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Matrix Metalloproteinases and Lysosomal Cysteine Proteases in Osteoclasts Contribute to Bone Resorption through Distinct Modes of Action

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The effects of inhibitors of matrix metalloproteinases (MMPs) and lysosomal cysteine proteases on osteoclastic pit formation in dentine slices were investigated. A nonspecific cysteine protease inhibitor, E-64, inhibited pit formation on naked slices in a concentration-dependent manner, and at 10 μM E-64 reduced the pit volume by 70%. However, up to 10 μM of the MMP inhibitor, BB-94, did not show any inhibition of pit formation. On the other hand, on slices coated with reconstituted basement membrane, both BB-94 and E-64 at 10 μM showed a marked decrease in pit volume by 73% and 68%, respectively. By a combination of treatment with both BB-94 and E-64, pit formation could be completely suppressed. These results suggest that MMPs are necessary for the migration of precursor and/or immature osteoclasts to bone surface through basement membranes, while cysteine proteases are essential for the osteoclastic degradation of bone collagen.

Bone matrix is composed of hydroxyapatite and fibrous proteins, mainly type I collagen. Degradation of the bone collagen, in addition to demineralization of the inorganic mineral component, is a primary process of bone resorption by osteoclasts [1, 2]. Both *in vitro* and *in vivo* studies suggest that the degradation of bone collagen by osteoclasts is mainly carried out by two types of proteases, the matrix metalloproteinases (MMPs) and lysosomal cysteine proteases [1, 3-5]. Of these proteases, particularly MMP-9 (gelatinase B, EC 3.4.24.35) and cathepsin K (EC 3.4.22.38) have been shown to be abundantly expressed in human and rabbit osteoclasts and the multinucleate giant cells of human osteoclastoma [6, 7]. We recently demonstrated that cathepsin K antisense oligodeoxynucleotide inhibited the osteoclastic bone resorption in a pit formation assay [8]. Votta *et al.* [9] also reported that the cathepsin K inhibitor, Cbz-Leu-Leu-Leu-H, effectively reduced bone resorption both *in vitro* and *in vivo.* Cathepsin K is a characteristic protease, digesting native type I collagen which is resistant to proteolysis by most proteases, including MMP-1 [10, 11]. Taken together, these results strongly suggest that cathepsin K contributes to osteoclastic bone resorption by directly digesting native type I collagen. On the other hand, MMP-9 cleaves the native form of type IV collagen, a specific component of the basement membranes [12], and denatured type I collagen (gelatin), but not the native form of type I collagen which has a triple-helix structure [13]. Expression of MMP-9 by osteoclasts has been found in mouse, rabbit, and human [7, 14-16]. Considering the difference in substrate specificity between these two proteases, MMP-9 is expected to play a distinct role to cathepsin K in the process of osteoclastic bone resorption. The strong proteolytic activity of MMP-9 against type IV collagen raises the possibility that osteoclasts and/or preosteoclasts may require this enzyme for migration to the bone surface through the basement membrane from the blood vessel lumen. However, this possibility has not been investigated to date.

In this study, we developed a modified pit formation assay system using a confocal scanning laser microscope that allows more quantitative analysis than was previously possible [5, 17-21]. We evaluated the effects of inhibitor of MMPs and cysteine proteases on osteoclastic bone resorption using dentine slices which

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were either naked or coated with reconstituted basement membrane. We indicated that MMPs contributed to osteoclast precursors/immature osteoclasts migration from basement membrane matrix to bone surface, while cysteine proteases contributed to osteoclastic bone resorption.

MATERIALS AND METHODS

Materials. Acid hematoxylin, E-64 (trans-epoxy-succinyl-L-leucylamido(4-guanidino)-butane) and BB-94 (batimastat) were purchased from the Sigma Chemical, Co. (St. Louis, MO, USA). Alpha-minimum essential medium (α-MEM) was obtained from Flow Laboratories (McLean, VA, USA) and fetal bovine serum (FBS) was from Gibco (Grand Island, NY, USA).

Preparation of osteoclasts. Long bones were prepared from 10-day-old rabbits. After carefully removing the adherent soft tissue, the bones were minced with scissors for 10 min in α-MEM containing 5% FBS. Bone cells were dissociated from the bone particles by sedimentation under normal gravity for 3 min after mild stirring for 30 sec. The supernatant was a suspension of unfractionated bone cells including osteoclasts. The cells were cultured on dentine slices (150 μm thick and 6 mm diameter) at a concentration of 4×10^5 cells/slice in 96-well tissue culture plates in the presence or absence of various concentrations of E-64 or BB-94 in α-MEM containing 5% FBS. Cells were cultured for 24 h in an atmosphere of 10% CO_2 and 90% air at 37°C.

