10

Generating Murine Osteoclasts from Bone Marrow

Naoyuki Takahashi, Nobuyuki Udagawa, Sakae Tanaka, and Tatsuo Suda

1. Introduction

Osteoclasts, the multinucleated giant cells that resorb bone, originate from hemopoietic cells of the monocyte–macrophage lineage *(1,2)*. We have developed a mouse bone marrow culture system, in which osteoclasts are formed in response to several bone-resorbing factors such as 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$], parathyroid hormone (PTH), prostaglandin E_2 (PGE_2) and interleukin-11 (IL-11) *(2,3)*. We also developed a mouse coculture system of primary osteoblasts and hemopoietic cells to examine the regulatory mechanism of osteoclastogenesis *(2,4)*. A series of experiments using the coculture system established the concept that osteoblasts/stromal cells have a key role in regulating osteoclast differentiation *(2)*. Macrophage colony-stimulating factor (M-CSF, also called CSF-1) produced by osteoblasts/stromal cells was shown to be an essential factor for differentiation of osteoclasts from osteoclast progenitors *(2,5)*. Recently, receptor activator of nuclear factor κB ligand (RANKL) was identified as another essential factor for osteoclastogenesis, which is expressed by osteoblasts/stromal cells in response to several bone-resorbing factors (*6,7*; *see* **Note 1**). Osteoclast precursors that possess RANK, a tumor necrosis factort (TNF) receptor family member, recognize RANKL through cell–cell interaction with osteoblasts/stromal cells, and differentiate into osteoclasts in the presence of M-CSF. Recent progress of molecular technology allows us to introduce foreign genes into mature osteoclasts for modulating their functions. Adenoviral vectors are quite useful for introducing foreign genes into osteoclasts *(8)*. We describe here the methods for osteoclast formation in mouse bone marrow cultures and for introduction of foreign genes into mature osteoclasts.

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2. Materials

2.1. Mice and Cell Lines

1. ddY mice (*see* **Note 2**).
2. Mouse bone marrow derived stromal cell lines, ST2 and MC3T3-G2/PA6 (RIKEN Cell Bank, Tsukuba, Japan).
3. Human embryonic kidney cell line 293 (American Type Culture Collection, Manassas, VA).

2.2. Reagents

1. Recombinant human M-CSF (Leukoprol; Kyowa Hakko Kogyo Co.Tokyo, Japan, or R &D systems, Minneapolis, MN) (*see* **Note 3**).
2. Recombinant mouse TNF-α, and human IL-1α (R&D Systems).
3. 1α,25-$(OH)_2D_3$ and PGE_2 (Wako Pure Chemical Industries, Ltd., Osaka, Japan).
4. PTH (Peptide Institute, Inc., Osaka) and IL-11 (R&D Systems).
5. Human osteoprotegerin (OPG) and a soluble form of human RANKL (Pepro Tech EC Ltd., London, UK).
6. Synthetic analogue of eel calcitonin (Elcatonin, Asahi Chemical Industry Co. Tokyo, Japan).
7. ^{125}I-labeled human calcitonin (Amersham Inc., Buckinghamshire, UK).
8. NR–M2 emulsion (Konica Co., Tokyo).
9. Rendol developer (Fuji Photo Film Co., Tokyo).
10. Type I collagen gel solution (cell matrix type IA; Nitta Gelatin Co., Osaka) (*see* **Note 4**).
11. Bacterial collagenase (Wako Pure Chemical Industries, Ltd.).
12. Tissue culture plastics (Corning).
13. α-Modification of minimum essential medium α-MEM), Dulbecco's modified Eagle's medium (DMEM), and Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline [PBS(–)] (Sigma Chemical Co., St. Louis, MO).
14. Fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS or Gibco BRL, Gaithersburg, MY).
15. Sterile instruments, syringes, and needles.
16. Other chemicals and reagents are of analytical grade.

2.3. Culture Media and Buffer Solutions

1. α-MEM containing 10% FBS for cultures of mouse bone marrow cells.
2. DMEM containing 10% FBS for cultures of 293 cells.
3. PBS(-) for washing cells.
4. α-MEM containing 0.2% bacterial collagenase for detachment of cells cultured on collagen gel coated dishes.
5. Trypsin–EDTA solution: PBS(-) containing 0.05% trypsin and 0.5 m*M* EDTA for detachment of cells from culture plates.
6. Pronase–EDTA solution: PBS(-) containing 0.001% pronase and 0.02% EDTA for removal of osteoblasts from cocultures. Pronase is dissolved in PBS(-) containing 0.02% EDTA just before use.

7. 0.1% Triton X-100 in PBS(-) for permeabilization of cells fixed with 3.7% formaldehyde in PBS(-).
8. Tartrate-resistant acid phosphatase (TRAP) staining solution: Five milligrams of naphthol AS–MX phosphate is dissolved in 0.5 mL of *N,N*-dimethyl formamide in a glass container. Thirty milligrams of fast red violet LB salt and 50 mL of 0.1 *M* sodium acetate buffer, pH 5.0, containing 50 m*M* sodium tartrate are added to the mixture. This solution is made up fresh before use. Further details on TRAP staining procedures can be found in Chapter 11 by van 't Hof and other chapters on osteoclast formation, *this volume*.
9. Type I collagen mixture for preparing collagen gelcoated dishes: Type I collagen solution (*see* **Subheading 3.3.**), 5× conc. α-MEM, and 200 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 2.2% $NaHCO_3$ (7:2:1, by vol) are quickly mixed at 4°C just before use.
10. 0.1 *M* cacodylate buffer, pH 7.4, containing 1% formaldehyde and 1% glutaraldehyde for fixation of cells for autoradiography.

3. Methods

3.1. Marrow Culture

The mouse bone marrow culture system was developed for examining effects of bone-resorbing factors on osteoclast formation *(3)*. Discovery of the RANKL–RANK interaction for osteoclastogenesis indicated that the growth of stromal cells is an essential step for osteoclast development in bone marrow cultures *(6,7)*.

1. Tibiae are removed aseptically from 7- to 9-wk-old male mice and the bone ends are cut off with scissors. The marrow cavities are flushed with 1 mL of α-MEM by injecting at one end of the bone using a sterile 27-gauge needle.
2. Bone marrow cells are washed once with α-MEM, suspended in α-MEM containing 10% FBS, and cultured at 1.0×10^6 cells/0.5 mL/well in 24-well plates (Corning, Corning, NY) in a humidified atmosphere of 5% CO_2. Cultures are fed every 2–3 d by replacing 0.4 mL of old medium with fresh medium *see* **Note 5**).
3. Osteotropic factors such as 10^{-8} *M* 1α,25-$(OH)_2D_3$, 100 ng/mL of PTH, 10^{-6} *M* PGE_2, and 10 ng/mL of IL-11 induce osteoclast formation in this marrow culture. These factors are usually added at the beginnning of culture and at each time of medium change.
4. Cells are fixed, and stained for TRAP (a marker enzyme of osteoclasts) as described in section **Subheading 3.5.1.**

