

Tumor Necrosis Factor α Stimulates Osteoclast Differentiation by a Mechanism Independent of the ODF/RANKL-RANK Interaction

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Abstract

Osteoclast differentiation factor (ODF, also called RANKL/TRANCE/OPGL) stimulates the differentiation of osteoclast progenitors of the monocyte/macrophage lineage into osteoclasts in the presence of macrophage colony-stimulating factor (M-CSF, also called CSF-1). When mouse bone marrow cells were cultured with M-CSF, M-CSF-dependent bone marrow macrophages (M-BMM ϕ) appeared within 3 d. Tartrate-resistant acid phosphatase-positive osteoclasts were also formed when M-BMM ϕ were further cultured for 3 d with mouse tumor necrosis factor α (TNF- α) in the presence of M-CSF. Osteoclast formation induced by TNF- α was inhibited by the addition of respective antibodies against TNF receptor 1 (TNFR1) or TNFR2, but not by osteoclastogenesis inhibitory factor (OCIF, also called OPG, a decoy receptor of ODF/RANKL), nor the Fab fragment of anti-RANK (ODF/RANKL receptor) antibody. Experiments using M-BMM ϕ prepared from TNFR1- or TNFR2-deficient mice showed that both TNFR1- and TNFR2-induced signals were important for osteoclast formation induced by TNF- α . Osteoclasts induced by TNF- α formed resorption pits on dentine slices only in the presence of IL-1 α . These results demonstrate that TNF- α stimulates osteoclast differentiation in the presence of M-CSF through a mechanism independent of the ODF/RANKL-RANK system. TNF- α together with IL-1 α may play an important role in bone resorption of inflammatory bone diseases.

Key words: bone resorption • tumor necrosis factor receptor • nuclear factor- κ B • macrophage colony-stimulating factor • interleukin-1

Introduction

Osteoclasts, the multinucleated giant cells that resorb bone, develop from hemopoietic cells of the monocyte/macrophage lineage (1, 2). Osteoblasts or bone marrow stromal cells are involved in osteoclastogenesis through a mechanism involving cell-to-cell contact with osteoclast progenitor cells. We previously proposed a hypothesis that osteoblasts/stromal cells express osteoclast differentiation factor (ODF)¹

in response to several osteotropic factors in supporting osteoclast differentiation from their precursors (1, 3, 4). This working hypothesis was proved by the recent discovery of a new member of the TNF ligand family, [ODF (5), also called osteoprotegerin ligand (OPGL) (6)/TNF-related activation-induced cytokine (TRANCE) (7)/receptor activator of nuclear factor- κ B ligand (RANKL) (8)] as a

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¹Abbreviations used in this paper: 1 α ,25(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; JNK, c-Jun NH₂-terminal kinase; M-BMM ϕ , M-CSF-dependent

bone marrow macrophages; M-CSF, macrophage CSF; NF- κ B, nuclear factor κ B; OCIF, osteoclastogenesis inhibitory factor; ODF, osteoclast differentiation factor; OPGL, osteoprotegerin ligand; RANKL, receptor activator of NF- κ B ligand; RT-PCR, reverse-transcriptase PCR; TRAP, tartrate-resistant acid phosphatase.

membrane-associated factor (3, 4). Osteoclast precursors that express RANK (9–11), a TNF receptor family member, recognize ODF/RANKL through cell-to-cell interaction with osteoblasts/stromal cells, and differentiate into osteoclasts in the presence of macrophage CSF (M-CSF, also called CSF-1) (5, 6). M-CSF has been shown to be an essential factor for osteoclast formation *in vivo* and *in vitro* (12, 13). Osteoclastogenesis inhibitory factor [OCIF, also called osteoprotegerin (OPG)/TNF receptor-like molecule 1] is a soluble decoy receptor for ODF/RANKL (14–18). Nuclear factor κ B (NF- κ B) and c-Jun NH₂-terminal kinase (JNK) activated by the RANK-mediated signals in osteoclast precursors appear to be involved in their differentiation into osteoclasts (7, 8).

We have also shown that osteoblasts/stromal cells play an integral role in inducing osteoclast function (19, 20). When purified osteoclasts formed in cocultures were further cultured on dentine slices, they rapidly underwent apoptosis and failed to form resorption pits on the slices. Osteoblasts/stromal cells added to purified osteoclasts greatly stimulated the survival and resorption activity of osteoclasts through cell-to-cell contact. Both IL-1 α and soluble ODF/soluble RANKL (sODF/sRANKL) prolonged the survival of purified osteoclasts and induced their pit-forming activity (9, 21). OCIF/OPG inhibited both the survival and resorption activity of osteoclasts supported by not only sODF/sRANKL (9), but also osteoblasts/stromal cells (4). Fuller et al. (22) reported that TRANCE (ODF/RANKL) is involved in osteoclast activation induced by osteoblasts treated with parathyroid hormone. It was also demonstrated that OPGL (ODF/RANKL) activated mature osteoclasts *in vitro*, and that administration of OPGL induced rapid increases in blood-ionized calcium levels in mice (23). These results suggest that ODF/RANKL expressed by osteoblasts/stromal cells is responsible for inducing the differentiation, survival, and activation of osteoclasts (3, 4). IL-1 α can be substituted for ODF/RANKL in inducing the survival and activation of osteoclasts (21).

