbreviations:

Z-Arg-Arg-AFC:Cbz-L-arginyl-L-arginine-7-amino-(4-trifluoromethyl)coumarin,Z-Phe-Arg-AFC:Cbz-L-phenylalanyl-L-arginine-7-amino-(4-trifluoromethyl)coumarin, AFC: 7-amino-(4-trifluoromethyl) coumarin, Z-Phe-Ala-CHN_2: benzyloxycarbonyl-L-phenylalanyl-alanyl diazomethane, Z-Phe-Phe-CHN_2: benzyloxycarbonyl-L-phenylalanyl-phenylalanine diazomethane, E-64: trans-epoxy-succinyl-L-leucylamido-(4-guanidino)-butane.

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and thus, have been identified as collagenolytic cathepsins (12-14). Cathepsin L appears as the most potent, since it has the highest capacity to degrade insoluble collagen (14-16). The cathepsins have been shown to degrade collagen by removal of its telopeptide extensions (17). The effects of cysteine proteinase inhibitors were examined with disaggregated chick osteoclasts on dentin (7). Resorption was markedly reduced, but a generalized cathepsin proteinase inhibitor was used and, therefore, it was difficult to identify which enzyme made the more significant contribution. Furthermore, there have been no investigations to assay the activities of cysteine proteinases from highly enriched preparations of osteoclasts.

Through the use of synthetic substrates and inhibitors, much kinetic information has been obtained on cathepsins B and L. These inhibitors include peptidyl-diazomethanes, such as Z-Phe-Ala-CHN_2 (18); and L-trans-epoxy-succinylpeptides, such as E-64 (19). Selective inhibitors of cathepsin L, notably, Z-Phe-Phe-CHN_2, can also be used to differentiate cathepsin L activity from cathepsin B (20) . Cathepsin B has been assayed with the substrate, Z-Arg-Arg-AFC, at a ratio of specific activity equal to 200:2:1 for cathepsins B, L, and H (16,21,22). However, no specific substrate has yet been identified for cathepsin L (16). Nonetheless, Z-Phe-Arg-AFC, at 1 μM, provides a much more sensitive assay for cathepsin L than cathepsin B (16). Unlike cathepsin S (23), the activity of cathepsin N cannot be readily determined, since it lacks activity on synthetic substrates (15,16). Furthermore, cathepsins N and S have not been identified in all tissues; cathepsin S has been identified only in spleen (24) and lymphoid tissue (25).

Thus, the objective of this study will focus on the more extensively characterized cathepsins, B and L. We have used the disaggregated rat osteoclast system to study the effect of Z-Phe-Phe-CHN_2 on resorption. However, this model does not provide large numbers of purified osteoclasts. Thus, we have also used isolated osteoclasts from a vitamin D-deficient chicken model to provide large numbers of purified cells (26). We have used these chicken osteoclasts to measure and compare cathepsin B and L activities and to examine the effect of inhibitors.

MATERIALS AND METHODS

Materials: Cbz-L-arginyl-L-arginine-7-amino-(4-trifluoromethyl)coumarin-AFC, Z-Arg-Arg-AFCandCbz-L-phenylalanyl-L-arginine-7-amino-(4-trifluoromethyl)coumarin-AFC,Z-Phe-Arg-AFC, were purchased from Enzyme Systems Products, (Livermore, CA) and were used as the substrates for cathepsins B and L, respectively. 7-amino-(4-trifluoromethyl)coumarin was purchased from Enzyme Systems Products (Livermore, CA) and used as the standard. The cysteine proteinase inhibitors, Z-Phe-Ala-CHN_2 (benzyloxycarbonyl-L-phenylalanyl-alanyl diazomethane) and Z-Phe-Phe-CHN_2 (benzyloxycarbonyl-L-phenylalanyl-phenylalanine diazomethane) were purchased from Enzyme Systems Products (Livermore, CA). The cysteine proteinase inhibitor, E-64 (trans-epoxy-succinyl-L-leucylamido-(4-guanidino)-butane), was

purchased from Sigma Chemical Company (St. Louis, MO). The rachitogenic test diet was a product from United States Biochemical Corporation (Cleveland, OH). Spectrum Medical Industries (Los Angeles, CA) supplied the polyester and polypropylene filters. Fetal bovine serum, Hank's balanced salt solution, Spinner's salt solution, Medium 199, and penicillin/ streptomycin were purchased from Gibco Laboratories (Grand Island, NY).