RNA isolation and RT-PCR. The expression of following genes; MMP-9, cathepsin K, tartrate resistant acid phosphatase (TRAP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed by reverse transcription (RT)-polymerase chain reaction (PCR). Poly(A)$^+$ RNA was prepared from rabbit isolated osteoclasts [22] using QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech Inc., Uppsala, Sweden). The first strand cDNAs were synthesized in a total volume of 20 μl containing 20 units of RNase inhibitor, 2.5 pmol oligo dT primer, 20 nmol of each deoxyribonucleotide triphosphate mixture (dNTP) and 0.1 μg of poly(A)$^+$ RNA. After the denaturation of 3 min at 72°C, 5 units of Avian Myeloblastosis Virus (AMV) reverse transcriptase XL (Takara Shuzo, Kyoto, Japan) was added. The incubation for 1 h at 50°C was followed by an inactivation step for 3 min at 95°C. The PCR was performed in 50 μl containing 10 nmol of dNTP, 50 pmol of each set of gene specific primers, 2.5 units of LA Taq DNA polymerase (Takara Shuzo) and 1 μl from RT products with a GeneAmp 9600 PCR system (Perkin Elmer Cetus, Foster City, CA, USA). After an initial denaturation of for 3 min at 95°C, a total of 30 cycles was performed with a denaturation step of 15 sec at 96°C and annealing-extension step of 5 min at 68°C. The PCR products were analyzed in a 1% agarose gel containing 0.5 μg/ml ethidium bromide. All gene specific primers used in this study were listed in Table 1.

Measurement of resorption pit. After a 24 h cultivation period, the cells were brushed off the dentine slices with a rubber policeman, and the slices stained with acid hematoxylin for 2 min. The total number of pits on the dentine slices were counted manually under a light microscope (Nikon, Tokyo, Japan) equipped with a micrometer on the eyepiece (objective lens, x20).

For the measurement of the pit volume, a confocal scanning laser microscope 1LM21 (Lasertec, Osaka, Japan) was used. The 633 nm He-Ne laser beam was raster scanned at NTSC TV rates. The reflected light was received by a 1000 pixel CCD linear array. The z axis of the stage was stepped in 100 Å increments, thereby obtaining a vertical series of horizontal slices which were combined in memory producing a 3-dimensional composite image. The objective lens was a x 20/0.70 dry objective. The images were transferred to an image processor (Lasertec & Mitani Corp., Osaka, Japan) and smoothed to remove background noise. Pit volume was then calculated by image analysis soft-ware, SALT (Mitani Corp., Osaka, Japan). In these measurements, 20 areas were selected from each dentine slice, each area corresponding to the size of a video TV frame. These areas were selected repeatedly from each dentine slice by an auto microprocessor-controlled stage (MINI-60XY and PC-5K, SIGMA KOKI, Osaka, Japan). Total pit volume on each dentine slice was calculated by

$$\text{Total pit volume } (\mu m^3) = \text{Average pit volume (in 20 areas)} \times \text{Total pit number,}$$

where total pit number on dentine slice was counted manually under a light microscope.

Matrigel coating of dentine slices. The capability of osteoclasts to penetrate the basement membrane was evaluated by a pit assay using dentine slices coated with reconstituted basement membrane, MATRIGEL Basement Membrane Matrix (Becton Dickinson Labware, MA, USA). The surface of dentine slices in 96-well plates were coated with 50 μl of MATRIGEL (1 mg/ml) and allowed to polymerize for 5 h at 37°C before using for pit assay.

Statistical analysis. Data are expressed as mean ± SE. The statistical significance between the control and the experimental group was assessed by Student's *t* test. $P < 0.05$ was considered to be significant.

RESULTS

Expression Analysis by RT-PCR in Osteoclasts

We examined by RT-PCR whether gelatinase B (MMP-9) and cathepsin K were expressed in rabbit osteoclasts we isolated. By the RT-PCR analysis with the sets of specific primers for each gene (listed in Table 1), the bands of 663 bp and 218 bp in length were detected for MMP-9 and cathepsin K, respectively (Fig. 1). PCR products amplified from TRAP mRNA, as a marker gene for osteoclasts, and GAPDH mRNA, as a housekeeping gene, were also detected as the bands of 189 bp and 576 bp in length, respectively (Fig. 1). All these PCR products were subjected to DNA sequencing analysis to confirm that they were amplified from the intended mRNAs (data not shown).