TNF- α , which is produced by many types of cells, including monocytes and macrophages, has been proposed to be involved in bone resorption, particularly in inflammatory bone diseases. Merkel et al. (24) demonstrated that TNF- α is involved in orthopedic osteolysis induced by implant-derived particles. It was also reported that TNF- α and IL-1 α play important roles in bone loss associated with osteoporosis and periodontitis (25, 26). Lipopolysaccharide-stimulated osteoclastogenesis has been shown to be mediated by TNF- α (27). In addition, Pfeilschifter et al. (28) reported that human TNF- α and IL-1 α stimulated formation of multinucleated cells with the characteristics of osteoclasts in human bone marrow cultures. TNF- α induces a number of biological responses via two cell-surface receptors termed TNFR1 and TNFR2 (also called TNFR p55 and TNFR p75, respectively) (29–31). Both TNFR1 and TNFR2 can transduce intracellular signals that stimulate the proteolytic breakdown of I κ B, a cytoplasmic inhibitor of NF- κ B (32–35). The activated NF- κ B is then translocated into the nucleus, where it induces the transcription of several TNF- α -respon-

sive genes. In addition, the binding of TNF- α to TNFR1 triggers programmed cell death in many cells. This process depends on the presence of the “death domain” located in the cytoplasmic region of TNFR1, which is absent in TNFR2 (36, 37). It is well established that mouse TNF- α binds to both mouse TNFR1 and TNFR2 with high affinity, while human TNF- α binds to mouse TNFR1 with higher affinity than to mouse TNFR2 (29, 30).

In the present study, the role of TNF- α in osteoclast differentiation was studied in detail. Mouse TNF- α strongly stimulated the differentiation of M-CSF-dependent mouse bone marrow macrophages (M-BMM ϕ) into osteoclasts in the presence of M-CSF. Human TNF- α induced only a few osteoclasts in the M-BMM ϕ cultures. OCIF/OPG did not inhibit TNF- α -induced osteoclast formation. We report here that the ODF/RANKL–RANK interaction is not the sole pathway that leads osteoclast progenitors to differentiation into osteoclasts *in vitro*; the TNF- α –TNFR1/TNFR2 interaction can be substituted for the ODF/RANKL–RANK interaction in inducing osteoclast differentiation under some pathological conditions.

Materials and Methods

Antibodies and Chemicals. Recombinant mouse sODF/sRANKL and human OCIF/OPG were prepared as described previously (5, 16). Recombinant proteins of mouse and human TNF- α , and human IL-1 α were obtained from R & D Systems, Inc. Human M-CSF was obtained from Yoshitomi Pharmaceutical Co. Anti-mouse TNFR1 and TNFR2 antibodies were purchased from Genzyme Diagnostics. The Fab fragment of anti-RANK polyclonal antibody and nonimmunized rabbit immunoglobulin were prepared using the immunopure Fab preparation kit (Pierce) as described previously (10). Anti-Mac-1, Moma-2, and F4/80 rat monoclonal antibodies were obtained from Serotec. (3-[¹²⁵I]iodotyrosyl¹²) Human calcitonin (specific activity, 74 TBqmmol) was obtained from Amersham International.

Mouse M-BMM ϕ Culture. 5–8-wk-old male ddY and C57BL/6J mice and newborn ddY mice were obtained from Sankyo Labo Service Co. C57BL/6J mice, in which the TNFR1 or TNFR2 gene had been deleted, were obtained from Jackson Immuno-Research Laboratories, Inc. Bone marrow cells prepared from the tibia of ddY mice, TNFR1-deficient mice [TNFR1(–/–)], or TNFR2-deficient mice [TNFR2(–/–)] were suspended in α MEM containing 10% fetal bovine serum (JRH Biosciences), and cultured in 48-well plates (1.5×10^5 cells/0.25 ml per well) in the presence of M-CSF (100 ng/ml). After culturing for 3 d, nonadherent cells were completely removed from the culture by pipetting. Characteristics of adherent cells were examined by staining with antibodies against Mac-1, Moma-2, and F4/80 antigens using a Vectastain ABC AP kit and Vector Red (Vector Laboratories, Inc.). Positive cells were stained red (see Fig. 1). As almost all of the adherent cells were positive for these antibodies, we called the adherent cells M-BMM ϕ . M-BMM ϕ were further cultured for 3 d with either cytokine of sODF/sRANKL, mouse TNF- α , human TNF- α , or IL-1 α in the presence or absence of OCIF/OPG and/or M-CSF. Some cultures were also treated with anti-mouse TNFR1 or TNFR2 antibody, or the Fab fragment of anti-RANK antibody. Cells were then fixed and stained for tartrate-resistant acid phosphatase (TRAP) as described previously (38). Cells were also stained for alkaline phosphatase (a