TRAP-positive mononuclear cells appear on d 3–4 and multinucleated cells on d 4–5 in the presence of bone-resorbing factors. The number of TRAP-positive multinucleated cells reaches a maximum on d 6–8. TRAP-positive osteoclasts are formed only near the colonies of alkaline phosphatase (ALP)-positive osteoblasts in the culture treated with PTH (**Fig. 1A,B**; *see* **Note 6**). OPG completely inhibited the TRAP-positive cell formation induced by PTH

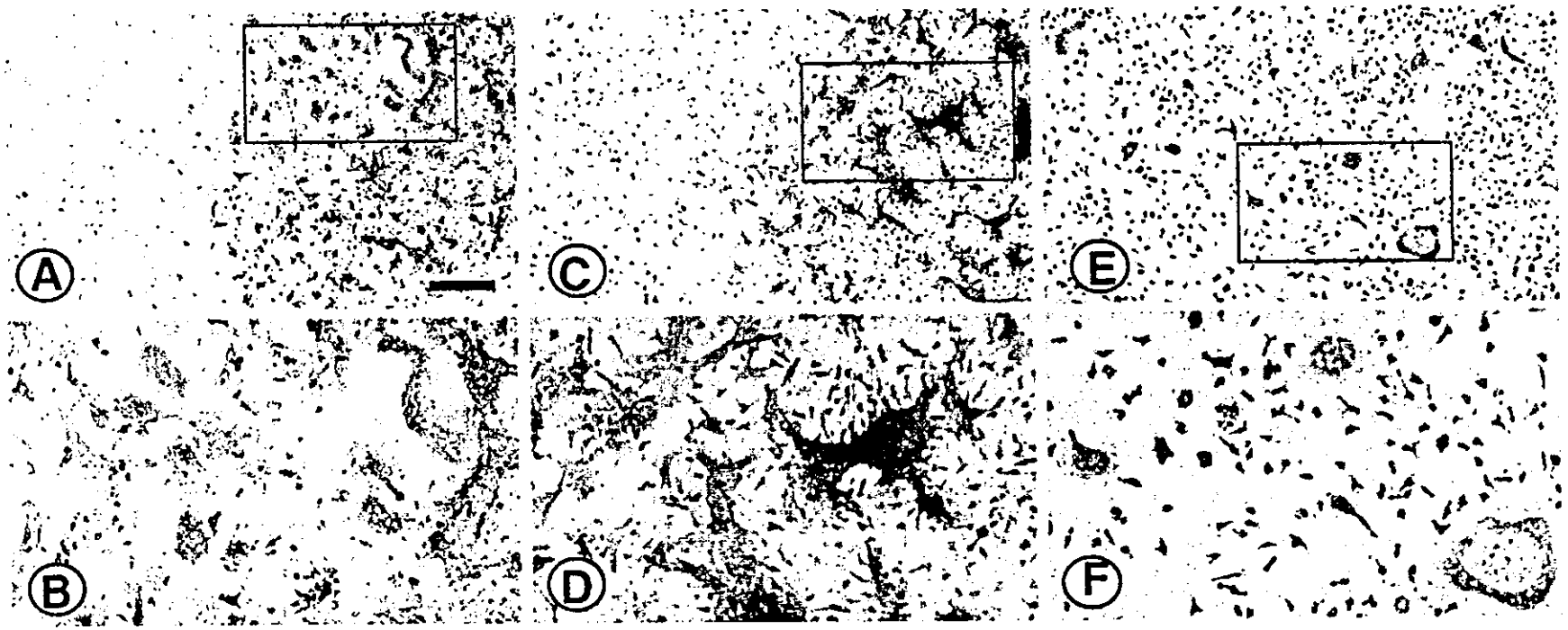

Fig. 1. Enzyme histochemistry for TRAP and ALP in mouse bone marrow cultures. Bone marrow cells of ddY mice were cultured for 7 d with 100 ng/mL of (**A**) PTH, 100 ng/mL of PTH plus 100 ng/mL of OPG (**C**), or 100 ng/mL of RANKL plus 50 ng/mL of M-CSF (**E**). Marrow cultures were then fixed and double-stained for TRAP and ALP. TRAP-positive cells appeared as red cells and ALP-positive cells as blue cells. (**B**), (**D**), and (**F**) show high power views of portions in (**A**), (**C**), and (**E**), respectively. Note that TRAP-positive cells formed in the culture are observed near or within the colonies of ALP-positive cells in the presence of PTH (**B**). In contrast, TRAP-positive cells are distributed uniformly on the culture dish in the presence of both RANKL and M-CSF (**F**). Adding OPG completely suppressed the formation of TRAP-positive cells induced by PTH (**D**). Scale bar = 200 μm.

in bone marrow cultures (**Fig. 1C, D**). Osteoclasts are also formed when mouse bone marrow cultures are treated with 50 ng/mL of M-CSF and 100 ng/mL of RANKL (**Fig. 1E, F**). In this culture, osteoclasts are formed uniformly all over the culture dish. Cocultures of primary osteoblasts with bone marrow cells produce more osteoclasts than bone marrow cultures alone do *(4)*. A protocol for osteoclast formation in coculture is given in Chapter 11 by van 't Hof, *this volume*.

3.2. Bone Marrow Macrophage Culture

Macrophages appearing in bone marrow cultures are the precursors of osteoclasts. We have modified the mouse bone marrow culture system to prepare highly purified osteoclast precursors *(9)*.

1. Bone marrow cells are treated with 100 ng/mL of M-CSF in αMEM containing 10% FBS in 48-well plates (3×10^5 cells/0.5 mL/well) (*see* **Note 7**).
2. Cells are cultured for 3 d, and nonadherent cells are completely removed from the culture by pipetting. Adherent cells strongly express macrophage specific antigens such as Mac-1, Moma-2, and F4/80. Therefore, adherent cells are called "M-CSF-dependent bone marrow macrophages (M-BMMϕ)." Typically, 1×10^4 M-BMMϕ are obtained when 1×10^5 bone marrow cells are cultured for 3 d in the presence of M-CSF.
3. When M-BMMϕ are further cultured with 100 ng/mL of RANKL and 100 ng/mL of M-CSF, TRAP-positive mononuclear and multinucleated cells are formed within 3 d (**Fig. 2B**) (*see* **Note 7**).

3.3. Collagen Gel Culture

Osteoclasts formed on plastic culture dishes are very difficult to detach by the treatment with either trypsin–EDTA or bacterial collagenase. To obtain functionally active osteoclasts formed in cocultures with osteoblasts, a collagen gel culture is recommended *(10,11)*.

