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p38 MAPK-Mediated Signals Are Required for Inducing Osteoclast Differentiation But Not for Osteoclast Function

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Receptor activator of nuclear factor-κB ligand (RANKL)-induced signals play critical roles in osteoclast differentiation and function. SB203580, an inhibitor of p38 MAPK, blocked osteoclast formation induced by 1α,25-dihydroxyvitamin D_3 and prostaglandin E_2 in cocultures of mouse osteoblasts and bone marrow cells. Nevertheless, SB203580 showed no inhibitory effect on RANKL expression in osteoblasts treated with 1α,25-dihydroxyvitamin D_3 and prostaglandin E_2. RANKL-induced osteoclastogenesis in bone marrow cultures was inhibited by SB203580, suggesting a direct effect of SB203580 on osteoclast precursors, but not on osteoblasts, in osteoclast differentiation. However, SB203580 inhibited neither the survival nor dentine-resorption activity of osteoclasts induced by RANKL. Lipopolysaccharide (LPS), IL-1, and TNFα all stimulated the survival of osteoclasts, which was not inhibited by SB203580. Phosphorylation of p38 MAPK was induced by RANKL, IL-1, TNFα, and LPS in osteoclast precursors but not in osteoclasts. LPS stimulated phosphorylation of MAPK kinase 3/6 and ATF2, upstream and downstream signals of p38 MAPK, respectively, in osteoclast precursors but not in osteoclasts. Nevertheless, LPS induced degradation of IκB and phosphorylation of ERK in osteoclasts as well as in osteoclast precursors. These results suggest that osteoclast function is induced through a mechanism independent of p38 MAPK-mediated signaling. (*Endocrinology* 143: 3105–3113, 2002)

O STEOCLASTS, MULTINUCLEATED CELLS responsible for bone resorption, develop from hemopoietic cells of the monocyte-macrophage lineage under the control of bone microenvironment (1–4). A coculture system of mouse osteoblasts and hemopoietic cells was developed to examine the regulatory mechanisms of osteoclast differentiation and function (5, 6). A series of experiments using the coculture system have shown that osteoblasts are critically involved in osteoclast development (7, 8). Studies of macrophage colony-stimulating factor (M-CSF)-deficient op/op mice showed that M-CSF produced by osteoblasts is an essential factor for osteoclastogenesis (9–11). Recently, the gene for another essential factor for osteoclastogenesis, receptor activator of nuclear factor-κB ligand (RANKL), was cloned (12–16). RANKL is a new member of the TNF-ligand family and is expressed by osteoblasts in response to many bone-resorption-related factors. Osteoclast precursors that express RANK, a TNF receptor family member, recognize RANKL expressed by osteoblasts and differentiate into osteoclasts in the presence of M-CSF (1–4, 16). Osteoprotegerin (OPG), which is produced by many types of cells, including osteoblasts, is a soluble decoy receptor for RANKL, thus inhibiting osteoclastogenesis *in vivo* and *in vitro* (17–19).

The cytoplasmic tail of RANK interacts with TNF-associated factor (TRAF)1, TRAF2, TRAF3, TRAF5, and TRAF6 (20–23). Among these TRAFs, TRAF6 seems to play important roles in osteoclast differentiation and function (24–26). Recent studies have shown that lipopolysaccharide (LPS) and inflammatory cytokines such as TNFα and IL-1 regulate osteoclast differentiation and function independently of the RANKL-RANK interaction (27–30). It was also shown that Toll-like receptor 4 (TLR4) is a receptor of LPS, and the signaling cascade of TLR4 is quite similar to that of IL-1 receptors, both of which use TRAF6 as a common signaling molecule (31–33). Thus, TRAF6-mediated signals seem to play central roles in the regulation of osteoclast differentiation and function.

Mice deficient in both p50 and p52 subunits of nuclear factor-κB (NF-κB) develop severe osteopetrosis (34, 35). Mice lacking c-Fos also develop osteopetrosis (36, 37). RANK-mediated signals have been shown to activate NF-κB and c-Jun N-terminal kinase (JNK) in the target cells, including osteoclasts (15, 38). The dimeric transcription factor, activator protein-1 (AP-1), is composed of Fos proteins and Jun proteins. These results suggest that NF-κB- and AP-1-mediated signals play important roles in osteoclast differentiation induced by RANKL.

MAPK family members, which are proline-directed

serine/threonine kinases, function in various signaling cascades, including TRAF-mediated ones (39, 40). MAPK family members are classified into three groups: the ERK, JNK, and p38 MAPK groups. p38 MAPK was originally identified as the target of pyridinylimidazole compounds that inhibit the production of inflammatory cytokines in monocytes (41). Phosphorylation of p38 MAPK by MAPK kinase (MKK) 3/6 results in the activation of p38 MAPK. Activated p38 MAPK then phosphorylates transcription factor ATF2, which, in turn, induces transcription of the target genes (39, 40). It was shown that the expression of dominant-negative forms of p38 MAPK and MKK 6 in RAW264 cells inhibited RANKL-induced differentiation of RAW264 cells into osteoclasts (42). Pyridinylimidazole SB203580, a specific inhibitor of p38 MAPK (43), has been widely used to investigate the roles of p38 MAPK in the regulation of cell differentiation and function (39, 40, 44). Using SB203580, p38 MAPK-mediated signals were shown to be involved in osteoclastic bone resorption induced by IL-1 and TNFα in fetal rat long bones (44). These results suggest that p38 MAPK-mediated signals regulate osteoclast differentiation or function, or both.

In the present study, we explored the roles of p38 MAPK-mediated signals in the differentiation, survival, and activation of osteoclasts. We found that p38 MAPK-mediated signals were essential for RANKL-induced osteoclast differentiation, but not for RANKL-induced osteoclast function. LPS, IL-1, TNFα, and RANKL all stimulated the survival of osteoclasts, but these factors failed to induce phosphorylation of p38 MAPK in osteoclasts. These experimental results suggest that osteoclast function is regulated through a mechanism involving TRAF6 but independent of p38 MAPK-mediated signals.

Materials and Methods

Animals and chemicals

Five- to 8-wk-old male ddY mice and newborn ddY mice were obtained from Shizuoka Laboratories Animal Center (Shizuoka, Japan). All procedures for animal care were approved by the Animal Management Committees of Matsumoto Dental University and Showa University. Recombinant human M-CSF (leukoprol) was obtained from Kyowa Hakko Kogyo Co. (Tokyo, Japan). Recombinant soluble RANKL was purchased from PeproTeck EC Ltd. (London, UK). Mouse TNFα and IL-1 were obtained from Genzyme/Techne (Minneapolis, MN). 1α,25-Dihydroxyvitamin D$_3$ [1α,25-(OH)$_2$D$_3$], and prostaglandin E$_2$ (PGE$_2$) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Human PTH (1–34) was obtained from Peptide Institute (Osaka, Japan). SB203580 was purchased from Calbiochem (La Jolla, CA). LPS was purified from *Escherichia coli* strain K235 as described previously in our laboratory (45). Eel calcitonin was kindly provided by Asahi Chemical Industry Co. (Tokyo, Japan). ^{45}CaCl$_2$ was obtained from Amersham International (Buckinghamshire, UK). Anti-phospho-p38 MAPK and p38 MAPK rabbit polyclonal antibodies, anti-phospho-MKK3/6 and MKK3 rabbit polyclonal antibodies, anti-phospho-ATF-2 and ATF-2 rabbit polyclonal antibodies, anti-phospho-ERK and ERK rabbit polyclonal antibodies, and anti-IκB-α rabbit polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Specific PCR primers for mouse RANKL, OPG, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by Life Technologies, Inc. (Tokyo, Japan). Other chemicals and reagents were of analytical grade.

Osteoclast differentiation assay

Bone marrow cells were obtained from tibiae of 5- to 8-wk-old adult mice. Primary osteoblasts were prepared from calvariae of newborn ddY

mice as previously described (46). Mouse bone marrow cells (1.5 × 10^5 cells/well) and primary osteoblasts (3 × 10^3 cells/well) were cocultured for 7 d in the presence of 1α, 25-(OH)$_2$D$_3$ (10^{-8} M) and PGE$_2$ (10^{-7} M) in αMEM (Sigma, St. Louis, MO) supplemented with 10% FBS (JRH Biosciences, Lenexa, KS) in 48-well plates (46). Some cocultures were treated with SB203580 at 10^{-7} M or 10^{-6} M. In other experiments, bone marrow cells (1.5 × 10^5 cells/well) were cultured for 7 d with RANKL (200 ng/ml) and M-CSF (100 ng/ml) in 48-well plates in the presence or absence of SB203580 at 10^{-7} M or 10^{-6} M. Cells were then fixed and stained for tartrate-resistant acid phosphatase (TRAP; a marker enzyme of osteoclasts) as described (46). TRAP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclasts, under microscopic examination. The results were expressed as the means ± SEM of three cultures.

PCR amplification of reverse-transcribed mRNA

For semiquantitative RT-PCR analysis, osteoblasts were cultured in αMEM containing 10% FBS with 1α, 25-(OH)$_2$D$_3$ (10^{-8} M) and PGE$_2$ (10^{-7} M), with or without SB203580 (10^{-6} M), on 100-mm-diameter dishes. After cells were cultured for 48 h, total cellular RNA was extracted from osteoblasts using TRIzol solution (Life Technologies, Inc.). First-strand cDNA was synthesized from total RNA with random primers and subjected to PCR amplification with EX *Taq* polymerase (Takara Biochemicals, Shiga, Japan) using specific PCR primers: mouse RANKL, 5'-CGCTCTGTTC CTGTACTTTCGAGCG-3' (forward, nucleotides 195–219) and 5'-TCGTGCTCCCTCCTTTCATCAGGTT-3' (reverse, nucleotides 757–781); mouse OPG 5'-CAGAGACTAATAGATCAAAG-GCAGG-3' (forward, nucleotides 135–159) and 5'-ATGAAGTCTCAC-CTGAGAAGAACC-3' (reverse, nucleotides 742–765); and mouse GAPDH, 5'-ACCACAGTCCATGCCATCAC-3' (forward, nucleotides 566–585) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse, nucleotides 998-1017). The PCR products were separated by electrophoresis on 2% agarose gels and were visualized by ethidium bromide staining with UV light illumination. The sizes of the PCR products for mouse RANKL, OPG, and GAPDH were 587 bp, 380 bp, and 452 bp, respectively.

