Secretion and processing mechanisms of procathepsin L in bone resorption

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Abstract Secretion of procathepsin L into the culture medium from a bone cell mixture was markedly enhanced by addition of parathyroid hormone (PTH), 1α,25-$(OH)_2D_3$ or tumor necrosis factor α (TNFα). These stimulators of secretion of procathepsin L enhanced bone pit formation, which was inhibited by E-64, but not by CA-074, a specific inhibitor of cathepsin B. Procathepsin L may thus participate in the process of bone collagenolysis during bone resorption. Procathepsin L partially purified from rat long bones under cold conditions was rapidly converted to the mature form under acidic conditions at room temperature. This conversion was inhibited by E-64, suggesting that the procathepsin L secreted into lacunae is catalytically converted to the mature enzyme by cysteine proteinase(s).

Key words: Procathepsin L; Cysteine proteinase; Bone resorption; PTH; 1α, 25-$(OH)_2D_3$; TNFα

1. Introduction

Osteoclasts are multinucleated cells mainly responsible for the degradation of both mineral and organic components during the process of bone resorption [1–3]. Activated osteaclasts excrete protons and lysosomal proteinases into the extracellular microenvironment called Howship's lacunae under their ruffled borders. These protons are thought to be involved in not only the degradation of bone mineral, chiefly solid calcium hydroxyapatite, but also in activation of cysteine proteinases by acidification of the lacunae. The lysosomal proteinases secreted into the lacunae, possibly cysteine proteinases, have been thought to play an important role in osseous collagenolysis [1,3–8]

We previously reported [9] that the bone pit formation stimulated by PTH was significantly suppressed by specific inhibitors of cathepsin L and L-type proteinases, but not by those of cathepsins B and D. Moreover, we also demonstrated [10] that the increase in pit formation stimulated by PTH paralleled the increase of a 39 kDa precursor form of cathepsin L secreted into media, and that both increases induced by PTH were markedly inhibited by addition of calcitonin. These findings indicate that the secretion of procathepsin L from osteoclasts is an important process in PTH-induced bone resorption.

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Abbreviations: Z, benzyloxycarbonyl; MCA, methylcoumaryl-amide; E-64-a, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl-L-leucine-4-aminobutylamide; CA-074, *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline. *Enzyme:* cathepsin L, EC 3.4.22.15.

In the present study, designed to clarify the mechanism and regulation of both the secretion of procathepsin L and its processing in lacunae, we examined the secretion of procathepsin L induced by 1α,25-$(OH)_2D_3$, TNFα or by PTH, and also the effects of the cysteine proteinase inhibitors, E-64 and CA-074, on the pit formation induced by these effectors. Furthermore, to clarify the participation of cysteine proteinase(s) in processing from the precursor to the active form, the inhibitory effect of E-64 on the processing of procathepsin L partially purified from rat long bones was also examined.

2. Experimental

2.1. Materials

E-64-a and CA-074 were kindly supplied by Taisho Pharmaceutical Co. (Saitama, Japan). Rabbit anti-rat cathepsin L was donated by Dr. E. Kominami (Department of Biochemistry, Juntendo University, School of Medicine, Japan). Fetal bovine serum (FBS) was from Gibco (Grand Island, NY), 1α,25-$(OH)_2D_3$ and TNFα was purchased from Funakoshi Co. (Tokyo, Japan), and human PTH (1–34) from Peninsula Laboratories Inc. (Belmond, CA). All other chemicals were of analytical grade. Sephadex G-75 and S-Sepharose were purchased from Pharmacia (Uppsala, Sweden). YM-10 membrane was purchased from Amicon (Japan). Male Sprague–Dawley (SD) rats (1–2 days and 6–7 weeks old) were purchased from Charles River, Japan. Low calcium diet (ca. 0.02–0.03%), a slight modified Diet-11, was purchased from Nippon Clea (Osaka, Japan).