1. A 10-cm culture dish (Corning) is coated with 4 mL of the type I collagen mixture at 4°C. The dish is put in a CO_2 incubator for 10 min to make the aqueous type I collagen gelatinous at 37°C.
2. Primary osteoblasts (2×10^6 cells; *see* the chapter by Bakker and Klein-Nulend, *this volume*) and bone marrow cells (2×10^7 cells; *see* **Subheading 3.1.**) are cocultured on a collagen gel coated dish in 15 mL of α-MEM containing 10% FBS and 10^{-8} *M* 1α,25-$(OH)_2D_3$. The medium is changed every 2–3 d.
3. After culture for 7 d, the dish is treated with 4 mL of 0.2% collagenase solution for 20 min at 37°C in a shaking water bath (60 cycles/min). The culture dishes are carefully placed on a sheet of aluminum foil put on the water surface of the water bath to maintain the sterile condition of the dishes.
4. The cells released from the dish are collected by centrifugation at 250*g* for 5 min and suspended in 10 mL of α-MEM containing 10% FBS (the crude osteoclast preparation). Usually, 4×10^4–1×10^5 osteoclasts are recovered from a 10 cm

Fig. 2. Effects of RANKL, mouse TNF-α , and IL-1 on TRAP-positive cell formation in M-BMMϕ cultures. Bone marrow cells of ddY mice were cultured with M-CSF for 3 d to prepare M-BMMϕ. M-BMMϕ were further cultured for 3 d without (**A**) or with either 100 ng/mL of RANKL (**B**), 20 ng/mL of mouse TNF-α (**C**), or 10 ng/mL of human IL-1 (**D**) in the presence of 100 ng/mL of M-CSF. Cells were then fixed and stained for TRAP. Scale bar = 100 μm.

collagen gel coated dish, and the purity of osteoclasts is 2-3% in this crude preparation.

5. The crude osteoclast preparation is used for biological and biochemical studies of osteoclasts.

3.4. Purification of Osteoclasts Formed In Vitro

Because the purity of osteoclasts in the crude osteoclast preparation is only 2–3%, further purification is essential for biochemical studies of osteoclasts. Osteoclasts are easily purified from the crude osteoclast preparation placed on plastic dishes by treatment with pronase–EDTA solution *(12,13)*. This procedure is identical to that described in the chapter by Coxon et al. to obtain pure mature rabbit osteoclasts.

1. Ten milliliters of the crude osteoclast preparation is placed on a 10-cm culture dish (Corning) for 6–15 h in the presence of 10% FBS (**Fig. 3A**).
2. Adherent cells are washed with α-MEM, and treated with 8 mL of pronase–EDTA solution for 10 min.
3. Osteoblasts are then removed by gentle pipetting. More than 90% of the adherent cells on the dishes are TRAP-positive mononuclear and multinucleated cells (**Fig. 3B**).

Using the purified osteoclast preparation, we have shown that osteoclasts possess phosphatidylinositol-3 kinase *(14)*, *rho* p21 *(15)*, and $p60^{c\text{-}src}$ *(16)*. We have also reported that osteoclasts express IL-1 receptors *(17)*, TNF type I and type II receptors *(18)*, and RANK *(18)*, and they respond to cytokines through these receptors.

It is difficult to obtain a highly enriched preparation of functionally active osteoclasts (*see* Chapter 7 by Coxon et at. and Chapter 6 by Collin-Osdoby et al., *this volume*). Using the disintegrin "echistatin," highly purified mononuclear and binuclear prefusion osteoclasts (pOCs) can be obtained from the coculture of mouse bone marrow cells and mouse osteoblastic MB1.8 cells treated with 10^{-8} *M* 1α,25-$(OH)_2D_3$ *(19)*. The purity of pOCs in the preparation is about 95% as determined by staining for TRAP. We have shown that pOCs themselves fail to form resorption pits on dentine slices, but they form resorption pits in the presence of RANKL or IL-1 *(17,18)*.

3.5. Identification of Osteoclasts Formed In Vitro

3.5.1. TRAP staining (see **Note 8**)

Cytochemical staining for TRAP is widely used for identifying osteoclasts in vivo and in vitro *(2,7,13)*.

1. Cells are fixed with 3.7% (v/v) formaldehyde in PBS(-) for 10 min, fixed again with ethanol–acetone (50:50, v/v) for 1 min, and incubated with the TRAP-staining solution for 10 min at room temperature.

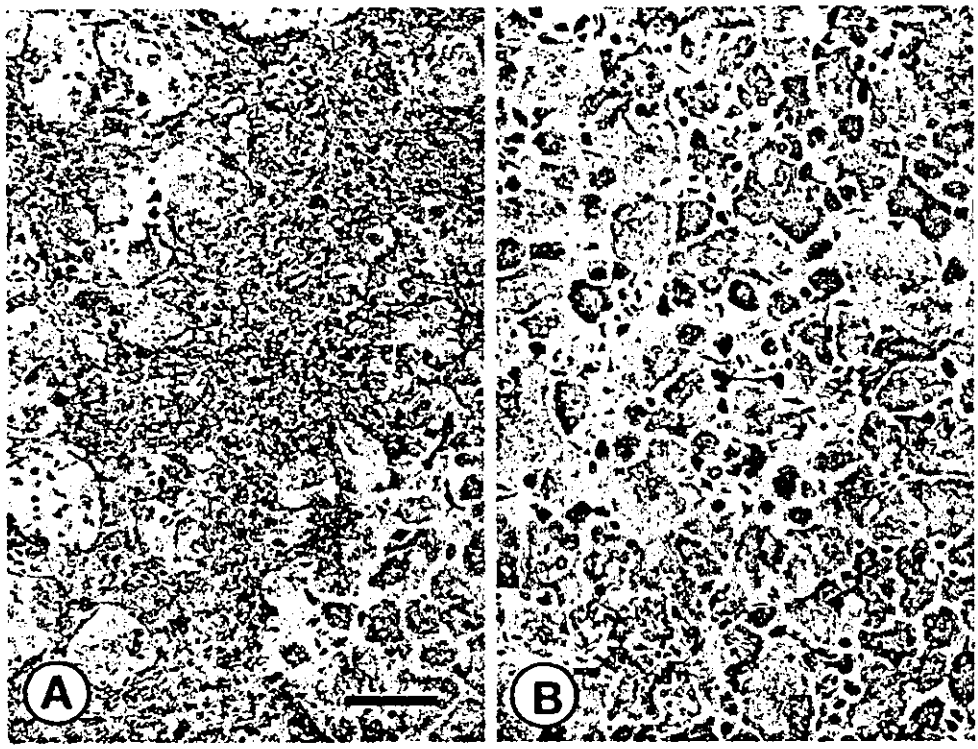

Fig. 3. Purified TRAP-positive osteoclasts formed in cocultures of mouse osteoblasts and bone marrow cells. Primary osteoblasts (2×10^6 cells) and bone marrow cells (2×10^7 cells) were cocultured for 7 d on a collagen gel coated dish. The dish was then treated with 0.2% collagenase solution to recover all the cells from the dish. The cells released from the dish were collected by centrifugation and suspended in 10 mL of α-MEM containing 10% FBS (the crude osteoclast preparation). The crude osteoclast preparation was placed on a 10-cm culture dish for 10 h in the presence of 10% FBS (**A**). The purity of osteoclasts in this crude preparation was only 2–3%. Adherent cells were washed with α-MEM, then treated for 10 min with 8 mL of pronase–EDTA solution. Osteoblasts were then removed by gentle pipetting. More than 90% of the adherent cells on the dish were TRAP-positive mononuclear and multinucleated cells **(B)**. Scale bar = 200 μm.

