Absence of platelet-activating factor receptor protects mice from osteoporosis following ovariectomy

Hisako Hikiji,1,2 Satoshi Ishii,2 Hideo Shindou,2 Tsuyoshi Takato,1 and Takao Shimizu2

1Department of Oral and Maxillofacial Surgery and 2Department of Biochemistry and Molecular Biology, Faculty of Medicine, The University of Tokyo, Bunkyo, Tokyo, Japan.

Introduction

Postmenopausal osteoporosis is a condition caused principally by an acute decrease in serum estrogen levels after cessation of ovarian function. Estrogen deficiency results in an increase in bone turnover (1) and a bone-remodeling imbalance, leading to bone resorption and an increased risk of fracture. Approximately 100 million women worldwide suffer from postmenopausal osteoporosis, and therapies such as hormone replacement therapy have been widely tested as prevention methods (2–4).

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent phospholipid mediator with various biologic activities, including platelet activation, airway constriction, hypotension, and vascular permeation (5, 6) via activation of a G-protein–coupled PAF receptor (PAFR) (7, 8). It has been reported that PAF is produced in inflamed human gingival tissue (9) and detected in the arthritic joint fluids of rabbits with acute antigen-induced arthritis (10). Peripheral plasma concentrations of PAF were also noted to be high in patients suffering from hypercalcemia of malignancy (11). Thus, PAF may be implicated in diseases associated with bone resorption. Rabbit osteoclasts responded to PAF in vitro with an increase in bone pit area as well as an elevation in intracellular Ca2+ concentration ([Ca2+]i) and a transient retraction of pseudopods (12). In spite of this circumstantial evidence of PAF actions on bone metabolism, the precise role of PAF in postmenopausal osteoporosis remains unclear. By using PAFR knockout (PAFR-KO) mice, we show here the important role of PAF in the development of osteoporosis in vivo. Several in vitro data also illustrate the role of PAF as an autocrine activator of osteoclasts.

Results

Bone density in ovariectomized PAFR-KO mice. In soft x-ray radiograph analysis, radioluency of the excised femurs and tibiae was increased in ovariectomized PAFR-WT mice compared with sham-operated PAFR-WT mice (Figure 1A). However, ovariectomized PAFR-KO mice showed little increase in radiolucency. To quantitate these observations, the bone mineral density (BMD) of the entire femurs in the ovariectomized mice was compared with that of the sham-operated mice. Differences in BMD observed by dual x-ray absorptiometry (DXA) measurements were significant between the ovariectomized group and sham-operated group in PAFR-WT mice (Figure 1B). DXA analysis also demonstrated that the BMD of the femurs in ovariectomized PAFR-KO mice was comparable to that in sham-operated PAFR-KO mice. There were no differences in the levels of Ca, phosphate, or parathyroid hormone in the sera of both genotypes, either with or without ovariectomy (data not shown).

Bone histomorphometry in ovariectomized PAFR-KO mice. It was apparent from the histologic observations that the bone volume in the metaphyseal region of the tibiae was reduced in ovariectomized PAFR-WT mice, but ameliorated in ovariectomized PAFR-
KO mice, compared with sham-operated mice (Figure 2A). Histomorphometric analysis of the metaphyseal region in the tibial bone confirmed these results (Figure 2B). Trabecular bone volume (BV/TV) was significantly reduced in PAFR-WT mice, but not in PAFR-KO mice, by ovariectomy. Other indices related to BV/TV, trabecular number (Tb.N), and trabecular separation (Tb.Sp) also indicated that the bone volume of ovariectomized PAFR-KO mice was amended to the level of the sham-operated PAFR-KO mice. The reduction in bone mass in PAFR-WT mice was associated with an increase in bone turnover because osteoid thickness (O.Th) and osteoclast surface per bone surface (Oc.S/BS) mice were increased significantly in ovariectomized PAFR-WT relative to sham-operated PAFR-WT mice.