Quantitative Pit Formation Assay

To establish and confirm the pit assay used in this study, we first examined the effect of E-64, a non-specific inhibitor of cysteine proteases, on osteoclastic bone resorption, because several studies have clearly demonstrated that it suppresses osteoclastic bone resorption. The sum of the 20 areas selected corresponded to approximately 20% of the total surface area of a dentine slice. Without E-64, the average number of pits was 37 ± 3.5, and the average volume of each pit was 625.2 ± 48.6 μm^3 (Fig. 2A, n = 4). In this assay system, E-64 markedly decreased osteoclast pit formation in a concentration-dependent manner (Fig. 2A). At 10 μM E-64 reduced the average pit volume by 43%. The IC_{50} value was determined to be 21.5 ± 5.4 nM using the curve fitting program, GraFit Version 3.0 (Erithacus Software Ltd., Staines, U.K.).

TABLE 1

Nucleotide Sequence of Primers Used for RT-PCR

Gene	Nucleotide sequence	Product size (bp)
MMP-9	Forward[a] 5′-TGGCCGGCCACTGTGCGCCCCTCCGAG-3′	663
	Reverse[b] 5′-CACTAGGTTCACCTCGTTCCGGGTACT-3′	
cathepsin K	Forward[c] 5′-AGCTGGGGAGAAAGCTGGGGAAACAAAG-3′	218
	Reverse[d] 5′-AGGCACAAACAAATGGGGAAACCAAACA-3′	
TRAP	Forward[e] 5′-AAGGAGGACTACGTGCTCGTGGCCGGC-3′	189
	Reverse[f] 5′-TCCACTCAGCACGTAGCCCACGCCGTT-3′	
GAPDH	Forward[g] 5′-AGGGCTGCTTTTAACTCTGGCAAAGTGG-3′	576
	Reverse[h] 5′-AGTGGAGGCAGGGATGATGTTCTGGGCG-3′	

[a] Corresponding to the sequence from nucleotide 1416 to 1442 of MMP-9.
[b] Corresponding to the sequence from nucleotide 2052 to 2078 of MMP-9.
[c] Corresponding to the sequence from nucleotide 913 to 940 of cathepsin K.
[d] Corresponding to the sequence from nucleotide 1157 to 1130 of cathepsin K.
[e] Corresponding to the sequence from nucleotide 51 to 77 of TRAP.
[f] Corresponding to the sequence from nucleotide 213 to 239 of TRAP.
[g] Corresponding to the sequence from nucleotide 126 to 153 of GAPDH.
[h] Corresponding to the sequence from nucleotide 674 to 701 of GAPDH.

When 40 areas were randomly selected corresponding to approximately 40% of the total area of a dentine slice, the average control pit volume was 702 ± 40.2 μm^3 (n = 4), and 10 μM E-64 reduced the average pit volume by 41% (IC_{50} = 27.8 ± 13.0 nM) (Fig. 2B). Further, when all pits on each dentine slice were analyzed, the average pit volume of control was 759 ± 79.1 μm^3 (n = 4), and inhibition in average pit volume was 40% (IC_{50}=26.2 ± 5.1 nM) (Fig. 2C). Thus, almost equal results in the maximum inhibition and the IC_{50} values were obtained using three distinct experimental protocols, suggesting that the assay in a selection of only 20 pieces of area on each dentine slice is sufficient to obtain reliable and reproducible data.

Effects of BB-94 and E-64 in Pit Number and Area on Bone Resorption

Having established that our pit measurement system was reliable, we investigated the effects of BB-94, a non-specific inhibitor of MMPs, on osteoclastic bone resorption in comparison to the effect of E-64. Firstly, the changes in pit number and pit area caused by these inhibitors were examined. E-64 reduced both pit number and area formed by osteoclasts in a concentration-dependent manner (Fig. 3A). At 10 μM E-64, inhibited the pit number and area by 53% and 46%, respectively. However, BB-94 did not show any reduction in either pit number or area up to 10 μM (Fig. 3B).

