Involvement of p38 Mitogen-activated Protein Kinase Signaling Pathway in Osteoclastogenesis Mediated by Receptor Activator of NF-κB Ligand (RANKL)*

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The receptor activator of NF-κB ligand (RANKL) induces osteoclast differentiation from bone marrow cells in the presence of macrophage colony-stimulating factor. We found that treatment of bone marrow cells with SB203580 inhibited osteoclast differentiation via inhibition of the RANKL-mediated signaling pathway. To elucidate the role of p38 mitogen-activated protein (MAP) kinase pathway in osteoclastogenesis, we employed RAW264 cells which could differentiate into osteoclast-like cells following treatment with RANKL. In a dose-dependent manner, SB203580 but not PD98059, inhibited RANKL-induced differentiation. Among three MAP kinase families tested, this inhibition profile coincided only with the activation of p38 MAP kinase. Expression in RAW264 cells of the dominant negative form of either p38α MAP kinase or MAP kinase kinase (MKK) 6 significantly inhibited RANKL-induced differentiation of the cells. These results indicate that activation of the p38 MAP kinase pathway plays an important role in RANKL-induced osteoclast differentiation of precursor bone marrow cells.

Bone morphogenesis, remodeling, and resorption are controlled in part by osteoclasts. These cells differentiate from hematopoietic myeloid precursors of monocyte/macrophage lineage under control of osteotropic hormones and local factors produced by supporting cells such as osteoblasts and stromal cells (1–9).

The receptor activator of NF-κB ligand (RANKL)1 (10), also referred to as osteoclast differentiation factor (11), tumor necrosis factor-related activation-induced cytokine (12), or osteoprotegerin ligand (13), was shown to be highly expressed in osteoclasts (7, 13–15). To describe RANKL-induced osteoclastogenesis, the sequential phenotype progression model was proposed. The model includes the appearance of mononuclear osteoclasts, the fusion process prior to multinucleated osteoclast formation, and the osteoclast maturation process (6). Moreover, it has been shown that mutant mice disrupted with either RANKL or its receptor RANK revealed severe osteopetrosis and osteoclast defects (16, 17), indicating that the RANKL-RANK signaling system plays an essential role in osteoclast differentiation.

It has been shown recently that RANK is associated with tumor necrosis factor receptor-associated factors (TRAFs) (18–21). The intracellular domain of RANK contains two distinct TRAF-binding domains, each of which recognizes different TRAF proteins specifically (18, 19). While the C-terminal region of RANK interacts with TRAF2 and TRAF5, the TRAF6-binding domain resides in the middle of the RANK intracellular region. Overexpression of RANK C-terminal deletion mutants has revealed that activation of the RANK-mediated signaling pathway results in the activation of NF-κB and c-Jun N-terminal kinase (JNK) which correlate with the TRAF6 interaction activity of mutants (18, 19). In addition, mice with disrupted TRAF6 gene exhibit an osteopetrotic phenotype due to a defect in bone resorption (22, 23). Therefore it is speculated that JNK might play an important role in osteoclast differentiation.

Mitogen-activated protein (MAP) kinases are proline-directed serine/threonine kinases that are important in cell growth, differentiation, and apoptosis (24–27). They become activated by phosphorylation on threonine and tyrosine in response to external stimuli. Three major subfamilies of MAP kinase have been identified in mammalian cells: 1) extracellular signal regulated kinases (ERKs), 2) JNKs, and 3) p38 MAP kinases. It is widely accepted that peptide growth factors and phorbol esters preferentially activate ERKs, while cellular stresses, such as hyperosmolarity or reactive oxygen species, potently activate JNKs and p38 MAP kinases (28–30).

In this paper, we have investigated the possible involvement of MAP kinase families in the RANKL-RANK signaling pathway that leads to osteoclast differentiation. Here we demonstrate that a p38 MAP kinase inhibitor, SB203580, inhibits RANKL-induced osteoclast differentiation. In addition, we show that the expression of a dominant negative form of either p38 MAP kinase or MAP kinase kinase (MKK) 6 partially inhibits differentiation. Our results indicate for the first time that activation of the p38 MAP kinase pathway plays a role in RANKL-induced osteoclast differentiation.