Acetyl-CoA:lyso-PAF acetyltransferase activity and cytosolic phospholipase A₂ expression in bone cells. To understand the cell lineages that have the potential for PAF synthesis in bone tissues, the activity of acetyl-CoA:lyso-PAF acetyltransferase (lyso-PAF acetyltransferase), which catalyzes the final reaction for PAF synthesis in the remodeling pathway (13), was measured in cultured bone cells (Figure 3A). Osteoclasts derived from RAW 264.7 mouse macrophage cells,
spleen-derived osteoclasts, and bone marrow–derived osteoclasts had high lyso-PAF acetyltransferase activities. Treatment with TNF-α and IL-1β increased enzyme activities significantly in both spleen- and bone marrow–derived osteoclasts. On the other hand, MC3T3-E1 mouse osteogenic cells and primary mouse osteoblasts displayed significantly lower activities than osteoclasts, even after cytokine stimulation. Western blot analysis showed that primary osteoclasts expressed higher amounts of cytosolic phospholipase A₂ (cPLA₂) than the osteoblasts (Figure 3B); cPLA₂ is an important PLA₂ for lyso-PAF production in the remodeling pathway (14, 15).

Expression of PAF receptor in bone cells. To analyze cell lineages expressing PAFR mRNA in bone tissues, Northern blot analysis was performed in cultured bone cells (Figure 4). Osteoclasts derived from RAW 264.7 cells, spleen-derived osteoclasts, and bone marrow–derived osteoclasts accumulated significant amounts of PAFR mRNA. Consistently, PAF raised the [Ca²⁺] level in spleen-derived osteoclasts of PAFR-WT mice (Figure 5A) but not in those of PAFR-KO mice (Figure 5B). In contrast, MC3T3-E1 osteoblastic cells and primary mouse osteoblasts expressed much smaller amounts of PAFR mRNA (Figure 4). Even after cytokine stimulation, PAFR mRNA expression was not induced in MC3T3-E1 cells. Furthermore, in the primary osteoblasts, PAFR activation neither elevated [Ca²⁺], (Figure 5C) nor induced receptor activator of NF-κB ligand (RANKL) mRNA expression (Figure 5D).

Effects of PAFR on osteoclast survival and Ca resorption. Purified mature osteoclasts readily underwent apoptosis (16, 17). Thus, the effect of PAFR stimulation on the apoptosis of osteoclasts was examined using methylcarbamoyl PAF (mc-PAF), a nonhydrolyzable agonist of PAFR. Although a number of osteoclasts promptly became apoptotic after purification, 1 μM of mc-PAF enhanced survival (Figure 6A). The administration of 1 μM PAF also had a survival effect on the osteoclasts but was less potent than 1 μM mc-PAF. This is possibly because PAF is degraded by acetylhydrolases during the 24-hour incubation in serum (18). WEB 2086, an antagonist of PAFR, inhibited the stimulatory effects of mc-PAF on the survival of osteoclasts (Figure 6B). M-CSF is well known to induce the survival of osteoclasts (19). Simultaneous administration of mc-PAF and M-CSF increased the osteoclast survival rate more than a sole administration of mc-PAF or M-CSF (Figure 6B). The stimulatory effects of mc-PAF on the survival of osteoclasts were not found in osteoclasts of PAFR-KO mice (Figure 6C). IL-1β also enhanced the survival of osteoclasts and their Ca resorptive activity, and these parameters were significantly suppressed by WEB 2086 treatment (Figure 7, A and B) and genetic PAFR deficiency (Figure 7, C and D).

Next, we examined the effect of PAFR on IL-1β–mediated bone resorption in an organ culture system of parietal bones.

Figure 3
Lyso-PAF acetyltransferase activity and cPLA₂ expression in bone cells. (A) Lyso-PAF acetyltransferase activity. MC3T3-E1 mouse osteogenic cells and primary mouse osteoblasts (gray bars) had low lyso-PAF acetyltransferase activities even after stimulation with TNF-α and IL-1β as designated by (+). Osteoclasts derived from RAW 264.7 cells, spleen-derived osteoclasts, and bone marrow–derived osteoclasts (black bars) had significantly higher lyso-PAF acetyltransferase activities compared with osteoblasts. In addition, cytokine stimulation, denoted by (+), raised the activities. Values are mean ± SD measured in triplicate or quadruplicate. Similar results were obtained in another experiment. *P < 0.05 vs. primary osteoblasts; †P < 0.05 vs. unstimulated spleen-derived osteoclasts; †P < 0.05 vs. unstimulated bone marrow–derived osteoclasts. (B) Western blot analysis of cPLA₂. The cell lysates (16 μg of protein) were subjected to SDS-PAGE and immunoblotted with an anti-mouse cPLA₂ monoclonal antibody. Spleen-derived osteoclasts and bone marrow–derived osteoclasts expressed higher amounts of cPLA₂ than primary osteoblasts. Similar results were obtained in another experiment.