Survival assay of mature osteoclasts

Osteoblasts (1.5 × 10^6 cells) and mouse bone marrow cells (10^7 cells/dish) were cocultured in αMEM supplemented with 10% FBS, 1α,25-(OH)$_2$D$_3$ (10^{-8} M), and PGE$_2$ (10^{-7} M) in 100-mm-diameter dishes precoated with type I collagen gel (cell matrix type-IA; Nitta Gelatin, Inc., Osaka, Japan) (45, 46). Osteoclasts were formed within 7 d in the coculture, and all cells were removed from the dishes by treatment with 0.1% collagenase (Wako Pure Chemical Industries Ltd.). The purity of osteoclasts in this preparation was about 5% (47). The crude osteoclast preparation was replated in culture dishes. After the cells were cultured for 6–8 h, osteoblasts were removed by treatment with 0.05% trypsin and EDTA for 5 min (Life Technologies, Inc.) (46). The purity of osteoclasts in this preparation was about 95%. The purified osteoclasts were further cultured for 48 h with vehicle (control), RANKL (200 ng/ml), M-CSF (100 ng/ml), LPS (2 μg/ml), IL-1 (10 ng/ml), or TNFα (10 ng/ml) in the presence or absence of SB203580. In experiments in which osteoclasts were treated with SB203580 together with those factors, the cells were pretreated for 30 min with SB203580 alone. After the cells were cultured for 48 h, they were fixed and stained for TRAP. Preliminarily experiments showed that pretreatment of TRAP-positive MNCs with 0.05% trypsin and EDTA for 5 min does not seem to affect their survival induced by RANKL or M-CSF. TRAP-positive MNCs containing more than three nuclei were counted as viable osteoclasts.

Pit formation assay by osteoclasts

For the resorption pit assay, aliquots of the crude osteoclast preparations, described above, were placed on dentine slices that had been placed in 96-well plates (46). After preincubation for 1 h, dentine slices were transferred to 48-well plates (1 dentine slice/well) containing 0.3 ml αMEM containing 10% FBS, and they were further cultured with or without SB203580 at 10^{-7} M or 10^{-6} M for 48 h. Dentine slices incubated with calcitonin (10^{-8} M) for the same period were regarded as the positive control. Resorption pits on dentine slices were visualized by

staining with Mayer's hematoxylin solution (Sigma) as described (46). The number of resorption pits on each slice was counted.

Fetal long-bone organ culture system

Bone-resorption activity was measured using a modification of Raisz's organ culture method (19, 48). Pregnant ddY mice were injected sc with 25 μCi of ^{45}Ca on d 16 of gestation. Twenty-four hours after the injection, the shafts of the radii and ulnae were dissected from fetuses, cleaned free of surrounding muscle and fibrous tissues, and precultured in serum-free BGJb medium (Life Technologies, Inc.). After preincubation for 48 h, bones were transferred into 0.5 ml BGJb medium containing 0.2% BSA and incubated for 72 h in the presence or absence of PTH, with or without SB203580, at 10^{-6} M and 10^{-7} M. At the end of the culture, ^{45}Ca was counted, respectively, in the medium and in the bone. Bone-resorbing activity was expressed as the percent release of ^{45}Ca from prelabeled bones using the following formula (19, 49):

$$^{45}\text{Ca release (\%)} = \frac{^{45}\text{Ca in medium}}{(^{45}\text{Ca in medium} + {}^{45}\text{Ca in bone})} \times 100.$$

Western blot analysis

Bone marrow cells (5 × 10^6 cells) were cultured in αMEM containing 10% FBS, in the presence of M-CSF (100 ng/ml), on 60-mm-diameter dishes. After the cells were cultured for 3 d, nonadherent cells were completely removed from the cultures by pipetting (29). Almost all of the adherent cells expressed macrophage-specific antigens such as Mac-1, Moma-2, and F4/80 (29). These macrophages and purified osteoclasts, purified on 60-mm dishes, were further incubated with test materials in the presence of 10% FBS, and then washed twice with PBS and lysed in cell lysate buffer [0.5 M Tris-HCl (pH 6.8, 2 ml), 10% SDS (4 ml), 2-mercaptoethanol (1.2 ml), glycerol (2 ml), H$_2$O (0.8 ml), bromophenol blue (10 mg)]. Whole-cell extracts were electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Millipore Corp., Bedford, MA). After blocking with 5% skim milk in Tris-buffered saline containing 0.5% Tween 20, the antibodies for p38 MAPK, MKK3/6, ATF2, ERK, or IκB-α were added in Tris-buffered saline containing 0.5% Tween 20 containing 5% BSA, and bound antibodies were visualized by using the enhanced chemiluminescence assay with reagents from Amersham Pharmacia Biotech(Arlington Heights, IL) and by exposure to x-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Results

TRAP-positive osteoclasts were formed in the cocultures of mouse calvarial osteoblasts and bone marrow cells in the presence of 1α,25-(OH)$_2$D$_3$ and PGE$_2$ (Fig. 1, A and B). SB203580, a specific inhibitor of p38 MAPK added to the coculture, strongly inhibited osteoclast formation induced by 1α,25-(OH)$_2$D$_3$ and PGE$_2$. The inhibitory effect of SB203580, at 10^{-7} M, on osteoclast formation was as strong as that at 10^{-6} M. Osteoclasts were also formed in mouse bone marrow cultures treated with RANKL together with M-CSF, even in the absence of osteoblasts (Fig. 1C). Osteoclast formation induced by RANKL and M-CSF was inhibited by the addition of SB203580 as well. Expression of RANKL mRNA in osteoblasts was increased, within 24 h, by the treatment with 1α,25-(OH)$_2$D$_3$ and PGE$_2$; and the expression level was still high, even after treatment for 48 h (Fig. 1D). In contrast, expression of OPG mRNA was down-regulated in osteoblasts by the addition of 1α,25-(OH)$_2$D$_3$ and PGE$_2$. Neither RANKL nor OPG mRNA expression regulated by 1α,25-(OH)$_2$D$_3$ and PGE$_2$ in osteoblasts was affected by SB203580 (Fig. 1D). These results suggest that SB203580 acts directly on osteoclast progenitors, rather than on supporting osteoblasts, to inhibit osteoclast formation.

We next examined the effects of SB203580 on the survival

and pit-forming activity of mature osteoclasts. We previously reported that osteoclasts spontaneously died in the absence of osteoblasts, but cytokines such as RANKL, M-CSF, IL-1, and TNFα stimulated the survival of osteoclasts (28, 29, 38). When osteoblasts were removed from the cocultures, most of the osteoclasts died within 48 h (Fig. 2, A and B). The survival of osteoclasts was stimulated by the addition of either RANKL or M-CSF (Fig. 2, A and B). SB203580 showed no inhibitory effects on RANKL- or M-CSF-enhanced survival of osteoclasts. When the crude osteoclast preparation with 5% purity was placed, for 48 h, on dentine slices, many resorption pits were formed on dentine slices (Fig. 2, C and D). Osteoblasts coexisting in this osteoclast preparation stimulated the pit-forming activity of the osteoclasts. Calcitonin strongly inhibited pit formation by osteoclasts (Fig. 2, C and D). In contrast, SB203580 showed no inhibitory effect on the pit-forming activity of osteoclasts. These results suggest that SB203580 does not inhibit osteoclast function supported by RANKL, M-CSF, or osteoblasts.

Figure 3 shows the effects of SB203580 on bone resorption induced by PTH in our mouse organ culture system. PTH markedly stimulated the release of ^{45}Ca from the prelabeled mouse long bones in organ culture (Fig. 3). The addition of SB203580 to the organ culture caused no inhibitory effect on bone resorption induced by PTH. Thus, SB203580 failed to inhibit osteoclast function, though it strongly inhibited osteoclast differentiation. These results suggest that p38 MAPK is involved in osteoclast differentiation but not in osteoclast function.

We next examined the effects of RANKL and other factors on the phosphorylation of p38 MAPK in osteoclasts and osteoclast precursors. Purified osteoclast preparations were obtained from cocultures by removing osteoblasts. Bone marrow macrophages were prepared from bone marrow cultures treated with M-CSF, and used as osteoclast precursors. Figure 4 shows the time course of change in the phosphorylation of p38 MAPK in response to RANKL in bone marrow macrophages and osteoclasts. Similar amounts of p38 MAPK protein were present in bone marrow macrophages and osteoclasts. p38 MAPK was phosphorylated within 20 min, in response to RANKL in bone marrow macrophages. The phosphorylation reached a maximal level within 40 min and then returned to the basal level at 60 min (Fig. 4). In contrast, p38 MAPK was not phosphorylated at all, even in osteoclasts treated with RANKL throughout the culture period of 60 min. The total amounts of the p38 MAPK protein in bone marrow macrophages and osteoclasts were unchanged in the presence and absence of RANKL throughout the experimental period (Fig. 4).