marker enzyme of osteoblasts) as described previously (38). Positive cells appeared as blue cells. The number of TRAP-positive cells, including mononuclear and multinucleated cells, was scored under microscopic examination. In some experiments, TRAP-positive cells containing more than three nuclei were also counted as TRAP-positive multinucleated cells.

Autoradiography for Calcitonin Binding. Bone marrow cells of ddY mice (2×10^5 cells/chamber) were plated on Lab-Tek 8-chamber slides (Nalge Nunc International). Cells were first cultured with M-CSF (100 ng/ml) for 3 d, and further cultured with or without cytokines for 3 d. Cultures were then treated with 0.2 nM [125 I]-human calcitonin for 1 h at room temperature. After washing twice with PBS, cells were fixed with 0.1 M cacodylate buffer, pH 7.4, containing 1% formaldehyde and 1% glutaraldehyde, stained for TRAP, and processed for autoradiography as described previously (38). Nonspecific binding of [125 I]-labeled calcitonin was assessed in the presence of an excess amount (200 nM) of unlabeled eel calcitonin (Asahi Chemical Industry).

Pit Formation Assay. To determine resorption activity of TRAP-positive cells, bone marrow cells of ddY mice (2×10^5 cells/well) were plated on dentine slices (4 mm in diameter) that had been placed in 48-well culture plates. Bone marrow cells were first cultured with M-CSF (100 ng/ml) for 3 d. The slices, on which M-BMM ϕ were formed, were then well washed with α MEM to remove nonadherent cells, and further cultured with or without cytokines for an additional 3 d. Some dentine slices were subjected to TRAP staining. The remaining slices were cleaned by ultrasonication in 1 M NH_4OH to remove adherent cells and stained with Mayer's hematoxylin to visualize resorption pits as described (38). The number of TRAP-positive cells on the slices and that of resorption pits were counted under microscopic examination.

Survival Assay of Osteoclasts. Osteoblasts obtained from the calvaria of newborn ddY mice and bone marrow cells from ddY mice were cocultured in α MEM containing 10% fetal bovine serum, $1\alpha,25$ -dihydroxyvitamin D_3 [$1\alpha,25(\text{OH})_2\text{D}_3$] (10^{-8} M) (Wako Pure Chemical Co.), and prostaglandin E_2 (10^{-6} M) (Sigma Chemical Co.) in 100-mm-diameter dishes coated with Type I collagen gels (Nitta Gelatin Co.) as described previously (38). Osteoclasts formed within 6 d were recovered from the dishes by treating with 0.2% collagenase (crude osteoclasts). To purify osteoclasts, the crude osteoclast preparation was replated and cultured for 8 h. Osteoblasts were then removed by treating with PBS containing 0.001% pronase E and 0.02% EDTA (38). Some of the cultures were then stained for TRAP. The other cultures were further incubated for 36 h in the presence or absence of mouse TNF- α (20 ng/ml), human TNF- α (20 ng/ml), IL-1 α (10 ng/ml), or sODF/sRANKL (100 ng/ml), and stained for TRAP. TRAP-positive multinucleated cells containing more than three nuclei were counted as living osteoclasts.

Polymerase Chain Reaction Amplification of Reverse-transcribed mRNA. For semiquantitative reverse-transcriptase PCR (RT-PCR) analysis, total RNA was extracted using Trizol solution (GIBCO BRL) from freshly isolated bone marrow cells, M-BMM ϕ , and purified osteoclasts. Purified osteoclasts were obtained from cocultures of mouse bone marrow cells and calvarial osteoblasts as described previously (21). First strand cDNA was synthesized from total RNA with random primers and was subjected to PCR amplification with EX Taq polymerase (Takara Shuzo) using specific PCR primers: mouse TNFR1, 5'-GAGTGCTGACCTA-TAACACATTCCT-3' (forward, nucleotides 3152-3176) and 5'-CATCTTGCCAGTTCAAACTAAGT-3' (reverse, nucleotides 3553-3577); mouse TNFR2, 5'-CATTCTAAGAACAATT-CCATCTGCT-3' (forward, nucleotides 246-270) and 5'-GGG-TACTGGAGACAGGAGAACTAA-3' (reverse, nucleotides 961-984); mouse RANK, 5'-ACACCTGGAATGAAGAAGATAA-ATG-3' (forward, nucleotides 260-284) and 5'-AGCCACTAC-TACCACAGAGATGAAG-3' (reverse, nucleotides 684-708); mouse c-Fms, 5'-AACAAAGTTCTACAACTGGTGAAGG-3' (forward, nucleotides 2653-2677) and 5'-GAAGCCTGTAGTCT-AAGCATCTGTC-3' (reverse, nucleotides 3381-3405); mouse glyceraldehyde-3-phosphate dehydrogenase, 5'-ACCACAGTCC-ATGCCATCAC-3' (forward, nucleotides 566-585) and 5'-TCC-ACCACCTGTTGCTGTA-3' (reverse, nucleotides 998-1017). The PCR products were separated by electrophoresis on a 2% agarose gel.

