Sphingomyelinase and ceramide inhibit formation of F-actin ring in and bone resorption by rabbit mature osteoclasts

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Abstract **Recent studies have demonstrated that ceramide plays an important role as a second messenger in many kinds of cells. However, it is not known whether apoptosis of and bone resorption by mature osteoclasts are mediated via sphingomyelinase (SMase) and ceramide. Thus, we examined the possible involvement of SMase and ceramide in the induction of apoptosis in and bone resorption by rabbit mature osteoclasts. SMase and C2-ceramide inhibited strongly F-actin ring formation of and bone resorption by the osteoclasts. However, the osteoclast apoptosis was not induced by C2-ceramide. The ceramide inhibition of the bone resorption was suppressed by DL-*threo*-dihydrosphingosine, an inhibitor of sphingosine kinase. In addition, we observed that sphingosine-1-phosphate is able to inhibit bone resorption by the osteoclasts. These results suggest an important role of the sphingomyelin pathway in bone resorption by rabbit mature osteoclasts.**

Key words: Ceramide; F-actin ring; Bone resorption; Osteoclast

1. Introduction

Many recent studies [1–9] have demonstrated that the sphingomyelin pathway is a new transducing pathway mediating the action of several biological and physical stimulators. This signal pathway is triggered by activation of a neutral sphingomyelinase (SMase) that hydrolyzes membrane sphingomyelin to ceramide. Recently, also, it has been shown that ceramide acts as a second messenger via activation of a ceramide-activated protein kinase. On the other hand, interesting studies [10–16] have shown that membrane-permeable ceramide is able to mimic the biological action of several stimulators such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), lipid A, and so on.

Osteoclasts are multinucleate cells that dissolve bone matrix and minerals and play a central role in bone remodeling. The bone-resorbing activity of osteoclasts is closely regulated by systemic and local factors such as retinoic acid, vitamin D_3, prostaglandins, transforming growth factor β (TGF-β), TNF-α and interleukin 1 [17–23]. In addition, the cell activity is also controlled by mechanical stress. Since these observations suggested the possibility that ceramide may be involved in the activity of osteoclasts, it is of interest to examine whether ceramide acts as a potent regulator of bone resorption by osteoclasts. So, in the present study, we investigated the effect of SMase and ceramide on the bone-resorbing activity of rabbit mature osteoclasts. We show here that ceramide inhibits this activity via disruption of the ringed structure of F-actin (actin ring) that is contained in the podosomes of their bone-resorbing cells.

2. Materials and methods

2.1. Reagents

Sphingomyelinase, DL-*threo*-dihydrosphingosine (DL-dihydrosphin.), and sphingosine-1-phosphate (Sph.-1-phos.) were purchased from Sigma Chem. Co. (St. Louis, MO, USA). C2-ceramide was from Molecular Probes, Inc. (Eugene, OR, USA).

2.2. Osteoclast isolation and bone-resorbing activity on dentine slice

Osteoclasts were obtained from minced long bones of 10-day-old Japanese white rabbits (Saitama Experimental Animal Supply Co., Saitama, Japan) according to the modified method as described originally by Hattersley et al. [24]. The cell suspension was separated on a Percoll density gradient at 1.07 *g*/ml. The upper band, containing mature osteoclasts, was recovered and washed three times with α-MEM. This cell population was plated on dentine slices in each well of Falcon 24-well flat multiplates and incubated in 10% fetal calf serum (FCS)-containing α-MEM. After 1 h of incubation, the dentine slices were washed to remove tissues debris and non-adherent cells, and then the slices bearing adherent osteoclastic cells were incubated in 10% FCS-containing α-MEM supplemented with or without test samples. After 16 h of incubation, the pit number was counted as the bone-resorbing activity of mature osteoclasts. The results are expressed as the mean ± S.D. of quadruplicate cultures.

2.3. Agarose gel electrophoresis for DNA fragmentation

Osteoclast DNA fragmentation was assessed as described previously [25]. Briefly, the osteoclasts were treated or not with C2-ceramide and then lysed by incubation in digestion buffer. The DNA was extracted with phenol/chloroform, precipitated with 0.5 M NaCl and ethanol, and electrophoresed on 3% agarose gel containing ethidium bromide. The DNA fragments were then visualized under UV light.

