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FEBS Letters 342 (1994) 308–312

FEBS 13869

FEBS LETTERS

The mechanisms and regulation of procathepsin L secretion from osteoclasts in bone resorption

Kahori Tagami[a], Hisao Kakegawa[b], Hiroshi Kamioka[a], Koji Sumitani[a], Terushige Kawata[a], Brigita Lenarčič[c], Vito Turk[c], Nobuhiko Katunuma[b,*]

[a]*Department of Orthodontics, School of Dentistry, The University of Tokushima, Kuramoto-cho, Tokushima 770, Japan*
[b]*Institute for Health Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770, Japan*
[c]*Department of Biochemistry, J. Stefan Institute, Jamova, 39, 61000 Ljubljana, Slovenia*

Received 28 February 1994

Abstract

The secretion mechanisms of cathepsin L from osteoclasts in the process of bone resorption were investigated. The increases in bone pit numbers formed take place by PTH addition in parallel with the increases of cathepsin L and/or L-like proteinase activities in the culture medium of bone cells, and these were suppressed by the addition of calcitonin. The Z-Phe-Arg-MCA hydrolysing activity increased in the medium through the effect of PTH is considered to be a kind of procathepsin L by Western blotting analysis, and was suppressed by calcitonin addition. Furthermore, monensin inhibited not only the PTH-induced pit formation, but also cysteine proteinase activity in osteoclasts. Therefore, the procathepsin L excreted might be transferred from endothelial reticulum via Golgi and/or via lysosomes.

Key words: Procathepsin L; Cysteine proteinase; Parathyroid hormone; Calcitonin; Monensin; Bone resorption

1. Introduction

The degradation of the bone organic matrix mainly consisting of collagen during the process of bone resorption occurs in the extracellular acidified microenvironment (Howship's lacuna) under the ruffled border of the osteoclasts [1–4]. Recently, lysosomal cysteine proteinases in osteoclasts, especially cathepsins L and B, have been thought to participate in the osseous collagenolysis [2,4–9]. Kominami et al. immunohistochemically demonstrated the presence of cathepsin B and L in rat osteoclasts of the bone surface, suggesting the participation of these proteinases in the degradation of the organic constituents of the bone matrix [9]. We previously reported that rat osteoclastic bone resorption induced by parathyroid hormone (PTH) was markedly inhibited by pig leucocyte cysteine proteinase inhibitor (PLCPI) [10,11], a specific inhibitor of cathepsin L, and by chymostatin, a selective inhibitor of cathepsin L, but not by CA-074 [12], a specific inhibitor of cathepsin B [13]. These facts suggest that cathepsin L or L-like proteinase, including procathepsin L, is the main proteinase responsible for bone collagen degradation.

It is important to clarify the individual functions of the various cells responsible for the secretion of cysteine proteinases related to bone resorption, such as osteoclasts, osteoblasts and osteocytes. The lysosomal cysteine proteinases of osteoclasts are synthesized in the endothelial reticulum and transported via Golgi toward the ruffled border membrane and secreted into the bone resorbing lacuna [1–4]. Everts et al. indicated that the release of β-glucronidase as a marker of the exocytosis of lysosomal enzymes induced by PTH in the mouse calvarial system and the amount of calcium released in the medium were both significantly decreased in the presence of calcitonin, which binds to the receptor on the surface of osteoclasts to suppress osteoclastic bone resorption [14]. The aim of the present work is to clarify the mechanisms of PTH-induced secretion of collagenolytic cysteine proteinases into the culture medium. We investigated the relationship between pit formation and the increase in cysteine proteinase activity induced by PTH into the culture medium of rat bone marrow cells, and the sup-

*Corresponding author. Fax: (81) (886) 22 3217.

Abbreviations: Z, benzyloxycarbonyl; MCA, methylcoumarylamide; E-64-a, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucine-4-aminobutyl-amine; CA-074, *N*-(L-3-*trans*-propyl-carbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline.

Enzymes: cathepsin B, EC 3.4.22.1; cathepsin L, EC 3.4.22.15.

SSDI 0014-5793(94)00254-S

pression of these phenomena by calcitonin. Furthermore, the effect of monensin [15,16] on rat osteoclastic bone resorption induced by PTH was evaluated.

