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

XIAOTONG LI, NOBUYUKI UDAGAWA, KANAMI ITOH, KOJI SUDA, YOSHIYUKI MURASE, TATSUJI NISHIHARA, TATSUO SUDA, AND NAOYUKI TAKAHASHI

Institute for Oral Science (X.L., N.T.), the Department of Biochemistry (N.U.), Matsumoto Dental University, Nagano 399-0781, Japan; the Department of Biochemistry (K.I., K.S.), School of Dentistry, Showa University, Tokyo 142-8555, Japan; the Department of Periodontology (Y.M.), Aichi Gakuin University, Nagoya 464-8651, Japan; the Department of Oral Microbiology (T.N.), Kyushu Dental College, Fukuoka 803-8580, Japan; and the Research Center for Genomic Medicine (T.S.), Saitama Medical School, Saitama 350-1241, Japan

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)

OSTEOCLASTS, 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

Abbreviations: AP-1, Activator protein-1; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; MKK, MAPK kinase; MNC, multinucleated cell; NF-κB, nuclear factor-κB; OPG, osteoprotegerin; PGE_2, prostaglandin E_2; RANKL, receptor activator of nuclear factor-κB ligand; TLR4, Toll-like receptor 4; TRAF, TNF-associated factor; TRAP, tartrate-resistant acid phosphatase.

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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-$(OH)_2D_3$], 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-$(OH)_2D_3$ (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-$(OH)_2D_3$ (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′-CAGAGACTAATAGATCAAAGGCAGG-3′ (forward, nucleotides 135–159) and 5′-ATGAAGTCTCACCTGAGAAGAACC-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-$(OH)_2D_3$ (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

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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_2O (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-$(OH)_2D_3$ 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-$(OH)_2D_3$ 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-$(OH)_2D_3$ 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-$(OH)_2D_3$ and PGE_2. Neither RANKL nor OPG mRNA expression regulated by 1α,25-$(OH)_2D_3$ 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.

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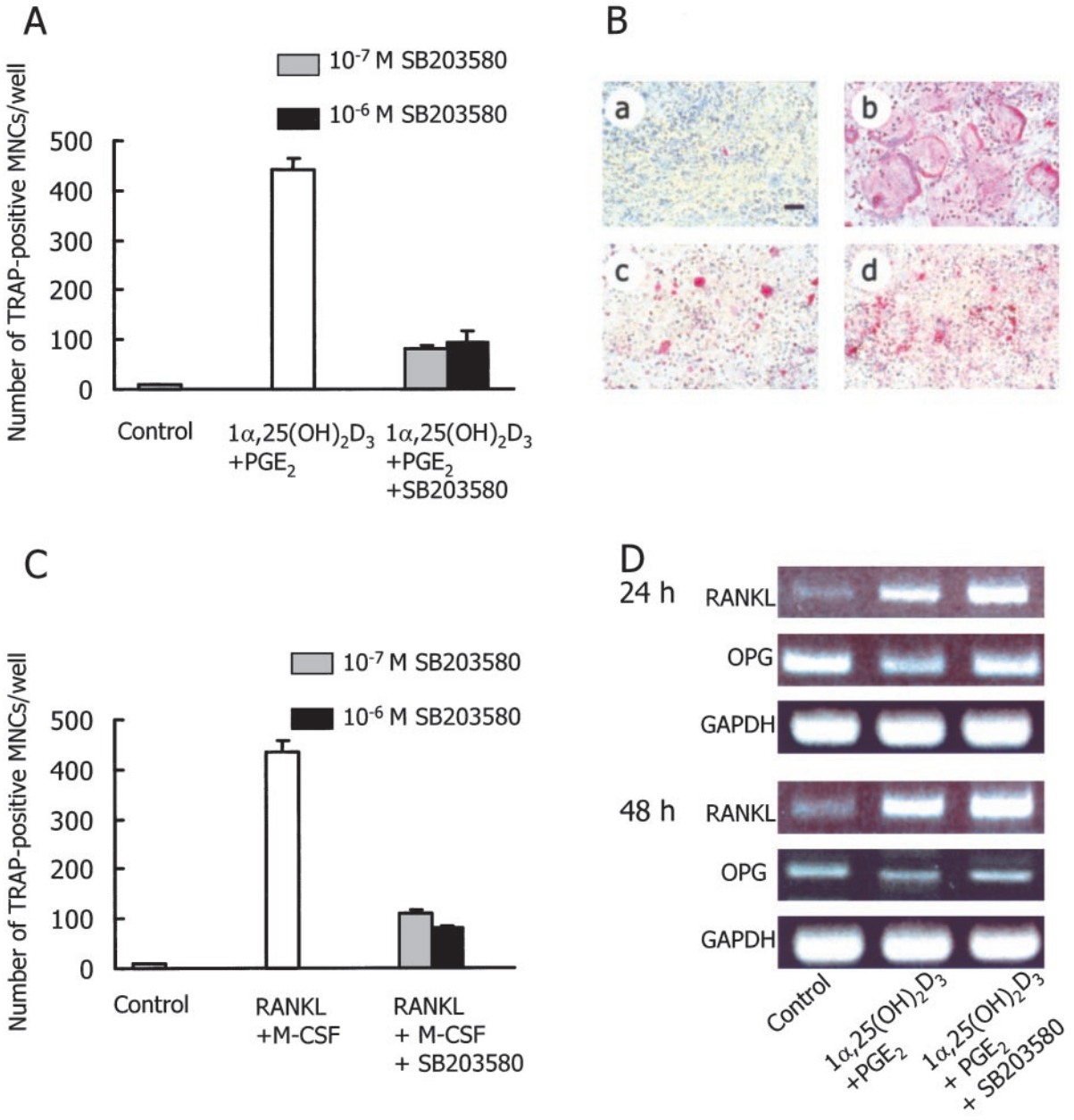