Electrophoretic Mobility Shift Assay. Mouse M-BMM ϕ were treated with sODF/sRANKL (100 ng/ml), mouse TNF- α (20 ng/ml), or human TNF- α (20 ng/ml) for 1 h. Nuclear extracts were then prepared from M-BMM ϕ as described previously (39). An NF- κB -binding oligonucleotide sequence 5'-AGCTTGGGGACTT-TCCGAG-3' was used as a radioactive DNA probe. DNA binding reaction was performed at room temperature in a volume of 30 μl , which contained the binding buffer (10 mM Tris/HCl, pH 7.5, 1 mM EDTA, 4% glycerol, 100 mM NaCl, 5 mM DTT, 100 mg/ml of BSA), 3 μg of poly(dI-dC), 10^5 cpm of a ^{32}P -labeled probe, and 8 μg of nuclear proteins. After incubation for 15 min, the samples were electrophoresed on native 5% acrylamide/0.25 \times Tris-borate-EDTA gels. The gels were dried and exposed to x-ray film.

Statistical Analysis. Significance of differences was determined using Student's *t* test.

Results

When mouse bone marrow cells were cultured with M-CSF for 3 d, adherent cells of uniform size and shape appeared on the culture plate. Almost all of the adherent cells strongly expressed antigens such as Mac-1, Moma-2, and

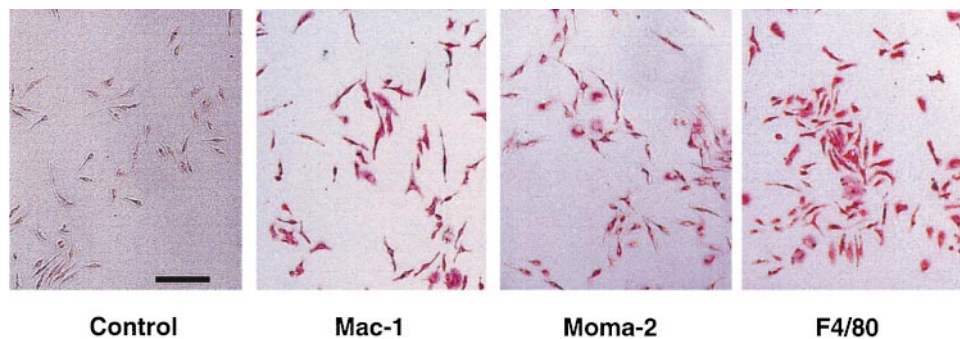


Figure 1. Expression of macrophage-associated antigens by M-BMM ϕ . Mouse bone marrow cells of ddY mice were cultured with M-CSF (100 ng/ml). After culturing for 3 d, nonadherent cells were completely removed, and remaining adherent cells were fixed and stained with antibodies against Mac-1, Moma-2, and F4/80 antigens. Cells expressing each antigen were stained red. Note that almost all of the adherent cells are positive for Mac-1, Moma-2, and F4/80. Bar, 100 μm .

F4/80, which are specific for macrophages (Fig. 1). In this study, these adherent cells were called M-BMM ϕ . Typically, 10^4 M-BMM ϕ were obtained when 10^5 bone marrow cells were cultured for 3 d in the presence of M-CSF. There were very few alkaline phosphatase-positive stromal cells detectable in the M-BMM ϕ preparation (data not shown).

When M-BMM ϕ were further cultured with sODF/sRANKL and M-CSF, TRAP-positive mononuclear and multinucleated cells were formed within 3 d (Fig. 2, A and B). In the absence of M-CSF, most of the M-BMM ϕ died

within 3 d. No TRAP-positive cells were formed when M-CSF was not added, even in the presence of sODF/sRANKL. Although varied in each experiment, 40–80% of the M-BMM ϕ usually differentiated into TRAP-positive cells in response to sODF/sRANKL and M-CSF. When nonadherent cells, which were recovered from bone marrow cultures treated for 3 d with M-CSF, were further cultured for 3 d with ODF/RANKL and M-CSF, only a few TRAP-positive cells were formed (data not shown). This suggests that osteoclast progenitors are present preferen-