2. TRAP-positive osteoclasts appear as red cells. An incubation period longer than 10 min should be avoided, since cells other than osteoclasts become weakly positive with time.
3. After staining, cells are washed with distilled water, and TRAP-positive multinucleated cells having three or more nuclei are counted as osteoclasts under a microscope.

3.5.2. Autoradiography for Calcitonin Receptors

Osteoclasts have been shown to possess abundant calcitonin receptors *(13,20)*. Expression of calcitonin receptors is one of the most reliable markers

for identifying osteoclasts. Here we give a method for detection of calcitonin receptors by autoradiography, but immunocytochemical detection has been described also (*see* **Note 9**).

1. For autoradiography with ^{125}I-labeled human calcitonin, cultures are prepared on plastic coverslips (ϕ13.5 mm) placed in 24-well culture plates.
2. Cells grown on the coverslips are washed with α-MEM, and incubated with 0.2 n*M* ^{125}I-calcitonin in the presence or absence of 200 n*M* unlabeled salmon calcitonin in α-MEM containing 0.1% bovine serum albumin (BSA) for 1 h at 20°C.
3. Cells are washed three times with PBS(-) and fixed for 5 min with 0.1 *M* cacodylate buffer, pH 7.4, containing 1% formaldehyde and 1% glutaraldehyde.
4. The specimens are fixed again with ethanol–acetone for 1 min, and stained for TRAP.
5. The coverslips are then mounted on a glass slide, dipped in NR–M2 emulsion, and stored in a dark box at 4°C.
6. After incubation for 14 d, slides are developed in Rendol. Calcitonin receptors are identified by accumulation of dense grains due to ^{125}I-calcitonin binding, which disappear from the specimen when incubated with excess unlabeled calcitonin.

3.5.3. Pit Formation Assay

When osteoclasts are placed on dentine slices, they form resorption pits within 24 h. A reliable pit formation assay was established using the crude osteoclast preparation and dentine slices ***(13,21)***.

1. Dentine slices (ϕ 4 mm, 200 μm thick) are prepared from ivory blocks using a band saw (BS-3000, Exakt, Germany) and a cutting punch.
2. Dentine slices are cleaned by ultrasonication in distilled water, sterilized using 70% ethanol, and dried under ultraviolet light.
3. Dentine slices are placed in 96-well plates containing 0.1 mL/well of α-MEM with 10% FBS (a slice/well). A 0.1-mL aliquot of the crude osteoclast preparation is transferred onto the slices.
4. After a setting period of 60 min at 37°C, slices are removed, and placed onto 24-well plates containing α-MEM with 10% FBS (0.5 mL/slice/well).
5. After incubation for 24–48 h, the medium is removed and 1 *M* NH_4OH (1 mL/well) is added to the wells for 30 min.
6. Dentine slices are then cleaned by ultrasonication, stained with Mayer's hematoxylin (Wako Pure Chemical Industries) for 35–45 s, and washed with distilled water.
7. Resorption pits are clearly visualized with Mayer's hematoxylin under transmitted light.
8. The number of resorption pits formed on dentine slices is counted under a light microscope. Alternatively, the resorbed area is measured using an image analysis system linked to the light microscope. For a detailed description of such a method using reflected light microscopy *see* Chapter 11 by van 't Hof, *this volume*.

3.6. Introduction of Foreign Genes into Osteoclasts (see Note 10)

3.6.1. Preparation of Adenovirus Vector

An adenoviral vector system is useful for introducing foreign genes into osteoclasts to study the regulation of osteoclast function *(8)*.

1. The recombinant adenovirus carrying a foreign gene under the control of the CAG (cytomegalovirus IE enhancer + chicken β-actin promoter + rabbit β-globin poly[A] signal) promoter is constructed by homologous recombination between the expression cosmid cassette and the parental virus genome in 293 cells *(22)*.
2. Titers of virus stocks are determined by an endpoint cytopathic effect assay with the following modifications. Fifty microliters of DME containing 10% FBS is dispensed into each well of a 96-well plate, then eight rows of threefold serial dilutions of the virus starting from 10^{-4} dilutions are prepared.
3. To each well 3×10^5 293 cells in 50 μL of DMEM containing 10% FBS is added. The plate is incubated at 37°C in 5% CO_2 in air, and 50 μL of DME containing 10% FBS is added to each well every 3 d.
4. After culture for 12 d, the endpoint of the cytopathic effect is determined by microscopy, and the 50% tissue culture infectious dose ($TCID_{50}$) is calculated. One $TCID_{50}$ approx corresponds to one plaque forming unit (PFU)/mL.
5. The efficiency of infection is affected not only by the concentration of viruses and cells, but also by the ratio of viruses to cells, the multiplicity of infection (MOI). MOI is expressed as a measure of titer how many PFU are added to each cell.

3.6.2. Infection of Adenovirus Carrying Foreign Genes to Osteoclasts

1. Incubate mouse cocultures on d 4, when osteoclasts begin to appear, with a small amount of α-MEM containing the recombinant adenoviruses for 1 h at 37°C at a suitable MOI. We usually employ an MOI of 100 in our experiments.
2. Wash the cells twice with PBS(-) and incubate further with α-MEM with 10% FBS at 37°C. Experiments are preformed 24 h after the infection.

Using recombinant adenovirus carrying the *lacZ* gene, we have shown that the adenovirus carrying the *lacZ* gene can effectively infect osteoclasts with no apparent morphological changes or cellular toxicity *(8)* (**Fig. 4**). A high level of β-galactosidase activity is observed in mouse osteoclasts infected with the recombinant adenovirus carrying the *lacZ* gene. The proportion of β-galactosidase-positive osteoclasts increases in an MOI dependent manner, and > 80% of osteoclasts are positively stained at 100 MOI (**Fig. 4**).

4. Notes

1. In 1997, osteoprotegerin (OPG) and osteoclastogenesis inhibitory factor (OCIF), which inhibit osteoclast development in vivo and in vitro, respectively, were cloned independently by two different research groups *(23,24)*. Incidentally, OPG and OCIF were the same protein molecule. OPG/OCIF is a member of the TNF