LPS, as well as RANKL, TNFα, and IL-1, supported the survival of osteoclasts (Fig. 5A). Again, SB203580 showed no inhibitory effect on the survival of osteoclasts supported by RANKL, TNFα, IL-1, or LPS (Fig. 5A). These factors all stimulated phosphorylation of p38 MAPK in bone marrow macrophages, but p38 MAPK was not phosphorylated at all, even in osteoclasts treated with RANKL, TNFα, IL-1, or LPS (Fig. 5B). The amounts of p38 MAPK proteins remained unchanged in macrophages and osteoclasts treated with those factors. Thus, the phosphorylation system of p38 MAPK might be blocked in osteoclasts.
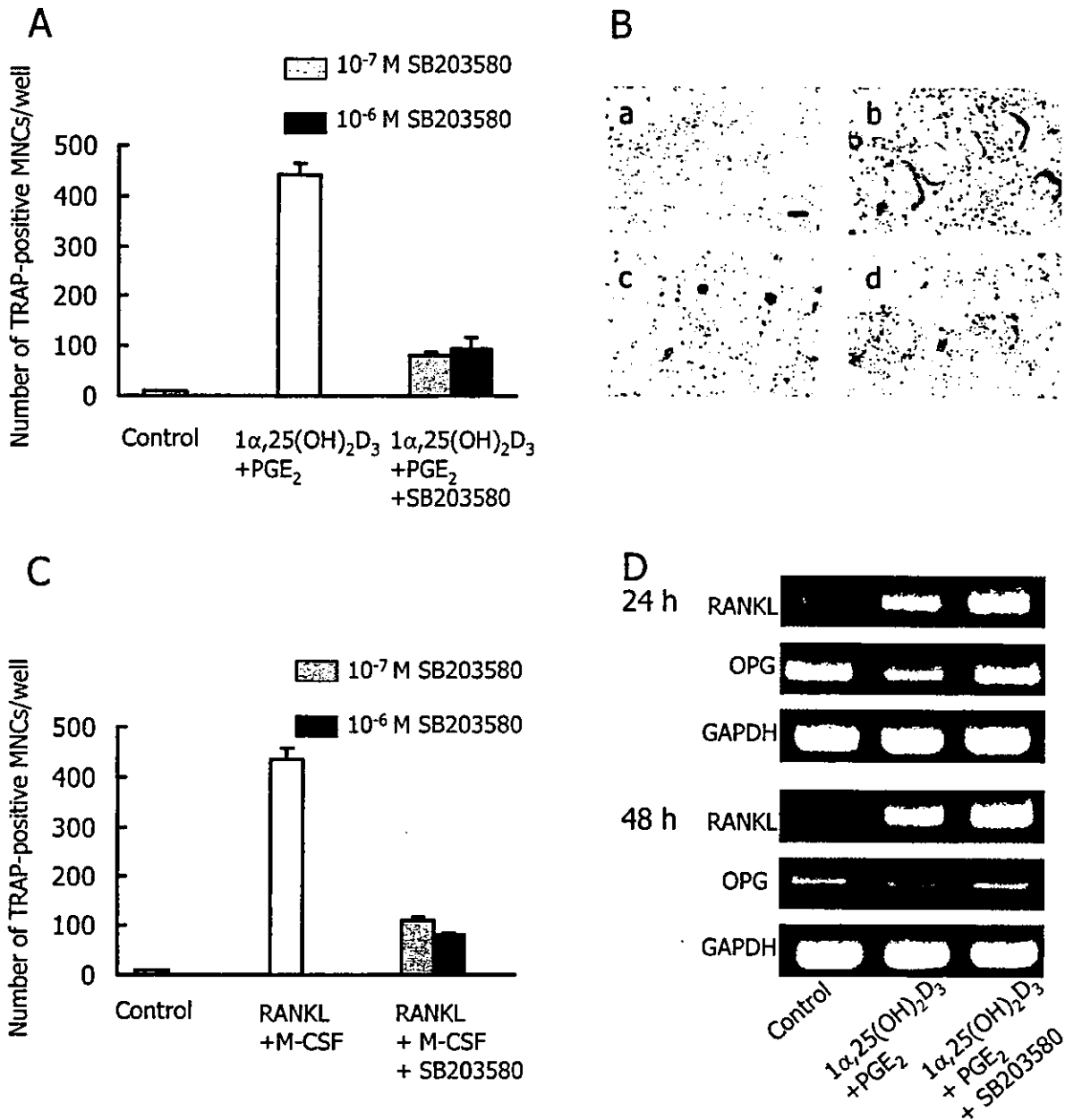
FIG. 1. Effects of SB203580 on osteoclast differentiation of mouse bone marrow cells and on the expression of RANKL mRNA in osteoblasts. A, Mouse calvarial osteoblasts and bone marrow cells were cocultured, in 48-well culture plates, in the presence of $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) and PGE_2 (10^{-7} M). SB203580, at 10^{-7} M or 10^{-6} M, was added to some cocultures. After the cells were cultured for 7 d, they were fixed and stained for TRAP. TRAP-positive MNCs containing three or more nuclei were counted as osteoclasts. The results were expressed as the means $\pm$ SEM of three cultures. B, TRAP-staining of cocultures: a, control coculture; b, coculture treated with $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) and PGE_2 (10^{-7} M); c, coculture treated with $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) and PGE_2 (10^{-7} M) plus SB203580 (10^{-7} M); d, coculture treated with $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) and PGE_2 (10^{-7} M) plus SB203580 (10^{-6} M). TRAP-positive MNCs appeared as red cells with clear peripheries. Bar, 100 μm. C, Bone marrow cells were cultured, in 48-well culture plates, in the presence of RANKL (200 ng/ml) and M-CSF (100 ng/ml). Some cultures were also treated with SB203580 at 10^{-7} M or 10^{-6} M. After cells were cultured for 7 d, they were fixed and stained for TRAP. TRAP-positive MNCs containing three or more nuclei were counted as osteoclasts. The results were expressed as the means $\pm$ SEM of three cultures. D, Mouse calvarial osteoblasts were cultured, for 24 h or 48 h, in the presence of $1\alpha,25\text{-}(OH)_2D_3$ (10^{-8} M) and PGE_2 (10^{-7} M). Some cultures were pretreated for 30 min with SB203580 (10^{-6} M) before addition of $1\alpha,25\text{-}(OH)_2D_3$ and PGE_2, and further treated for 24 h or 48 h with SB203580 in the presence of $1\alpha,25\text{-}(OH)_2D_3$ and PGE_2. Total RNA was then extracted from osteoblasts, and the expression of RANKL and OPG mRNAs was analyzed by RT-PCR. The PCR products for RANKL and OPG were 587 bp and 380 bp, respectively. Similar results were obtained from three independent experiments.

We previously reported that RANKL and IL-1 induced the activation of NF-κB and JNK in osteoclasts (38, 50). Therefore, we next examined the phosphorylation of MKK3/6 and ATF2, signaling molecules of upstream and downstream of p38 MAPK, respectively, in bone marrow macrophages and osteoclasts (Fig. 6). Phosphorylation of another MAPK, ERK, and degradation of IκB were also examined in both types of cells. The phosphorylation of

A

B

C

D
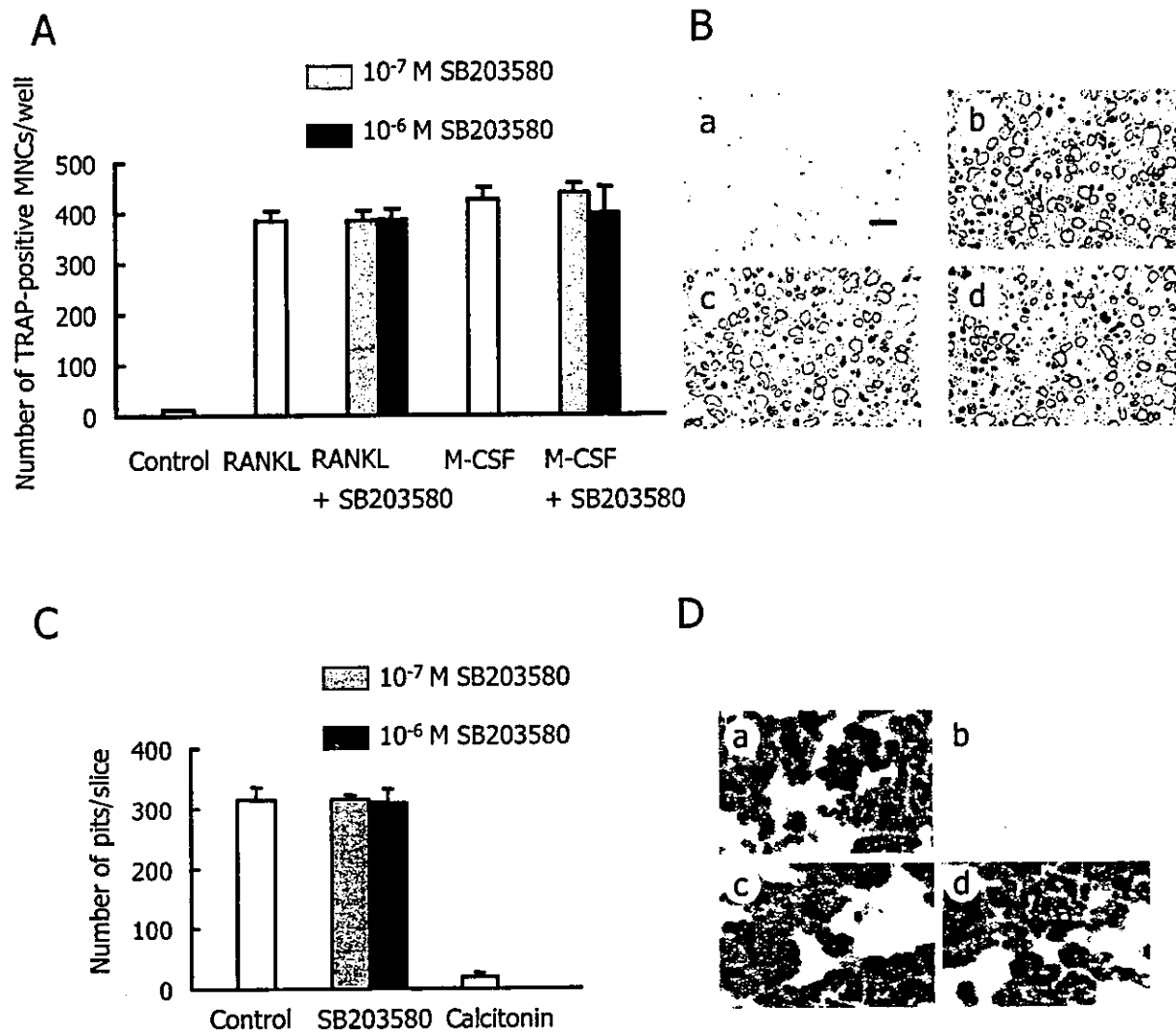
FIG. 2. Effect of SB203580 on the survival and function of osteoclasts. A, Crude osteoclast preparations with 5% purity were cultured for 6 h in 24-well plates. Osteoblasts were then removed with 0.05% trypsin/EDTA to obtain highly purified osteoclasts. Purified osteoclasts with 95% purity were pretreated for 30 min with or without SB203580 at 10^{-7} M or 10^{-6} M. Cells were further cultured for 48 h, in the presence or absence of RANKL (200 ng/ml) or M-CSF (100 ng/ml) together with or without SB203580 at 10^{-7} M or 10^{-6} M. Cells were then fixed and stained for TRAP. TRAP-positive MNCs containing three or more nuclei were counted as osteoclasts. The results were expressed as the means ± SEM of three cultures. B, TRAP staining of purified osteoclast cultures: a. control culture; b. culture treated with RANKL; c. culture treated with RANKL (200 ng/ml) plus SB203580 (10^{-7} M); d. culture treated with RANKL (200 ng/ml) plus SB203580 (10^{-6} M). *Bar*, 100 μm. C, Effect of SB203580 on pit-forming activity of osteoclasts. Aliquots of the crude osteoclast preparation were cultured on dentine slices in the presence or absence of SB203580 at 10^{-7} M or 10^{-6} M. Some cultures were also treated with calcitonin (10^{-8} M). After cells were cultured for 48 h, they were removed from the dentine slices, resorption pits formed on the slices were stained with Mayer's hematoxylin solution, and the number of resorption pits was counted. The results were expressed as the means ± SEM of three cultures. D, Resorption pits formed on dentine slices: a, control culture; b, culture treated with calcitonin (10^{-8} M); c, culture treated with SB203580 (10^{-7} M); d, culture treated with SB203580 (10^{-6} M). *Bar*, 100 μm. Similar results were obtained from three independent experiments.