2. Experimental

2.1. Materials

Z-Phe-Arg-MCA was purchased from the Peptide Institute (Osaka, Japan). E-64-a and CA-074 were kindly supplied by Taisho Pharmaceutical Co., (Saitama, Japan). PLCPI was prepared as described previously [10,11]. Fetal bovine serum (FBS) was from Gibco (Grand Island, NY), while human PTH (1–34) and salmon calcitonin were from Peninsula Laboratories Inc. (Belmond, CA). Monensin was from Wako Pure Chemical Co., Osaka. All other chemicals were of analytical grade. Rat liver cathepsin L was purified according to the published procedure [19]. Rabbit anti-cathepsin L was donated by Dr. E. Kominami (Department of Biochemistry, Juntendo University School of Medicine, Japan). Sprague–Dawley (SD) rats (1–2 days old) were used for preparation of unfractionated bone cells, and male SD rats (6–8 weeks old) were purchased from Charles River, Japan. Bovine compacted bone slices (150 μm thick) were punched out as circles 6 mm in diameter. Goat anti-mouse IgG labeled alkaline phosphatase (ALP) was purchased from EY Laboratories Inc.

2.2. Enzyme assay

Cathepsin activities were measured with Z-Phe-Arg-MCA at pH 5.5, as described previously [13]. Briefly, E-64, CA-074 and PLCPI were preincubated with enzyme and the reactions were started by the addition of substrates. The fluorescence of 7-amino-4-methylcoumrin liberated from the substrate was monitored by a fluorescence spectrometer (Hitachi F-2000).

2.3. Assay of bone resorption

The bone resorption assay was carried out according to the method of Mcsheehy and Chambers [18], with certain modifications as described previously. For the preparation of bone cells, the long bones from 1- to 2-day-old Sprague–Dawley rats were minced by scissors in alpha minimum essential medium (α-MEM, Flow Laboratories, McLean, VA) containing 10% FBS. Two-hundred μl of the cell suspension (1×10^6 cells per well) were added to each well of a 96-well plate containing a bovine bone slice in each well, and the cells were incubated at 37°C in a CO_2 incubator for 40 min. After the slices were rinsed with α-MEM, five slices were transferred to 500 μl of fresh medium containing 100 nM of PTH and 1 or 10 nM of calcitonin but not containing FBS in each well of a 24-well plate, and were incubated for 24–72 h. Osteoclast-induced pits on slices were stained with acid hematoxylin for 5 min. The total area of pits was measured under an optical microscope.

2.4. Cysteine proteinase activity in rat osteoclasts and macrophages

Rat osteoclasts were purified according to the method of Tezuka et al. as described previously [13]. After purification, 90% of cells were multinucleate and had tartrate-resistant acid phosphatase activity. Rat peripheral macrophages were obtained from whole blood of male SD rats. The blood was collected into heparinized tubes from the aorta descendens, and centrifuged at 900 rpm for 15 min. After removal of the platelet fraction, the buffy coat was collected, and then diluted 1:4 with RPMI 1640 containing 100 units of kanamycin. The diluted buffy coat was layered onto the lymphocyte separation medium (LymphoSep, ICN Flow, Norway), and centrifuged at 900 rpm for 20 min. The leucocyte fraction collected was suspended in RPMI 1640 containing 10% fetal bovine serum, and transferred to 100-nm culture dishes, and incubated at 37°C in a CO_2 incubator (5% CO_2/95% air) for 2 h. Non-adherent cells were then removed by washing the cells five times with phosphate-buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). The isolated cells were sonicated, and centrifuged. Each supernatant was provided to the assay. The cysteine proteinase activities in the supernatants were assayed as described above. Protein concentration in a supernatant was determined by the BCA method (Pierce Chemical. Co., Rockford, IL), with bovine serum albumin as a standard.

2.5. Effect of monensin on Z-Phe-Arg-MCA hydrolytic activities in rat osteoclasts

Rat bone marrow cells prepared as described above were incubated in a 100-mm dish (CORNING, NY) in α-MEM containing 10% FBS, 50 nM of PTH and 1×10^{-8} M of monensin for 24 h at 37°C in a CO_2 incubator. Osteoclasts were then purified and the Z-Phe-Arg-MCA hydrolytic activity in the cells was measured as described above.