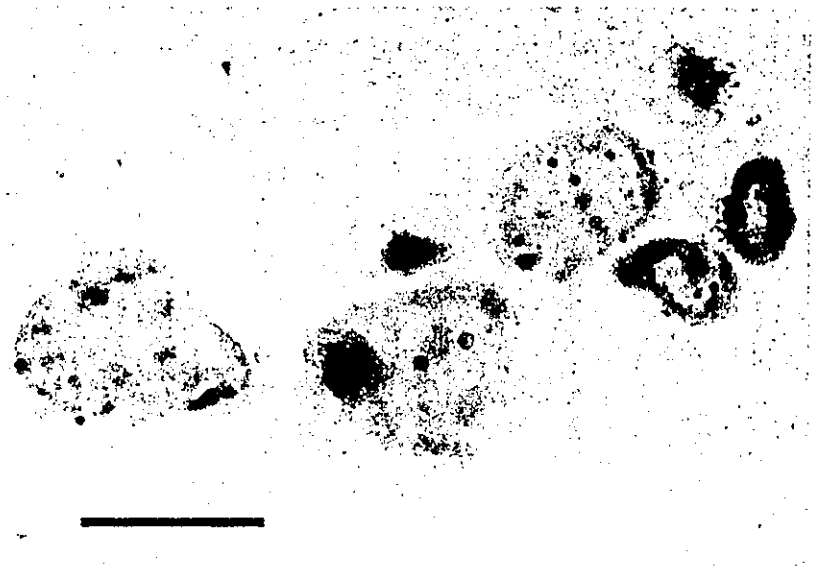

Fig. 4. An efficient adenovirus vector-mediated gene transfer to mouse osteoclasts as evidenced by cytochemical staining for β-galactosidase activity. Mouse cocultures on d 4 were incubated with a small amount of α-MEM containing the recombinant adenovirus vector encoding the *lacZ* gene (*AxCASLacZ*) for 1 h at 37°C at an MOI of 100. The cells were then washed twice with PBS(-) and further cultured for 2 d with α-MEM with 10% FBS at 37°C. Then, the cells were fixed for 10 min in 3.7% (v/v) formaldehyde in PBS(-) and washed in PBS(-). β-Galactosidase activity was detected by immersing the cells into a staining solution. Cells expressing β-galactosidase activity were stained as blue cells. More than 80% of multinucleated osteoclasts were transfected with the adenovirus carrying the *lacZ* gene at an MOI of 100. Scale bar = 100 μm.

receptor family, but it does not have a transmembrane domain, suggesting that OPG/OCIF functions as a circulating soluble factor. Subsequently, the cDNA encoding the binding molecule of OPG/OCIF was isolated from an expression library of the mouse stromal cell line ST2 and was named as osteoclast differentiation factor (ODF) *(25)*. A ligand for OPG/OCIF was also cloned from an expression library of the mouse myelomonocytic cell line 32D, and was named as OPG ligand (OPGL) *(26)*. OPGL was found to be identical to ODF. The binding molecule of OPG/OCIF was a membrane-associated protein of the TNF ligand family. ODF/OPGL was also identical to TNF-related activation-induced cytokine (TRANCE) *(27)* and RANKL *(28)*, which were independently cloned from mouse T cell hybridomas and mouse dendritic cells, respectively. RANK is the transmembrane receptor of ODF/OPGL/TRANCE/RANKL, which is expressed by osteoclast precursors and mature osteoclasts (**Fig. 5**). OPG/OCIF is a decoy receptor of ODF/OPGL/TRANCE/RANKL. Thus, ODF, OPGL, TRANCE, and RANKL are different names for the same protein, which is essential for the development and function of osteoclasts (**Fig. 5**). The terms "RANKL," "RANK," and "OPG" are used in this chapter according to the guide-

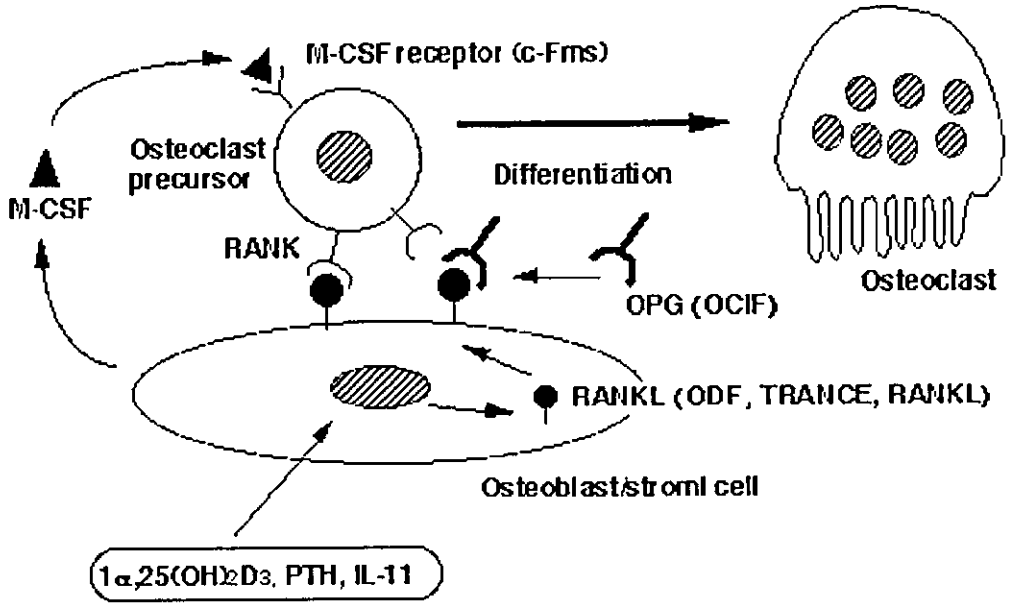

Fig. 5. Schematic representation of osteoclast differentiation regulated by osteoblasts/stromal cells. Recent studies have revealed the role of new TNF receptor-ligand family members responsible for osteoclast formation. Osteotropic factors such as $1\alpha,25\text{-}(OH)_2D_3$, PTH, and IL-11 stimulate expression of RANKL in osteoblasts/stromal cells as a membrane-associated cytokine for induction of osteoclast differentiation in bone marrow cultures or in cocultures of osteoblasts and hemopoietic cells. OPG, a soluble decoy receptor of RANKL, is produced mainly by osteoblasts. OPG strongly inhibits the RANKL-induced differentiation of osteoclast precursors into osteoclasts. The terms "RANKL," "RANK," and "OPG" are used in this chapter according to the guideline of the American Society for Bone and Mineral Research (ASBMR) President's Committee on Nomenclature *(29)*.

line of the American Society for Bone and Mineral Research (ASBMR) President's Committee on Nomenclature *(29)*. When spleen cells are treated with RANKL in the presence of M-CSF, osteoclasts are formed even in the absence of osteoblasts. OPG completely inhibits osteoclast formation in bone marrow cultures treated with osteotropic factors. RANKL and OPG are expressed mainly by stromal cells in bone marrow cultures. Osteotropic factors stimulate the expression of RANKL and suppress OPG expression by stromal cells in mouse bone marrow cultures.

2. Other strains of mice such as BALB/c, C57BL, and ICR can also be used for mouse osteoclast formation.
3. Human M-CSF is effective in both human and murine cells, whereas murine M-CSF is effective in murine cells but not in human cells.
4. Only this type of collagen (cell matrix type IA; Nitta Gelatin Co.) is suitable for this procedure.
5. Because FBS is one of the important factors that affect osteoclast formation, careful batch testing of FBS is recommended.