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1 The abbreviations used are: RANKL, receptor activator of NF-κB ligand; RANK, receptor activator of NF-κB; M-CSF, macrophage colony-stimulating factor; MAP, mitogen-activated protein; MKK, MAP kinase kinase; RT-PCR, reverse transcription-polymerase chain reaction; TRAF, tumor necrosis factor receptor-associated factor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; OPG, osteoprotegerin; TRAP, tartrate-resistant acid phosphatase; NEAA, non-essential amino acid; GST, glutathione S-transferase.
RANKL-induced Osteoclastogenesis

EXPERIMENTAL PROCEDURES

Cell Culture—Bone marrow cells were prepared by removing femurs from 6–8-week-old ddY mice and flushing the bone marrow cavity with RPMI 1640 (Life Technologies Inc., Gaithersburg, MD) containing 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 μg/ml kanamycin, 1% non-essential amino acid (NEAA), 1% sodium pyruvate, and 5 μM β-mercaptoethanol. After lysing erythrocytes with a lysing buffer (17 mM Tris, pH 7.5, 0.75% NP-40, 0.5% SDS, 5% β-mercaptoethanol), the red blood cells were washed twice with 0.5 M NaCl in 24-well plates in the presence of human recombinant soluble RANKL (rRANKL, Peprotech EC Ltd., London, United Kingdom) and/or murine M-CSF (Genzyme, Cambridge, MA). The culture medium was replaced every 3 days with a fresh complete medium containing the appropriate regents. After 8–10 days, cells were washed and subjected to a tartrate-resistant acid phosphatase (TRAP) assay (32). In brief, adherent cells were trypsinized and starved for 5 h in serum-free minimal essential medium/NEAA media and then cultured for 5 days in MEM/NEAA containing 2% fetal bovine serum and rRANKL.

Materials—Polyclonal antibodies against p38 MAP kinase, phosphorylated p38 MAP kinase Thr180/Tyr182, JNKs (34), and phosphorlated JNKs were purchased from New England Biolabs Inc. (Beverly, MA). Monoclonal anti-phosphorylated p38 MAP kinase (Thr180/Tyr182), JNKs (Thr183/Tyr185), and phosphorlated JNKs (Thr183/Tyr185) were purchased from New England Biolabs. The polyclonal anti-MAPKAP kinase-2 and MAPKAP kinase substrate peptide were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Recombinant mouse osteoprotegerin (OPG), Fc chimera, and monoclonal anti-RANKL antibody were from New England BioLabs. The polyclonal anti-MAPKAP kinase-2 and MAPKAP kinase substrate peptide were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Recombinant mouse osteoprotegerin (OPG), Fc chimera, and monoclonal anti-RANKL antibody were from New England BioLabs. Recombinant mouse osteoprotegerin (OPG), Fc chimera, and polyclonal anti-RANK antibody were from R & D Systems Inc. (Minneapolis, MN). The p38 MAP kinase expression plasmid was prepared as described previously (31). The MKK6 expression plasmid was a kind gift provided by Dr. H. Sakurai (Tanabe Pharmaceutical Co., Ltd., Osaka, Japan).

Western Blot Analysis—Immunoblotting and immunoprecipitations were performed as described (35). In brief, cells were lysed in a lysis buffer (20 mM Heps, pH 7.4, 2 mM EDTA, 50 mM β-glycerophosphate, 1.0% Triton X-100, 10% glycerol, 1 μM dithiothreitol, 1 μM leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). Whole cell extracts were prepared by centrifugation at 10,000 × g for 15 min at 4 °C. Whole cell extracts (30 μg) were electrophoresed on a 10% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membranes. Immunoblot detection was performed with the corresponding rabbit antisera or mouse monoclonal antibody using a ECL detection kit (Amer sham Pharma Biotech, Buckinghamshire, UK).

