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Mechanisms involved in the enhancement of osteoclast formation by enamel matrix derivative

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Background and Objective: Enamel matrix derivative (EMD) is used clinically to promote periodontal tissue regeneration, and it has been reported that EMD can induce the formation of osteoclasts in mouse marrow cultures. In the present study, we investigated the mechanisms of EMD-induced osteoclast formation using a mouse monocytic cell line, RAW 264.7.

Material and Methods: Bioactive fractions were purified from EMD by reverse-phase HPLC using a C_{18} hydrophobic support, following which RAW 264.7 cells were cultured with EMD or its purified fractions in the presence of receptor activator of nuclear factor-κB ligand (RANKL) for 8 d. Following staining with tartrate-resistant acid phosphatase (TRAP), TRAP-positive multinucleated cells were counted. The expression of receptor activator of nuclear factor-κB (RANK), as well as phosphorylation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein (MAP) kinase, in RAW 264.7 cells were detected using immunoblotting. To determine whether EMD has an effect on osteoclast function, differentiated RAW 264.7 cells were cultured on Osteologic™ Multitest slides with RANKL in the presence of EMD.

Results: Purified EMD fractions (fraction numbers 21–25; EMD peak 2) were found to enhance the formation and function of RAW 264.7 cells induced by RANKL. Moreover, EMD peak 2 enhanced the levels of phosphorylation of ERK p38 and RANK in RAW 264.7 cells stimulated with RANKL.

Conclusion: Our results indicate that EMD induces the formation of osteoclasts through interaction with RANKL, while ERK and p38 MAPK may play a critical role in the enhancement of osteoclast formation in RAW 264.7 cells.

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Hemmerström and colleagues published a series of studies on enamel matrix derivative (EMD) prepared from acid extracts of porcine enamel proteins (1–4), after which a number of research groups provided basic and clinical evidence of periodontal regeneration elicited by EMD. Clinical studies have shown that EMD successfully promotes cementum and alveolar bone formation in periodontal tissues (5,6). In addition, other clinical studies of EMD treatment for intrabony periodontal defects found that it led to regeneration of the periodontal ligament, and markedly enhanced clinical attachment and alveolar bone growth (7,8).

Osteoclasts are multinucleated giant cells that are derived from hematopoietic precursors of monocytes/macrophages and primarily involved in bone resorption. Proteins involved in osteoclast differentiation have been identified and the committee on nomenclature has proposed the name receptor activator of nuclear factor-κB ligand

(RANKL) for these proteins (9). RANKL are membrane-bound proteins of the tumor necrosis factor (TNF) ligand superfamily that are expressed on the surface of osteoblastic cells and bind to its receptor (RANK) on osteoclast progenitors, which leads to osteoclast differentiation, in cooperation with macrophage colony-stimulating factor (10). In a previous study, we found that EMD induced osteoclast formation through RANK–RANKL interactions in mouse marrow cultures. Those results suggested that EMD provides a local environment suitable for bone regeneration in periodontal tissues through the communication of osteoblasts and osteoclasts (11).

RANKL triggers osteoclastogenesis by forming a complex with RANK, a member of the TNF receptor family, and the binding of RANKL to RANK results in a cascade of intracellular events, including activation of the intracellular adaptor protein family in pre-osteoclastic cells (12–14). Previous reports have strongly suggested that RANKL expression on osteoblasts or stromal cells is essential for osteotropic factor-mediated osteoclast formation under physiological conditions (15).

Mitogen-activated protein kinase (MAPK) family members are proline-directed serine/threonine kinases that are important for cell growth, differentiation, and apoptosis (16–19), and become activated by phosphorylation of threonine and tyrosine in response to external stimuli. MAPK family members are classified into the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK groups, and it is widely considered that peptide growth factors and phorbol esters preferentially activate ERK, while cellular stress, such as that caused by hyperosmolarity or reactive oxygen species, potently activates JNK and p38 MAP kinases (20–23).