6. Osteoclast development proceeds within a local microenvironment of bone. This process can be reproduced ex vivo in a coculture of mouse calvarial osteoblasts and hemopoietic cells. Some mouse stromal cell lines such as MC3T3-G2/PA6 and ST2 are capable of supporting osteoclastogenesis when cultured with mouse spleen cells *(2,30)*. In such cocultures, osteoclasts are formed in response to various osteotropic factors including $1\alpha,25\text{-}(OH)_2D_3$, PTH, PGE_2 and IL-11. Cell-to-cell contact between osteoblasts/stromal cells and osteoclast progenitors is required to induce osteoclastogenesis *(2,7)*. Subsequent experiments have established that the target cells of osteotropic factors for inducing osteoclast formation in vitro are osteoblasts/stromal cells *(2,7)*. In bone marrow culture, stromal cells present in bone marrow support osteoclast formation from the progenitors in response to osteotropic factors. Therefore, the growth of stromal cells is one of the determinants for osteoclast formation in bone marrow cultures *(2,7)*.
7. Treatment of bone marrow cells with a high concentration of M-CSF (100 ng/mL) for 3 d stimulates the proliferation of macrophages without growth of stromal cells. ALP-positive cells are seldom observed in the M-BMMϕ preparation. In the absence of M-CSF, most of the M-BMMϕ rapidly die within 3 d. No TRAP-positive cells are formed even in the presence of RANKL, when M-CSF is not added to the culture. Mouse TNF-α (20–100 ng/mL) also stimulates formation of osteoclasts from M-BMMϕ in the presence of M-CSF (**Fig. 2C**), but human TNF-α shows only weak activity in inducing TRAP-positive cell formation from M-BMMϕ even at a higher concentration (100 ng/mL). Osteotropic hormones and cytokines including $1\alpha,25\text{-}(OH)_2D_3$, PTH, PGE_2 and IL-1 (**Fig. 2D**) fail to induce osteoclast formation in M-BMMϕ cultures.
8. Various alternatives to the TRAP staining protocol given here are described in the other chapters on osteoclasts in this volume. All are equally useable.
9. Recently, Quinn et al. (*31*) developed a method for immunostaining of murine calcitonin receptors using polyclonal antibodies against rat calcitonin receptors. Osteoclasts (both mononuclear and multinucleated) formed in the coculture of mouse bone marrow cells and osteoblasts were specifically immunostained by the antibodies.
10. To investigate the molecular mechanism of osteoclast function, it is necessary to modulate gene expression in osteoclasts by introducing foreign genes into the cells. Adenovirus vectors have several advantages *(8)*. First, these vectors are capable of infecting a variety of terminally differentiated cells including neurons, hepatocytes, and osteoclasts. Second, recombinant adenovirus can be amplified easily to a very high titer in vitro. Third, adenovirus infection to the cells has been reported to require the interaction of the RGD sequence in the penton base of the virus with the cell surface of osteoclasts. We have successfully introduced such foreign genes into osteoclasts as epidermal growth factor receptor *(8)*, C-terminus Src family kinase (csk) *(32)*, dominant negative ras *(33)*, constitutively active MEK1 (MAP kinase kinase) *(33)*, dominant negative IκB kinase 2, and constitutively active IκB kinase 2 *(33)*.

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Destruxins, cyclodepsipeptides, block the formation of actin rings and prominent clear zones and ruffled borders in osteoclasts

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Abstract

Bone-resorbing osteoclasts exhibit polarized morphological structures such as actin rings, clear zones, and ruffled borders. To gain insight into the mechanism of bone-resorbing activity of osteoclast and to discover new types of anti-resorptive agents, we have screened for natural compounds that inhibit the bone-resorbing activity of osteoclast-like multinucleated cells (OCLs). Destruxin B (DestB) and E (DestE), cyclodepsipeptides, were found to inhibit pit formation without affecting osteoclast differentiation and survival. Destruxins reversibly induced morphological changes in OCLs in a dose-dependent manner (DestB, 0.2–1 μM; DestE, 0.01–0.05 μM) and inhibited pit formation. Destruxin-induced morphological changes were accompanied by disruption of the actin rings in OCLs. The formation of actin rings in OCLs after adhesion was also inhibited by destruxins. Electron microscopical analysis revealed that destruxin-treated OCLs on dentine slices have no prominent clear zones and ruffled borders. The effective concentrations of destruxins on the morphological changes were almost the same as those that inhibited bone resorption in organ culture system. These results suggest that the anti-resorptive effects of destruxins result from induction of a disorder of the morphological structures in polarized OCLs.

vwords: Destruxin; Osteoclast; Bone resorption; Actin ring; Ruffled border

Introduction

Osteoclasts are multinucleated cells that play a crucial role in bone resorption [1,2]. They are formed by the fusion of mononuclear osteoclasts derived from osteoclast precursors of the monocyte/macrophage lineage [1]. Enhanced bone resorption and recruitment of osteoclasts are the key pathophysiological events in many metabolic bone disorders in the adult human, such as osteoporosis, hyperparathyroidism, metastastic bone disease, and hypercalcemia of malignancy. Bone-resorbing osteoclasts, in contrast to non-resorbing osteoclasts, exhibit highly polarized morphological features: ring-like structures consisting of F-actin dots (actin rings), separator between the extracellular space and the resorption lacuna (clear zones), and the vacuolar-type proton-ATPase (V-ATPase)-concentrated late endosome-like membrane (ruffled borders), which together are a marker of functional osteoclast [3–7]. The resorption lacuna are formed by targeted secretion of protons and proteases through the ruffled border after tight attachment of osteoclasts to mineralized bone surface through their integrins and the following polarization [6,7]. The resorption function is carried out in the developed clear zone-separated extracellular space between the ruffled border membrane and the bone surface [2,3].

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Calcitonin (CT) directly inhibits osteoclastic bone resorption without affecting osteoclast survival and is widely used to treat metabolic bone disorders, such as osteoporosis [8]. Although it inhibits bone resorption by disrupting the morphological structures in polarized osteoclasts [9–11], the detailed mechanism of its activity in the relationship between the polarization and the function of osteoclasts is not fully understood. It is also recognized that continuous treatment with CT eventually leads to decreased inhibitory effects on osteoclastic bone resorption (escape phenomenon) [12,13]. The escape phenomenon is known to be involved in the down-regulation of the CT receptor (CTR) which is mediated through activation of the protein kinase A (PKA) pathway via the cell surface CTR [12]. Low-molecular-weight compounds that exert calcitonin-like activity without inducing activation of the PKA pathway would be viewed as new types of anti-resorptive agents to treat osteoporosis. Therefore, to discover low-molecular-weight compounds with calcitonin-like activity and to gain new insights about the relationship between polarization and function of osteoclasts, we screened for natural compounds that inhibit the bone-resorbing activity of OCLs without affecting their survival. Among approximately 300 purified low-molecular-weight natural compounds, destruxin B and E, which were isolated from an entomopathogenic or phytopathogenic fungus [14,15], were found to inhibit bone resorption by OCLs without affecting their survival. In the present study, we characterized the behaviors of destruxins on OCLs cultured on plastic plates or dentine slices.