FIG. 1. Effects of SB203580 on osteoclast differentiation of mouse bone marrow cells and on the expression of RANKL mRNA in osteoblasts. A, Mouse calvarial osteoblasts and bone marrow cells were cocultured, in 48-well culture plates, in the presence of 1α,25-$(OH)_2D_3$ (10^{-8} M) and PGE_2 (10^{-7} 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 ± SEM of three cultures. B, TRAP-staining of cocultures: a, control coculture; b, coculture treated with 1α,25-$(OH)_2D_3$ (10^{-8} M) and PGE_2 (10^{-7} M); c, coculture treated with 1α,25-$(OH)_2D_3$ (10^{-8} M) and PGE_2 (10^{-7} M) plus SB203580 (10^{-7} M); d, coculture treated with 1α,25-$(OH)_2D_3$ (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 ± SEM of three cultures. D, Mouse calvarial osteoblasts were cultured, for 24 h or 48 h, in the presence of 1α,25-$(OH)_2D_3$ (10^{-8} M) and PGE_2 (10^{-7} M). Some cultures were pretreated for 30 min with SB203580 (10^{-6} M) before addition of 1α,25-$(OH)_2D_3$ and PGE_2, and further treated for 24 h or 48 h with SB203580 in the presence of 1α,25-$(OH)_2D_3$ 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

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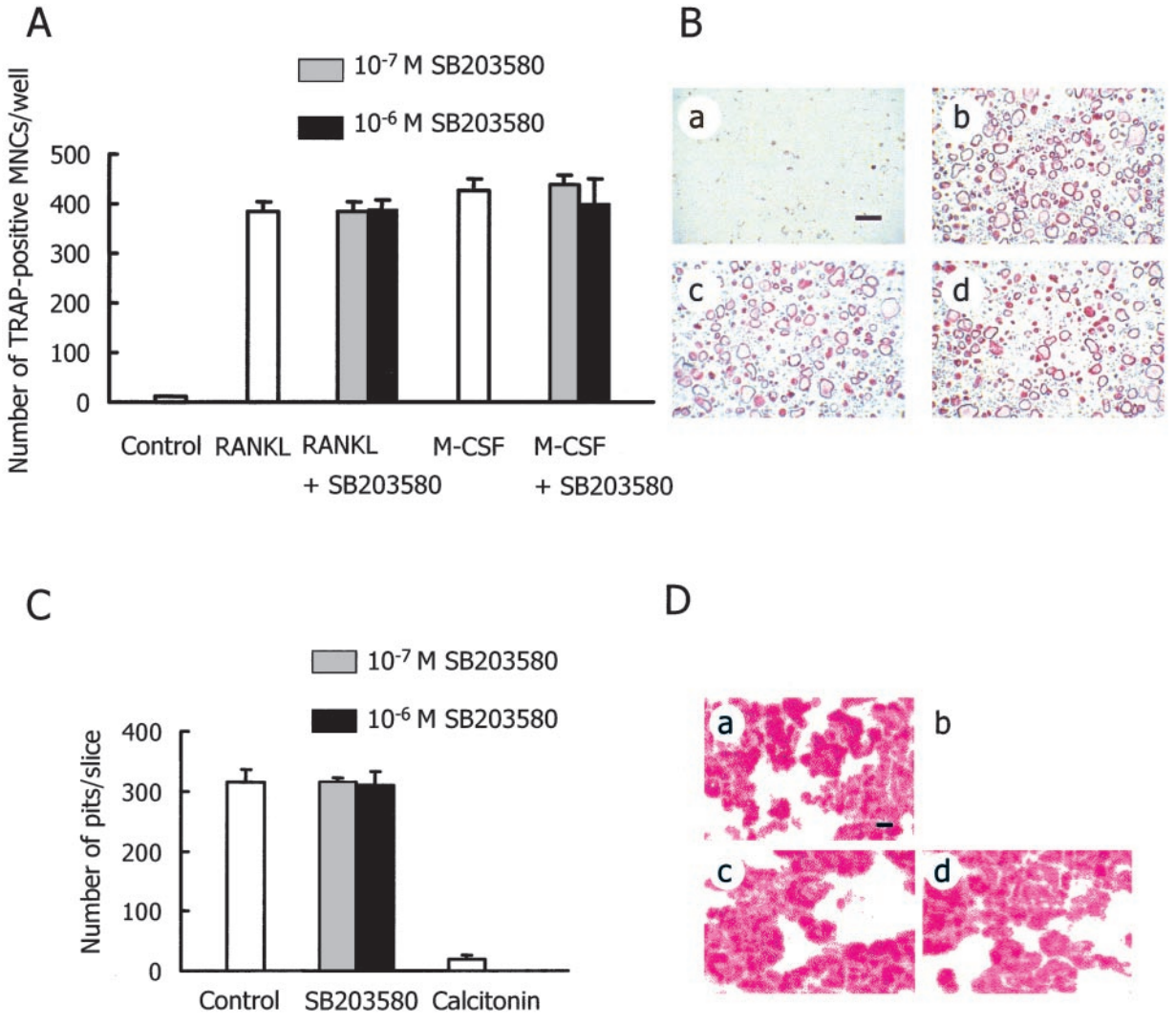

