Activation of p38 mitogen-activated protein kinase is crucial in osteoclastogenesis induced by tumor necrosis factor

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Abstract Tumor necrosis factor (TNF) induces osteoclast differentiation from bone marrow cells in the presence of macrophage colony-stimulating factor. Treatment of bone marrow cells with SB203580 but not PD98059 inhibited TNF-induced osteoclast differentiation. In RAW264 cells which differentiate into osteoclast-like multinucleated cells by TNF treatment alone, activation of p38 mitogen-activated protein (MAP) kinase induced by murine TNF was comparable to and independent of the receptor activator of necrosis factor-κB ligand. Moreover, the number of multinucleated osteoclasts induced by TNF in bone marrow cell cultures derived from p38 MAP kinase gene deficient mice was significantly less than that from control mice. These results indicate that the p38 MAP kinase pathway plays a crucial role in TNF-mediated osteoclast differentiation.

Key words: Osteoclastogenesis; p38 mitogen-activated protein kinase; Receptor activator of necrosis factor-κB ligand; Tumor necrosis factor

1. Introduction

Osteoclasts which differentiate from hematopoietic myeloid precursors of monocyte/macrophage lineage, play pivotal roles in bone morphogenesis, remodeling, and resorption in concert with other supporting cells such as osteoblasts and stromal cells [1–6].

The receptor activator of necrosis factor (NF)-κB ligand (RANKL) [7], a member of tumor necrosis factor (TNF) ligand family, is highly expressed in supporting cells and directly induces the differentiation and maturation of osteoclasts [8]. It has been shown that mutant mice disrupted with either RANKL [9] or its receptor RANK [10] revealed severe osteopetrosis, indicating that the RANKL–RANK signaling system plays an essential role in the osteoclast differentiation. In our recent work, we investigated the signal transduction mechanism of RANKL-induced osteoclastogenesis and found that the p38 mitogen-activated protein (MAP) kinase signaling pathway plays an important role in the differentiation of bone marrow cells into osteoclast-like multinucleated cells [11].

TNF, a monocyte/macrophage-derived cytokine, was originally identified as a factor with anti-tumor activity in vitro and in vivo, but is now known to be implicated in diverse biological processes including inflammation, immunoregulation, anti-viral defense, endotoxic shock, angiogenesis, and mitogenesis [12,13]. Moreover, it has been reported that in the presence of macrophage colony-stimulating factor (M-CSF), TNF directly acts on osteoclast progenitors and induces osteoclast differentiation [14–16]. Since chronic inflammatory diseases such as rheumatoid arthritis and periodontal diseases are associated with the accumulation of TNF, it is generally believed that TNF is one of the mediators of local bone destruction seen in these diseases [17]. In addition, it has been reported that the injection of anti-TNF monoclonal antibody is effective for the improvement of rheumatoid arthritis.[17,18]. Since osteoclasts have been identified in tissue sections from sites of bone erosion in rheumatoid arthritis and animal models of inflammatory arthritis [19], it is essential to elucidate the mechanism of TNF action on osteoclasts for the development of reagents which improve the diseases.

In the current study, we have investigated the signal transduction mechanism of TNF-mediated osteoclastogenesis. Our data reveal that activation of p38 MAP kinase plays a pivotal role in TNF-induced osteoclast differentiation.

2. Materials and methods

2.1. Materials

Recombinant murine TNF-α (mTNF) was purchased from R&D systems Inc. (Minneapolis, MN, USA) and recombinant soluble human RANKL (sRANKL) from Peprotech EC Ltd. (London, UK). Murine M-CSF and recombinant human interleukin-1β (IL-1β) from Genzyme (Cambridge, MA, USA). Polyclonal antibodies against p38 MAP kinase and phosphorylated p38 MAP kinase (Thr^{180}/Tyr^{182}) and GST–ATF2 protein were purchased from New England Biolabs Inc. (NEB, Beverly, MA, USA). The polyclonal anti-MAPKAP kinase-2 and MAPKAP kinase substrate peptide were obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Recombinant mouse osteoprotegerin (OPG)-Fc chimera protein was from R&D systems Inc. (Minneapolis, MN, USA). SB203580 and PD98059 were purchased from Calbiochem Corp. (La Jolla, CA, USA) and NEB, respectively.