ERK was induced in macrophages and osteoclasts in response to LPS (Fig. 6). LPS also stimulated the degradation of IκB in bone marrow macrophages and osteoclasts, indicating that NF-κB was activated in osteoclasts, as well as in macrophages, in response to LPS. In contrast, MKK3/6, a kinase responsible for the activation of p38 MAPK, was phosphorylated in response to LPS in macrophages but not in osteoclasts (Fig. 6). Similarly, LPS-induced phosphorylation of ATF2, which acts downstream of p38 MAPK, was observed in bone marrow macrophages treated with LPS but not in osteoclasts.

Discussion

SB203580, a selective inhibitor of p38 MAPK, strongly inhibited osteoclast differentiation, not only in cocultures of mouse osteoblasts and bone marrow cells treated with 1α,25-(OH)$_2$D$_3$ and PGE$_2$ but also in bone marrow cultures treated with RANKL and M-CSF. We previously reported that TNFα stimulates osteoclast differentiation in mouse bone marrow macrophage cultures in the presence of M-CSF through a mechanism independent of the RANKL-RANK interaction (29). Osteoclast formation induced by TNFα and M-CSF was
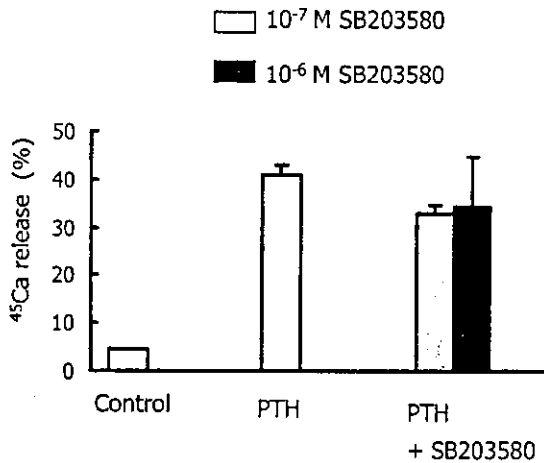
FIG. 3. Effect of SB203580 on bone resorption in fetal mouse long bone cultures. Fetal bones (radii and ulnae) prelabeled with ^{45}Ca were cultured for 72 h in the presence or absence of PTH (100 ng/ml) together with or without SB203580 at 10^{-7} M or 10^{-6} M. Bone-resorption activity was expressed as the percent release of ^{45}Ca from prelabeled bones.
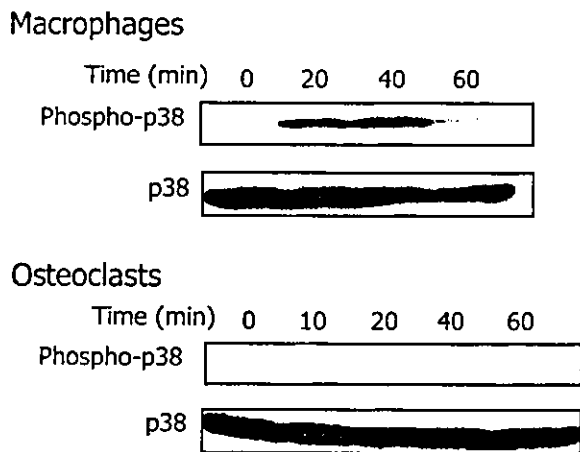
Macrophages

Osteoclasts

FIG. 4. Time course of change in the phosphorylation of p38 MAPK in macrophages and osteoclasts after treatment with RANKL. Mouse bone marrow macrophages (osteoclast precursors) were prepared from bone marrow cultures treated with M-CSF. Osteoclasts were purified from crude osteoclast preparations by the removal of osteoblasts using 0.05% trypsin/EDTA. Bone marrow macrophages (*upper panel*) or purified osteoclasts (*lower panel*) were incubated with RANKL (200 ng/ml) for the indicated periods, and total cell lysates were prepared. The total cell lysates were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with anti-phospho-p38 MAPK antibodies (phospho-p38) or anti-p38 MAPK antibodies (p38).

A

B

Macrophages

Osteoclasts

FIG. 5. Effects of RANKL, TNFα, IL-1, and LPS on the survival of osteoclasts, and on the phosphorylation of p38 MAPK in macrophages and osteoclasts. A, Purified osteoclasts were prepared in 24-well culture plates and further cultured for 48 h in the presence or absence of RANKL (200 ng/ml), TNFα (10 ng/ml), IL-1 (10 ng/ml), or LPS (2 μg/ml). For treatment of cells with SB203580, together with those factors, cells were preincubated for 30 min with SB203580 (10^{-6} M). After cells were cultured for 48 h, they were fixed and stained for TRAP. TRAP-positive MNCs containing three or more nuclei were counted as osteoclasts. The results were expressed as the means ± SEM of three cultures. B, Mouse bone marrow macrophages were prepared from bone marrow cultures treated with M-CSF. Osteoclasts were purified from crude osteoclast preparations. Bone marrow macrophages and purified osteoclasts were incubated for 20 min with or without RANKL (200 ng/ml), TNFα (10 ng/ml), IL-1 (10 ng/ml), or LPS (2 μg/ml). Total cell lysates were prepared, separated by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with anti-phospho-p38 MAPK antibodies (phospho-p38) or anti-p38 MAPK antibodies (p38).

also inhibited by the addition of SB203580 (data not shown). p38 MAPK was phosphorylated in bone marrow macrophages in response to RANKL but not in mature osteoclasts. These results suggest that SB203580 acts directly on osteoclast precursors, rather than on osteoblasts, to inhibit osteoclast differentiation. These results are also consistent with the findings that SB203580 inhibited osteoclast differentiation induced by RANKL in RAW264 cells, and that the expression of dominant-negative forms of p38 MAPK and MKK 6 in RAW264 cells significantly inhibited the RANKL-induced differentiation of the RAW cells (42). These findings,
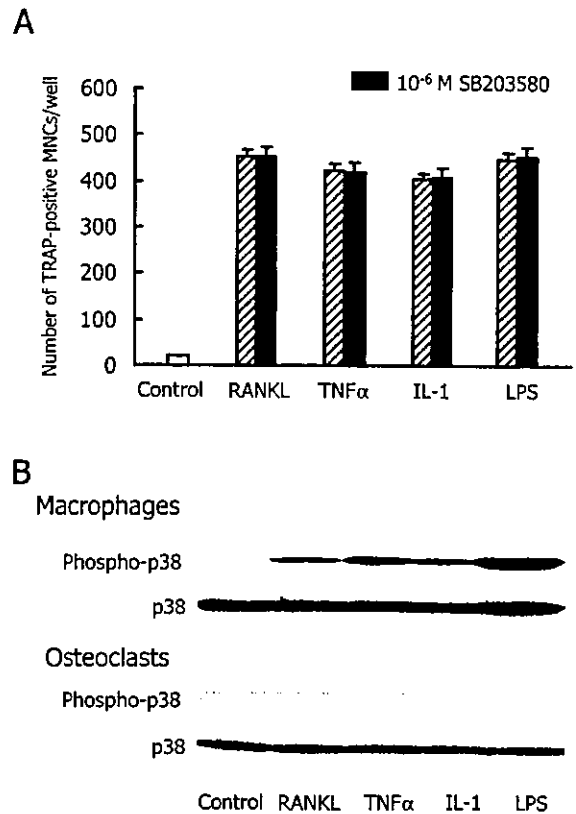
together with the present study, suggest that p38MAPK-mediated signals are of fundamental importance for the differentiation of osteoclast precursors into osteoclasts (Fig. 7).