Figure 4
Northern blot analysis of PAFR mRNA in bone cells. MC3T3-E1 cells and primary mouse osteoblasts expressed little PAFR mRNA. Even after stimulation with TNF-α and IL-1β, MC3T3-E1 cells did not increase mRNA expression as denoted by (+). On the contrary, RAW 264.7 cells, osteoclasts derived from RAW 264.7 cells, spleen-derived osteoclasts, and bone marrow–derived osteoclasts had high amounts of PAFR mRNA. Total RNA (10 μg) was applied in each lane. Similar results were obtained in another experiment.
Semiquantitative grading of the cultured parietal bones revealed an increase in the degree of osteoclastic bone resorption after IL-1β treatment. In line with the results of the bone cell experiments described above (Figure 7), both WEB 2086 treatment and genetic ablation of PAFR conferred resistance to osteoclastic resorption in the cultured bones (Figure 8).

Discussion

This study is the first to demonstrate the critical effects of PAF on bone resorption in a murine model of postmenopausal osteoporosis (Figure 1). Markedly ameliorated bone resorption after ovariectomy in PAFR-KO mice suggests that PAF enhances bone resorption through a receptor. Ovariectomy did not affect indices related to bone volume (BV/TV, Tb.N, and Tb.Sp) or those related to bone turnover (O.Th and Oc.S/BS) in PAFR-KO mice (Figure 2). Prevention of increased O.Th may result from restrained bone turnover. PAFR deficiency did not affect bone turnover in mice with an intact ovarian function, as indicated by observations that sham-operated PAFR-KO mice showed similar bone volume and bone turnover to sham-operated PAFR-WT mice. Therefore, it is unlikely that PAF is required for normal trabecular bone metabolism.

It is interesting to note that PAFR deficiency completely prevented osteoporotic bone resorption. The prevention of bone loss observed after ovariectomy was reported in mice lacking TNF-α (20), IL-1 receptor (21), osteopontin (22), iNOS (23), and histidine decarboxylase (3). Therefore, these molecules, including PAFR, seem necessary, but not sufficient, for bone resorption following ovariectomy, because a deficiency in a single molecule entirely rescued the phenotype. As PAF is activated by oxidized phospholipids (6, 24), these ligands may also be involved in bone resorption.

PAF is synthesized via two major pathways: the de novo pathway and the remodeling pathway (13). In response to extracellular stimuli, cells produce PAF via the remodeling pathway involving PLA2 and lyso-PAF acetyltransferase. We previously reported that, among at least 19 isozymes, cPLA2α played a dominant role in the production of PAF in Ca ionophore–stimulated murine peritoneal exudate cells (14, 15). In the present investigation, we showed that osteoclasts had more prominent cPLA2α expression and higher lyso-PAF acetyltransferase activity compared with osteoblasts (Figure 3). These data indicate that PAF is produced mainly by osteoclasts in response to extracellular stimuli. However, the PAF production is below the detection limit in osteoclasts (data not shown), possibly because only a small number of osteoclasts were available, and PAF is rapidly degraded by acetylhydrolase enzyme in the culture system.

Estrogen is considered to preserve bone mass through the modulation of bone cell lifespan (25) and to decrease cytokine-
These results suggest that increased levels of TNF-α and IL-1β secretion of these cytokines (30, 31). We showed here that TNF-α, RANKL stimulates osteoclast bone-resorbing activities without the activation of osteoblast/stromal cells (33, 34). While osteoblasts expressed a small amount of PAFR mRNA, osteoclasts expressed high levels (Figure 4). Consistent with the mRNA expression profiles, the levels of [Ca^2+]i was elevated in PAFR-WT osteoclasts in response to PAF; no Ca response was observed in osteoblasts or PAFR-KO osteoclasts (Figure 5, B and C). In addition, mc-PAF had no effect on the mRNA expression of RANKL in osteoblasts (Figure 5D). Thus, PAF may be a novel factor that acts on osteoclasts in an autocrine/paracrine manner without affecting osteoblasts.

We demonstrated that PAF has a survival effect on osteoclasts through PAFR (Figure 6). The survival of mature osteoclasts relies on several factors, such as RANKL, IL-1, TNF-α, and M-CSF (35). Furthermore, TNF-binding protein and decoy IL-1 receptor reduced bone loss due to estrogen deficiencies (36, 37). Several intracellular signaling pathways, including the activations of extracellular signal regulated kinase (16, 38), NF-κB (39), and PI3K (16, 40) have been shown to be involved in the survival of osteoclasts. PAF is reported to activate these signaling molecules in several cell lines (7) and to evoke Ca responses in osteoclasts (41). While the intracellular signaling pathways of PAFR that regulate cell survival in osteoclasts remain to be elucidated, PAF is likely to affect the resorptive activity by increasing osteoclast number.