Disaggregated rat osteoclasts and bone resorption assay:

Rat osteoclasts were disaggregated as described (27) and assayed for resorptive activity (28). Aliquots (100 ul) from the resulting cell suspension were immediately transferred to 96-well tissue culture plates (Corning, NY), each containing a single bone slice. Bone slices were previously prepared as transverse slices (4.5 mm^2 x 0.1 mm^2) from dense cortical bovine bone using a low speed diamond saw (Buehler Isomet, Lake Bluff, IL). Following a settling period of 30 minutes at 37°C, the slices were stained with toluidine blue, 1% (v/v), for 4 minutes. This staining provided a light microscopic method of quantitating bone resorption (28). The total area resorbed per slice has been shown to correlate strongly with pit number (27).

Isolation of chicken osteoclasts:

Chicken osteoclasts were isolated and purified by sequential filtration as previously described (26). An enriched population of osteoclasts was obtained with a purity of approximately 85-90%, as indicated by tartrate resistant acid phosphatase staining and point counting. Cell suspensions were stored frozen until ready for cathepsin assays.

Assay for cathepsin B and L:

Enzyme activities were assayed as previously described (22). The hydrolysis of substrates with the release of AFC was measured over a linear range at 25°C with an excitation of 400 nm and an emission of 505 nm. Protein determinations followed the protocol of Lowry et al. (29).

RESULTS

The effect of Z-Phe-Phe-CHN_2 was examined with disaggregated rat osteoclasts **(Figure 1).** In fact, the response of these cells to this inhibitor appeared dose-dependent. A 1 μM concentration inhibited resorption approximately 50%; 5 μM approximately 85%; and at 10 μM, inhibition reached 100%. No apparent change in osteoclast numbers was noted with Z-Phe-Phe-CHN_2 at 1 and 5 μM, whereas at 10 μM, osteoclast numbers declined approximately 15 percent (data not shown). An inhibition of cathepsin L activity markedly inhibited bone resorption in this system.

There was a marked difference between the activities of cathepsins B and L from chicken osteoclasts **(Figure 2).** Cathepsin L activity was approximately 25-fold greater than cathepsin B ($p < 0.001$). Chicken osteoclasts treated with Z-Phe-Phe-CHN_2 showed reductions in both cathepsin B and L activities **(Figure 3).** At 1, 5, and 10 μM, cathepsin B was inhibited approximately 20, 40, and 80%, respectively. However, at the same concentrations, cathepsin L was inhibited approximately 55, 85, and 100%, respectively. The inhibitory effect of Z-Phe-Phe-CHN_2 was significantly greater ($p < 0.01$) on cathepsin L than cathepsin B activity at 1 and 5 μM.

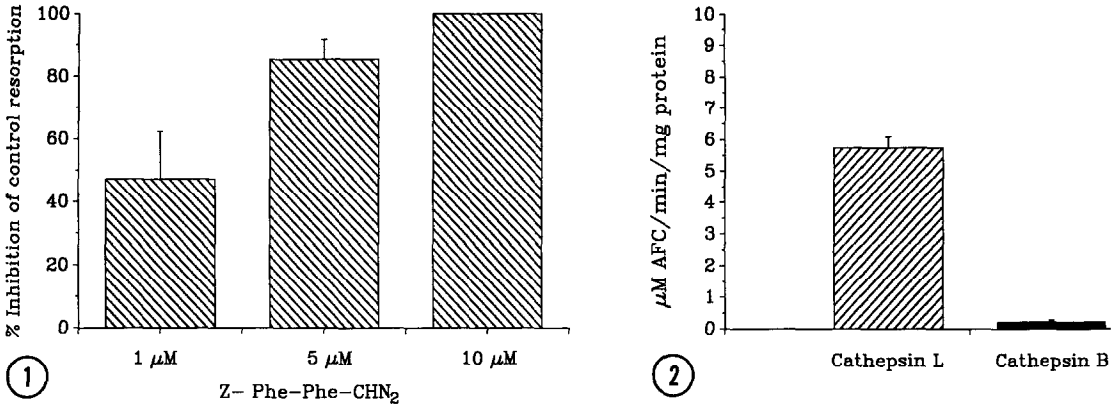

Figure 1. Effect of Z-Phe-Phe-CHN_2, a selective cathepsin L inhibitor, on bone resorption. Disaggregated rat osteoclasts were incubated with inhibitor for 24 h. Results are expressed as % inhibition of control resorption and analyzed as the mean ± SEM (n=6) for each concentration.