2.2. Assay of bone resorption

Bone resorption was carried out according to the method of McSheehy and Chambers [11] with modifications as described previously [9]. For Western blotting analysis, the media were collected at 72 h after addition of bone resorption factor, concentrated 50 times and subjected to SDS-PAGE.

2.3. Electrophoresis and Western blotting analysis

SDS-PAGE was performed by the method of Laemmli [12] in a 15–25% gradient gel containing 0.1% SDS and 5% 2-mercaptoethanol at room temperature, and transferred electrophoretically to an Immobilon transfer membrane (Millipore). The SDS-PAGE low-range standards (Bio-Rad, Richmond) used as molecular weight markers were BSA (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa) and lysozyme (18.5 kDa). Immunoblotting was developed using rabbit anti-rat cathepsin L as the first antibody, and bound antibodies were detected by goat anti-rabbit IgG-conjugated alkaline phosphatase. The alkaline phosphatase reaction was performed using Picoblue Immunoscreening Kits (Stratagene, CA).

2.4. Enzyme preparation

The long bones, excised from male SD rats receiving a low calcium diet for one week, were homogenized and sonicated. The homogenate was centrifuged for 10 min at 12,000 rpm, and the supernatant then treated with $(NH_4)_2SO_4$. The material precipitated with 20–70% saturation was resuspended in 50 mM sodium acetate buffer, pH 5.5. Insoluble materials were removed by centrifugation. The suspension was applied to a column of Sephadex G-75 equilibrated with the same

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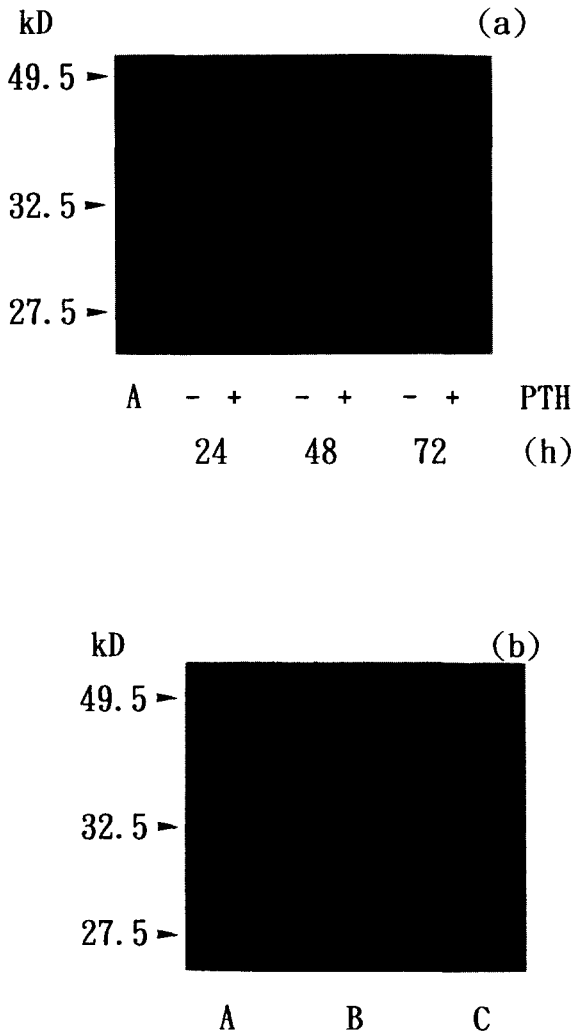

Fig. 1. (a) Time-dependent secretion of procathepsin L by PTH from bone cell mixture. (Lane A) Rat liver cathepsin L (b) Increase in secretion of procathepsin L by 1α,25-$(OH)_2D_3$ and TNFα. Lane A = Untreated-group; lane B = 1α,25-$(OH)_2D_3$-plus-group; lane C = TNFα-plus-group. The culture media were concentrated 50 times, and were subjected to SDS-PAGE in the presence of 5% 2-mercaptoethanol. Western blotting analysis using rabbit anti-rat cathepsin L IgG was carried out as described in section 2.

buffer. The enzyme fractions having the activity of cathepsin L were collected and concentrated by ultrafiltration on an Amicon YM-10 membrane (Amicon, Japan). The Sephadex G-75 fraction was applied to a column of S-Sepharose. The column was washed with two bed volumes of the same buffers, and then developed with a linear gradient of 0.1–0.7 M NaCl (total volume; 360 ml). The fractions having cathepsin L activity were combined and concentrated.