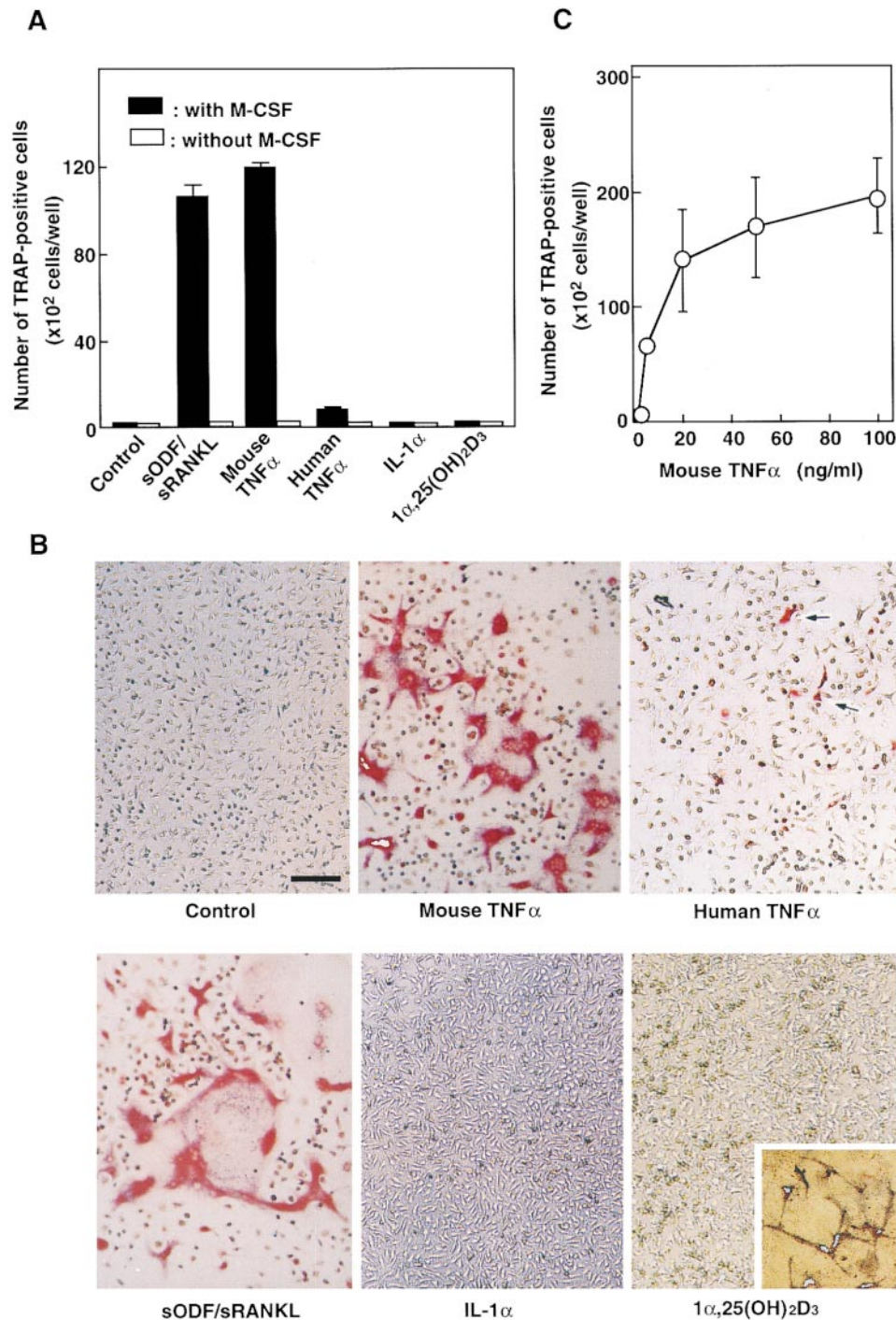


Figure 2. Effects of sODF/sRANKL, and mouse and human TNF- α on TRAP-positive cell formation in M-BMM ϕ cultures. Bone marrow cells of ddY mice were cultured with M-CSF for 3 d to prepare M-BMM ϕ . (A) M-BMM ϕ were further cultured for 3 d with either sODF/sRANKL (100 ng/ml), mouse TNF- α (20 ng/ml), human TNF- α (20 ng/ml), IL-1 α (10 ng/ml), or 1 α ,25(OH)₂D₃ (10⁻⁸ M) in the presence and absence of M-CSF (100 ng/ml). Cells were then fixed and stained for TRAP and the number of TRAP-positive cells were scored. (B) TRAP staining of M-BMM ϕ cultures treated with vehicle (control), sODF/sRANKL (100 ng/ml), mouse TNF- α (20 ng/ml), human TNF- α (20 ng/ml), or IL-1 α (10 ng/ml) in the presence of M-CSF (100 ng/ml) for 3 d. Arrows in the culture treated with human TNF- α indicate TRAP-positive mononuclear cells. M-BMM ϕ cultures treated with 1 α ,25(OH)₂D₃ (10⁻⁸ M) in the presence of M-CSF (100 ng/ml) for 3 d were double stained for TRAP and alkaline phosphatase. The inset shows alkaline phosphatase staining of primary osteoblasts cultured for 3 d in the presence of 1 α ,25(OH)₂D₃ (10⁻⁸ M) as the control. Bar, 100 μ m. (C) M-BMM ϕ were further cultured for 3 d with increasing concentrations of mouse TNF- α in the presence of M-CSF (100 ng/ml). Cells were then fixed and stained for TRAP, and the number of TRAP-positive cells was scored. Results were expressed as the means \pm SEM of three to four cultures. Similar results were obtained in three independent experiments.