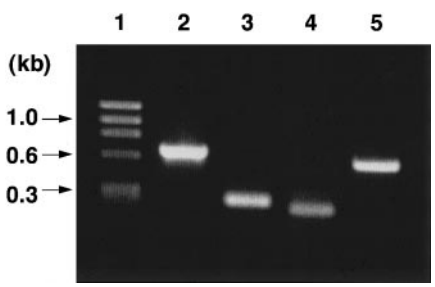

FIG. 1. Expression of MMP-9, cathepsin K, TRAP and GAPDH mRNAs in rabbit osteoclasts. Rabbit osteoclasts were subjected to RNA isolation and RT-PCR with primers specific for MMP-9, cathepsin K, TRAP and GAPDH. PCR products were separated by agarose-gel electrophoresis containing ethidium bromide. Lane 1, molecular weight marker; Lane 2, MMP-9 (663 bp); Lane 3, cathepsin K (218 bp); Lane 4, TRAP (189 bp) and Lane 5, GAPDH (576 bp).

Effects of BB-94 and E-64 in Pit Volume on Bone Resorption

To further investigate the total effects of BB-94 and E-64 on bone resorption, total pit volume on a dentine slice was calculated as described in MATERIALS AND METHODS. E-64 showed an inhibitory effect on pit volume in a concentration-dependent manner and, at 10 μM, inhibited the pit volume by 70% (Fig. 4A). However, BB-94 showed no inhibitory effect on pit volume up to 10 μM (Fig. 4B). These results indicate that MMPs do not contribute to the pit formation by osteoclasts adhered to dentine surface.

Invasion Assay of Osteoclasts

On matrigel-coated dentine slices, BB-94 reduced pit number and volume by 65% and 73% respectively, compared to controls (Fig. 5, $P < 0.05$). Furthermore, E-64 reduced pit number and volume by 61% and 68%, respectively, compared to controls (Fig. 5, $P < 0.05$). These results, taken together with the result that E-64, but not BB-94, inhibited pit formation on naked dentine slices, suggests that MMPs were necessary for osteoclasts (precursors or immature one) to migrate through basement membrane matrix while the activity

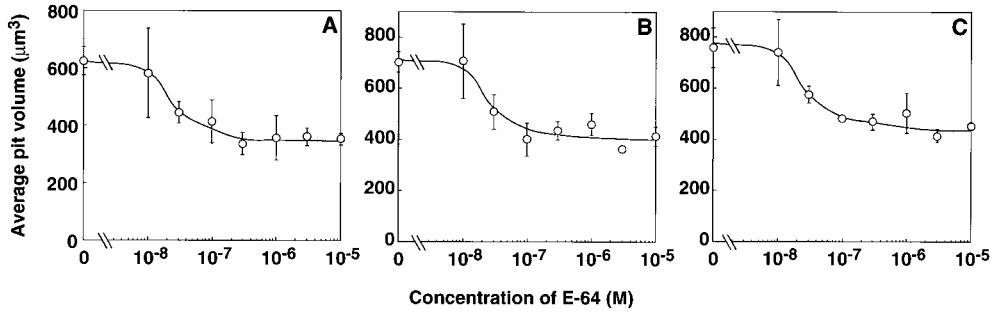

FIG. 2. Establishment of pit formation assay. To find out the suitable conditions of measuring the pit volume, the effects of E-64 on bone resorption were investigated as a reference compound. In the cases of 20 areas (A), 40 areas (B) and all areas (C) picked up from each dentine slice, E-64 showed almost same inhibition manner obtaining close maximum inhibition percentage (%) and IC_{50} values in average pit volume. Data are expressed as mean ± SE of four experiments.

of cysteine proteases was essential for bone degradation by osteoclasts to adhere to the bone surface. By the combination of treatment with both BB-94 (10 μM) and E-64 (10 μM), both pit number and volume were almost completely inhibited (Fig. 5, $P < 0.05$).

DISCUSSION

To date, both MMPs and lysosomal cysteine proteases have been considered to be involved in bone resorption by osteoclasts. In fact, of these proteases, MMP-9 [7] and membrane type 1 (MT1)-MMP [23] as matrix metalloproteinases, and cathepsin K [24] as a cysteine protease have been found to be abundantly expressed in rabbit osteoclasts (and present data). Recently, we have obtained evidence that cathepsin K is directly involved in osteoclastic bone resorption based on the effective inhibition of osteoclastic pit formation by cathepsin K antisense oligodeoxynucleotide [8]. Indeed, in the present study, a non-selective cysteine protease inhibitor, E-64, was shown to inhibit pit formation by rabbit osteoclasts on both naked and matrigel-coated dentine slices, presumably by inhibiting cathepsin K activity (Figs. 3–5).