2.5. Effect of E-64 on the processing of procathepsin L

The enzyme preparation was carried out as described above in the presence of 10^{-5} M E-64. The S-Sepharose fraction concentrated 100 times in the absence or presence of E-64 was incubated for 30 min at 37°C in 50 mM sodium acetate buffer, pH 4.5, subjected to SDS-PAGE, and transferred to a transfer membrane. Western blotting analysis using rabbit anti-rat cathepsin L was performed as described above.

2.6. Enzyme assay

Cathepsin L activities were measured with Z-Phe-Arg-MCA as substrate at pH 5.5 [13]. The enzyme fraction was preincubated with 10^{-7} M of CA-074 for 5 min at 37°C in 0.1 M sodium acetate buffer, pH 5.5, containing 8 mM cysteine and 1 mM EDTA. The mixture was then incubated with 2×10^{-5} M substrate for 10 min at 37°C. The fluorescence of 7-amino-4-methylcoumaryl-amine liberated from the substrate was monitored by a fluorescence spectrometer (Hitachi F-2000). The Z-Phe-Arg-MCA hydrolizing activity in the presence of E-64 subtracted from that in the presence of CA-074 corresponds to the activity of cathepsin L and L-type proteinase.

3. Results and discussion

3.1. Enhancement of procathepsin L secretion by bone resorption factors

We previously reported [10] that a 39 kDa cysteine proteinase which seems to be a precursor form of mature cathepsin L (30 kDa), was secreted into culture media of bone cell mixture at 30 h after addition of PTH, during the process of pit formation. The amount of procathepsin L secreted was increased by addition of PTH, and this increase was markedly suppressed by calcitonin. Fig. 1a shows the time-dependency of the secretion of 39 kDa procathepsin L into the culture medium and the enhancement of its secretion by PTH. The effects of 1α,25-$(OH)_2D_3$ and TNFα on the secretion of procathepsin L were also examined by Western blotting. The secretion of the proteinase into the culture medium was enhanced by addition of these bone resorption stimulators (Fig. 1b). These findings indicate that the secretory cysteine proteinase is procathepsin L, which is secreted from osteoclasts indirectly activated by PTH, 1α,25-$(OH)_2D_3$ and TNFα via osteoblastic functions.

We also examined the effects of E-64 and CA-074 on pit formation induced by 1α,25-$(OH)_2D_3$, TNFα and PTH. The areas of resorptive pits were markedly enhanced by addition of these enhancers of procathepsin L excretion. E-64 inhibited the pit formation enhanced by these factors in a dose-dependent manner at concentrations ranging from 10^{-7} to 10^{-6} M. On the

Table 1
Effects of E-64-a and CA-074 on the pit formation induced by 1α,25-$(OH)_2D_3$, TNFα or PTH

Stimulator	Inhibitor	Concentration (M)	Relative pit formation (%)
1α,25-$(OH)_2D_3$	Blank†	–	14.3 ± 1.7**
	Control	–	100.0 ± 8.3
	+E-64-a	10^{-7}	71.8 ± 7.2*
		10^{-6}	38.5 ± 3.5**
	+CA-074	10^{-7}	106.5 ± 12.1
		10^{-6}	121.3 ± 7.7
TNFα	Blank†	–	21.4 ± 2.5**
	Control	–	100.0 ± 10.4
	+E-64-a	10^{-7}	71.0 ± 12.2
		10^{-6}	19.1 ± 5.2**
	+CA-074	10^{-7}	105.9 ± 9.0
		10^{-6}	88.3 ± 6.8
PTH	Blank†	–	10.8 ± 2.7**
	Control	–	100.0 ± 4.1
	+E-64-a	10^{-7}	61.6 ± 9.1**
		10^{-6}	48.6 ± 4.9**
	+CA-074	10^{-7}	94.2 ± 26.9
		10^{-6}	73.1 ± 29.7