Signals mediated by p38 MAPK have been shown to regulate the function of osteoblasts (51, 52). SB203580 inhibited the synthesis of vascular endothelial growth factor induced by PGE$_1$ in osteoblastic MC3T3-E1 cells (51). The inhibitory effect of sphingosine on PGF$_2$α-induced inositol phosphate formation in MC3T3-E1 cells was markedly reduced by the addition of SB203580 (52). Kumar *et al.* (44) also reported that SB203580 inhibited IL-6 production induced by IL-1 and TNFα in osteoblasts and chondrocytes. However, the present study showed that SB203580 did not affect, at all, 1α,25-(OH)$_2$D$_3$- and PGE$_2$-induced up-regulation of RANKL mRNA expression or down-regulation of OPG mRNA ex-
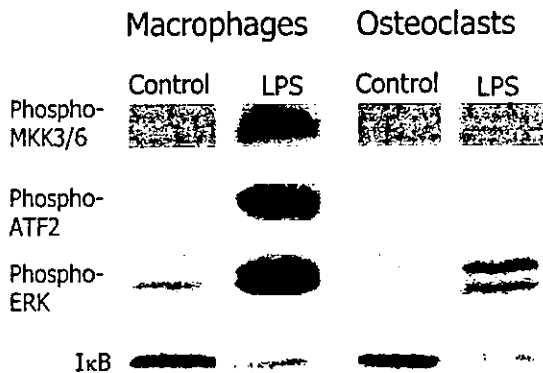
FIG. 6. Effects of LPS on the phosphorylation of MKK3/6, ERK, and ATF2, and the degradation of IκB in macrophages and osteoclasts. Mouse bone marrow macrophages were prepared from bone marrow cultures treated with M-CSF. Osteoclasts were purified from crude osteoclast preparations. Bone marrow macrophages and purified osteoclasts were incubated for 20 min with LPS, and total cell lysates were prepared. The lysates were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and immunoblotted with anti-phospho-MKK3/6 antibodies (phospho-MKK3/6), anti-phospho-ATF2 antibodies (phospho-ATF2), anti-phospho-ERK antibodies (phospho-ERK), or anti-IκB-α antibodies (IκB).
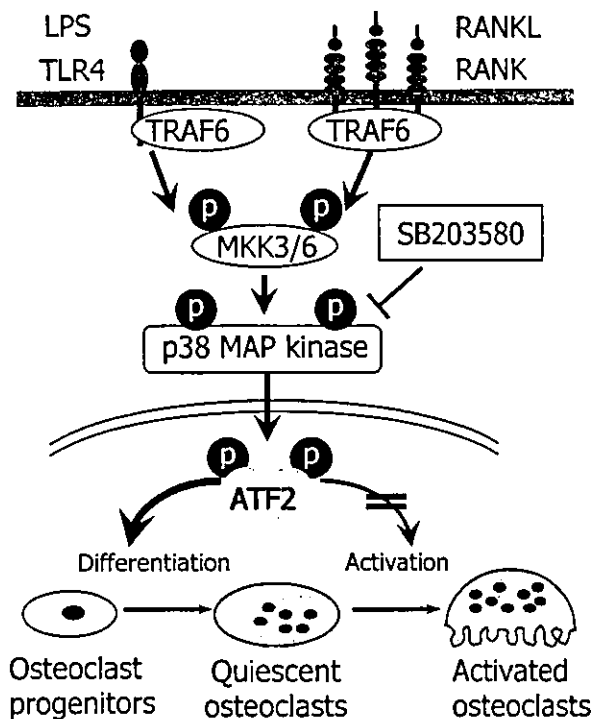

FIG. 7. A schematic representation of the p38 MAPK signal pathway in osteoclast differentiation and activation regulated by RANKL and LPS. RANKL and LPS independently stimulate the p38 MAPK-mediated signal pathway. TRAF6 may act as a common signal transducer for RANK and TLR4. SB203580, a selective inhibitor of p38 MAPK, blocks p38 MAPK-induced activation of its downstream signals, such as ATF2. SB203580 inhibits RANKL-induced osteoclast differentiation but not osteoclast activation. Sequential phosphorylations of MKK3/6, p38 MAPK, and ATF2 are involved in RANKL-induced differentiation of macrophages into osteoclasts. Both RANKL and LPS induce phosphorylations of MKK3/6, p38 MAPK, and ATF2 in macrophages but not in osteoclasts. Thus, osteoclast function is induced through a mechanism independent of p38 MAPK-mediated signals.

pression in primary osteoblasts. These results suggest that, although p38 MAPK-mediated signals regulate several aspects of osteoblast function, this signaling cascade is not involved in the regulation of bone resorption-related functions of osteoblasts such as RANKL and OPG expression.

Although SB203580, at 10^{-7} M, almost completely inhibited osteoclast differentiation, this compound showed no inhibitory effect, even at 10^{-6} M, on the survival and pit-forming activity of osteoclasts induced by RANKL. Interestingly, osteoclasts expressed a certain amount of p38MAPK but failed to phosphorylate p38 MAPK in response to any stimuli examined. This finding explains why SB203580 showed no inhibitory effect on the function of mature osteoclasts (Fig. 7). Bone resorption induced by PTH in fetal mouse long bone cultures was not affected by the addition of SB203580, suggesting that activation of preexisting osteoclasts, but not formation of new osteoclasts, predominantly occurs in response to PTH in mouse long bone cultures.

We previously reported that osteoclasts expressed RANK mRNA, and treatment with RANKL rapidly induced activation of NF-κB and JNK in osteoclasts (38). It has also been shown that osteoclasts express functional IL-1 type 1 receptors (28, 50). IL-1 induces rapid translocation of NF-κB from the cytosol to the nuclei of osteoclasts. Suda et al. (27) reported that LPS induced degradation of IκB in mononuclear prefusion osteoclasts, and stimulated their survival, fusion, and pit-forming activity. In the present study, TNFα, IL-1, and LPS as well as RANKL, supported the survival of osteoclasts, which was not inhibited by the addition of SB203580. These factors failed to induce phosphorylation of p38 MAPK in osteoclasts. Phosphorylation of MKK3/6 and ATF2 was not induced in osteoclasts either, suggesting that the entire p38 MAPK signaling pathway is nonfunctional in osteoclasts. In contrast, activation of ERK and JNK was rapidly induced in osteoclasts in response to LPS and RANKL. These results suggest that three subtypes of MAPKs (p38

MAPK, ERK, and JNK) are regulated independently of one another in osteoclasts.

Phosphorylation of p38 MAPK was similarly induced in bone marrow macrophages in response to IL-1, TNFα, RANKL, and LPS. This suggests that osteoclast precursors lose the ability to phosphorylate p38 MAPK during their differentiation into osteoclasts. Signals mediated by p38 MAPK have been shown to regulate the production of inflammatory cytokines (such as IL-1, TNFα, and IL-6) in several types of cells. We recently found that bone marrow macrophages produced inflammatory cytokines (such as IL-1, TNFα, and IL-6) in response to LPS, but osteoclasts did not (Itoh, K., et al., in preparation). This suggests that p38 MAPK-mediated signals may be involved in the production of those inflammatory cytokines in response to LPS. Further studies will be necessary to elucidate the reason why p38MAPK signals have been shut down in osteoclasts.

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Review

The molecular mechanism of osteoclastogenesis in rheumatoid arthritis

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Abstract

Bone-resorbing osteoclasts are formed from hemopoietic cells of the monocyte–macrophage lineage under the control of bone-forming osteoblasts. We have cloned an osteoblast-derived factor essential for osteoclastogenesis, the receptor activator of NF-κB ligand (RANKL). Synovial fibroblasts and activated T lymphocytes from patients with rheumatoid arthritis also express RANKL, which appears to trigger bone destruction in rheumatoid arthritis as well. Recent studies have shown that T lymphocytes produce cytokines other than RANKL such as IL-17, granulocyte–macrophage colony-stimulating factor and IFN-γ, which have powerful regulatory effects on osteoclastogenesis. The possible roles of RANKL and other cytokines produced by T lymphocytes in bone destruction are described.

Keywords: granulocyte–macrophage colony-stimulating factor, IFN-γ, IL-17, IL-18, RANKL

Introduction

Bone-resorbing osteoclasts originate from hemopoietic cells probably of the colony-forming-unit megakaryocyte (CFU-M)-derived monocyte–macrophage family. Osteoclasts are large multinucleated giant cells that express tartrate-resistant acid phosphatase (TRAP) activity and calcitonin receptors, and they have the ability to form resorption pits on bone and dentine slices. The characteristics of osteoclasts thus differ from those of macrophage polykaryons.

We have developed a mouse coculture system of hemopoietic cells and primary osteoblasts to investigate osteoclast formation *in vitro* [1–3]. In this coculture system, several systemic and local factors induced formation of TRAP-positive multinucleated cells, which satisfied most of the osteoclast criteria [4]. Subsequent experiments established that the

target cells of osteotropic factors for inducing osteoclast formation in the coculture were osteoblasts/stromal cells but not osteoclast precursors. In the coculture system, cell-to-cell contact between osteoblastic cells and osteoclast progenitors was essential for inducing osteoclastogenesis.

From these experimental findings, we proposed in 1992 that osteoblastic cells induce osteoclast differentiation factor as a membrane-associated factor in response to various osteotropic factors [4]. Six years later, we succeeded in the molecular cloning of osteoclast differentiation factor from a cDNA library of mouse stromal ST2 cells treated with bone-resorbing factors [5]. Osteoclast differentiation factor is a member of the tumor necrosis factor (TNF) ligand family, and was found to be identical to RANKL, TNF-related activation-induced cytokine and

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CFU-M = colony-forming-unit megakaryocyte; COX-2 = cyclooxygenase; ELISA = enzyme-linked immunosorbent assay; GM-CSF = granulocyte–macrophage colony-stimulating factor; HuPBL-NOD/SCID = human peripheral blood lymphocyte-nonobese diabetic/severe combined immunodeficiency; IFN = interferon; IL = interleukin; MAP = mitogen-activated protein; M-CSF = macrophage colony-stimulating factor; NF = nuclear factor; OA = osteoarthritis; OPG = osteoprotegerin; PBMC = peripheral blood mononuclear cell; PCR = polymerase chain reaction; PGE_2 = prostaglandin E_2; RA = rheumatoid arthritis; RANK = receptor for RANKL; RANKL = receptor activator of NF-κB ligand; sIL-6R = soluble IL-6 receptors; TNF = tumor necrosis factor; TNFR1 = TNF receptor type 1 (p55); TNFR2 = TNF receptor type 2 (p75); TRAF = TNF receptor-associated factor; TRAP = tartrate-resistant acid phosphatase.

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osteoprotegerin (OPG) ligand, which were independently identified by three other research groups [5-9]. The *ad hoc* Committee of the American Society of Bone and Mineral Research has recommended using RANKL as the standardized name [10].

RANKL induced osteoclast differentiation from mouse hemopoietic cells and human peripheral blood mononuclear cells (PBMCs) in the presence of macrophage colony-stimulating factor (M-CSF) [5,8,11,12]. RANK, a receptor for RANKL, is the sole signaling receptor for RANKL in inducing development and activation of osteoclasts [9] (Fig. 1). OPG, which is produced by a variety of cells including osteoblasts, is a soluble decoy receptor for RANKL. OPG inhibits osteoclastogenesis to compete against RANK [9]. The present review article describes the possible roles of members of the TNF receptor and ligand superfamily in osteoclastic bone resorption, especially in rheumatoid arthritis (RA).