We do not have a clear explanation for the data that 1 μM mc-PAF or PAF was required for the survival assay (Figure 6A). A 24-hour incubation may result in the chemical and enzymic degradation of the compounds. Nevertheless, these effects are receptor-mediated processes as they were not observed in WEB 2086–treated or PAFR-KO cells (Figure 6, B and C). It is of interest that both treatment with PAFR antagonist and the genetic ablation of PAFR reduced survival rates of IL-1β–stimulated osteoclasts (Figure 7, A and C). These data suggest that IL-1β promotes the production of PAF, which in turn increases osteoclast survival through PAFR.

It is possible that PAF enhances osteoclastogenesis, osteoclast recruitment, and resorptive activity in vivo because positive effects of PAF on osteoclast motility and resorptive activity in vitro have been reported (12, 41). Our study indeed demonstrated that WEB 2086 treatment and PAFR deficiency reduced the stimulatory effect of IL-1β on Ca resorption in the coculture system of both osteoclasts and osteoblasts (Figure 7, B and D). Therefore, PAFR activation may lead to enhancement of osteoclastic bone resorptive activity and/or osteoclast motility in addition to prolongation of cell survival. As to osteoclastogenesis, we were not able to detect any stimulatory effects of PAF on RAW 264.7 cells or cocultured bone marrow cells (data not shown). Moreover, there is a possibility of an indirect mechanism through which PAFR may alter osteoclastic resorption. For example, PAF may act on other bone marrow cells, for example, the B lymphocyte lineage to induce osteoclastogenesis.

To establish an experimental link between the phenotypes of ovariecetomized mice and the in vitro data from isolated bone cells, the organ culture experiments were conducted with parietal bones. Consequently, we found that PAFR mediated the IL-1β–stimulated bone resorption (Figure 8), the data further supporting a role for the PAF-PAFR system in the exacerbation of osteoporosis.

In conclusion, we propose a model for PAF action in bone resorption (Figure 9). Withdrawal of estrogen after ovarian dysfunction increases the levels of cytokines such as TNF-α and IL-1 in blood and bone marrow, as described previously (30, 31, 42). These cytokines appear to enhance PAF production in osteoclasts by activating lyso-PAF acetyltransferase. Thus, PAF activates PAFR on osteoclasts in an autocrine/paracrine manner and affects osteoclastic cell functions including cell survival, cell motility, and...
bone-resorption activity. The autocrine/paracrine action of PAF on osteoclasts is noteworthy because PAF could be a candidate for targeting the prevention of osteoporosis without affecting osteoblasts. Furthermore, these findings suggest that PAF inhibition would be beneficial for diseases such as periodontitis, arthritis, and hypercalcemia of malignancy, in which PAF is abundantly produced (9–11, 43).

Methods
Materials. αMEM was purchased from Invitrogen Corp. (Carlsbad, California, USA) and FBS from Bioserum (Victoria, Australia). A monoclonal antibody directed against mouse cPLA$_2$ (catalog no. sc-454) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA), and peroxidase-conjugated anti-mouse IgG antibody from Amersham Biosciences Corp. (Piscataway, New Jersey, USA). Recombinant murine M-CSF and murine soluble RANKL were from Techne Inc. (Burlington, New Jersey, USA) and PeproTech (London, United Kingdom), respectively. Recombinant human TNF-α and human IL-1β were from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan) and Techne Inc., respectively. Bac- terial collagenase from Clostridium histolyticum and 1,25-(OH)$_2$D$_3$ were purchased from Wako Pure Chemicals Industries Inc. (Osaka, Japan). Dispase and collagen gel (cell matrix type IA) were from Nitta Gelatin Inc. (Osaka, Japan). [3H]Acetyl-coenzyme A (acetyl-CoA), 185 GBq/mmol, was from Amersham Biosciences. PAF (C16) and 1-O-hexadecyl-sn-glycero-3-phosphocholine (lyso-PAF, C16) were from Cayman Chemical (Ann Arbor, Michigan, USA). WEB 2086 was a generous gift from Boehringer Ingelheim GmbH (Ingelheim, Germany). Bondapak C-8 (dimethyloctylsilyl-bonded amorphous silica, 37–55 μm) was from Millipore Corp. (Bedford, Massachusetts, USA). Naphtol AS-MX phosphate was purchased from Sigma-Aldrich (St. Louis, Missouri, USA), and dissolved in N,N-dimethylformamide from Sigma-Aldrich. Other chemicals were of analytical grade.