Materials and methods

Animal and chemicals

Newborn Std.ddY mice and 6- to 9-week-old male Std.ddY mice were purchased from Japan SLC Co. (Hamamatsu, Japan). Fast red violet LB salt and naphthol AS-MX phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Collagenase, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], and eel calcitonin were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dispase was purchased from Godo Shusei (Tokyo, Japan). Collagen gel solutions (Cellmatrix, Type I-A) were from Nitta Gelatin Co. (Osaka, Japan). Alexa488-conjugated phalloidin was from Molecular Probes, Inc. (Eugene, OR). Destruxin B (DestB) and destruxin E (DestE) were generous gifts from Dr. A. Takatsuki (The Institute of Physical and Chemical Research, Saitama, Japan) and K. Hasumi (Tokyo University of Agriculture and Technology, Tokyo, Japan), respectively. Recombinant human M-CSF (Leukoprol) and recombinant human soluble form of RANKL (sRANKL) were purchased from Welfide, Co. (Osaka, Japan) and Pepro Tech EC Ltd. (London, UK), respectively. Human PTH (1–34) was purchased from Peptide Institute (Osaka, Japan). $^{45}CaCl_2$ was from Amersham Pharmacia Biotech (Buckinghamshire, UK).

Preparation of crude OCLs

Murine tartrate-resistant acid phosphatase-positive [TRAP(+)] osteoclast-like multinucleated cells (OCLs) were prepared from a coculture system as previously described [16]. In brief, primary mouse osteoblastic cells were obtained from calvariae of newborn ddY mice and mouse bone marrow cells were from the femora and tibiae of 6- to 9-week-old male ddY mice. Bone marrow cells (2×10^7 cells) and osteoblastic cells (1×10^6 cells) were cocultured for 7 days in α-minimal essential medium (α-MEM) (Gibco BRL, Life Technology Inc., Gathersburg, MD) containing 10% fetal calf serum (Asahi Techno Glass Ltd., Chiba, Japan) on 100-mm tissue culture dishes (Corning Inc., Corning, NY) (15 ml/dish) precoated with 2.5 ml of 0.2% collagen gel matrix. Half of the medium was changed after coculture for 2 and 5 days. For the last 5 days, cells were cultured in the presence of 10 nM 1α,25$(OH)_2D_3$ and 1 μM PGE_2. OCLs were formed within 7 days of culture and released from dishes by treating with 1.5 ml of 0.2% collagenase and 0.1% dispase. Cells were collected by centrifugation (1000 rpm, 5 min) and placed on 96-well culture plates (Corning Inc.). The purity of OCLs in this culture was about 5% (Crude OCL preparation).

Preparation of pOCs and preparation of OCLs from pOCs culture

TRAP-positive mononuclear osteoclasts (pOCs) were prepared from a coculture system as previously described [17]. Briefly, bone marrow cells (2×10^7 cells) and osteoblastic cells (2×10^6 cells) were cocultured as noted under Preparation of crude OCLs. After coculture for 2 days, half of the medium was changed and fresh medium containing 10 nM 1α,25$(OH)_2D_3$ and 1 μM PGE_2 was added. After coculture for 4 days, floating cells were removed and mononuclear cells attached to the osteoblastic cell layer were recovered as pOCs by gentle pipetting with fresh α-MEM. pOCs were collected by centrifugation (1000 rpm, 5 min) and 3×10^5 cells were placed on 96-well culture plates. The purity of pOCs in this culture was about 50–60% (pOC preparation). pOC preparations placed on 96-well culture plates were cultured in the presence of M-CSF (20 ng/ml) and sRANKL (100 ng/ml) and OCLs were formed within 24 h of culture. In some experiments, pOCs cultured in the presence of M-CSF and sRANKL for 6 h were further cultured in fresh medium containing 100 ng/ml of M-CSF and OCLs were formed within 24 h of culture. The purity of OCLs in this culture was more than 70% (OCL preparation).

Cell staining

OCL preparations were placed on 96-well culture plates. After preculture for 24 h, the cells were treated with vehicle [dimethyl sulfoxide (DMSO)] or destruxins for 24 h and stained for TRAP, a typical marker enzyme of osteoclasts. TRAP staining was carried out as described previously [16].

In brief, the cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS)(−) for 15 min. After treatment with ethanol/acetone (1/1) for 1 min, the well surface was dried and treated with the TRAP staining solution [0.1 M sodium acetate buffer (pH 5.0) containing 50 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate, and 1 mg/ml fast red violet LB salt] for 10 min. In some experiments, cells were further stained with Alexa488-conjugated phalloidin for F-actin. After TRAP staining, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. After treatment with Alexa488-conjugated phalloidin, the distribution of F-actin was visualized and detected under a fluorescence microscope (Cart Zeiss, Axioskop 2, Germany). The fluorescence images were acquired using IPLab Spectrum Ver 3.2.4.

Assay for the viability of osteoblastic cells

Osteoblastic cells (4×10^4 cells/well) were placed on 96-well culture plates and precultured for 24 h. Then, the cells were cultured with 1% (v/v) DMSO (vehicle) or 100 μM destruxins (DestB and DestE) for 24 h. After the culture, the cells were incubated in 100 μl of 0.1% crystal violet in PBS(−) for 15 min. After the cells were washed with PBS(−), the dye incorporated into the cytoplasm of living cells was eluted with 100 μl of methanol, and then absorbance at 595 nm was measured. Viability of osteoblastic cells was expressed as a percentage of the absorbance in control culture.

Pit formation assay

Inhibitory effects of destruxins on pit-forming activity of OCLs were determined by the method previously reported [17]. Briefly, crude OCL preparations were placed on 96-well culture plates containing dentine slices (4 mm in diameter) and culture medium with 1% (v/v) DMSO (vehicle) or destruxins. At the end of culture period, a freeze-thaw cycle at −20°C and room temperature was performed to remove adherent cells on the slices. The slices were stained with Mayer's hematoxylin (Sigma Chemical Co.) to visualize resorption pits and the number of pits on slices was counted by microscopical observation. The images of pits were acquired using IPLab Spectrum Ver 3.2.4. Data were expressed as the mean ± SD of four cultures.

Assay for ^{45}Ca release from long bone in an organ culture system

Pregnant ddY mice were injected sc with 25 μCi of ^{45}Ca on Day 16 of gestation. Twenty-four hours after injection, shafts of radii and ulnae were dissected from fetuses, cleaned free of surrounding muscle and fibrous tissues, and precultured in serum- or BSA-free BGJb medium (Life Technologies, Grand Island, NY). After preincubation for 48 h, the bones were transferred into 0.5 ml BGJb medium containing 0.2% BSA and incubated for 72 h in the presence of destruxin E or eel calcitonin (eCT). At the end of culture period, the bones were extracted in 0.1 N HCl. Radioactivity in media and bone extracts was determined by liquid scintillation spectrophotometry. Bone-resorbing activity was expressed as the percentage of the incorporated ^{45}Ca that was released into the medium. Data were expressed as the mean ± SD of four cultures.

Assay for pOCs formation

Bone marrow cells (1×10^4 cells/well) were cultured in 96-well culture plates in the presence of M-CSF (20 ng/ml) for 24 h to prepare osteoclast precursor cells which are TRAP-negative macrophage-like cells (TRAP-negative osteoclast precursor cells) [1]. The osteoclast precursor cells were cultured for 48 h with M-CSF (20 ng/ml) and sRANKL (100 ng/ml) to induce pOCs, and were cultured with 0.02% (v/v) DMSO (vehicle), 2 μM destruxin B, or 0.1 μM destruxin E for the last 24 h. After the culture, the cells were fixed and stained for TRAP for 1 h. The number of pOCs formed in the culture was counted. Data were expressed as the mean ± SD of four cultures.