Protein Kinase Assays—p38 MAP kinase and JNK activities were measured in an immunocomplex kinase assay. Phosphorylated p38 MAP kinase was immunoprecipitated by incubation with anti-phosphorylated p38 MAP kinase (p38) monoclonal antibody immobilized on agarose beads (anti-p38 Ab-agarose), and phosphorylated JNK with GST-c-Jun fusion protein bound to glutathione-Sepharose beads (GST-c-Jun-Sepharose). After 16 h of incubation at 4 °C, the immunoprecipitates were collected, washed twice with whole cell extract lysate buffer, and then twice with a kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM MgCl2). Immunoprecipitates of anti-p38 Ab-agarose were mixed with 2 μg of GST fused to ATP2 (GST-ATP2) protein as a substrate and 200 μM ATP in 50 μl of kinase buffer. Pellets precipitated with GST-c-Jun-Sepharose were mixed with 100 μM ATP in 50 μl of kinase buffer. Then the reaction mixtures were further incubated for 30 min at 30 °C. The kinase reaction was terminated by boiling in an appropriate volume of 2× SDS sample buffer. An aliquot of the sample buffer (10 μl) was loaded onto P81 phosphocellulose filters and radioactivity incorporated into the substrate peptide was determined by liquid scintillation spectrometry after washing the filter five times in 0.75% phosphoric acid.

For MAPKAP kinase-2 assay, immunoprecipitates were incubated for 30 min at 30 °C with 100 μM ATP and 1 μM of (γ-32P)ATP in 30 μl of the kinase buffer (50 mM β-glycerophosphate, pH 7.0, 0.1 mM EDTA, 10 mM magnesium acetate, and 0.1 mM Na3VO4). The reactions were terminated by adding 10 μl of 1% orthophosphoric acid containing 1 mM ATP and 1% bovine serum albumin. An aliquot (25 μl) was then spotted onto PS1 phosphocellulose filters and radioactivity incorporated into the substrate peptide was determined by liquid scintillation spectrometry after washing the filter five times in 0.75% phosphoric acid.

Preparation of High Titer Retroviral Particles and Infection—Ecopack 293 packaging cells (CLONTECH) were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum and 100 μg/ml kanamycin. Cells to be transfected were plated 24 h before transfection at a density of 2 × 106 cells per 100-mm dish. Using a calcium phosphate precipitation protocol, Ecopack 293 cells were co-transfected with expression plasmids and pSV-S (as an empty vector) to result RAW264 cells which was a kind gift from H. Miyoshi (The Salk Institute). Supernatants from the transfected 293 cells were collected 72 h after transfection and concentrated by centrifugation (8,000 × g). High titer virus particles were used for infection to target cells after filtration through 0.45-μm filters. Transfectants were selected in cultures supplemented with the appropriate concentration of G418.

RESULTS

Characterization of Osteostat Differentiation from Bone Marrow Cells Induced by M-CSF and sRANK—It is well known that the treatment of bone marrow cells with M-CSF and RANKL induces osteoclast differentiation (6, 10–15). Fig. 1A shows the typical morphological changes of murine bone marrow cell-derived adherent cells into TRAP-positive cells when treated with these cytokines. Treatment of the cells with M-CSF alone resulted in an increase in the number of surviving cells compared with control cells (Fig. 1A, b). However, no TRAP-positive cells were observed in the culture. On the other hand, RANKL induced the appearance of TRAP-positive mononuclear cells without increase in the number of adherent cells (Fig. 1A, d). In the presence of M-CSF and sRANKL, drastic morphological changes of adherent cells were observed, and TRAP-positive mononuclear and multinucleated cells appeared in the culture (Fig. 1A, c).

To determine the possible involvement of MAP kinase signaling pathways in RANKL-induced bone marrow cell differentiation into TRAP-positive cells, bone marrow cells were treated with SB203580, a specific inhibitor of p38 MAP kinase.
M-CSF (20 ng/ml) and sRANKL (50 ng/ml) (Fig. 3). To this end the cells were treated with M-CSF in the presence or absence of inhibitors was incubated for 8 days and subjected to the analysis as described under “Experimental Procedures.” Control cells were harvested without cytokine treatment at day 0.