Mice. All animal studies were conducted in accordance with the guidelines for animal research at The University of Tokyo and were approved by the University of Tokyo Ethics Committee for Animal Experiments. PAFR-KO mice were established using a gene-targeting strategy (44). PAFR-KO mice and the corresponding PAFR-WT mice have been backcrossed for ten generations onto a C57BL/6N genetic background. Mice were fed with a standard laboratory diet and water ad libitum. Mice aged 8–11 weeks old were used. PAFR-KO mice had body weights (mean ± SD = 18.8 ± 2.4 g, n = 9) indistinguishable from those of PAFR-WT mice (17.2 ± 2.0 g, n = 11). C57BL/6N mice from Charles River Japan (Tokyo, Japan) were also used for in vitro studies.

Ovariectomy. Age-matched PAFR-KO and PAFR-WT female mice received a bilateral ovariectomy or a sham procedure in which the ovaries were exteriorized but not removed. Mice were sacrificed 4 weeks after the surgical procedure.

Radiographic analysis. Radiographs of the femurs and tibiae were taken by soft x-ray (model CMB-2; SOFTEX Co. Ltd., Tokyo, Japan). The BMD of the femurs was measured by DXA (model DCS-600R; ALOKA Co. Ltd., Tokyo, Japan). The BMD was calculated by dividing the bone mineral content of the measured area by the area.

Figure 8
Effect of PAFR on osteoclastic resorption stimulated by IL-1β. (A) Radiographic images of parietal bones cultured in the presence of IL-1β (0.3 ng/ml) and/or WEB 2086 (100 μM). Scale bar: 1.0 mm. (B) and (C) Assessments of osteoclastic bone resorption. The resorption levels of the cultured parietal bones were semiquantitatively scored as described in Methods, and scores for individual bone tissues are presented in each graph. Bars depict means of groups. *P < 0.0001 vs. control; #P < 0.005 vs. IL-1β–treated osteoclasts in (B) or IL-1β–treated PAFR-WT osteoclasts in (C).

Figure 9
Proposed model of PAF action in bone resorption. For details, see Discussion.
Histomorphometry. Tibiae were fixed with 70% ethanol and embedded in glycol methacrylate, and undecalcified 3-μm sections were prepared. The sections were stained with toluidine blue and subjected to histomorphometric analyses using a light microscope with a micrometer and an image analyzer (Osteoplan II; Carl Zeiss Japan, Tokyo, Japan). BV/TV (%, Tb.N (per mm), Tb.Sp (μm), O.Th (μm), and Oc.S/BS (%) were measured in an area 1.2 mm in length from 0.3 mm below the growth plate at the proximal metaphysis of the tibiae. The quantitative histomorphometric analyses were performed in a blinded fashion.

Osteoclast culture. Calvariae dissected from 1-day-old mice were washed in PBS and digested with 0.1% collagenase and 0.2% dispase for 10 min five times, and cells from fractions 2–5 were pooled and cultured for 7 days in αMEM containing 10% FBS. MC3T3-E1 mouse osteogenic cells (RIKEN, Saitama, Japan) were also cultured in αMEM containing 10% FBS. BMD3T3-E1 mouse osteogenic cells (RIKEN, Saitama, Japan) were also cultured in αMEM containing 10% FBS. 

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Measurement of lys-PAF acetyltansferase activity. The collected cells, in a buffer containing 20 mM Tris-HCl (pH 7.4), 0.3 M sucrose, 5 mM 2-mercaptoethanol, 20 μM 4-aminophenylmethanesulfonyl fluoride (APMSF, Sigma-Aldrich) and 1× Complete (a protease inhibitor mixture; Roche Diagnostics GmbH, Mannheim, Germany), were sonicated and centrifuged at 800 g for 10 min. The supernatant was used for the enzyme reaction, which was performed in a 96-well round-bottom microplate (no. 25850; Corning Inc., Corning, New York, USA) in a water bath at 37°C. The reaction mixture contained 20 mM Tris-HCl (pH 7.4), 5 mM 2-mercaptoethanol, 3 mM CaCl2, 20 μM APMSF, 1 mg/ml phosphatidylcholine (Sigma-Aldrich), and 100 μM [3H]acetyl-CoA (1.11 GBq/mmol) with or without 20 μM lys-PAF in a total volume of 0.1 ml. After a 15-min incubation, 0.1 ml of methanol was added to terminate the reaction. The radioactivity of Bondapack C-8 resin (2 mg)-adsorptive products were measured according to the method of Kume et al. (49), except for washing the resin one time before and eight times after application of the reaction mixture with 50% methanol in 20 mM Tris-HCl (pH 7.4). The enzyme activity was estimated by the increase in the radioactivity due to the addition of lys-PAF to the reaction mixture.