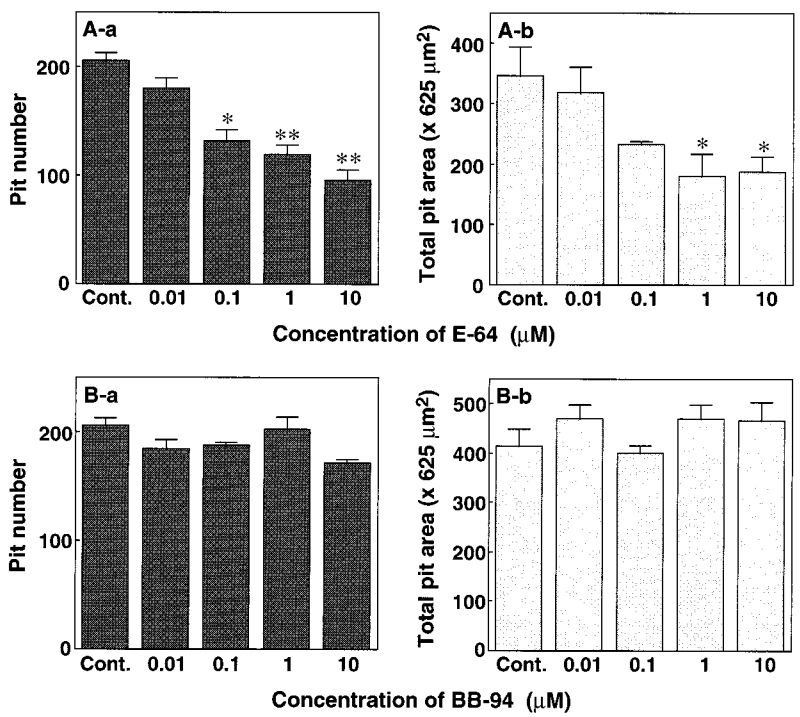

FIG. 3. The effects of E-64 and BB-94 on osteoclastic bone resorption in pit number and area. E-64 showed the concentration-dependent inhibition in both pit number (A-a) and area (A-b), however, BB-94 did not show any inhibitory effect in either pit number (B-a) or area (B-b). Data are expressed as mean ± SE of four experiments. ** $P < 0.01$, * $P < 0.05$.

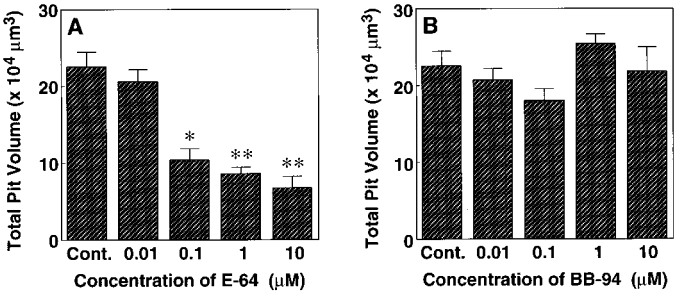

FIG. 4. The effects of E-64 and BB-94 on osteoclastic bone resorption in pit volume. E-64 showed the concentration-dependent inhibition in pit volume (A), while BB-94 did not show any inhibitory effect in pit volume (B). Data are expressed as mean ± SE of four experiments. ** $P < 0.01$, * $P < 0.05$.

Contrary to the clear-cut results for cathepsin K, the contribution of various MMPs to osteoclastic bone resorption has remained controversial. Delaissé *et al.* reported that the bone resorption in neonatal mouse calvariae was reduced by a protease inhibitor, CI-1, selective for collagenase (MMP-1) *in vivo* [4], while this inhibitor did not inhibit the bone resorption by isolated osteoclasts [18]. Based on the result they proposed that collagenase (MMP-1) is secreted from osteoblasts, but not osteoclasts, and is necessary for the removal of the thin layer of osteoid (undemineralized bone) that separates resting osteoclasts from the bone mineral [25]. On the other hand, Hill *et al.* [26] examined the effects of two low-molecular-mass compounds, CT1166 and Ro 31-7467, which are concentration-dependent selective inhibitors of the gelatinases (MMP-2 and MMP-9) and collagenase (MMP-1) respectively, on bone resorption by several lines of *in vitro* studies. They found that these inhibitors significantly inhibited pit formation on dentine slices by isolated osteoclasts stimulated with interleukin-1α (IL-1α), and concluded that MMPs might be released by osteoclasts and participate in bone collagen degradation within the resorption lacunae. However, the conditions under which they performed pit formation assay are far from physiological in that the osteoclasts were treated with a high dose of IL-1α, and thus their results may not reflect events occurring *in vivo.*