To confirm that SB203580 interferes with the RANKL-mediated signal transduction system, bone marrow cells were treated sequentially with M-CSF and sRANKL (Fig. 3). To this end the cells were treated with M-CSF in the presence or absence of SB203580 and incubated for 3 days. After removing the reagents by washing, cells were further incubated for 2 days with each cytokine. Sequential treatment with M-CSF and sRANKL resulted in the appearance of TRAP-positive mononuclear cells, whereas M-CSF and sRANKL without SB203580 resulted in the appearance of TRAP-positive mononuclear cells. When cells were pretreated with M-CSF and then incubated in the presence of sRANKL and SB203580, few TRAP-positive mononuclear cells were observed. In contrast, after treatment with M-CSF and SB203580, sRANKL induced the appearance of TRAP-positive cells, indicating that SB203580 interfered with sRANKL-mediated, but not M-CSF-mediated, signaling pathways. Taken together, the results described above strongly suggest that activation of the p38 MAP kinase signaling pathway is involved in the RANKL-induced differentiation of bone marrow cells into osteoclasts.

SB203580 Inhibits the RANKL-induced Differentiation of RAW264 Cells—To further examine the role of action of p38 MAP kinase in sRANKL-mediated osteoclast differentiation, we employed the murine monocyte cell line, RAW264, to simplify the assay system, since this cell line is known to express RANK and differentiate into TRAP-positive osteoclasts. We next examined whether or not p38 MAP kinase signaling pathway is involved in the RANKL-induced differentiation of RAW264 cells.

Activation of MAP Kinases by sRANKL—We next examined whether or not p38 MAP kinase was indeed activated by sRANKL in RAW264 cells (Fig. 5). Activation of sRANKL was monitored by immunoblot analysis employing the anti-p38 MAP kinase antibody. Kinetic analysis revealed phosphorylated p38 MAP kinase to be detectable within 5 min, reached a plateau at 15 min after addition of sRANKL, and 4. The effect of sRANKL was prominent at a concentration of 5 ng/ml and reached a maximum level at 50 ng/ml (Fig. 4B). SB203580 inhibited the activation of TRAP-positive cells in a dose-dependent manner with IC50 being 0.56 μM (Fig. 4C).

Activation of MAP Kinases by sRANKL—We next examined whether or not p38 MAP kinase was indeed activated by sRANKL in RAW264 cells (Fig. 5). Activation of p38 MAP kinase was monitored by immunoblot analysis employing the anti-p38 MAP kinase antibody. Kinetic analysis revealed phosphorylated p38 MAP kinase to be detectable within 5 min, reached a plateau at 15 min after addition of sRANKL, and...
gradually declined to a basal level in up to 2 h. In contrast, total amounts of p38 MAP kinase protein were not affected by sRANKL treatment as shown by the anti-p38 MAP kinase antibody. We also measured the in vitro kinase activity employing the GST-ATF2 fusion protein as a substrate and found that the phosphorylation of p38 MAP kinase correlated well with the kinase activity (data not shown).

To confirm the in vivo activation of p38 MAP kinase, we next measured the activation of MAPKAP kinase-2, a downstream substrate of p38 MAP kinase in RAW264 cells treated with sRANKL. RAW264 cells were treated with sRANKL (50 ng/ml) for the indicated times in the presence (closed circle) or absence (open circle) of 5 μM SB203580. Cell lysates were then immunoprecipitated with anti-MAPKAP kinase-2 antibody followed by the kinase reaction. The MAPKAP kinase-2 activity was determined by measuring radioactivity of incorporated into MAPKAP kinase-2 substrate peptide (see “Experimental Procedures”). B, inhibition of MAPKAP kinase-2 activity by SB203580. RAW264 cells were pretreated with various concentrations of SB203580 for 50 min, and MAPKAP kinase-2 activity was determined by immuno-precipitation with anti-MAPKAP kinase-2 antibody. C, inhibition of MAPKAP kinase-2 activity by OPG. RAW264 cells were treated with 50 ng/ml sRANKL in the presence of various concentrations of OPG for 15 min and immunoprecipitated with anti-MAPKAP kinase-2 antibody. Results represent the mean ± S.D. of triplicate determinations.