Western blot analysis. Cells were lysed with a buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 0.25 M sucrose, and 1× Complete. The protein concentration in the cell lysate was measured using a Protein Assay Kit (Bio-Rad Laboratories, Hercules, California, USA) using BSA (fraction V, fatty acid-free; Sigma-Aldrich) as a standard. Sixteen micrograms of protein was separated by 8% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After blocking with Block Ace (Dainippon Pharmaceutical Co. Ltd.), the membrane was incubated with a mouse monoclonal antibody against cPLA2α and then with peroxidase-conjugated anti-mouse IgG antibody. Immunoreactive proteins were visualized by the ECL chemiluminescence reaction (Amersham Biosciences) following the manufacturer's instructions.

Osteoclast survival assay. A coculture system of mouse osteoblasts and bone marrow cells has been developed to examine the regulatory mechanisms of osteoclast function (46, 52). Removal of osteoblast/stromal cells from cocultures induces apoptosis of osteoclasts (19, 52). Mouse osteoblasts (1×10⁶ cells/dish) and bone marrow cells (2×10⁶ cells/dish) from femurs and tibiae of 7- to 8-week-old C57BL/6J mice were prepared as described above and were cocultured on 10-cm culture dishes coated with 0.24%
collagen gel matrix. The culture medium, αMEM with 10% FBS, 100 nM 1,25-(OH)₂D₃, and 1 μM PGE₂, was changed every 2–3 days. After 7 days of culture, dishes were treated with 4 ml of 0.2% collagenase in αMEM for 20 min at 37°C, and the collected cells were suspended in αMEM containing 10% FBS. An aliquot of the crude osteoclast preparation was cultured in 24-well plates. After incubation for 2 hours, the plates were treated with PBS containing 0.1% collagenase and 0.2% dispase to remove osteoblast/stromal cells. After the purification procedure, 88% of the total cells were osteoclasts and 97% were TRAP positive. The purified osteoclasts were cultured with mc-PAF (1 μM), M-CSF (100 ng/ml), or IL-1β (1 ng/ml) for 24 hours in the presence or absence of WEB 2086 (10 μM). To calculate the survival rate, the numbers of surviving osteoclasts were counted under light microscopy (BH-2; Olympus, Corp.) before and after the incubation.

Ca resorption assay. An aliquot of cocultured osteoblast/osteoclast preparation with αMEM containing 10% FBS (0.25 ml) was transferred into a CaPO₄-coated dish (BioCoat Osteologic bone culture system; BD Biosciences, Bedford, Massachusetts, USA). After 48-hour culture with 0.3–1.0 ng/ml IL-1β and/or 10 μM WEB 2086, cells were removed with a bleach solution (6% NaOCl and 5.2% NaCl). The dishes were washed with water and photographed under light microscopy (BH-2; Olympus). The resorbed area (mm²) with cleared CaPO₄ resorption pits was measured using Photoshop 7.0 software (Adobe Systems Inc., San Jose, California, USA).

Organ culture of parietal bone. The bone organ culture was performed as described previously (53). Briefly, paired parietal bones from 1-day-old mice were aseptically harvested and cultured for 24 hours in 2 ml BGJ medium (Invitrogen Corp.) containing 1 ml/mg BSA (fraction V; Serological Proteins Inc., Kankakee, Illinois, USA). Then, each half parietal bone was transferred to fresh culture medium containing 0.3 ng/ml IL-1β and/or 100 μM WEB 2086, and cultured for an additional 2 days with a medium change every 48 hours. To detect bone resorption, parietal bones were stained with cleared CaPO₄ and their resorption area was measured under light microscopy (BH-2; Olympus). The resorption rate (μM) was calculated as the sum of the two scores.

Address correspondence to: Satoshi Ishii, Department of Biochemistry and Molecular Biology, Faculty of Medicine, The University of Tokyo, Hongo 7-3-1, Bunkyo, Tokyo 113-0033, Japan. Phone: 81-3-5802-2925; Fax: 81-3-3813-8732; E-mail: mame@m.u-tokyo.ac.jp.