FIG. 2. Effect of SB203580 on the survival and function of osteoclasts. A, Crude osteoclast preparations with 5% purity were cultured for 6 h in 24-well plates. Osteoblasts were then removed with 0.05% trypsin/EDTA to obtain highly purified osteoclasts. Purified osteoclasts with 95% purity were pretreated 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-$(OH)_2D_3$ 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

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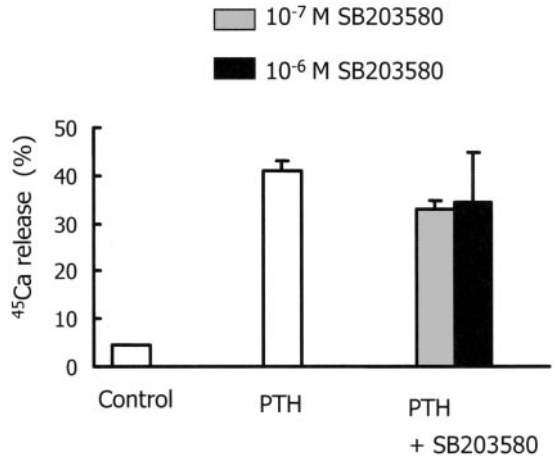

FIG. 3. Effect of SB203580 on bone resorption in fetal mouse long bone cultures. Fetal bones (radii and ulnae) prelabeled with ^{45}Ca were cultured for 72 h in the presence or absence of PTH (100 ng/ml) together with or without SB203580 at 10^{-7} M or 10^{-6} M. Bone-resorption activity was expressed as the percent release of ^{45}Ca from prelabeled bones.

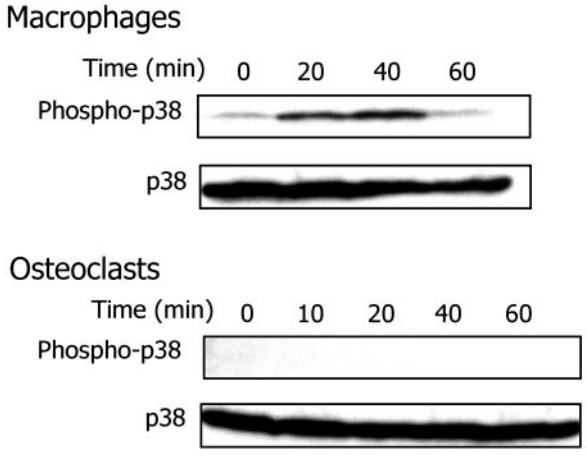

FIG. 4. Time course of change in the phosphorylation of p38 MAPK in macrophages and osteoclasts after treatment with RANKL. Mouse bone marrow macrophages (osteoclast precursors) were prepared from bone marrow cultures treated with M-CSF. Osteoclasts were purified from crude osteoclast preparations by 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).