## Possible roles of TNF receptor and ligand superfamily members in osteoclastic bone resorption in RA

RA is a chronic inflammatory disease characterized by the destruction of articular cartilage and bone in a chronic phase. Although histologic analyses of periarticular trabecular bone have demonstrated that osteoclastic bone resorption is greatly stimulated in RA patients, the mechanism of the joint destruction in RA patients remains to be elucidated.

The levels of monocyte/macrophage-derived cytokines such as IL-1 and IL-6, together with soluble IL-6 receptors (sIL-6R), are significantly elevated in the synovial fluids of patients with RA compared with those patients with osteoarthritis (OA) [13]. These cytokines may play important roles not only in immune responses and in development of inflammation, but also in joint destruction.

The role of T cells in the pathogenesis of RA at a chronic stage, however, has not yet been determined, because T-cell-derived cytokines such as IL-2 and IFN-γ are undetectable in the synovial tissues and fluids [14]. We recently reported that T cells indirectly regulate osteoclastogenesis via IL-17 and IL-18. IL-18 inhibits osteoclast formation by inducing granulocyte–macrophage colony-stimulating factor (GM-CSF) in T cells [15,16]. In contrast, IL-17 first acts on osteoblastic cells, then stimulates cyclooxgenase (COX)-2-dependent prostaglandin E_2 (PGE₂) synthesis, and subsequently induces RANKL gene expression, which in turn induces differentiation of osteoclast progenitors into mature osteoclasts [17].

It has been reported that RANKL is expressed in activated T cells as well as in osteoblastic cells [6,7,18]. These activated T cells are in fact capable of triggering osteoclasto-
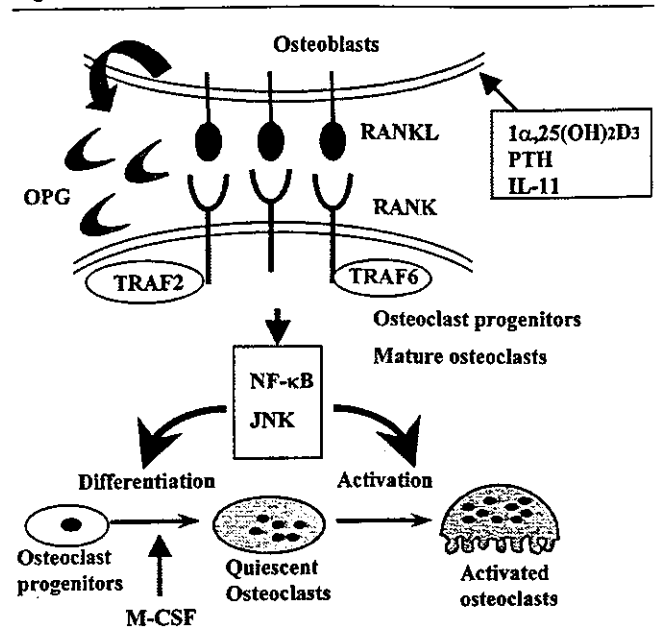
A schematic representation of osteoclast differentiation supported by osteoblasts/stromal cells. RANKL, which is induced by bone resorbing factors such as 1-α,25(OH)₂D₃, parathyroid hormone (PTH) and IL-11 on the plasma membrane of osteoblasts/stromal cells, binds its receptor RANK present in osteoclast progenitors and mature osteoclasts. Osteoprotegerin (OPG), a decoy receptor for RANKL, strongly and competitively inhibits the RANKL–RANK interaction. The RANK signaling is transduced via TNF receptor-associated factor 2 (TRAF2) and TNF receptor-associated factor 6 (TRAF6), leading to the activation of NF-κB and Jun kinase (JNK), which in turn stimulates differentiation and activation of osteoclasts. M-CSF, macrophage colony-stimulating factor.

genesis directly through RANKL [18-20]. Kong *et al.* [8] found that systemic activation of T cells *in vivo* leads to a RANKL-mediated increase in osteoclastogenesis and bone loss. In a T-cell-dependent model of rat adjuvant arthritis characterized by severe joint destruction, OPG treatment prevented bone destruction but not inflammation [18]. In addition, we demonstrated that the level of the soluble form of RANKL is elevated, while the level of OPG is decreased in synovial fluids from RA patients [20]. It is thus possible to postulate that T cells directly and indirectly stimulate osteoclastogenesis. Takayanagi *et al.* [21] recently reported that T-cell production of IFN-γ strongly suppresses osteoclastogenesis by disrupting the RANKL–RANK signaling pathway. They showed that there is a crosstalk between the TNF and IFN families of cytokines in bone resorption.

A potential role of IL-17 in joint destruction of RA patients

We previously reported that IL-6 alone did not induce osteoclast formation, but sIL-6R together with IL-6 markedly stimulated osteoclast formation in a mouse

coculture system [22,23]. We also examined whether sIL-6R and IL-6 are involved in joint destruction in RA patients [13]. The number of osteoclast-like multinucleated cells found in the synovial tissues derived from the knee joint was greater in RA patients than in OA patients. These multinucleated cells from RA synovial tissues expressed the osteoclast-specific phenotypes such as TRAP, carbonic anhydrase II, vacuolar type proton-ATPase and vitronectin receptors at similar levels to those from a human giant cell tumor of bone. The concentrations of both IL-6 and sIL-6R were significantly higher in the synovial fluids of patients with RA than in those of patients with OA. The concentrations of IL-6 and sIL-6R were correlated well with the roentgenologic grades of joint destruction [13]. These results suggest that IL-6 in RA synovial fluids is responsible, at least in part, for joint destruction in the presence of sIL-6R through osteoclastogenesis (Fig. 2).

IL-17 is a newly discovered cytokine that is secreted by activated memory CD4+ T cells and modulates an early stage of immune responses [24]. Rouvier et al. [25] cloned cytotoxic T-lymphocyte-associated antigen 8 (rat IL-17) from a T-cell subtraction library. Mouse IL-17 was subsequently cloned from a thymus-derived, activated T-cell cDNA library [26]. Fossiez et al. [27] reported that IL-17 stimulated epithelial cells, endothelial cells and fibroblastic stromal cells to secrete several cytokines, including IL-6, IL-8, granuloctye colony-stimulating factor and PGE_2. In addition, IL-17 greatly promoted the proliferation of CD34+ hemopoietic progenitors in cocultures with synovial fibroblastic cells collected from RA patients [27].

We examined potential roles of IL-17 in osteoclastogenesis using a mouse coculture system. IL-17 greatly stimulated osteoclast formation via a cell-to-cell interaction between osteoclast progenitors and osteoblasts [17]. IL-17 increased PGE_2 synthesis in cultures of osteoblasts. In addition, IL-17 induced the expression of RANKL mRNA in osteoblasts. Like OPG, NS398 (a selective inhibitor of COX-2) completely inhibited IL-17-induced osteoclast differentiation in the cocultures.

IL-17 levels were significantly higher in the synovial fluids of RA patients compared with those of OA patients. Anti-IL-17 antibody significantly inhibited osteoclast formation induced by conditioned media of the cultures of RA synovial tissues in cocultures. Immunostaining of the synovial tissues from RA patients demonstrated that IL-17-positive cells were detected in a subset of CD4+, CD45RO+ T cells in the specimens [17]. These findings suggest that IL-17 first acts on osteoblasts, which stimulates COX-2-dependent PGE_2 synthesis, and it then induces RANKL gene expression, which in turn induces differentiation of osteoclast progenitors into mature osteoclasts. It is probable that IL-17 is a crucial cytokine for osteoclastic bone resorption in RA patients (Fig. 2).

Figure 2
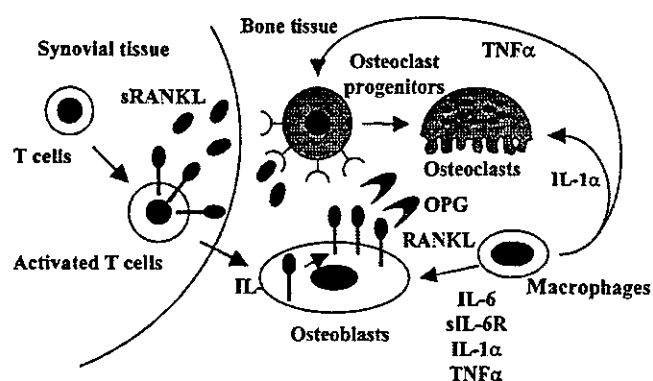
A possible mechanism of osteoclast formation by activated T cells in rheumatoid arthritis. Activated T cells present in the synovial tissues also produce membrane-associated RANKL, some of which are cleaved enzymatically from the plasma membrane, resulting in soluble RANKL (sRANKL). Activated T cells also produce IL-17, which induces RANKL via prostaglandin E_2 synthesis in osteoblasts. IL-6 together with soluble IL-6 receptors (sIL-6R), IL-1-α and TNF-α derived from macrophages induce RANKL in osteoblasts. In addition, TNF-α directly acts on osteoclast progenitors, which then differentiate into osteoclasts by a mechanism independent of the RANKL–RANK interaction. IL-1 also induces osteoclast activation directly. OPG, osteoprotegerin.

Chabaud and co-workers [28,29] examined the contribution of soluble factors in the interaction between T cells and synoviocytes in RA patients. IL-17 induced production of IL-6 and leukemia inhibitory factor in synovial fibroblasts [28]. IL-17 increased bone resorption and decreased bone formation in human RA bone explants [29]. Chabaud et al. also reported that IL-17 was spontaneously produced in organ cultures of synovial tissues derived from RA patients.

Addition of anti-inflammatory cytokines IL-4 and IL-13 completely inhibited the production of IL-17 in synovial tissues [30]. Lubberts et al. [31] recently reported the IL-4 gene therapy for collagen-induced arthritis in mice, using a gene transfer with an IL-4-expressing adenoviral vector. Local treatment with IL-4 greatly prevented joint damage and bone erosion, although severe inflammation remained unchanged. The protective effect of IL-4 was associated with the decreased formation of osteoclasts and the downregulation of IL-17 mRNA and RANKL protein expression [31].