Figure 2. Cathepsin B and L activities in isolated chicken osteoclasts. Osteoclasts were isolated from vitamin D-deficient chickens and assayed for cathepsin B and L activities. Values represent the mean ± SEM (n=6).

Chicken osteoclasts were also treated with Z-Phe-Ala-CHN_2. Unlike Z-Phe-Phe-CHN_2, this inhibitor was less selective in its action and inhibited both cathepsins B and L to the same extent **(Figure 4).** At 1, 5, and 10 μM, cathepsin B was inhibited approximately 60, 90, and 100% respectively. At the same concentrations, cathepsin L was inhibited approximately 70, 95, and 100%. Chicken osteoclasts were treated with E-64 to determine whether noncysteine

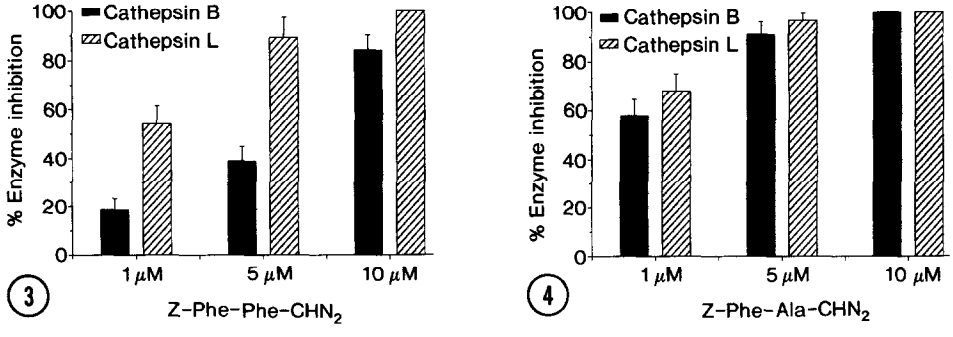

Figure 3. Effect of Z-Phe-Phe-CHN_2 on cathepsin B and L activities in isolated chicken osteoclasts. Enzyme activities were assayed in the spectrofluorometer cuvette at 25°C with inhibitor at final concentrations of 1, 5, and 10 μM. Values represent the mean ± SEM (n=6).

Figure 4. Effect of Z-Phe-Ala-CHN_2 on cathepsin B and L activities in isolated chicken osteoclasts. Values represent the mean ± SEM (n=6).

proteinases were active. E-64 (1 μM) completely abolished both cathepsin B and L activities (data not shown).

DISCUSSION

Our data suggested that cathepsin L may play a role in bone resorption. However, the significance of its presence and role remain open for further study. Rat osteoclasts treated with a selective inhibitor of cathepsin L, Z-Phe-Phe-CHN_2, showed a reduction in resorption and it also inhibited cathepsin L activity to a greater extent in avian osteoclasts. Furthermore, cathepsin B activity was minimal in comparison to cathepsin L in chicken osteoclasts **(Figure 2)**. Two possibilities may explain this observation: (1) cathepsins other than cathepsin B may have catalyzed the cathepsin B substrate, although Z-Arg-Arg-AFC is highly selective for cathepsin B. Such a possibility would imply that cathepsin B activity is not present. In fact, Sannes et al. (30) have shown that it was not present in osteoclasts with histochemical techniques; or (2) cathepsin B may indeed be a small part of total cathepsin activity. Cathepsin B has also been shown to activate latent collagenase (31,32). Recently, Baron et al. (33) localized collagenase in the subosteoclastic zone. However, if cathepsin B activity is minimal, then it may be highly unlikely that the activation of collagenase is mediated through it. Cathepsin L appears as the most potent of the collagenolytic cathepsins (14-16). The inhibition of cathepsin L activity and the associated inhibition of bone resorption in our cultured osteoclasts may be unrelated to collagenase, since cathepsin L can directly degrade collagen. This argument receives support from Delaisse et al. (7) in which the addition of TIMP, a tissue inhibitor of metalloproteinases, did not inhibit osteoclast-mediated resorption in vitro. Nonetheless, collagenase activity may play a role in degrading collagen fibers exposed, but perhaps not degraded, after the osteoclast has moved from its site of resorption. This site would then likely contain a neutral pH, optimizing collagenase activity. Thus, the role of collagenase and its precise relationship to cathepsins in bone resorption will require further clarification.

Our studies do not rule out the possibility that other cathepsins, like cathepsin N, may play a role in both mammalian and avian osteoclasts. However, since this enzyme cannot act on synthetic substrates (15,16), its activity cannot be measured and, therefore, its exact role in bone resorption is not yet defined.

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