2.6. Gel electrophoresis and western blotting analysis

SDS-PAGE was carried out by the method of Laemmli [19] in a 15–25% gradient gel containing 0.1% SDS at room temperature. The SDS-PAGE low-range standards (Bio-Rad, Richmond) used as molecular weight markers were phosphorylase B (106 kDa), BSA (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa) and lysozyme (18.5 kDa). For Western blot analysis, the media were concentrated 40 times, subjected SDS-PAGE, and transferred electrophoretically to an Immobilon transfer membrane (Milipore). Immunoblotting was developed using a 1:2000 dilution of rabbit anti-rat cathepsin L as the first antibody, and bound antibodies were detected by goat anti-IgG Fab'-conjugated alkaline phosphatase. The alkaline phosphatase reaction was performed using Picoblue Immunoscreening Kits (Stratagene, CA).

3. Results and discussion

3.1. Cysteine proteinase activities of osteoclasts in comparison with those of macrophages

The contents of various cysteine proteinases in osteoclasts using Z-Phe-Arg-MCA hydrolytic activities were compared with those of macrophages using their homogenates from the isolated osteoclasts and macrophages. Specific inhibitors for individual cathepsins were used to determine the contents of respective cathepsins, such as CA-074 [12], a specific inhibitor of cathepsin B, E-64 [20], a general inhibitor cysteine proteinases, and pig leucocyte cysteine proteinase inhibitor (PLCPI) [10,11], a specific inhibitor of cathepsin L and cathepsin L-like proteinase. The results are shown in Table 1.

The value of the activity in the presence of 1×10^{-7} M of CA-074 subtracted from the total activity was 0.62 mU/mg, which corresponds to the activity of cathepsin

Table 1
The characteristic profiles of Z-Phe-Arg-MCA hydrolytic activities of rat osteoclasts and macrophages

	Z-Phe-Arg-MCA hydrolytic activities (mU/mg)	
Inhibitor	Osteoclasts	Macrophages
None	1.48	1.53
CA-074[a]	0.86	0.14
E-64[b]	0.56	0.09
E-64[c]	0.11	0.03
CA-074[a] + PLCPI[b]	0.48	0.06

[a] 1×10^{-7}M, [b] 1×10^{-6} M, [c] 1×10^{-5} M.
The values of the activities in the presence of 1×10^{-7} M of CA-074 subtracted from those in the absence of inhibitors correspond to cathepsin B activities. The values of the activities in the presence of 1×10^{-6} M of E-64 subtracted from those in the presence of 1×10^{-7} M of CA-074 correspond to cathepsin L and/or L-like proteinase activities. One unit corresponds to the production of 1 μmol methylcoumarylamine per min.

B, since CA-074 of this concentration inhibits only cathepsin B among all cysteine proteinases. The value of the activity in the presence of 1×10^{-6} M of E-64 subtracted from that in the presence of 1×10^{-7} M of CA-074 was 0.30 mU/mg, which was totally inhibited by 1×10^{-6} M of PLCPI, a specific inhibitor of cathepsin L and L-like proteinase. It has been established that 1×10^{-6} M of E-64 almost completely inhibits both activities of cathepsins B and L, but not cathepsin J. These facts indicate that this part in Z-Phe-Arg-MCA hydrolytic activities corresponds to the activity of cathepsin L and/or L-like proteinase. The remaining activity in the presence of 1×10^{-6} M of E-64 was 0.56 mU/mg, which was inhibited by a higher concentration of E-64, indicating that this part of activity may be due to the activity of cathepsin J [21], which has been reported to be inhibited by more than 1×10^{-6} M of E-64, and/or the unknown cysteine proteinases. It is established that macrophages and osteoclasts are differentiated from the same origin, granulo-monocyte colony forming units (GM-CFU). In the case of macrophages, the value of total Z-Phe-Arg-MCA hydrolytic activity minus the remaining activity with CA-074 was 1.39 mU/mg, which corresponds to cathepsin B. On the other hand, the activities of cathepsin L and/or L-like proteinase were minimal. The contents of cathepsin L and/or L-like proteinase in osteoclasts are far more abundant than those in macrophages, although both osteoclasts and macrophages are differentiated from the same origin. These results support the important role of cathepsin L and/or L-like proteinase in bone resorption.