Jovanovic et al. [32] reported that IL-17 induced production of matrix metalloproteinase 9 in human monocyte/macrophages through PGE_2 synthesis. This stimulation was involved in both phosphorylation of p38 mitogen-activated protein (MAP) kinase and in NF-κB activation [32]. They also found that IL-17 stimulated the production and expression of inflammatory cytokines such as IL-1β, IL-6, and TNF-β by human macrophages [33]. Ziolkowska et al. [34] reported that high concentrations of IL-17 were

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strongly correlated with those of IL-15 in synovial fluids of RA patients. IL-15 stimulates IL-17 production by human PBMCs in primary cultures [34]. These results together with our recent findings suggest that IL-17 plays an important role in the joint destruction of RA patients.

Osteoclastogenesis by activated T cells in RA

Kong *et al.* [8] reported that RANKL knockout (–/–) mice showed severe osteopetrosis with total occlusion of the bone marrow space within endosteal bone. RANKL (–/–) mice lack osteoclasts but have normal osteoclast progenitors that can differentiate into functionally active osteoclasts when cocultured with wild-type osteoblasts. In addition, RANKL (–/–) mice exhibited defects in early differentiation of T cells and B cells, and they lacked all lymph nodes but had normal splenic structures and Peyer's patches [8]. These results suggest that RANKL is not only a prerequisite for osteoclast development, but that it also plays an important role in early differentiation of T cells and B cells.

Several reports have demonstrated that RANKL is detected in the synovial fibroblasts and activated T lymphocytes derived from RA patients [18,20,35–37]. Horwood *et al.* [19] reported that human PBMC-derived T cells activated by concanavalin A expressed RANKL, and that these cells supported osteoclast formation in cocultures with murine hemopoietic cells. Romas *et al.* [38] found that RANKL mRNA was highly expressed by the synovial cell infiltrate in arthritic joints, as well as by osteoclasts at the sites of bone erosion in collagen-induced arthritis. It was recently reported that the degree of bone erosion in RANKL (–/–) mice was greatly reduced in a serum transfer model of arthritis, when compared with the control mice [39].

To elucidate the direct effect of human T cells in inducing osteoclastogenesis in RA, we conducted coculture experiments of activated human T cells and human adherent PBMCs [20]. When PBMCs were cultured in the presence of M-CSF for 3 days and further cocultured for 7 days with activated CD3+ T cells, vitronectin receptor (CD51)-positive osteoclasts were formed even in the absence of exogenous RANKL. Osteoclast formation induced by activated T cells was completely inhibited by adding OPG.

Using an ELISA system, we measured the level of a soluble form of RANKL in the synovial fluids. Concentrations of soluble RANKL in the synovial fluids were significantly higher in patients with RA than in patients with other arthropathies including OA, gout, and trauma. In contrast, a decreased concentration of OPG was detected in the synovial fluids from RA patients. The ratio of the concentration of soluble RANKL to that of OPG

was significantly higher in the synovial fluids of RA patients than in those of patients with OA or gout [20]. These results suggest that excess production of RANKL by activated T lymphocytes may contribute to the pathogenesis of bone destruction in these patients (Fig. 2). Regulation of RANKL and/or OPG expression in RA patients will provide a clue for the strategy of the development of new treatment for inhibiting of bone destruction in this disease.
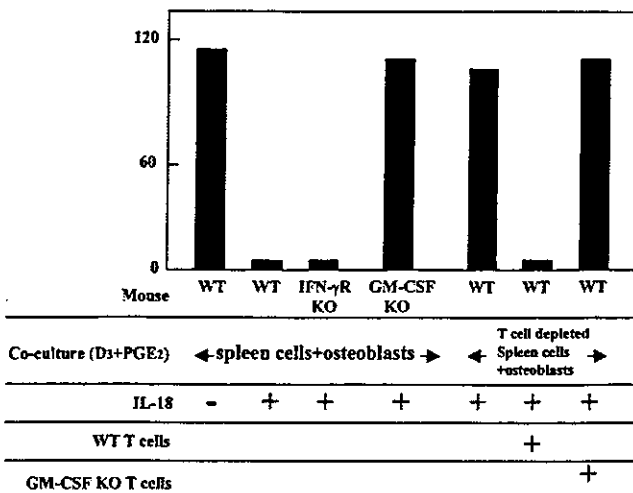
In a T-cell-dependent model of rat adjuvant arthritis characterized by severe joint inflammation and bone and cartilage destruction, OPG treatment at the onset of the disease prevented bone and cartilage destruction but not inflammation [18]. Teng *et al.* [40] also reported that CD4+ T-cell-mediated immunity is involved in the modulation of periodontal bone destruction in HuPBL-NOD/SCID mice after oral inoculation of *Actinobacillus actinomycetemcomitans*, a well-known Gram-negative anaerobic microorganism that causes human periodontitis. OPG treatment significantly reduced the number of osteoclasts at the sites of local periodontal infection.

Juji *et al.* [41] recently reported a simple and effective method of active immunization against self RANKL as a potential treatment of bone diseases. Immunization with RANKL vaccines almost completely prevented the bone destruction in RA model mice (SKG mice). These results suggest that a therapeutic vaccine approach targeting RANKL may be useful for inhibiting bone destruction in a variety of pathological bone diseases.

Inhibitory cytokines produced by T cells on osteoclast differentiation

We previously reported that bone-marrow-derived stromal cell lines, MC3T3-G2/PA6 and ST2, had the capacity to support osteoclast formation in cocultures with hemopoietic cells [2,3]. Chambers and co-workers established several bone-marrow-derived stromal cell lines from a transgenic mouse, in which the IFN-inducible major mouse histocompatibility complex H-2Kb promoter drives the temperature-sensitive immortalizing gene of simian virus 40 [42,43]. These cell lines differed in their osteoclast-inductive activity in cocultures with hematopoietic cells.

To identify genes in osteoblasts/stromal cells that are involved in the process of osteoclastogenesis, we used differential display of PCR to compare mRNA populations between osteoclast-inductive and osteoclast-noninductive cell lines [15]. Using this approach, we identified IL-18 (IFN-γ-inducing factor) as a product of osteoblastic stromal cells. IL-18 has been reported to induce production of IFN-γ and GM-CSF in T cells, both of which exhibit a potent inhibitory activity of osteoclastogenesis, at least *in vitro* [44]. IL-18 strongly inhibited osteoclast formation induced by bone-resorbing factors in cocultures. IL-18
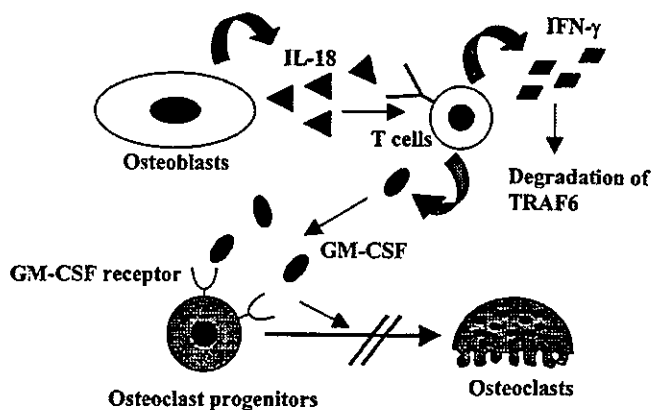
Figure 3

Mouse	WT	WT	IFN-γR KO	GM-CSF KO	WT	WT	WT
Co-culture (D3+PGE2)	◄ spleen cells+osteoblasts ►				◄ T cell depleted Spleen cells +osteoblasts ►		
IL-18	-	+	+	+	+	+	+
WT T cells						+	
GM-CSF KO T cells							+

Effects of IL-18 on osteoclast formation. Mouse spleen cells and osteoblasts from wild-type mice (WT), IFN-γ receptor type II-knockout mice (IFN-γR KO) or granulocyte–macrophage colony-stimulating factor-knockout mice (GM-CSF KO) were cocultured with 1-α,25(OH)$_2$D$_3$ and prostaglandin E$_2$ (PGE$_2$) in the presence or absence of IL-18. In some cocultures, T-cell-depleted spleen cells and osteoblasts were cocultured in the presence and absence of WT T cells or GM-CSF KO T cells. In each coculture, numbers of tartrate-resistant acid phosphatase-positive osteoclasts formed were scored.

Figure 4

A proposed mechanism of the inhibitory action of IL-18 on osteoclast differentiation. IL-18 secreted from osteoblasts acts on T lymphocytes, which generate granulocyte–macrophage colony-stimulating factor (GM-CSF) and IFN-γ. Both GM-CSF and IFN-γ are potent inhibitors of osteoclast formation, at least *in vitro*. When GM-CSF binds its receptor, GM-CSFR (present in osteoclast progenitors), osteoclast formation is completely inhibited. In contrast, the target molecule of IFN-γ is TNF receptor-associated factor 6 (TRAF6). The degradation of TRAF6 by IFN-γ leads to the inhibition of osteoclastogenesis. The inhibitory action of IL-18 on osteoclast differentiation occurs via GM-CSF, but not via IFN-γ.

was found to inhibit osteoclast formation in cocultures with osteoblasts and spleen cells from IFN-γ receptor type II-deficient mice, similarly to those with wild-type osteoblasts and spleen cells In contrast, IL-18 was unable to inhibit osteoclast formation in cocultures of osteoblasts and spleen cells from GM-CSF-deficient mice (Fig. 3).

Since T cells comprise a large proportion of the spleen cell population, the role of T cells in osteoclastogenesis was examined. T cells were removed from spleen cell preparations using a monoclonal antibody against Thy 1.2 membrane antigen, which was predominantly expressed on T lymphocytes. The complete absence of T cells abolished the action of IL-18 on osteoclast formation in cocultures of osteoblasts and spleen cells from wild-type mice (Fig. 3). Addition of wild-type T cells but not GM-CSF-deficient T cells to the coculture restored the inhibition by IL-18 of osteoclastogenesis (Fig. 3). These results suggest that IL-18 inhibits osteoclast formation by making T cells promote the release of GM-CSF, which then acts on osteoclast precursors to limit osteoclast differentiation [15,16] (Fig. 4).