2.4. Podosome formation in purified osteoclasts

Purified rabbit osteoclasts were prepared from cell suspensions of long bones according to the modified method of Tezuka et al. Briefly, the TRAP-positive bone cells (4×10^2 cells) were inoculated into each well of a LabTek chamber (Nunc, Naperville, IL). After 3 h of incubation, the cell monolayer was treated with 0.001% pronase E to remove the stromal cells. The remaining purified osteoclasts were cultured for selected times in 10% FCS-containing α-MEM supplemented or not with test samples. Thereafter, the osteoclasts were fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 10 min, and then washed with PBS. F-actin was stained for 30 min at room temperature with 0.3 mM fluorescence-conjugated phalloidin (Sigma). Distribution of F-actin was detected under a fluorescence microscope (Olympus BX60, Tokyo, Japan).

3. Results and discussion

3.1. SMase and ceramide inhibit F-actin ring formation by rabbit mature osteoclastic cells

Since it is well known that formation of F-actin rings in

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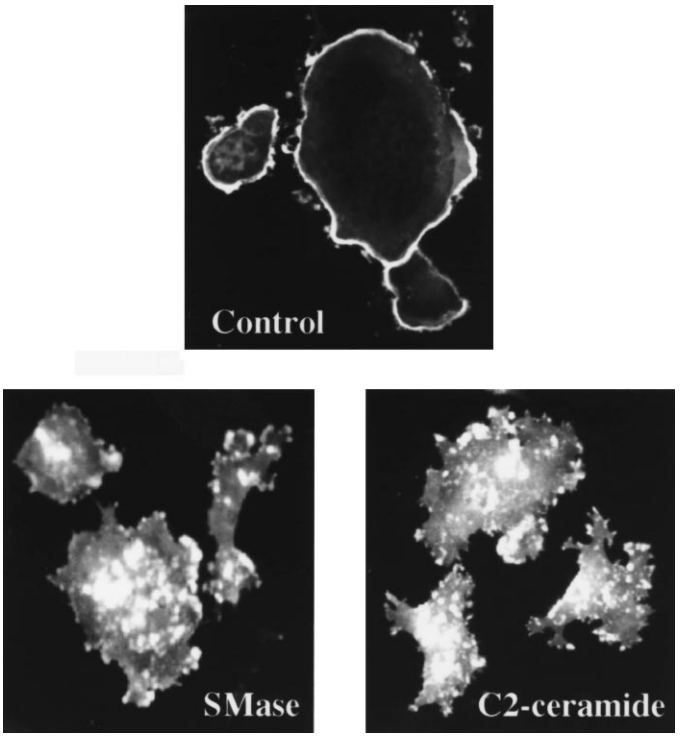

Fig. 1. SMase and C2-ceramide inhibit formation of F-actin ring in rabbit mature osteoclasts. Isolated osteoclasts prepared as described in Section 2, i.e. TRAP-positive bone cells (4×10^2 cells), were inoculated into each well of a LabTek chamber, and then treated or not with SMase (10 μM) or C2-ceramide (5 μM). After 3 h of incubation, the cells were fixed with 3% formaldehyde, and then washed. F-actin was stained for 30 min at room temperature with 0.3 mM fluorescence-conjugated phalloidin. Distribution of F-actin was detected under a fluorescence microscope. Three identical experiments independently performed gave similar results.

mature osteoclasts is essential for their bone-resorbing activity, we first examined the effect of SMase and C2-ceramide on F-actin ring formation by the mature osteoclasts. As shown in Fig. 1, the F-actin ring formation observed in control osteoclasts was clearly prevented by SMase or ceramide treatment. These observations suggested to us the possibility that SMase and ceramide may act as inhibitors of osteoclastic bone resorption, because F-actin ring formation plays an important role in osteoclastic bone resorption.

3.2. SMase and ceramide inhibit bone resorption of rabbit mature osteoclasts

We next examined the possibility that SMase and ceramide may inhibit bone resorption by rabbit mature osteoclasts. Fig. 2A,B shows the results. SMase and C2-ceramide inhibited their bone-resorbing activity in a dose-dependent manner. In addition, we also examined the kinetics of the inhibitory effect of ceramide on bone resorption. Significant inhibition by ceramide was observed at 12 h after the initiation of the treat-