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Abbreviations: JNK, c-Jun N-terminal kinase; M-CSF, macrophage colony-stimulating factor; MAP, mitogen-activated protein; RANKL, receptor activator of NF-κB ligand; TNF, tumor necrosis factor

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2.2. Cell culture

Bone marrow cells were prepared by removing femurs from 6–8-week-old mice and flushing the bone marrow cavity with RPMI-1640 (Life Technologies Inc. Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), 100 μg/ml kanamycin, 1% non-essential amino acid (NEAA), 1% sodium pyruvate and 5 μM β-mercaptoethanol [11]. After lysing erythrocytes with a lysing buffer (17 mM Tris, pH 7.65, 0.75% NH_4Cl), cells were seeded at 1.5×10^6 cells/well (0.5 ml) in 24-well plates in the presence of M-CSF. After a 3-day culture in a fresh complete medium containing the appropriate reagents, non-adherent cells were removed from the culture by pipetting and washing with PBS [15]. Adherent cells were further incubated for 4 days. Then cells were washed and subjected to a tartrate-resistant acid phosphatase (TRAP) assay (see below).

RAW264 (RIKEN, RCB0535 lot no.008) were grown in MEM media supplemented with 5% FBS and 1% NEAA. For the TRAP assay, RAW264 cells were trypsinized and starved for 5 h in serum-free MEM/NEAA media and then cultured for 5 days in MEM/NEAA containing 2% FBS and the desired cytokines.

2.3. Osteoclast differentiation assay

Osteoclast formation was measured by quantifying TRAP-positive cells as described [11]. In brief, adherent cells were fixed with 10% formaldehyde in PBS for 3 min. After treatment with ethanol/acetone (50:50 v/v) for 1 min, the well surface was air dried and incubated at room temperature in an acetate buffer (0.1 M sodium acetate, pH 5.0) containing 0.01% naphthol AS-MX phosphate (Sigma, St Louis, MO, USA) and 0.03% fast red violet LB salt (Sigma) in the presence of 50 mM sodium tartrate. Osteoclast-like TRAP-positive cells in each well were scored by counting the number of TRAP-positive mononuclear or multinucleated cells containing three or more nuclei.

2.4. Western blot analysis

Immunoblots and immunoprecipitations were performed as described [20,21]. In brief, cells were lysed in a lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 0.1% Triton X-100, 10% glycerol, 1 mM dithiothreitol (DTT), 1 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). Whole cell extracts (WCEs) sonicated for 5 s four times were prepared by centrifugation at $10\,000\times g$ for 15 min at 4°C. WCEs (30 μg) were electrophoresed on a 10% SDS–polyacrylamide gel and blotted onto polyvinylidene difluoride membranes. Immunoblot detection was performed with the corresponding antibodies using a ECL detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.5. Protein kinase assays

p38 MAP kinase activity was measured in an immune complex kinase assay. Phosphorylated p38 MAP kinase was immunoprecipitated by incubation with anti-phosphorylated p38 (pp38) monoclonal antibody immobilized on agarose beads (anti-pp38 Ab-agarose). After 16 h of incubation at 4°C, the immunoprecipitates were collected, washed twice with WCE lysis buffer, and then twice with a kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$). immunoprecipitates of anti-pp38 Ab-agarose were mixed with 2 μg of GST fused to ATF2 (GST–ATF2) protein as a substrate and 200 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 SDS sample buffer. The phosphorylated GST–ATF2 was detected by Western blot analysis with anti-phosphorylated ATF2 polyclonal antibody (NEB).

For MAPKAP kinase-2 assay, immunoprecipitates were incubated for 30 min at 30°C with 100 μM of ATP and 1 μCi of [γ-^{32}P]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 Na_3VO_4). The reactions were terminated by adding 10 μl of 1% orthophosphoric acid containing 1 mM ATP and 1% BSA. An aliquot (25 μl) was then spotted 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.