tially as adherent cells in M-CSF-treated bone marrow cells. Surprisingly, mouse TNF- α stimulated TRAP-positive cell formation from M-BMM ϕ in the presence of M-CSF in a dose-dependent manner (Fig. 2, A and C). Morphology of TRAP-positive cells induced by mouse TNF- α was quite similar to that induced by sODF/sRANKL (Fig. 2 B). Repeated experiments showed that 3–5% of the TRAP-positive cells induced by either mouse TNF- α or ODF/RANKL were multinucleated cells, which contained more than three nuclei. In contrast, human TNF- α at 20 ng/ml induced a few TRAP-positive mononuclear cells in M-BMM ϕ cultures (Fig. 2 B). Neither human nor mouse TNF- α induced apoptosis of M-BMM ϕ in the presence or absence of M-CSF. No TRAP-positive cells were formed in M-BMM ϕ cultures treated with IL-1 α at 10 ng/ml even in the presence of M-CSF (Fig. 2, A and B). Addition of 1 α ,25(OH) $_2$ D $_3$ at 10 $^{-8}$ M to the M-BMM ϕ cultures also failed to induce TRAP-positive cells, irrespective of the presence or absence of M-CSF (Fig. 2, A and B). Alkaline phosphatase-positive cells were not detected in the M-BMM ϕ preparation after culture for 3 d with 1 α ,25(OH) $_2$ D $_3$ and M-CSF (Fig. 2 B). Mouse TNF- α (20 ng/ml) stimulated TRAP-positive cell formation in cultures of mouse spleen

cells or whole bone marrow cells in the presence of M-CSF (100 ng/ml) (data not shown). We used M-BMM ϕ instead of whole bone marrow cells in this experiment to avoid contamination of cells other than osteoclast progenitors.

We previously reported that osteoclasts formed in the cocultures expressed mRNAs of TNFR1, TNFR2, and RANK (9). Using a semiquantitative RT-PCR, it was shown that not only purified osteoclasts but also M-BMM ϕ expressed these mRNAs (Fig. 3 A). Both M-BMM ϕ and osteoclasts expressed c-Fms mRNA as well. Freshly isolated bone marrow cells expressed mRNAs of TNFR1, TNFR2, RANK, and c-Fms only weakly, when compared with M-BMM ϕ and osteoclasts (Fig. 3 A). An electrophoretic mobility shift assay revealed that mouse TNF- α , human TNF- α , and sODF/sRANKL all activated NF- κ B in M-BMM ϕ (Fig. 3 B).

TRAP-positive cell formation induced by sODF/sRANKL was inhibited completely by simultaneously adding OCIF/OPG (Fig. 4 A, top). In contrast, TRAP-positive cell formation was similarly induced by mouse TNF- α irrespective of the presence or absence of OCIF/OPG (Fig. 4 A, bottom). Although these results suggest that TNF- α induces osteoclast differentiation through TNFR1/TNFR2, there were still two possibilities that TNF- α directly binds to RANK or TNF- α increases endogenous production of some factor(s) that can interact with RANK. To exclude these possibilities, the Fab fragment of polyclonal antibodies against the extracellular domain of RANK was used in the