It is clinically accepted that EMD has a beneficial effect in inducing periodontal wound healing and regeneration; however, no information concerning the biological mechanisms of EMD towards bone regeneration is currently available. In the present study, we found that purified EMD fractions enhanced osteoclast formation in RAW 264.7 cells stimulated with RANKL and report possible mechanisms involved in the enhancement of osteoclast formation by EMD. Our results indicate that EMD plays an important role during the process of osteoclastogenesis and may participate in bone regeneration through bone formation and bone resorption.

Material and methods

Reagents and antibodies

EMD (Biora, Malmo, Sweden) was generously provided by Seikagaku Corporation (Tokyo, Japan). Lyophilized EMD was dissolved in 0.1% trifluoroacetic acid (TFA) (40 mg/ml), after which reverse-phase high-performance liquid chromatography (HPLC) was carried out using a Waters system (Midford, MA, USA) and a C_{18} column (4.6 × 150 mm; Vydac, Hesperia, CA, USA) equilibrated with 0.1% TFA, as described previously (11). Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min and assayed for osteoclast formation as described below. Protein content was determined using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA, USA). Bioactive fractions, which induced osteoclast formation in the osteoclast differentiation assay described below, were lyophilized, dissolved in culture medium and used in the cell cultures. Recombinant human soluble RANKL was purchased from Pepro Tech EC Ltd (London, UK). The polyclonal antibody against RANK β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while polyclonal antibodies against p38 MAPK, phosphorylated p38 MAPK, ERK, and phosphorylated ERK were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Cell culture

Bone marrow cells were isolated from ddY mice, as described previously (24). The cells were suspended in α-minimal essential medium (α-MEM; Gibco BRL, Grand Island, NY, USA) containing 10% (v/v) fetal calf serum (FCS), penicillin G (100 U/ml) and streptomycin (100 μg/ml). Mouse monocytic RAW 264.7 cells (ATCC TIB 71) were maintained in α-MEM, containing 10% (v/v) FCS and antibiotics, in collagen-coated dishes (Asahi Techno Glass, Chiba, Japan).

Cell viability

RAW 264.7 cells were plated in 96-well plates, at a concentration of 5×10^2 cells/well, 1 d before starting the experiment, after which the cells were stimulated with RANKL and the test samples. The stimulated cells were cultured for 3, 4 or 5 d, and then a stock MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 2.5 mg/ml; 20 μl/well] (Sigma Chemical Co., St Louis, MO, USA) was added to the wells and the plates were incubated for 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added and mixed thoroughly, and the plates were read using a Multiskan Bichromatic microplate reader (Labsystems, Helsinki, Finland), with a test wavelength of 540 nm and a reference wavelength of 620 nm (25).

Differentiation of osteoclasts

Osteoclasts were detected using tartrate-resistant acid phosphatase (TRAP) staining (Sigma Chemicals), as described previously (26). In brief, RAW 264.7 cells were cultured in 96-well plates (5.0×10^2 cells/well) in the presence of RANKL (50 ng/ml) and test samples. After culture for 8 d, adherent cells were fixed and stained for TRAP activity. TRAP-positive multinucleated cells containing three or more nuclei were considered to be osteoclast-like cells and were counted using a microscope. To assay TRAP activity, RAW 264.7 cells were cultured for 3 d in 96-well plates (5.0×10^2 cells/well) in the presence of RANKL (50 ng/ml) and the test samples. Treated RAW 264.7 cells were suspended in 25 μl of phosphate-buf fered saline (PBS, pH 7.2), then frozen and thawed three times. TRAP activities in the supernatants were analyzed

using a phenyl-phosphate substrate kit (Sanseiphospha KII-Test-Wako; Wako, Osaka, Japan), according to the manufacturer's instructions (27).

Bone resorption assay

To examine bone resorption activity, RAW 264.7 cells (1×10^3/well) were cultured for 14 d with RANKL (50 ng/ml) and test samples on BD BioCoat™ Osteologic™ Multitest slides, which consisted of submicron synthetic calcium phosphate thin films coated onto various culture vessels (Becton Dickinson & Co., Bedford, MA, USA). The cells were removed using 6% (w/v) NaOCl and 5.2% (w/v) NaCl, and the number of resorption pits formed in each well were counted using a microscope (28).