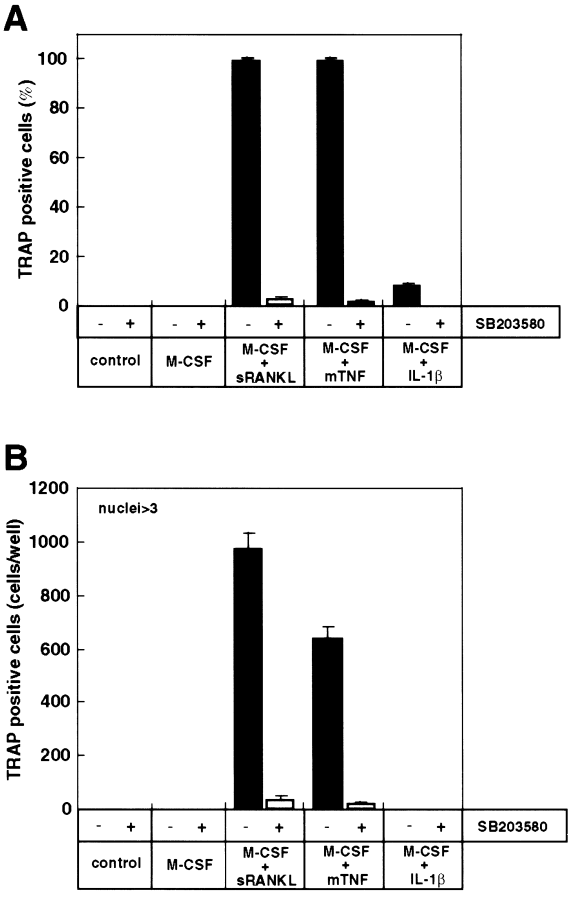

Fig. 1. Cytokine-induced formation of osteoclast-like mononuclear (A) and multinucleated (B) cells from murine bone marrow cells obtained from ddY mice. Bone marrow adherent cells were incubated for 7 days with no addition, M-CSF (20 ng/ml), M-CSF and sRANKL (100 ng/ml), M-CSF and mTNF (100 ng/ml), and M-CSF and IL-1β (100 ng/ml). The cells were treated with SB203880 (5 μM) 30 min prior to the addition of cytokines. After incubation, cells were subjected to the TRAP assay as described in Section 2. Cells containing three or more nuclei were scored as multinucleated cells in (B). Results represent the mean ± S.D. of triplicate determinations.

2.6. p38 MAP kinase deficient mice

p38 MAP kinase deficient mice were generated by gene targeting previously and backcrossed to C57BL/6J strain mice for four or five generations [22]. Genotyping was carried out by PCR using DNA templates prepared from mouse tails.

3. Results and discussion

It is recently reported that the treatment of bone marrow cells with M-CSF and TNF induces osteoclastogenesis [14–16]. It is also suggested that the intracellular signal through TNF is independent of that of RANKL since TNF and sRANKL synergistically induce osteoclastogenesis from bone marrow macrophages in the presence of M-CSF [14]. To test the possibility, we compared the morphological changes of murine bone marrow cell-derived adherent cells treated either with mTNF or sRANKL in the presence of M-CSF (Fig. 1). As shown previously [11], sRANKL (100 ng/ml) induced both TRAP-positive mononuclear and multinucleated cells in the presence of M-CSF. Comparable induction of TRAP-positive mononuclear cells was observed when