†Spontaneous bone resorption. Concentrations of 1α,25-$(OH)_2D_3$, TNFα and PTH were 10^{-8} M. Each value indicates the mean ± S.E.M. of 5–6 observations.
**$P < 0.01$, *$P < 0.05$; significant difference from control group (Student's t-test).

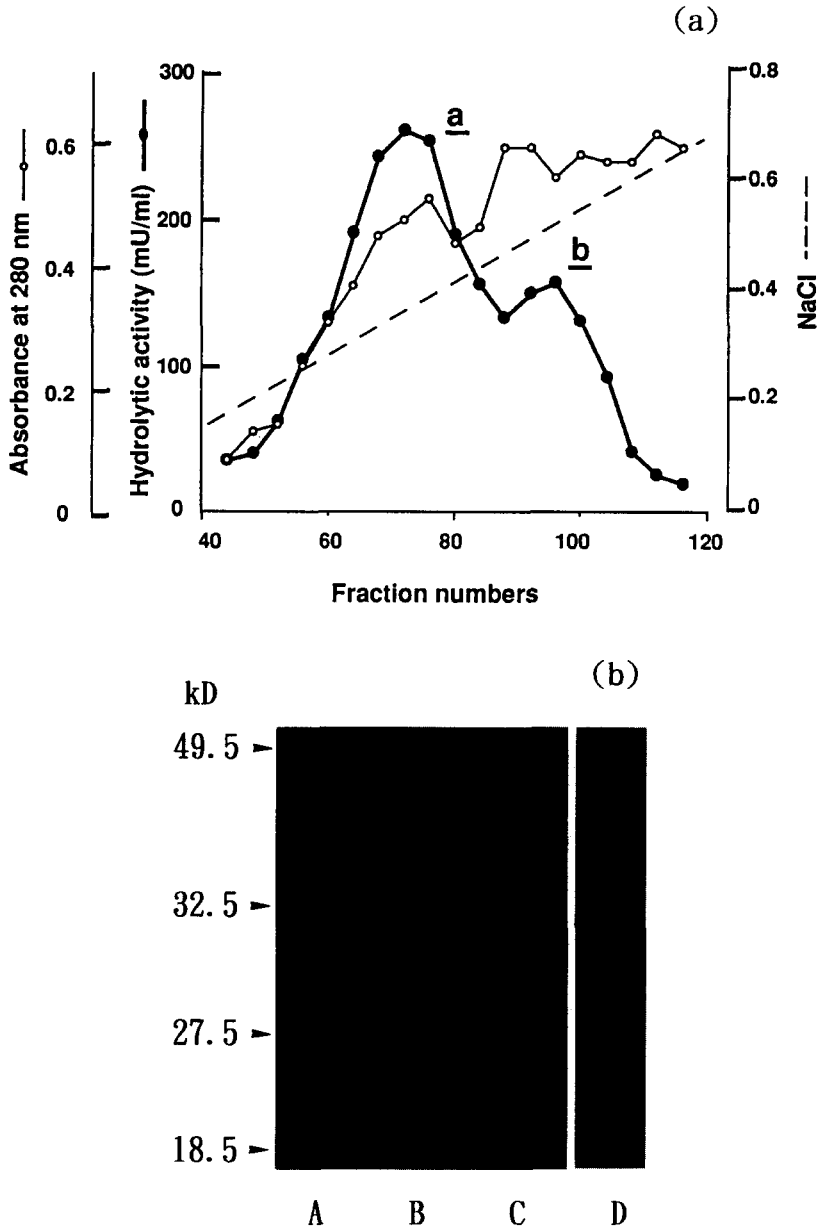

Fig. 2. (a) Chromatographic profile on a S-Sepharose column. A 45.0 mg sample of the Sephadex G-75 fraction was applied to a column (1.8 × 18 cm) of S-Sepharose equilibrated with 50 mM sodium acetate buffer, pH 5.5 The sample was eluted with a linear gradient of 0.1–0.7 M NaCl. (●) Cathepsin L activity (Z-Phe-Arg-MCA hydrolytic activity): (○) absorbance at 280 nm; (---) concentration of NaCl. (b) Western blotting analysis using rabbit anti-rat cathepsin L IgG on S-Sepharose fraction having cathepsin L activity. (Lane A) rat liver cathepsin L. (Lane B) S-Sepharose fraction 'a' (before concentration). (Lane C) S-Sepharose (after concentration at pH 5.5). (Lane D) S-Sepharose fraction 'b'.

other hand, no inhibition was observed with CA-074, a specific inhibitor of cathepsin B, in all of these cases. We previously reported that the bone resorption induced by PTH was markedly inhibited by pig leucocyte cysteine proteinase inhibitor (PLCPI), a specific inhibitor of cathepsin L, and by chymostatin, a selective inhibitor of cathepsin L, but not by CA-074. These findings indicate that cathepsin L and/or L-type cysteine proteinase, but not cathepsin B, is the main proteinase responsible for the PTH, 1α,25-$(OH)_2D_3$ and TNFα-induced bone collagenolysis. The present findings support the view that the bone collagenolysis induced by various bone resorption stimulators, such as 1α,25-$(OH)_2D_3$, TNFα and PTH, is mainly caused by stimulation of procathepsin L secretion.