Immunoblot analysis

RAW 264.7 cells (1×10^6/dish) were cultured in α-MEM containing 10% (v/v) FCS, in the presence of RANKL (50 ng/ml) and the test samples. Adherent cells were washed twice with PBS and lysed in a cell-lysis buffer [75 mM Tris-HCl containing 2% (w/v) sodium dodecyl sulphate (SDS) and 10% (v/v) glycerol, pH 6.8]. The protein contents were measured using a DC-protein assay kit (Bio-Rad, Hercules, CA, USA). The samples were electrophoresed on a 10% SDS–polyacrylamide gel, and then transferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore, Bedford, MA, USA). Nonspecific binding sites were blocked using 10% (v/v) skim milk in PBS for 1 h at room temperature, and then the membranes were washed four times in PBS, followed by incubation in diluted primary antibody for 2 h at room temperature. The primary antibodies used in this experiment were antibodies for ERK, phosphorylated ERK, p38 MAP kinase, phosphorylated p38 MAP kinase, and RANK, while the secondary antibodies were anti-mouse, anti-rabbit, and anti-goat immunoglobulin G horseradish peroxidase (HRP; Santa Cruz Biotechnology, Inc) conjugates. After washing the membrane, chemiluminescence was produced using an enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Uppsala, Sweden) and detected with Hyperfilm-ECL (Amersham Pharmacia Biotech).

Results

Purification of EMD

In our previous study we found two major peaks upon HPLC analysis, as well as some minor peaks. One of the major peaks, EMD P2 (fraction numbers 21–25; eluted at 39 to 42% acetonitrile in 0.1% TFA), showed a high level of specific activity to induce the formation of osteoclasts in mouse bone marrow cultures (11). The other major peak, EMD P1 (fraction numbers 15–18), was unable to induce osteoclast formation in mouse bone marrow cultures and was used as a negative control.

Effects of EMD P2 on cell growth of RAW 264.7 cells

We examined the effects of EMD P1 and EMD P2 on the proliferation of RAW 264.7 cells, in the presence or absence of RANKL, using an MTT viability assay. EMD P2 showed an inhibitory effect on the growth of RAW 264.7 cells after 4 and 5 d of culture, with or without RANKL. In contrast, EMD P1 had no effect on the proliferation of RAW 264.7 cells (Fig. 1).

EMD P2 enhanced TRAP-positive multinucleated cell formation stimulated with RANKL

RAW 264.7 cells were cultured, for 8 d, with EMD P2 (100 μg/ml), in the presence of RANKL (50 ng/ml), after which TRAP-positive multinucleated cells were detected, as shown in Fig. 2A. In addition, culturing with EMD P2 activated the differentiation of RAW 264.7 cells into osteoclasts in the presence of RANKL (50 ng/ml) (Fig. 2B), while EMD P1 had almost no effect. EMD P1 or P2 alone did not induce osteoclast formation in the absence of RANKL (data not shown). We also examined the effect of EMD P2, in the presence of RANKL (50 ng/ml), on the TRAP activity of RAW 264.7 cells using a phenyl-phosphate substrate method. When RAW 264.7 cells were cultured with both RANKL and EMD P2, the expression level of TRAP was 1.5-fold higher than that of