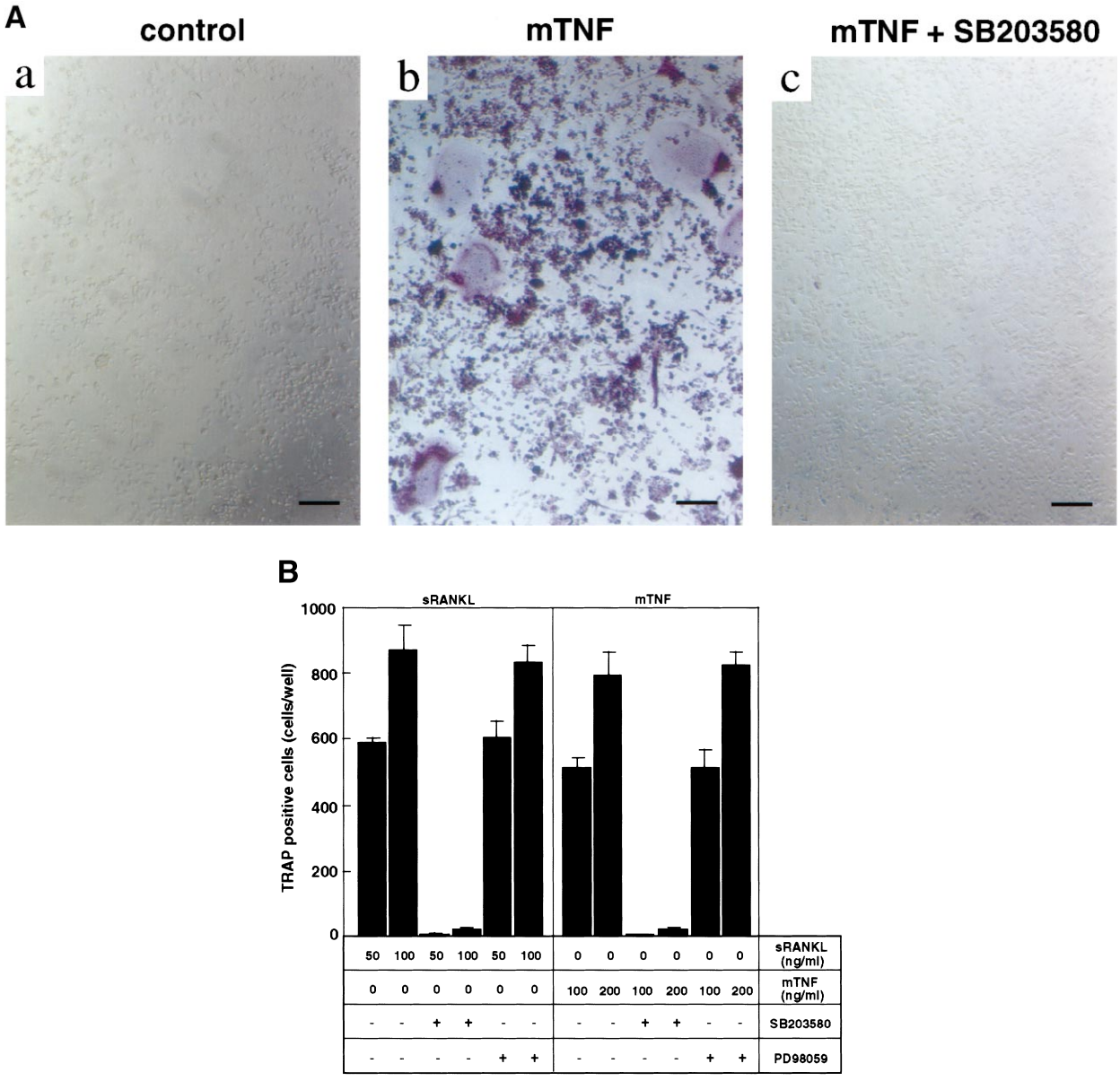

Fig. 2. Inhibition of mTNF-induced RAW264 cell differentiation by SB203580. A: RAW264 cells were seeded at 1×10^4 cells per well in 96-well plates and incubated for 6 days with: no addition (a), mTNF (100 ng/ml) (b), mTNF (100 ng/ml) and SB203580 (2.5 μM) (c). After incubation, cells were subjected to the TRAP assay as described in Section 2. Scale bar = 100 μm. B: Quantitative analysis of mTNF-induced differentiation of RAW264 cells into TRAP-positive multinucleated cells. The cells were incubated with various concentrations of mTNF in the presence or absence of inhibitors as indicated.

cells were treated with mTNF and M-CSF. On the other hand, mTNF (100 ng/ml) induced TRAP-positive multinucleated cells less efficiently and about 35% decrease in the appearance of the cells was observed. IL-1β had little effect on the induction of TRAP-positive cells in our assay system.