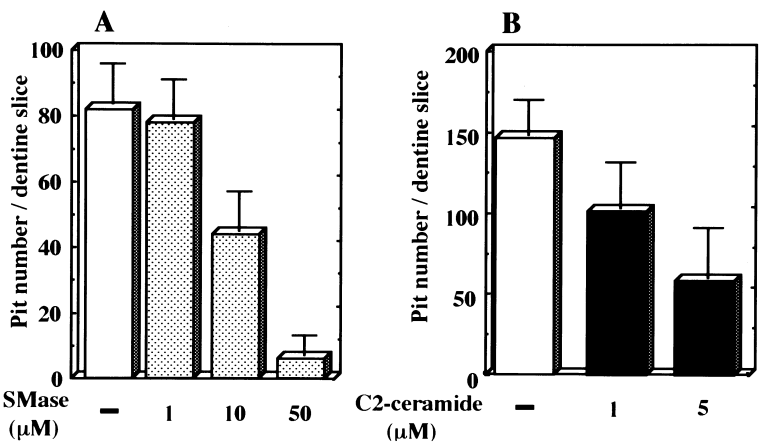

Fig. 2. Inhibitory effect of SMase and C2-ceramide on osteoclast-mediated bone resorption. TRAP-positive cells (1×10^3 cells) in a femoral bone cell suspension were inoculated onto each of several dentine slices, and the slices were rinsed 1 h later to remove the non-adherent stromal cells. Then the cells were treated or not with SMase (A) or C2-ceramide (B) at the indicated doses. After 18 h incubation, the resorption pit number was counted. The results are expressed as the mean ± S.D. of quadruplicate cultures. Three identical experiments independently performed gave similar results.

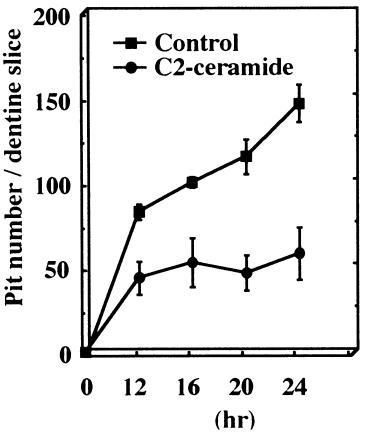

Fig. 3. Kinetics of C2-ceramide inhibition of osteoclast-mediated bone resorption. TRAP-positive cells (1×10^3 cells) in a femoral bone cell suspension were inoculated onto each of several dentine slices, and the slices were rinsed 1 h later to remove the non-adherent stromal cells. Then the cells were treated or not with C2-ceramide at 5 μM. After the selected times, the resorption pit number was counted. The results are expressed as the mean ± S.D. of quadruplicate cultures. Three identical experiments independently performed gave similar results.

ment, and thereafter the inhibitory activity of C2-ceramide was retained at least up to 24 h (Fig. 3). To ensure that the inhibitory effects were not the result of its cytotoxic action toward the cells, we incubated mature osteoclasts on dentine slice for 18 h with or without C2-ceramide (5 μM) and then counted the cell number reacting with TRAP, a marker enzyme of osteoclasts, in the cells. Although the data are not shown, the number of TRAP-positive cells was not affected by the treatment with ceramide. In addition, to know whether the

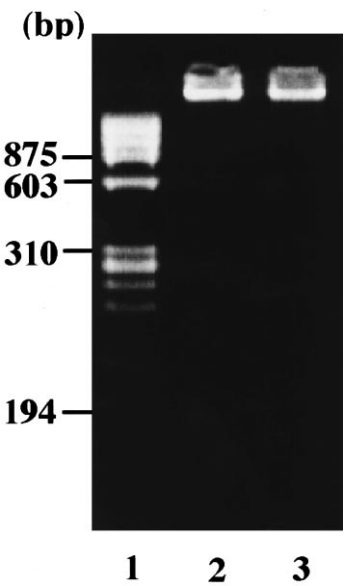

Fig. 4. C2-ceramide does not induce apoptosis of rabbit mature osteoclasts. TRAP-positive cells (1×10^4 cells) in a femoral bone cell suspension were inoculated into each well of a Lab-Tek chamber, and then isolated osteoclasts prepared as described in Section 2 were treated (lane 3) or not (lane 2) with C2-ceramide at 5 μM. The cells were lysed in digestion buffer 12 h later. Then their genomic DNA was extracted and subjected to agarose gel electrophoresis. ϕX174 RF DNA/*Hae*III fragments were used as molecular weight markers (lane 1).