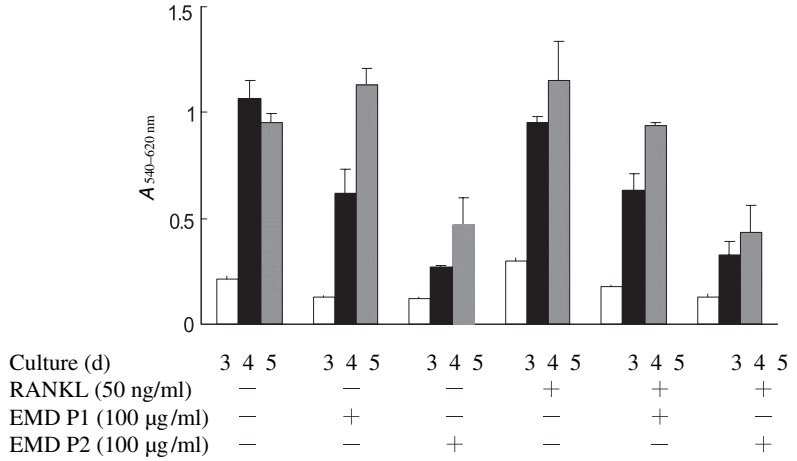

Fig. 1. Effects of purified enamel matrix derivative (EMD) on the growth of RAW 264.7 cells. RAW 264.7 cells were cultured with or without receptor activator of nuclear factor-κB ligand (RANKL) (50 ng/ml), EMD P1 (100 μg/ml) and EMD P2 (100 μg/ml) for the indicated number of days, after which cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Data are expressed as the mean ± standard deviation of triplicate cultures. Each experiment was performed three times, with similar results obtained on each occasion.