We then examined the effect of SB203580, a specific inhibitor of p38 MAP kinase [23–25], on the cytokine-induced differentiation of osteoclasts. As shown in Fig. 1, treatment of bone marrow cells with SB203580 inhibited the cytokine-induced appearance of both TRAP-positive mononuclear and multinucleated cells almost completely. In contrast, PD98059, a specific inhibitor of MAPK/ERK kinase had no effect on the cytokine-induced osteoclastogenesis. These results strongly suggest that the mTNF-mediated signal transduction pathway in osteoclastogenesis shares, at least in part, the p38 MAP kinase signaling pathway with that mediated by sRANKL. However, it was reported that while RANKL could mediate the functional maturation of osteoclasts, IL-1 was required for the TNF-mediated maturation of the cells [14,15]. These results suggest that activation of p38 MAP kinase is required for the early stage of osteoclast differentiation.

To examine the relationship between osteoclast differentiation and p38 MAP kinase activation, we employed the murine monocyte cell line, RAW264, since this cell line is known to differentiate into osteoclast-like multinucleated cells when treated with sRANKL [11,26]. M-CSF is not required for the differentiation. Fig. 2 shows the effect of mTNF on the morphological changes of RAW264 cells. mTNF induced the differentiation into TPAP-positive multinucleated cells in the absence of M-CSF. mTNF induced TPAP-positive multi-

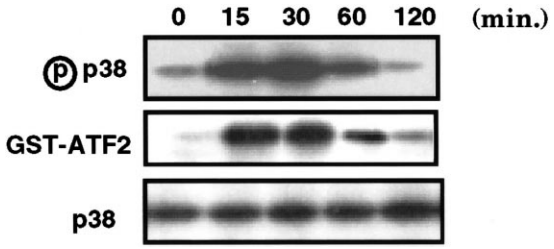

Fig. 3. Activation of p38 MAP kinase in RAW264 cells treated with mTNF. RAW264 cells (10^6 cells/culture) were treated with 100 ng/ml mTNF for the indicated times and then cells were lysed. Cell lysates (30 μg) were resolved by SDS–PAGE and immunoblotted with anti-pp38 MAP kinase (upper panel), or anti-p38 MAP kinase antibody (lower panel). In the middle panel, p38 MAP kinase activity was measured using GST–ATF2 protein as a substrate.

nucleated cells in a dose-dependent manner (Fig. 2B). However, when compared with sRANKL, it was slightly less. As expected, SB203580 but not PD98059 inhibited the appearance of TRAP-positive multinucleated cells almost completely.

Fig. 3 shows the kinetic analysis of p38 MAP kinase activation induced by mTNF. Phosphorylated p38 MAP kinase was detectable at least within 15 min, reached a plateau at 30 min after addition of mTNF and declined to a basal level in up to 2 h. We also measured the in vitro kinase activity employing GST–ATF2 fusion protein as a substrate and found that the kinase activity correlated well with the phosphorylation of p38 MAP kinase. On the other hand, total amounts of the p38 MAP kinase protein were not affected by mTNF treatment.

To confirm the in vivo activation of p38 MAP kinase by mTNF, we next measured the activation of MAPKAP kinase-2, a specific physiological substrate of p38 MAP kinase (Fig. 4) As in the case of sRANKL, activation of MAPKAP kinase-2 was first detectable at 15 min and reached to the maximum level at 60 min after addition of mTNF. As expected, pretreatment of SB203580 but not PD98059 inhibited the mTNF-stimulated MAPKAP kinase-2 in a dose-dependent manner with IC_{50} being 0.34 μM.