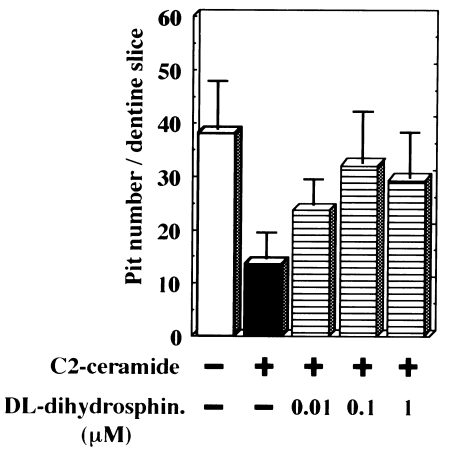

Fig. 5. DL-dihydrosphin. abolishes C2-ceramide inhibition of osteoclast-mediated bone resorption. TRAP-positive cells (1×10^3 cells) in a femoral bone cell suspension were inoculated onto each of several dentine slices and slices were rinsed 1 h later to remove the non-adherent stromal cells. Then the cells were pretreated or not with DL-dihydrosphin. at the indicated doses. C2-ceramide was added or not to the cell cultures 1 h later. After 18 h incubation, the resorption pit number was counted. The results are expressed as the mean ± S.D. of quadruplicate cultures. Two identical experiments independently performed gave similar results.

ceramide inhibition resulted from its stimulation of the osteoclast apoptosis, we explored the effect of C2-ceramide on this process. Fig. 4 shows that C2-ceramide did not induce any DNA fragmentation, a marker of apoptosis, in the osteoclasts. These observations strongly indicate that ceramide acts as second messenger to inhibit mature osteoclastic function.

3.3. *Sph.-1-phos. mediates ceramide inhibition of bone resorption by mature osteoclastic cells*

Since it is known that ceramide induces several biological activities via sph.-1-phos., we next examined the possible involvement of this glycolipid in the ceramide-induced inhibi-

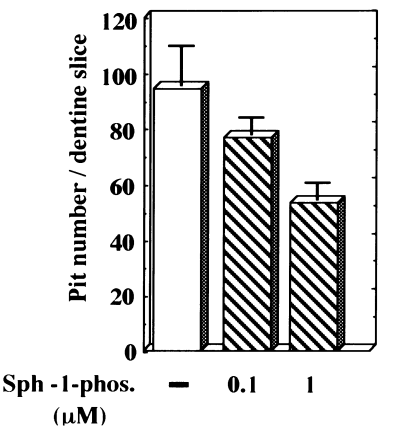

Fig. 6. Sph.-1-phos. inhibits osteoclast-mediated bone resorption. TRAP-positive cells (1×10^3 cells) in a femoral bone cell suspension were inoculated onto each of several dentine slices, and the slices were rinsed 1 h later to remove the non-adherent stromal cells. Then the cells were treated or not with sph.-1-phos. at the indicated doses. After 18 h incubation, the resorption pit number was counted. The results are expressed as the mean ± S.D. of quadruplicate cultures. Two identical experiments independently performed gave similar results.

tion of bone resorption. As shown in Fig. 5, ceramide inhibition of the osteoclastic bone resorption was eliminated in a dose-dependent fashion by DL-dihydrosphin., an inhibitor of sphingosine kinase and hence of sph.-1-phos. production. As these results suggested involvement of sph.-1-phos. in ceramide inhibition of the osteoclastic bone resorption, finally we examined whether sph.-1-phos. itself is able to inhibit bone resorption of the osteoclastic cells. Fig. 6 shows that this is indeed the case.

It is well known that F-actin ring formation is essential for ruffled border formation in and also bone resorption by osteoclasts. Interestingly, it has been shown that the ruffled border was not detected in osteoclasts of *c-src* knockout mice, which manifest a typical osteopetrosis [26]. As Boyce et al. [27] showed previously, we also observed that herbimycin A, a potent inhibitor of *c-src* tyrosine kinase, dramatically inhibited bone resorption by rabbit osteoclasts (unpublished data). These observations suggest that the oncogene tyrosine kinase is intimately involved in the ruffled border of and bone resorption by the osteoclasts. The present study showed that SMase and C2-ceramide inhibited bone resorption by rabbit osteoclasts through suppression of F-actin ring formation. It is of interest to elucidate which signal pathway of the ceramide, a second messenger, mediates the inhibitory action. Since sph.-1-phos. inhibited the bone resorption by the cells, we propose a signal pathway of ceramide inhibitory action of the bone resorption via sph.-1-phos., because the ceramide inhibition was abolished by treatment with DL-dihydrosphin. Since we now do not know the mechanism of SMase- and C2-ceramide-induced inhibition of F-actin ring formation in rabbit mature osteoclasts, further studies are required to elucidate this inhibitory mechanism.

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