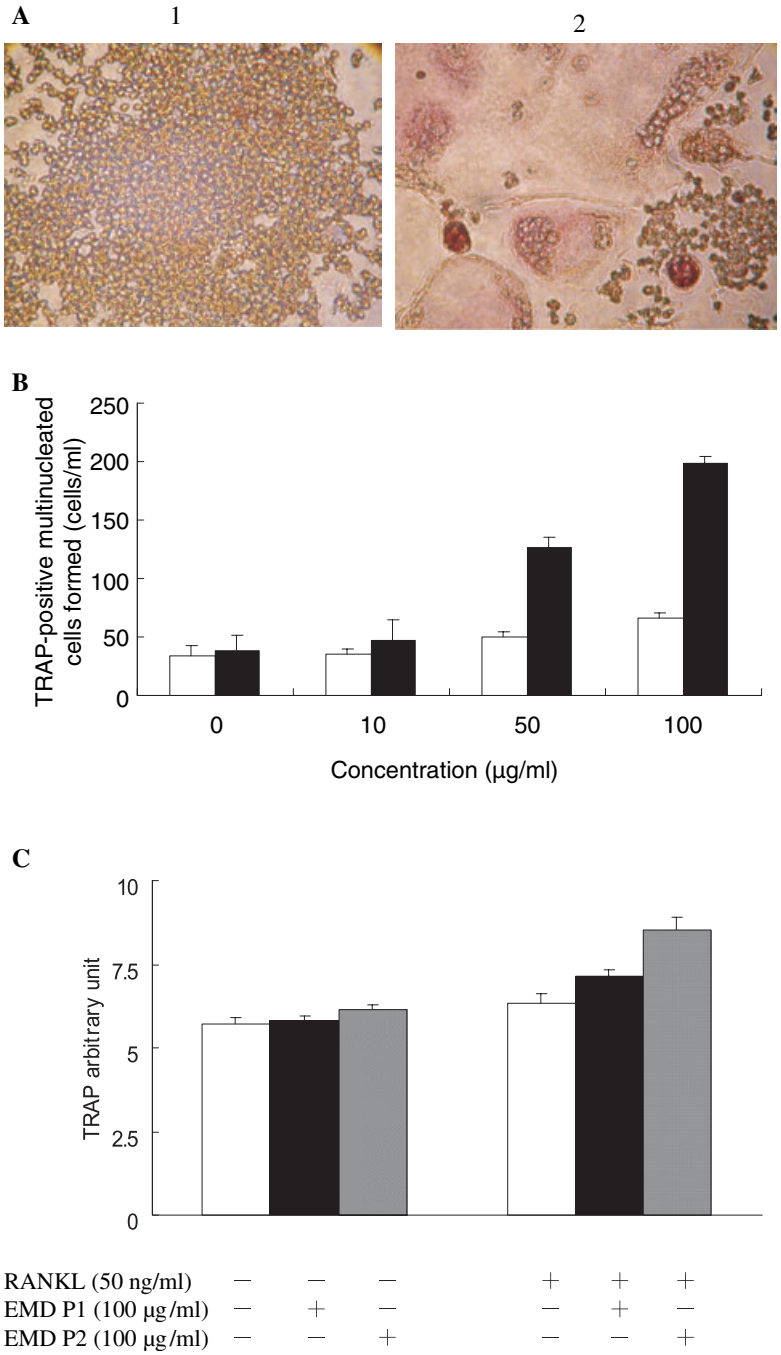

Fig. 2. Enhancement of osteoclast differentiation induced by receptor activator of nuclear factor-κB ligand (RANKL). RAW 264.7 cells were stimulated with RANKL (50 ng/ml) in the presence of enamel matrix derivative (EMD) P1 (100 μg/ml) or EMD P2 (100 μg/ml) for 8 d. (A) Images showing osteoclast formation. Image 1, no stimulant. Image 2, RANKL (50 ng/ml) and EMD P2 (100 μg/ml). (B) Number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts. White bar, RANKL (50 ng/ml) and EMD P1 (100 μg/ml). Black bar, RANKL (50 ng/ml) and EMD P2 (100 μg/ml). Data are expressed as the mean ± standard deviation of triplicate cultures. Each experiment was performed three times, with similar results obtained on each occasion. (C) RAW 264.7 cells were stimulated with RANKL (50 ng/ml), in the presence of EMD P1 (100 μg/ml) or EMD P2 (100 μg/ml), for 3 d, after which TRAP activity was determined as described in the Material and methods. Data are expressed as the mean ± standard deviation of triplicate cultures. Each experiment was performed three times, with similar results obtained on each occasion.

untreated cells. In contrast, EMD P1 had almost no effect on RANKL-induced TRAP activity in RAW 264.7 cells (Fig. 2C).

EMD P2-stimulated bone resorption induced by RANKL

To examine the effect of EMD P2 on osteoclast function, differentiated RAW 264.7 cells were cultured on Osteologic™ Multitest slides with RANKL (50 ng/ml) in the presence of EMD P1 or EMD P2 (100 μg/ml). EMD P2 enhanced the bone resorption activity of RAW 264.7 cells stimulated with RANKL, whereas EMD P1 had no such effect (Fig. 3).

Expression of RANK, ERK, and p38 in RAW 264.7 cells

We also examined the expression of RANK in RAW 264.7 cells by using immunoblot analysis. RAW 264.7 cells were cultured for 24 h in the presence or absence of RANKL, EMD P1, or EMD P2. Treatment with EMD P2 enhanced the expression level of RANK protein in RAW 264.7 cells treated with RANKL, whereas EMD P1 had no effect on the expression of RANK. Next, we examined the effects of EMD P1 and EMD P2 on the expression of ERK, phosphorylated ERK, p38, and phosphorylated p38 in RAW 264.7 cells treated with RANKL. When the cells were cultured with both RANKL and EMD P2, the phosphorylation level of ERK was higher than that in cells cultured with RANKL alone. In contrast, the total concentrations of ERK protein were not changed, even when the cells were treated with RANKL and EMD P2. We also examined the effects of EMD P2 on the phosphorylation of p38 MAPK in RAW 264.7 cells. Western blot analysis revealed that EMD P2 enhanced the RANKL-induced phosphorylation of p38 MAPK; however, there was no change in the level of phosphorylated p38 when the cells were treated with EMD P2 alone. Furthermore, the total concentration of the p38 MAPK protein in RAW 264.7 cells stimulated with RANKL