Fig. 4C shows the effect of OPG, a decoy receptor for RANKL [27], which inhibits the RANKL- but not TNF-induced osteoclastogenesis [14,15]. While OPG inhibited the RANKL-induced MAPKAP kinase-2 activation in a dose-dependent manner, it had no effect on that induced by mTNF, indicating that mTNF activated p38 MAP kinase independently of RANKL and mTNF-induced RANKL, if any, had little contribution to the p38 MAP kinase activation.

To further address the role of p38 MAP kinase in TNF-induced osteoclastogenesis, we next examined the osteoclastogenic effect of mTNF on bone marrow cells prepared from p38 MAP kinase deficient mice [22]. Since the null mutation of the gene is embryonic lethal, we prepared the cells from heterogeneously gene disrupted ($p38^{+/-}$) mice. It was shown that in comparison to wild type mice, the expression level of p38 MAP kinase was significantly reduced in $p38^{+/-}$ mice [22]. As shown in Fig. 5, repeated experiments indicated that number of mTNF-induced TRAP-positive multinucleated cells derived from $p38^{+/-}$ mice was significantly less than that from wild type littermates. On average, about 28% decrease in

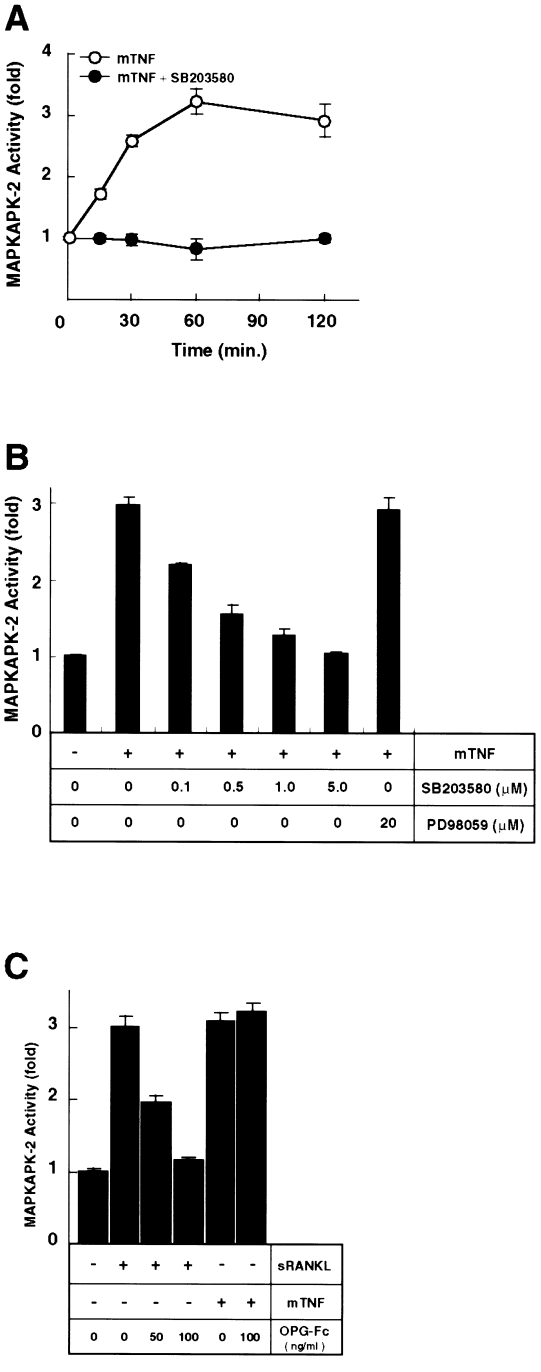

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Fig. 4. Activation of MAPKAP kinase-2 in RAW264 cells treated with sRANKL. A: RAW264 cells were treated with mTNF (100 ng/ml) for indicated times in the presence (closed circle) or absence (open circle) of SB203580 (5 μM) and cell lysates were immunoprecipitated with anti-MAPKAP kinase-2 antibody. B: Inhibition of MAPKAP kinase-2 activity by SB203580. RAW264 cells were pretreated with various concentrations of SB203580 for 30 min. Cells were then treated with 100 ng/ml mTNF, further incubated for 15 min and immunoprecipitated with anti-MAPKAP kinase-2 antibody. C: Inhibition of MAPKAP kinase-2 activity by OPG. RAW264 cells were treated either with mTNF (100 ng/ml) or sRANKL (100 ng/ml) 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.