Horwood et al. [45] found that, like IL-18, IL-12 strongly inhibited osteoclast formation in cocultures, as well as in spleen cell cultures treated with M-CSF and RANKL. An unknown inhibitory molecule was found to be secreted

from T cells in response to IL-12 and IL-18. Transwell experiments in which T cells were separated from hemo-poietic cells suggested that the inhibitory molecule was a secreted factor, but not a membrane-associated factor. Although a number of cytokines (IL-4, IL-10, IL-13, GM-CSF and IFN-γ) expressed by T cells have the capacity to inhibit osteoclast formation, the present inhibitory factor has not been identified. IL-12 and IL-18 are detected in the RA synovial membrane [46]. It was also reported that IL-18 stimulated expression of OPG mRNA in osteoblasts and bone marrow stromal cells [47]. IL-12 and IL-18 may therefore protect the joint destruction via osteoclast-mediated erosion. IL-18 is effective in inhibiting bone destruction in murine models of breast cancer metastasis in bone [48]. These results suggest that IL-12 and/or IL-18 therapy may be useful for reducing pathological bone loss.

Takayanagi et al. [21] demonstrated that activated T cells are capable of inhibiting osteoclastogenesis through IFN-γ production, which interferes with the RANKL–RANK signaling pathway. In that study, osteoclast formation was strongly inhibited in the coculture of activated T cells and bone marrow cells in the presence of RANKL and M-CSF [21]. When activated T cells were cocultured with bone marrow cells derived from IFN-γ receptor knockout mice in the presence of RANKL and M-CSF, the inhibitory effect of activated T cells was completely canceled.

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The expression of TNF receptor-associated factor (TRAF)6 was markedly inhibited by IFN-γ in osteoclast progenitors stimulated by RANKL and M-CSF, indicating that TRAF6 is a target molecule of IFN-γ. IFN-γ appears to inhibit osteoclastogenesis by decomposing TRAF6. In fact, TRAF6-deficient mice exhibited severe osteopetrosis [49,50]. It was also reported that IFN-γ receptor knockout mice developed collagen-induced arthritis more readily than wild-type mice [51]. These results suggest that TRAF6 is the critical target molecule in the IFN-γ-mediated suppression of osteoclast formation, and that the balance between RANKL and IFN-γ action may regulate osteoclastogenesis (Fig. 4).
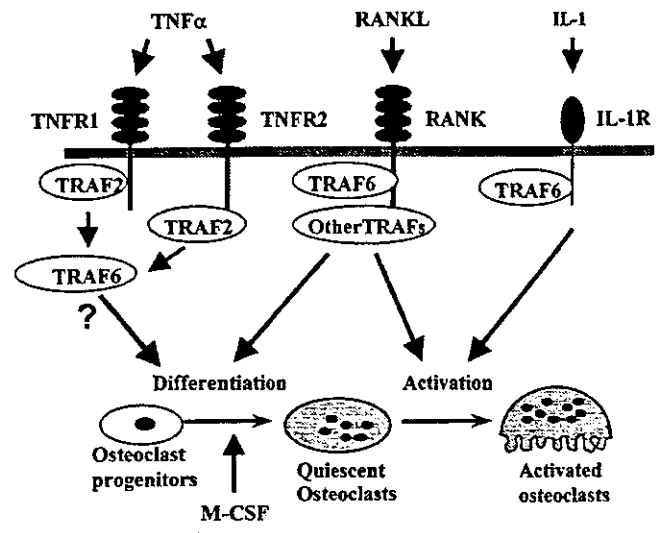
Possible roles of TNF-α in osteoclast differentiation

We have reported that TNF-α induced osteoclast formation via a mechanism independent of the RANKL–RANK signaling pathway [52] (Fig. 5). When mouse bone marrow cells were cultured with M-CSF for 3 days and nonadherent cells removed, adherent cells of uniform size and shape remained on the culture dish. The M-CSF-dependent bone marrow macrophage preparation contained no appreciable number of alkaline phosphatase-positive osteoblastic cells. When M-CSF-dependent bone marrow macrophages were further cultured for 3 days with several bone-resorbing cytokines, mouse TNF-α as well as RANKL induced osteoclast formation in the presence of M-CSF.

IL-1-α failed to induce osteoclast formation in macrophage cultures even in the presence of M-CSF. These osteoclast progenitors expressed not only RANK and c-Fms (M-CSF receptor), but also TNF receptor type 1 (TNFR1, p55) and TNF receptor type 2 (TNFR2, p75). Osteoclast formation induced by RANKL was completely inhibited by adding OPG, but osteoclastogenesis induced by TNF-α was not. Adding antibodies against TNFR1 and TNFR2 blocked osteoclast formation induced by TNF-α but not by RANKL. Bone marrow macrophages prepared from TNFR1 knockout mice differentiated into osteoclasts in response to RANKL, but they failed to differentiate into osteoclasts in response to TNF-α. Similarly, TNFR2 knockout mouse-derived bone marrow macrophages differentiated into osteoclasts in response to RANKL, but osteoclast differentiation induced by TNF-α was markedly decreased in TNFR2 knockout mouse-derived macrophage cultures [52].

These results suggest that TNF-α stimulates osteoclast formation via a mechanism independent of the RANKL pathway (Fig. 5). It was also shown that TNF-α as well as RANKL stimulated differentiation of RAW 264.7 cells into osteoclasts [53,54]. RANK-mediated signals for osteoclastogenesis are transduced via either TRAF6 or TRAF2, whereas TNFR1-mediated and TNFR2-mediated signals are transduced via TRAF2. TRAF-2-mediated signals may play important roles in osteoclast differentiation induced

by TNF-α. Using TRAF6-deficient mice, Kaji *et al.* [55] recently found that TRAF6 is also involved in TNF-α-induced osteoclastogenesis (Fig. 5). Further studies are necessary to determine the relationship between TRAF2 and TRAF6 in TNF-α-induced osteoclastogenesis.

To examine, whether TNF-α induces not only osteoclast differentiation, but also osteoclast activation, macrophages were cultured on dentine slices in the presence of TNF-α, M-CSF, and OPG [52]. Some cultures were also treated with IL-1-α. After culture for 6 days, similar numbers of osteoclasts were formed on dentine slices irrespective of the presence or absence of IL-1-α. However, no resorption pits were detected in macrophage cultures treated with TNF-α and M-CSF. Resorption pits on dentine slices were observed only in the presence of TNF-α and M-CSF together with IL-1-α.

These results suggest that TNF-α stimulates differentiation, but not activation, of osteoclasts. In contrast, IL-1-α does not induce differentiation of osteoclasts in macrophage cultures that do not contain osteoblasts/stromal cells, but it does stimulate pit-forming activity of the osteoclasts formed [52,56] (Fig. 5). Since IL-1R binds TRAF6 but not TRAF2, these results indicate that TRAF6 is a prerequisite for osteoclast activation.

Figure 6
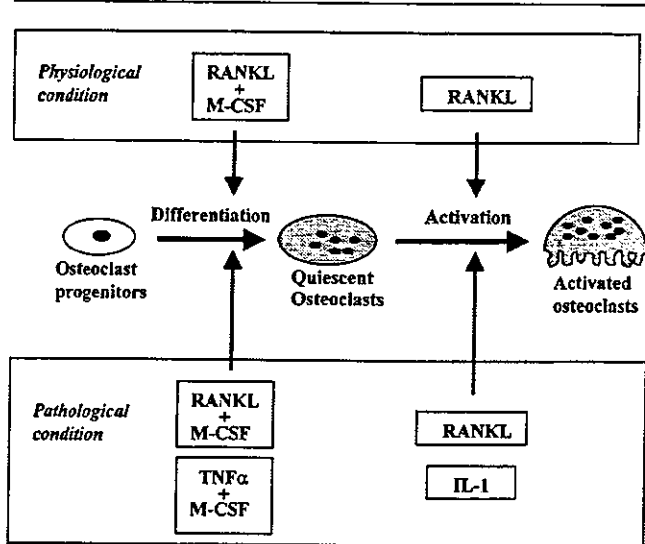
Involvement of TNF ligand family members in physiological and pathological bone resorption. RANKL appears to play a major role in physiological bone resorption. In contrast, both RANKL-dependent and RANKL-independent pathways appear to be involved in pathological bone resorption. At present, the contribution ratio of the RANKL-dependent and RANKL-independent pathways to the pathological bone resorption is not known. M-CSF, macrophage colony-stimulating factor.

Pacifici and co-workers [57,58] recently demonstrated that estrogen deficiency induces bone loss by enhancing TNF-α production by T cells. Ovariectomy failed to induce bone loss in T-cell-deficient athymic nude (nu/nu) mice as well as in TNFR1 knockout mice. They also found that ovariectomy increased the number of TNF-producing T cells in the bone marrow of normal mice without altering the TNF production per T cell [58]. These results suggest that T-cell-produced TNF and its interaction with TNFR1 play a key role in bone loss induced by estrogen deficiency.

Conclusion

Under physiological conditions, osteoclast formation requires cell-to-cell contact between hemopoietic cells (osteoclast progenitors) and osteoblastic cells, in which osteoblastic cells generate RANKL as a membrane-bound factor in response to several bone resorbing factors (Fig. 6). In contrast, like in RA, T cells appear to secrete a soluble form of RANKL in pathological bone resorption that acts directly on osteoclast progenitors without cell-to-cell contact. Furthermore, TNF-α directly stimulates osteoclast differentiation via a mechanism independent of the RANKL–RANK interaction. IL-1-α induces osteoclast activation via its own receptors (Fig. 6).

Takeuchi et al. [59] recently established a coculture system with nurse-like cells obtained from synovial tissues of patients with RA. These cells promote survival of B cells

and maintain the growth of myeloid cells. In addition, the nurse-like cells supported the generation of TRAP-positive osteoclasts from PBMCs [60]. These results suggest that, like bone-marrow-derived stromal cells, the nurse-like cells from RA synovial tissues also possess a novel ability to support osteoclast differentiation.

In conclusion, control of the production of RANKL, of OPG and of other T-cell-derived cytokines in RA patients will provide a clue for strategies of the development of new treatment for inhibiting bone destruction in this disease.

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