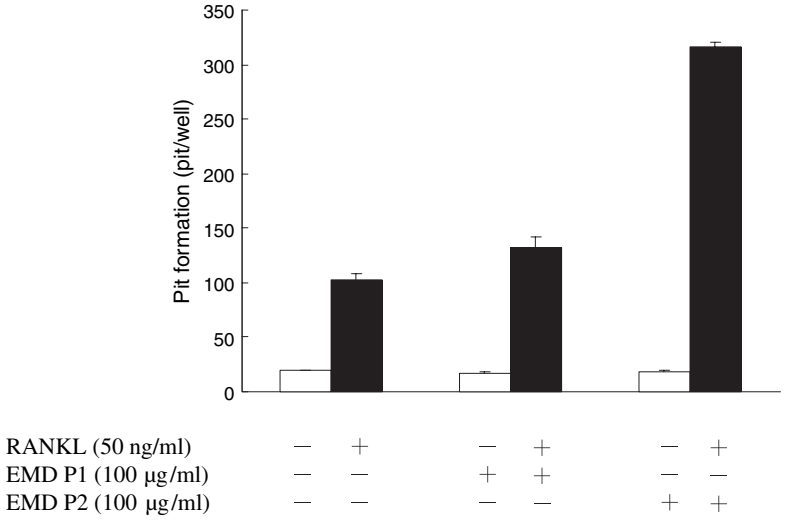

Fig. 3. Effects of enamel matrix derivative (EMD) P2 on bone resorption induced by receptor activator of nuclear factor-κB ligand (RANKL). RAW 264.7 cells were cultured with RANKL (50 ng/ml), in the presence of EMD P1 (100 μg/ml) or EMD P2 (100 μg/ml), on Osteologic™ plates, for 14 d. After removing the cells, the number of resorption pits was counted. Data are expressed as the mean ± standard deviation of triplicate cultures. Each experiment was performed three times, with similar results obtained on each occasion.

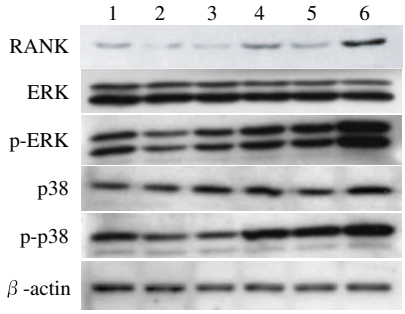

Fig. 4. Western blot analysis of RAW 264.7 cells treated with RANKL and purified enamel matrix derivative (EMD). RAW 264.7 cells were stimulated with receptor activator of nuclear factor-κB ligand (RANKL) (50 ng/ml), in the presence of EMD P1 (100 μg/ml) or EMD P2 (100 μg/ml), for 24 h, after which whole cell lysates were subjected to western blot analysis to detect the expression of receptor activator of nuclear factor-κB (RANK). To detect the expression of extracellular signal regulated kinase (ERK), phosphorylated ERK, p38, phosphorylated p38, and β-actin, RAW 264.7 cells were stimulated with RANKL (50 ng/ml), in the presence of EMD P1 (100 μg/ml) or EMD P2 (100 μg/ml), for 1 h. Lane 1, control; lane 2, EMD P1 (100 μg/ml); lane 3, EMD P2 (100 μg/ml); lane 4, RANKL (50 ng/ml); lane 5, RANKL (50 ng/ml) and EMD P1 (100 μg/ml); and lane 6, RANKL (50 ng/ml) and EMD P2 (100 μg/ml).

was unchanged, even when the cells were treated with EMD P2 (Fig. 4).

Discussion

A number of studies have reported the positive effects of EMD on periodontal regeneration, including the stimulation and differentiation of osteoblastic cells. However, scant attention has been paid to bone remodeling in regard to bone formation and resorption. In our previous study, we found that EMD promoted osteoclast formation in mouse bone marrow cultures and enhanced RANKL expression in primary osteoblastic cells, and we also clarified the effects of EMD on bone remodeling, in particular, osteoclast formation.

It is well known that EMD consists of predominantly mammalian proteins and peptides (29). However, controversy has arisen regarding whether EMD contains transforming growth factor-β (TGF-β) (4). In our previous study, we purified bioactive fractions that induced osteoclast formation in mouse bone marrow cultures by using reverse-phase HPLC and a C_{18} column, and identified fractions that induced osteoclast formation and enhanced the expression of RANKL in primary osteoblastic cells (11). Those findings indicated that EMD contains bioactive factors which induce osteoclast formation through RANK–RANKL interactions, and found no evidence of TGF-β activity in EMD P2 in an *in vitro* bioassay. It has been reported that EMD stimulates the proliferation of human periodontal ligament cells and induces matrix synthesis by periodontal fibroblasts (1–4). We have no ready explanation for the roles of EMD P1 and EMD P2 in periodontal tissue regeneration. Studies are underway to define more clearly the nature of EMD P1 and EMD P2 on the regeneration of periodontal tissue.