Assay for fusion of pOCs

pOC preparations (2×10^5 cells/well) placed on 96-well culture plates containing culture medium with 0.02% (v/v) DMSO (vehicle), 2 μM destruxin B, or 0.1 μM destruxin E were cultured in the presence of M-CSF (20 ng/ml) and sRANKL (50 ng/ml) for 24 h. After incubation, cells were fixed and stained for TRAP, and then the number of OCLs with 4–9 nuclei and more than 10 nuclei were counted. Data were expressed as the mean ± SD of four cultures.

Assay for OCL morphology

OCL with osteoblastic cells harvested from the cocultures were cultured again in 96-well culture plates in the presence of DMSO [1% (v/v)], destruxin B (2 μM), or destruxin E (0.1 μM) for the indicated periods. In some experiments, crude OCL preparations prepared from the cocultures and OCL preparations from pOCs culture on 96-well culture plates were cultured in the presence of 1% (v/v) DMSO or the indicated concentrations of destruxins. After the culture, cells were fixed and stained for TRAP, and then the number of OCLs having more than 10 nuclei with a clear cytoplasm and smooth periphery were counted. Data were expressed as the mean ± SD of the percentage of the total number of OCLs in the cultures.

Electron microscopic analysis

The cells cultured with or without 2 μM destruxin B for 2 h on dentine slices were fixed with a fixative [0.05 M

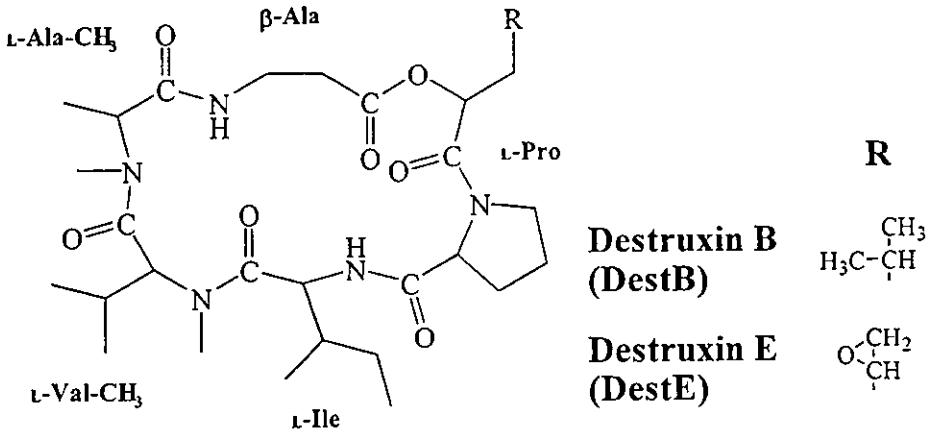

Fig. 1. Structures of destruxins (DestB and DestE).

sodium cacodylate buffer (pH 7.3) containing 2% formaldehyde, 2% acrolein, and 2.5% glutaraldehyde] for 2 h at 4°C. In immunocytochemical experiments, a fixative [0.1 M sodium cacodylate buffer (pH 7.3) containing 4% formaldehyde and 0.1% glutaraldehyde] was used. In some experiments, cells were further cultured for 4 h after removal of DestB from culture media.

For electron microscopic analysis, the specimens were decalcified in 10% EDTA solution (pH 7.3) for 2 weeks, and then postfixed with 1.5% potassium ferrocyanide-reduced 1% osmium tetroxide for 30 min at 4°C. They were block-stained with 1% uranyl acetate in 10% ethanol for 1 h at room temperature in the dark, dehydrated through a graded ethanol series, and embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Ultrathin sections were cut using a diamond knife on a Reichert-Jung Om-U4 and then stained with uranyl acetate and lead citrate.

For immunocytochemical analysis, the specimens were decalcified in 10% EDTA solution. They were then dehydrated through a graded ethanol series and embedded in medium-graded LR white resin (London Resin, Basingstoke, UK), which was polymerized at 20°C under ultraviolet rays. Ultrathin sections were cut as described above and mounted on Formvar-coated nickel grids. Furthermore, they were treated with a blocking buffer [0.01 M PBS containing 100 mg/ml bovine serum albumin (BSA)] for 1 h, and then incubated overnight at 4°C with rabbit polyclonal anti-V-ATPase [antiserum raised against 72-kDa subunit in V-ATPase donated by Dr. Moriyama (Hiroshima University, Hiroshima, Japan)], which was diluted 1:500 with 1% BSA/PBS. Nonimmune rabbit serum was used for negative control sections. After incubation, the sections were rinsed with PBS and incubated for 1 h with goat anti-rabbit IgG conjugated with 10 nm colloidal gold particles (BioCell Research Laboratories, Cardiff, UK), which was diluted 1:100 with PBS. A series of immunological procedures were carried out in a moisture chamber at room temperature. After immunological treatment, the sections were rinsed with PBS and then distilled water. Finally, the sections were stained with 2% uranyl acetate for 4 min.

Electron microscopic images were acquired using a Hitachi H-7000 electron microscope at 75 kV.

Results

Destruxins inhibit both pit formation on dentine slices and ^{45}Ca release in organ cultures

In our screening program, we found that destruxins (Fig. 1), cyclodepsipeptides, inhibited bone resorption by OCLs. OCLs placed on dentine slices formed resorption pits within 24 h, but OCLs cultured with destruxins did not (Fig. 2A). The culture of OCLs with destruxins inhibited pit formation in a dose-dependent manner (Figs. 2Ab–d). The inhibitory effect of 0.1 μM destruxin E was as strong as that of 0.4–2 μM destruxin B. In order to determine whether or not the inhibitory effects of destruxins resulted from cytotoxicity, we examined the cytotoxicty of destruxins on OCLs and osteoblastic cells on plastic plates. The cell viabilities OCLs and osteoblastic cells were not decreased by the concentrations of destruxins that inhibited pit formation (Fig. 2B). Furthermore, no significant decrease in the viability of osteoblastic cells was observed even when the cells were cultured with 100 μM destruxins (Fig. 2B). However, more than both 10 μM destruxin B and 0.5 μM destruxin E exerted cytotoxicity against OCLs (data not shown). These results suggest that destruxins inhibit pit formation by affecting the bone-resorbing function of OCLs. To investigate the inhibitory effect of destruxins on bone resorption in detail, ^{45}Ca released from the bone into the medium was measured in organ cultures. Destruxin E inhibited PTH-stimulated ^{45}Ca release in a dose-dependent manner (5–100 nM) (Fig. 2C). Although 100 nM destruxin E completely inhibited PTH-stimulated ^{45}Ca release, it did not affect the basal ^{45}Ca release. The same effect was also exerted by destruxin B (0.1–10 μM) (data not shown). The inhibitory effect of 100 nM destruxin E was as strong as that of 10 nM eel calcitonin (eCT) (Fig. 2D).