3.2. Participation of cysteine proteinase(s) in post-secretional processing of bone procathepsin L

Cathepsin L is synthesized as a preproenzyme, which is cotranslationally processed to a proenzyme and then located to the Golgi apparatus. Procathepsin L is targeted to the lysosome mediated by mannose-6-phosphate as the sorting signal [14]. During the process of targetting of procathepsin L to the lysosome in the case of hepatocytes and fibroblasts, the pro-

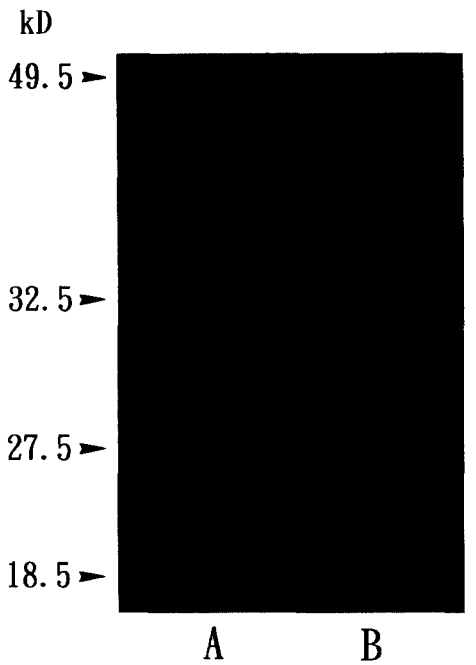

Fig. 3. Inhibitory effect of E-64 on the processing of procathepsin L. Procathepsin L was processed in 0.1 M sodium acetate buffer, pH 4.5, for 30 min in the presence (lane A) or absence (lane B) of 10^{-5} M E-64. Western blotting analysis was performed as described in section 2.

cathepsin L is converted to the mature enzyme by processing proteinase(s) located in the lysosomal membrane. On the other hand, some cells have been recently shown to secrete procathepsin L under regulation by physiological stimulators. For instance, the secretory protein appeared in the endometrium of estrogen-primed cats after progesterone administration, and cyclic protein-2 secreted by mature rat Sertoli cells has been reported to be a precursor form of cathepsin L [15,16]. The secretory proteins are generally transferred to secretion granules from the ER via the trans-Golgi apparatus. Procathepsin L, as is the case for secretory proteins in general, may be secreted via secretion granules.