Kusano *et al.* [16] have recently reported that the regulation of MMPs such as MMP-2, -3, -9, and -13 by IL-1 and IL-6 is associated with bone resorption in mouse calvaria. Especially, the induction of MMP-9 is closely linked to osteoclast formation. Okada *et al.* [15] detected the expression of MMP-9 at high levels in human osteoclasts in the bone tissues from normal subjects and patients with rheumatoid arthritis or metastatic carcinoma using immunohistochemistry, northern blot analysis, and *in situ* hybridization. MMP-9 was active on acid insoluble type I collagen at 37°C. These findings strongly suggest a key role of MMP-9 in bone resorption.

It is well agreed that osteoclasts are originated from a hematopoietic cell line, and therefore their precursor cells, including small immature osteoclasts, are considered to be recruited from blood. However, it is not well understood how these cells are able to migrate through basement membranes surrounding bone to reach bone surface. The migration mechanisms have been well studied for invasive cancer cells, which produce MMPs, including MMP-9 [12, 13, 27-29] and MT-MMPs [30], to degrade basement membranes of various tissues. Thus, we hypothesize that MMPs might also be involved in migration or invasion of osteoclast precursors/immature osteoclasts through the basement membranes. As expected, in the present study, a potent MMP inhibitor, BB-94, did not inhibit osteoclastic pit formation on naked dentine slices, while it apparently reduced the bone resorption by osteoclasts cultured on matrigel-coated dentine slices. Indeed, this study was not conducted to establish which MMP is involved in the migration/invasion process, since BB-94 is not a selective inhibitor of MMPs. However, taken together with the observations that MMP-9 is most abundantly expressed among all MMPs in osteoclasts [7, 14-16], and that it effectively cleaves a native type IV collagen, a major component of basement

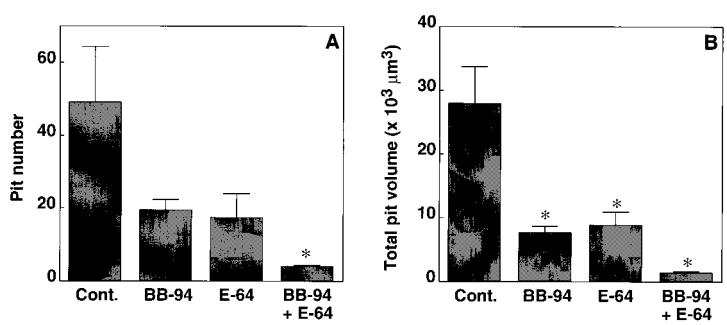

FIG. 5. The effects of E-64 and BB-94 on osteoclastic bone resorption in matrigel-coated dentine slices. On matrigel-coated slices, both 10 μM E-64 (A) and 10 μM BB-94 (B) showed significant reduction in pit volume. (C) The combination treatment of both E-64 and BB-94 caused complete inhibition in pit volume. Data are expressed as mean ± SE of four experiments. * $P < 0.05$.

membranes, it would seem likely that MMP-9 plays a key role in the migration/invasion process of osteoclast precursors/immature osteoclasts.

Sato *et al.* [31] have recently reported the observations similar to ours from their pit formation assays using collagen-coated dentine slices. When they cultured unpurified or purified osteoclasts on uncoated dentine slices, MMP inhibitors did not prevent pit formation. However, when they used dentine slices coated with type I collagen that was extracted in acid from porcine tendons, pit formation was highly inhibited by MMP inhibitors. Therefore, they concluded an important role of MMPs in migration and invasion of osteoclasts, but not in bone resorption. We postulate that binding of osteoclasts to basement membrane components as well as acid soluble type I collagen in the reconstituted form is necessary to induce MMP production and hence cell migration and invasion.

In conclusion, we developed the new pit assay system to measure the pit volumes on each dentine slice, which provided highly reliable and reproducible results to estimate the suppressive effects of factors on osteoclastic bone resorption *in vitro*. By this assay system, we found that MMPs were essential for the migration of osteoclast precursors, and possibly small preosteoclasts, to basement membranes, while cysteine proteases were necessary for osteoclastic bone resorption. Bifunctional inhibitors and/or a combination treatments of each inhibitor against MMPs and cysteine proteases might provide a therapeutic strategy for the treatment of osteoporosis.

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