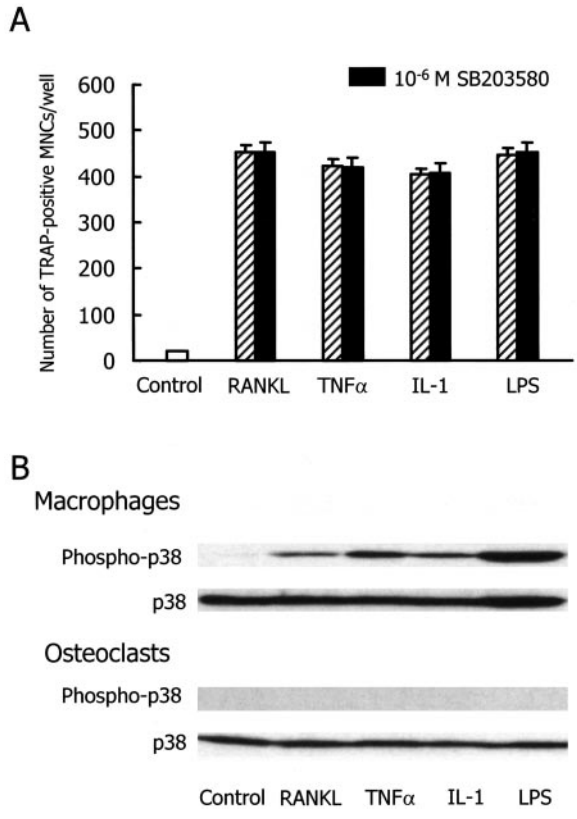

FIG. 5. Effects of RANKL, TNFα, IL-1, and LPS on the survival of osteoclasts, and on the phosphorylation of p38 MAPK in macrophages and osteoclasts. A, Purified osteoclasts were prepared in 24-well culture plates and further cultured for 48 h in the presence or absence of RANKL (200 ng/ml), TNFα (10 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 $PGF_2\alpha$-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-$(OH)_2D_3$- and PGE_2-induced up-regulation of RANKL mRNA expression or down-regulation of OPG mRNA ex-

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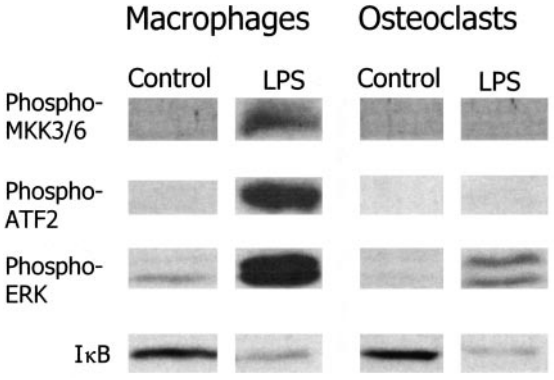

FIG. 6. Effects of LPS on the phosphorylation of MKK3/6, ERK, and ATF2, and the degradation of IκB in macrophages and osteoclasts. Mouse bone marrow macrophages were prepared from bone marrow cultures treated with M-CSF. Osteoclasts were purified from crude osteoclast preparations. Bone marrow macrophages 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).

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.

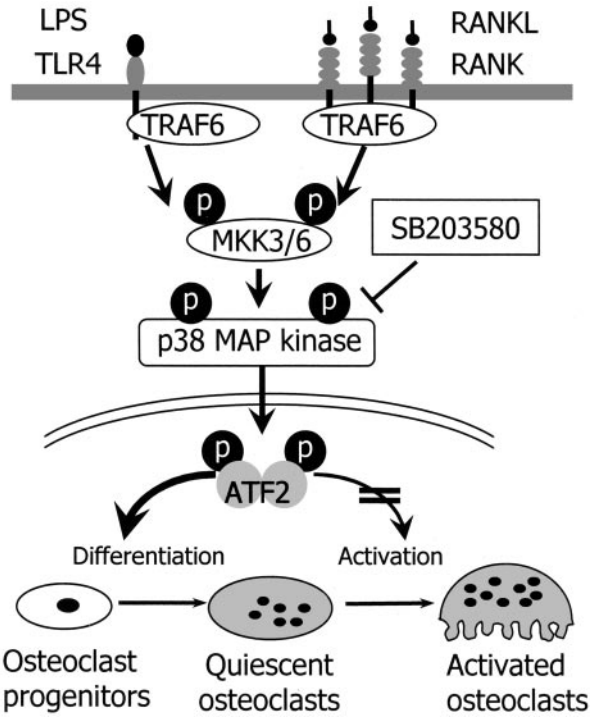

FIG. 7. A schematic representation of the p38 MAPK signal pathway in osteoclast differentiation and activation regulated by RANKL and LPS. RANKL and LPS independently stimulate the p38 MAPK-mediated signal pathway. TRAF6 may act as a common signal transducer for RANK and TLR4. SB203580, a selective inhibitor 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.

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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Address all correspondence and requests for reprints to: Naoyuki Takahashi, Ph.D., Institute for Dental Science, Matsumoto Dental University, 1780 Gobara, Hirooka, Shiojiri, Nagano 399-0781, Japan. E-mail: takahashinao@po.mdu.ac.jp.

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