To clarify the processing mechanism of bone procathepsin L after secretion, we partially purified the proteinase under cold conditions from long bones of rats receiving low calcium diets for one week to enhance the induction of the proteinase. We also examined the level of the precursor form of cathepsin L. Fig. 2a shows the chromatographic profile on a S-Sepharose column. The Z-Phe-Arg-MCA hydrolytic activity of the S-Sepharose fraction was resolved into two peaks, 'a' and 'b' at about 0.3 and 0.45 M NaCl, respectively. Fractions 68 to 72 (total 15 ml) were combined, and immediately subjected to Western blotting analysis using rabbit anti-rat cathepsin L. As shown in lane B, a 39 kDa protein as a precursor form of cathepsin L was detected. This fraction was then concentrated in sodium acetate buffer, pH 5.5, and subjected to Western blotting analysis again. As shown in lane C, the mature enzyme, but not the proform, was now detected. The K_m of this proteinase for Z-Phe-Arg-MCA, like rat liver cathepsin L, was 4.5 μM. These findings indicate that the rat bone procathepsin L was efficiently converted to mature cathepsin L in the acidic buffer, mediated by cysteine proteinase(s). The cathepsin L activity of fraction 'b' was due to a mature cathepsin L, because 27 kDa mature cathepsin L (heavy chain) was detected in this fraction by Western blotting analysis as shown in lane D.

Smith and Gottesman reported [17] that purified recombinant human cathepsin L expressed in *E. coli* was autocatalytically converted to the mature enzyme within 5 min below pH 5.0. Mason and Massey showed [18] that procathepsin L was auto-activated by contact with negatively charged materials such as dextran sulfate in acidic condition at pH 5.5. Ishidoh and Kominami also reported [19] that procathepsin L purified from v-*Ha-ras* transformed NIH3T3 cell conditioned medium was autocatalytically converted to the mature enzyme by incubation for 30 min at 37°C in sodium acetate buffer, pH 5.5, containing dextran sulfate and dithiothreitol. These results support the conclusion that rat bone procathepsin L is autoprocessed under acidic conditions. To confirm whether cysteine proteinases participate in the processing of bone procathepsin L, procathepsin L was partially purified in the presence or absence of 10^{-5} M E-64, incubated in acidic conditions at pH 4.5, and analysed by Western blotting. As shown in Fig. 3, E-64 partially inhibited the processing of prcathepsin L 37, 33 and 27 kDa (heavy chain) multistep processed intermediates being detected (lane A). These results suggest that procathepsin L secreted from osteoclasts is converted catalytically, perhaps autocatalytically, to a mature form in the acidified lacunae.

3.3. Conclusion

Using Western blotting analysis, the level of a 39 kDa precursor form of cathepsin L secreted from bone cells into the culture media has been found to be markedly enhanced by addition of bone resorption stimulators, such as PTH, $1\alpha,25\text{-}(OH)_2D_3$ or TNFα. The pit formation stimulated by these stimulators for the secretion of procathepsin L was inhibited by E-64, while no inhibition was observed by CA-074, a specific inhibitor of cathepsin B. Cathepsin L secreted as a proform from the activated osteoclasts induced by various enhancers may be the main proteinase responsible for bone collagen degradation. The procathepsin L partially purified from rat long bones was rapidly converted to a mature enzyme under acidic conditions and this conversion was inhibited by E-64. The protons released by osteoclasts participate in the hydrolysis of solid calcium hydroxyapatite, and also acidify lacunae to the optimum pH for cathepsin L. The procathepsin L secreted from osteoclasts may be autocatalytically converted to a mature form to degrade bone collagen in the acidified lacunae.

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