Osteoclasts (i.e. TRAP-positive multinucleated giant cells that resorb bone) develop from hemopoietic monocyte/macrophage lineage cells (10). Recently, the RANKL gene was cloned, and several researchers have reported that RANKL is expressed by osteoblastic cells in response to many bone-resorption mediators (15). Osteoclast progenitors have been shown to express RANK, which recognizes RANKL expressed by osteoblastic cells that then differentiate into osteoclasts in the presence of macrophage colony-stimulating factor (30). In the present study, we used murine monocytic RAW 264.7 cells to elucidate the direct effects of RANKL on osteoclast differentiation and function. This cell line is known to express RANK and to differentiate into TRAP-positive cells when cultured with RANKL (12), and the main advantage of this culture system is that it does not contain any osteoblastic/bone marrow stromal cells (28). Thus, in the present study, we used pre-osteoclastic RAW 264.7 cells to examine the effects of RANKL and purified EMD fractions on their differentiation and function.

In the present study, EMD P2 (fraction numbers 21–25) markedly inhibited the growth of RAW 264.7 cells in the presence or absence of RANKL, while it enhanced the formation of TRAP-positive multinucleated cells and TRAP

activities of RAW 264.7 cells stimulated with RANKL. On the other hand, EMD P1 (fraction numbers 15–18) showed no effect on osteoclastic cell growth and differentiation. In addition, EMD P2 markedly increased the expression of RANK protein in RAW 264.7 cells stimulated with RANKL. It is possible that EMD P2 changes RANK expression with an ultimate increase in osteoclast development. Bone resorption is a multistep process initiated by the differentiation of immature osteoclast precursors into mature osteoclasts, and degradation of the organic and inorganic phases of bone by mature resorptive cells. When being cultured with bone or dentin, osteoclasts excavate resorptive lacunae, which are similar to the structures formed when the cells degrade bone *in vivo* (31). In the present study, we used Osteologic™ slides coated with calcium phosphate substrate. As shown in Fig. 3, EMD P2 up-regulated the pit-forming activities of mature osteoclasts stimulated with RANKL. These findings suggest that EMD P2 enhances osteoclast formation by increasing the number of cells that undergo RANKL-mediated terminal differentiation to mature osteoclasts as a result of not stimulating the proliferation of monocyte/macrophage lineage cells.

MAPK family members are proline-directed serine/threonine kinases that become activated by the phosphorylation of threonine and tyrosine in response to external stimuli (17–19). MAPK family members are classified into the ERK, JNK, and p38 MAPK groups, and it is widely accepted that peptide growth factors and cytokines preferentially activate ERKs. In previous bone cell biological studies, some investigators have shown that activation of the ERK and p38 MAPK pathways plays an important role in RANKL-induced osteoclast differentiation of precursor bone marrow and RAW 264.7 cells (23,28). In the present study, RANKL-induced phosphorylation of ERK and p38 MAPK was clearly detected, and EMD P2 markedly enhanced the phosphorylation of both kinases in RAW 264.7 cells.

Our previous study demonstrated that EMD P2 induces osteoclast formation in a mouse culture system (11); however, relatively scant attention has been directed towards the differentiation and function of osteogenic cells in bone metabolism. In the present study, we found that EMD P2 enhanced osteoclast formation induced by RANKL in a RAW 264.7 cell culture system and stimulated the expression of RANK protein in those cells, indicating that EMD P2 promotes osteoclast cell formation by RANK–RANKL interactions in RAW 264.7 cells. Although there are no published reports concerning the biological effects of EMD on osteoclastogenesis or signal transduction by osteoclasts, we report here that EMD P2 enhances the phosphorylation of ERK and p38 MAPK in RAW 264.7 cells stimulated with RANKL. In addition, our data show that EMD is able to stimulate osteoclasts as well as osteoblasts, suggesting that it provides a local environment suitable for bone regeneration in periodontal tissues through bone remodeling activities, such as bone formation and resorption.

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