To confirm the in vivo activation of p38 MAP kinase, we next measured the activation of MAPKAP kinase-2, a downstream substrate of p38 MAP kinase and its inhibition by SB203580 (38). Activation of MAPKAP kinase-2 in RAW264 cells was first observed at 15 min and reached to the maximum level at 60 min after addition of sRANKL (Fig. 6A). Pretreatment of the cells with SB203580 inhibited the RANKL stimulated activity of MAPKAP kinase-2 in a dose-dependent manner with IC_{50} being 0.43 μM SB203580, indicating that SB203580 inhibits MAPKAP kinase-2 with a similar IC_{50} to that which inhibits osteoclast differentiation (Fig. 6B).

Since OPG is a decoy receptor for RANK and inhibits the RANKL-induced osteoclastogenesis (39, 40), we next examined the effect of OPG on RANKL-induced activation of p38 MAP kinase. As shown in Fig. 6C, in the presence of 50 ng/ml RANKL, 3-fold increase in the MAPKAP kinase-2 activity was observed. OPG inhibited the MAPKAP kinase-2 activity in a dose-dependent manner, further supporting the role of p38 MAP kinase in osteoclastogenesis.

Since RANKL is known to activate JNK (18–20, 22), we also measured the sRANKL-induced JNK activation in RAW264 cells (Fig. 7A). Treatment with sRANKL for time periods rang-
MAP kinases, SB203580 was included during the incubation period. When RAW264 cells (10^6 cells/culture) were treated with 50 ng/ml sRANKL, both p42 and p44 forms of ERK by sRANKL. RAW264 cells were then subjected to Western blot analysis employing an anti-phosphorylated c-Jun polyclonal antibody.

Effects of Expression of Dominant Negative Forms of p38 MAP Kinase and MKK6—In order to confirm the essential role of the p38 MAP kinase pathway, the cells were transfected with expression vectors encoding wild type MKK6 (CXMKK6 cell) or the dominant negative form of MKK6 (CXMKK6DN cell). As shown in Fig. 8B, about 50% decrease in the number of TRAP-positive cells induced by sRANKL was observed, indicating that a defect in the upstream regulator of p38 MAP kinase also caused the suppression of differentiation.

Further increase in the activity of p38 MAP kinase was observed in CX38 and CXMKK6 cells (4.2- and 4.1-fold, respectively) after treatment with sRANKL. In contrast, while the expression of the dominant negative form of p38 MAP kinase caused 26% decrease in the sRANKL-mediated enhancement of MAPKAP kinase-2 activity in CX38DN cells, that of the dominant negative form of MKK6 caused 41% decrease in CXMKK6DN cells, indicating some correlation between the p38 MAP kinase activity and the differentiation of the cells. Although not quantitative, comparable results were obtained by measuring the phosphorylation of p38 MAP kinase (Fig. 9A).

**DISCUSSION**

Osteoclasts differentiate from hematopoietic precursors through interaction with stromal and osteoblast cells, which provide the microenvironment essential for osteoclastogenesis (1–7, 15, 16). One of the critical factors produced by these two
supporting cells is M-CSF (41–44). In the presence of M-CSF, bone-resorbing factors such as prostaglandin E2 (45), vitamin D$_3$ (1,25-(OH)$_2$D$_3$) (46), and parathyroid hormone (47), induce the differentiation of precursor cells to mononuclear osteoclasts, followed by the generation of multinucleated osteoclasts. It was reported that the differentiation inducing activity of these reagents was mediated by RANKL, since the addition of OPG/OCIF, a decoy receptor for RANK, completely inhibited the generation of osteoclasts (39, 40). These results suggest that in combination with M-CSF, RANKL plays an essential role in the induction of osteoclast differentiation.