Table 2
Correlation between Z-Phe-Arg-MCA hydrolytic activities in culture medium of rat bone cells and bone resorption

Inhibitor	Z-Phe-Arg-MCA hydrolytic activities (mU/ml)		
	PTH minus	PTH plus	PTH and calcitonin plus
None[a]	6.85 ± 1.33**	14.00 ± 2.16	9.15 ± 1.01**
None – CA-074[b]	4.29 ± 0.94**	9.68 ± 1.69	6.12 ± 1.03**
CA-074 – E-64[c]	1.43 ± 0.16**	2.50 ± 0.44	1.55 ± 0.17**
	Bone resorption (number of pits/bone)		
	PTH minus	PTH plus	PTH and calcitonin plus
	211.8 ± 73.5**	668.4 ± 94.3	133.7 ± 41.1**

[a] Corresponding to total cysteine proteinase activities. [b]Corresponding to cathepsin B activity. [c]Corresponding to cathepsin L and/or L-like proteinase activities. Concentrations of CA-074 and E-64 were 1×10^{-7} and 1×10^{-6} M, respectively. Each value of Z-Phe-Arg-MCA hydrolytic activity indicates the mean ± S.D. of 5 observations. Each value of bone resorption indicates the mean ± S.D. of 20 observations. **$P < 0.01$; significant difference from PTH-plus-group (Student's t-test). Concentrations of PTH and calcitonin were 1×10^{-7} and 1×10^{-7} M, respectively.

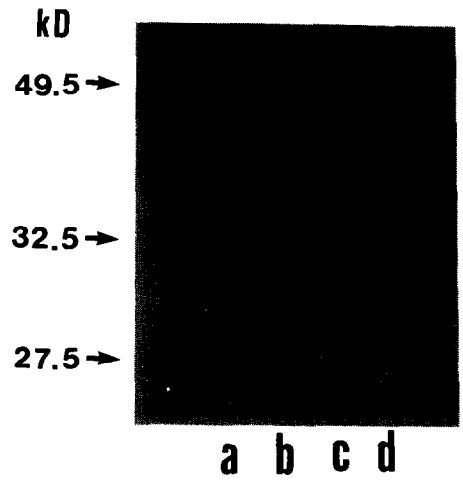

Fig. 1. Western blot analysis by anti-cathepsin L antibody on bone culture medium including PTH and calcitonin. (a) rat liver cathepsin L (heavy chain), (b) PTH-minus-group, (c) PTH-plus-group, (d) PTH and calcitonin-plus-group. Concentrations of PTH and calcitonin were 1×10^{-7} and 1×10^{-7} M, respectively.

3.2. *Regulation of cysteine proteinase secretion by PTH and calcitonin*

In the processes of bone resorption, the release of cathepsin L and/or L-like proteinase from osteoclasts seem to be essential processes. Vaes et al. have shown that several inhibitors of cysteine proteinases, such as leupeptin, antipain, tosyl-lysyl chloromethane (Tos-Lys-CH_2Cl), benzyloxycarbonyl-phenylalanyl-alanyl-diazomethane (Z-Phe-Ala-CHN_2) and E-64, markedly inhibited PTH-induced bone resorption in cultured mouse bone cells [4–6]. Moreover, we reported that the bone pit formation induced by PTH was strongly suppressed by PLCPI, a specific inhibitor of cathepsin L and/or L-like proteinase, and chymostatin, a selective inhibitor of cathepsin L, while no inhibition was observed by CA-074, a specific inhibitor of cathepsin B [13]. However, so far, there has been no apparent evidence that cathepsin L and/or L-like proteinase are secreted from the cells induced by PTH. Therefore, we subsequently examined the parallel movements of increases in the activities of cathepsins in culture medium and pit formation induced by PTH. The effects of calcitonin on both cases were also investigated. In the experiments, fetal bovine serum was not added to the medium, because FBS contains some inhibitors of cysteine proteinases. As shown in Table 2, the Z-Phe-Arg-MCA hydrolytic activity in the medium of the PTH-plus-group significantly increased 30 h after treatment with PTH, in contrast with that of the PTH-minus-group. Furthermore, both the values of the activity with CA-074 subtracted from total activity, correspond to cathepsin B activity, and the value of the activity in the presence of E-64 subtracted from that with CA-074, corresponds to cathepsin L and/or L-like proteinase activities, and are significantly elevated after the addition of PTH. The numbers of pit formation were