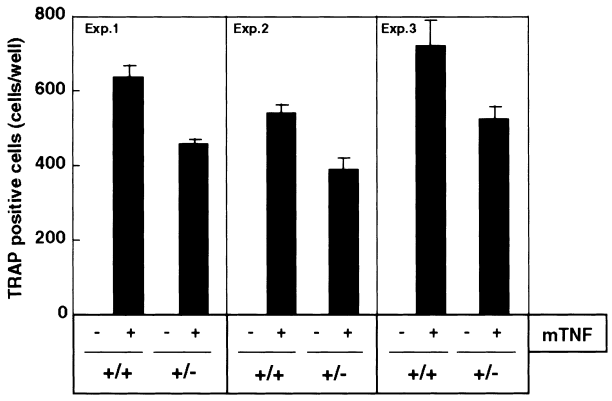

Fig. 5. mTNF-induced formation of osteoclast-like multinucleated cells from bone marrow cells prepared from $p38^{+/-}$ mice. Bone marrow adherent cells prepared either from $p38^{+/-}$ (+/−) mice or wild type littermates (+/+) were incubated for 7 days with M-CSF (20 ng/ml) and mTNF (100 ng/ml). After incubation, cells were subjected to the TRAP assay as described in Section 2. Results represent the mean ± S.D. of triplicate determinations of each experiment.

the appearance of TRAP-positive multinucleated cells was observed. These results suggest that the decreased expression of p38 MAP kinase in bone marrow cells caused the decrease in mTNF-mediated osteoclastogenesis. Similar results were obtained in RANKL-induced osteoclast differentiation (data not shown).

In this paper, we have examined the signal transduction mechanism of TNF-induced osteoclastogenesis. As in the case of sRANKL, mTNF-induced osteoclastogenesis was inhibited by SB203580, indicating for the first time that the p38 MAP kinase signaling pathway plays a pivotal role in TNF-mediated differentiation of bone marrow cells into osteoclasts. Decrease in the number of mTNF-induced osteoclasts derived from bone marrow cells of $p38^{+/-}$ mice support the notion.

According to the sequential phenotype progression model, osteoclastogenesis is divided into several stages [2–6,28]. The model includes the appearance of mononuclear osteoclasts, the fusion process prior to mulitinucleated osteoclast formation, and the osteoclast maturation processes. It was shown that while RANKL could mediate the functional maturation of mulitinucleated osteoclasts, in TNF-mediated osteoclastogenesis, IL-1 is required for the maturation [14,15]. Moreover, in our preliminary experiments, although human TNF induced considerable TRAP-positive mononuclear cells, significant decrease in the appearance of TRAP-positive multinucleated cells was observed when compared with mTNF, suggesting that at least two signals were required for the differentiation of bone marrow cells into TRAP-positive multinucleated cells [14–16]. It is conceivable that while signal through TNF receptor 1 alone is sufficient for the induction of TRAP-positive mononuclear cells, that through TNF receptor 2 is also required for the differentiation into multinuclear osteoclasts [14,15]. In this connection, it has been suggested that c-Jun N-terminal kinases (JNKs) play important roles in osteoclastogenesis [29–31]. Phosphorylation of JNKs was clearly detectable in RAW264 cells after treatment with mTNF (data not shown). To understand the signal transduction mechanisms of osteoclastogenesis exactly, we are now trying to elucidate the roles of these two receptors in detail.

It has been reported that TNF plays pivotal roles in the local bone destruction that is associated with chronic inflammatory diseases such as rheumatoid arthritis. Our present data may facilitate the development of new reagents which improve these diseases.

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