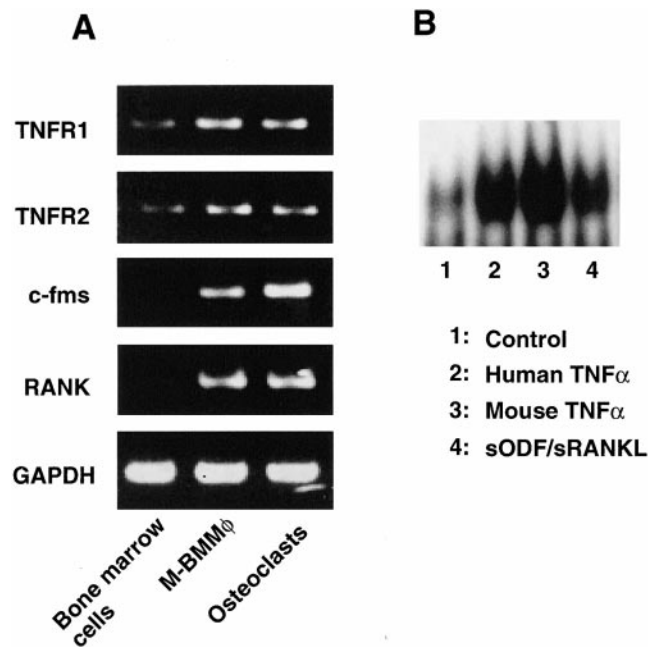


Figure 3. Expression of mRNAs of TNFR1, TNFR2, RANK, and c-Fms by M-BMM ϕ (A) and the effects of mouse and human TNF- α on the activation of NF- κ B in M-BMM ϕ (B). (A) Total RNA was extracted from bone marrow cells, M-BMM ϕ , and purified osteoclasts of ddY mouse origin, and subjected to semiquantitative PCR analysis for TNFR1, TNFR2, RANK, c-Fms, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the specific primer pairs described in Materials and Methods. PCR was performed under conditions determined to be in the linear range of product formation. (B) M-BMM ϕ were treated with sODF/sRANKL (100 ng/ml), mouse TNF- α (20 ng/ml), or human TNF- α (20 ng/ml) for 1 h. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay with an NF- κ B-binding oligonucleotide probe as described in Materials and Methods. Similar results were obtained in two independent experiments.

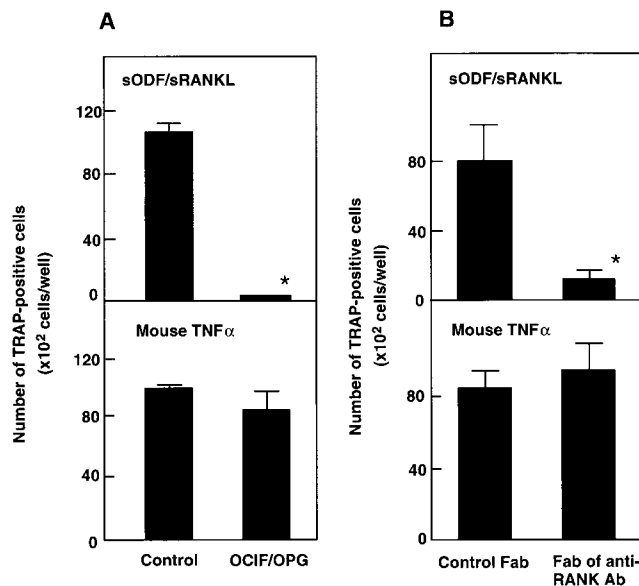


Figure 4. TRAP-positive cell formation induced by mouse TNF- α in M-BMM ϕ cultures caused by a mechanism independent of ODF/RANKL-RANK interaction. M-BMM ϕ prepared from ddY mice were further cultured for 3 d with sODF/sRANKL (100 ng/ml) (top) or mouse TNF- α (20 ng/ml) (bottom) in the presence of M-CSF (100 ng/ml). M-BMM ϕ were simultaneously treated with or without OCIF/OPG (100 ng) (A), or treated with the Fab fragment of anti-RANK antibody (10 μ g/ml) or the Fab fragment of control IgG (10 μ g/ml) (B). After culturing for an additional 3 d, cells were fixed and stained for TRAP. Results were expressed as the means \pm SEM of three cultures. Similar results were obtained in three independent experiments. *Significantly different from the control culture; $P < 0.01$.

next experiment. We previously reported that the anti-RANK antibody induced osteoclastogenesis in the presence of M-CSF, probably through clustering of RANK (10). In contrast, the Fab fragment of the antibody blocked the binding of ODF/RANKL to RANK, causing the inhibition of the sODF/sRANK-mediated osteoclastogenesis (10). The Fab fragment of the anti-RANK antibody inhibited TRAP-positive cell formation induced by sODF/sRANK (Fig. 4 B). In contrast, the Fab fragment of the anti-RANK antibody showed no inhibitory effect on mouse TNF- α -induced TRAP-positive cell formation in M-BMM ϕ cultures. These results indicate that TNF- α stimulates TRAP-positive cell formation by a mechanism independent of the ODF/RANKL-RANK interaction.