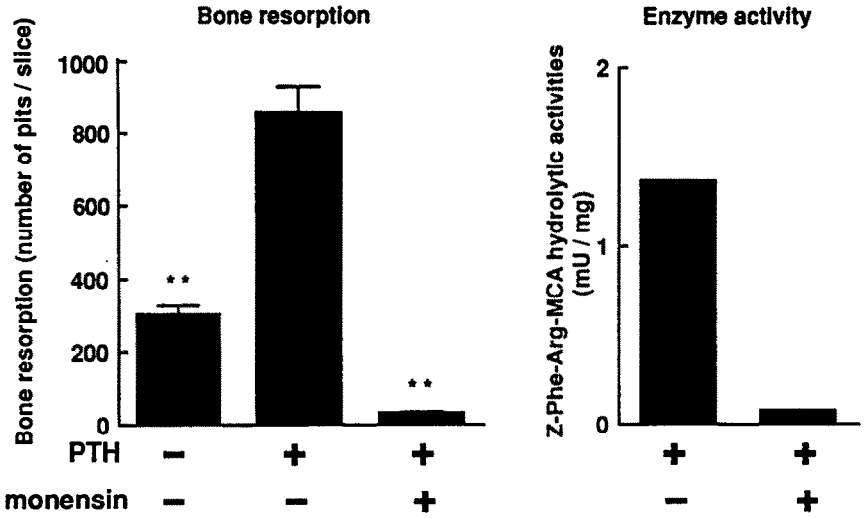

Fig. 2. Influence of monensin on the bone resorption inducted by PTH and the Z-Phe-Arg-MCA hydrolytic activities in rat osteoclasts. Each value of bone resorption indicates the mean ± S.D. of 5 observations. $^{**}P < 0.01$; significant difference from monensin-minus-group (Student's t-test). Concentrations of PTH and monensin were 5×10^{-8} and 1×10^{-8} M, respectively.

stimulated by PTH approximately 3-fold more than that of the PTH-minus-group. On the other hand, 1×10^{-7} M of calcitonin significantly suppressed the increases in both the cysteine proteinase activities secreted into the media and the pit formation stimulated by PTH. These results agree with the fact that calcitonin inhibits the release of both calcium, as a marker of bone resorption, and β-glucuronidase, as a marker of the exocytosis of lysosomal enzymes induced by PTH, from calvaria of mice into the cultured medium [14]. Calcitonin completely inhibited not only PTH-induced pit formation but also spontaneous bone resorption, while the increases in cysteine proteinase activities induced by PTH were unable to be inhibited by calcitonin to the level of the PTH-minus-group. This indicates that calcitonin can inhibit several functions of osteoclasts on the secretion of cysteine proteinases. These results suggest that PTH might induce secretion of lysosomal enzymes including cathepsin L from osteoclasts to degrade the osseous organic matrix.

3.3. *The identification of procathepsin L secreted into the culture medium*

Identification of the forms of cathepsin L and/or L-like proteinase in culture medium using Western blot analysis with rat anti-cathepsin L antibodies was carried out. As shown in Fig. 1, a 39 kDa protein was detected as a procathepsin L. The content of this proteinase secreted into the medium was significantly increased by addition of PTH, and the increase in secretion of procathepsin L induced by PTH was markedly suppressed by calcitonin. The bone resorption was equivalent to the secreted amounts of procathepsin L. A large number of calcitonin receptors were detected in osteoclasts, but not in osteoblasts [22]. Therefore it is possible to consider that procathepsin L might be secreted from osteoclasts, but not from osteoblasts. Procathepsin L secreted from osteoclasts is thought to be converted to the mature form by autolysis in the extracellular acidified lacuna, because when the 39 kDa proform partially purified from the bone of rats receiving a low calcium diet was incubated in acidic buffer, this enzyme was efficiently converted to mature cathepsin L (data is not shown).

3.4. *Effect of monensin on pit formation and cysteine proteinase activity in rat osteoclasts*

Furthermore, the effects of monensin [15,16] on the bone resorption induced by PTH and on the Z-Phe-Arg-MCA hydrolytic activity in isolated osteoclasts were investigated. As shown in Fig. 2, 1×10^{-8} M of monensin completely inhibited not only the PTH-induced pit formation but also the Z-Phe-Arg-MCA hydrolytic activities in osteoclasts. Since monensin itself does not inhibit cysteine proteinase activity, but inhibits the targetting of cystein proteinases into lysosomes to prevent the excretion of the cathepsins, the decreases in cathepsin L activities may result in the inhibition of translocation of the cysteine proteinases in the cells. These results confirm that the lysosomal cysteine proteinases in osteoclasts and their secretion from the cells may play an important role in the process of bone resorption.

Acknowledgements: We thank Dr. Kominami for providing us with rabbit anti-rat cathepsin L.

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