In this paper, we have confirmed that both M-CSF and sRANKL are required to induce terminal differentiation of bone marrow cells into multinucleated osteoclasts. Our results suggest that M-CSF and RANKL act on bone marrow cells sequentially to induce the terminal differentiation to osteoclasts and that RANKL is the differentiation inducing factor in our assay system since sRANKL alone could induce the TRAP-positive mononuclear cells in the absence of the increase in cell number. Several reports indicate that M-CSF plays an essential role in osteoclastogenesis through RANK induction, the stimulation of cell survival and proliferation, and by acting as a competence factor for differentiation (34, 48, 49).

It was quite unexpected for us that SB203580 but not PD98059 inhibited the M-CSF/sRANKL-induced differentiation of bone marrow cells into multinucleated osteoclasts. Since an increase in the number of adherent cells, albeit no appearance of TRAP-positive cells, was observed even in the presence of SB203580, we can speculate that SB203580 inhibits the differentiation of bone marrow cells by interfering with the RANKL-induced p38 MAP kinase activity.

To elucidate the causal link between the activation of the p38 MAP kinase pathway and RANKL-induced osteoclast differentiation, we employed RAW264 cells which expressed RANK and differentiated into TRAP-positive multinucleated cells through sRANKL treatment. As was the case with bone marrow cells, SB203580 but not PD98059 inhibited differentiation induced by sRANKL. In RAW264 cells, RANKL-induced activations of ERKs, JNKs, and p38 MAP kinase were clearly detected and SB203580 inhibited only the p38 MAP kinase activity. These results strongly suggest that at least in part p38 MAP kinase plays a critical role in RANKL-induced differentiation. However, we cannot rule out the possible involvement of ERK and JNK in osteoclastogenesis at present.

Expression of the dominant negative form of p38α MAP kinase resulted in a significant decrease in the number of TRAP-positive cells induced by RANKL. Significant decrease in the RANKL-induced p38 MAP kinase activation was also observed. These results further support the notion that the activation of the p38 MAP kinase signaling pathway is necessary for sRANKL-induced differentiation of bone marrow cells and RAW264 cells into osteoclasts. However, complete inhibition was not observed in CX38DN cells expressing the dominant negative form of p38α MAP kinase. It is conceivable that other isoforms of p38 MAP kinase could compensate for a possible defect in the signaling pathway through p38α MAP kinase. In addition to the α-type, at least three isoforms (β-, γ-, and δ-types) of p38 MAP kinase are reported (50–55). We detected the expression of the mRNA for β-type in RAW264 cells by RT-PCR (data not shown). Therefore it is possible that p38β MAP kinase functions in CX38DN cells. Because SB203580 inhibited osteoclastogenesis completely, it is likely that only p38α and p38β MAP kinases are involved in differentiation since these are the only isoforms inhibited by SB203580 at the low doses (38).

MKK6 is a direct and specific activator of all p38 MAP kinase isoforms so far identified (56, 57). Expression of the dominant negative form of MKK6 inhibited both sRANKL-induced differentiation of RAW264 cells and p38 MAP kinase activation. It is likely that the dominant negative form of MKK6 inhibits both α- and β-types of p38 MAP kinase, thus conferring a greater range of differentiation inhibition than the inhibition performed by the dominant negative form of p38α MAP kinase. The quantitative differences between the suppression of differentiation seen with dominant negative forms of p38α MAP kinase and MKK6 could be completely explained by the extent to which the transfected forms are able to suppress p38 activity in cells. Presumably if they were more efficient, they could both achieve complete suppression.

In conclusion, we have demonstrated in this paper that the p38 MAP kinase signaling pathway plays a crucial role in RANKL-mediated differentiation of bone marrow cells into osteoclasts. However, experiments of the overexpression of wild type kinases revealed no correlation between p38 MAP kinase activity and RANKL-induced differentiation, suggesting the role of other factors in the RANKL-mediated osteoclastogenesis. Since the activation of other MAP kinase families (i.e. ERKs and JNKs) during differentiation was clearly observed, the contribution of these kinases to osteoclast differentiation should be elucidated in the near future.

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