We next examined effects of blocking antibodies against TNFR1 or TNFR2 on TRAP-positive cell formation in M-BMM ϕ cultures treated with mouse TNF- α or sODF/sRANKL in the presence of M-CSF (Fig. 5 A). Both antibodies showed no inhibitory effect on the sODF/sRANKL-induced TRAP-positive cell formation, but each of the

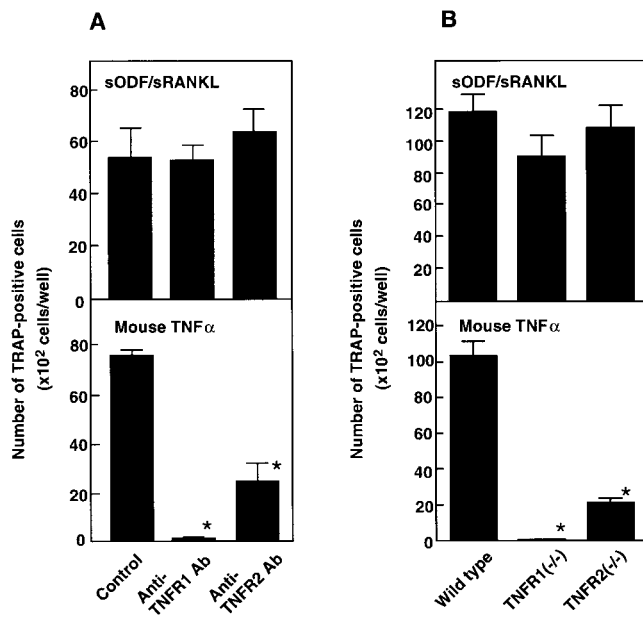


Figure 5. TRAP-positive cell formation induced by mouse TNF- α in M-BMM ϕ requires both TNFR1- and TNFR2-mediated signals. (A) M-BMM ϕ prepared from ddY mice were further cultured for 3 d with sODF/sRANKL (100 ng/ml) (top) or mouse TNF- α (20 ng/ml) (bottom) in the presence of M-CSF (100 ng/ml). M-BMM ϕ were simultaneously treated with or without control IgG (30 μ g/ml), anti-TNFR1 antibody (10 μ g/ml), or anti-TNFR2 antibody (30 μ g/ml). After culturing for an additional 3 d, cells were fixed and stained for TRAP. The number of TRAP-positive cells was scored. Results were expressed as the means \pm SEM of three cultures. Similar results were obtained in three independent experiments. (B) M-BMM ϕ were prepared from normal C57BL/6J mice (wild type), TNFR1-deficient C57BL/6J mice [TNFR1(-/-)] or TNFR2-deficient C57BL/6J mice [TNFR2(-/-)]. M-BMM ϕ were further cultured with sODF/sRANKL (100 ng/ml) (top) or mouse TNF- α (20 ng/ml) in the presence of M-CSF (100 ng/ml) (bottom). After culturing for an additional 3 d, cells were fixed and stained for TRAP. The number of TRAP-positive cells was scored. Results were expressed as the means \pm SEM of four mice in each group. *Significantly different from the control (A) or wild type (B); $P < 0.01$.

two antibodies strongly inhibited TRAP-positive cell formation induced by mouse TNF- α (Fig. 5 A). To further confirm the possibility that both TNFR1 and TNFR2 play important roles in TNF- α -induced TRAP-positive cell formation, M-BMM ϕ were prepared from TNFR1(-/-), TNFR2(-/-), and the control C57BL/6J (wild type) mice, and compared their capacity to differentiate into TRAP-positive cells in the presence of mouse TNF- α plus M-CSF or sODF/sRANKL plus M-CSF (Fig. 5 B). In the presence of sODF/sRANKL, the number of TRAP-positive cells formed from TNFR1(-/-) and TNFR2(-/-) M-BMM ϕ culture was similar to that from the control (wild type) M-BMM ϕ (Fig. 5 B). In contrast, no TRAP-positive cells were observed in TNFR1(-/-) M-BMM ϕ cultures treated with mouse TNF- α (Fig. 5 B). TRAP-positive cell formation induced by mouse TNF- α was markedly reduced in TNFR2(-/-) M-BMM ϕ cultures in comparison with that in the control (wild type) cultures. These results confirmed that both TNFR1- and TNFR2-mediated signals were important for TNF- α -induced osteoclast differentiation.

Autoradiography using [¹²⁵I]-labeled calcitonin showed that numerous grains, as a result of the binding of labeled calcitonin, accumulated on TRAP-positive cells induced by mouse TNF- α together with M-CSF even in the presence of OCIF/OPG (Fig. 6 A). In agreement with previous findings (5), numerous grains of the binding of labeled calcitonin were observed on TRAP-positive cells induced by sODF/sRANKL in the presence of M-CSF (Fig. 6 B). However, no calcitonin receptor-positive cells appeared in M-BMM ϕ cultures treated with sODF/sRANKL plus M-CSF in the presence of OCIF/OPG (Fig. 6 C). The binding of [¹²⁵I]-labeled calcitonin to TRAP-positive cells was inhibited by an excess amount of unlabeled calcitonin simultaneously added (data not shown), suggesting that TRAP-positive cells induced by mouse TNF- α as well as sODF/RANKL expressed calcitonin receptors.

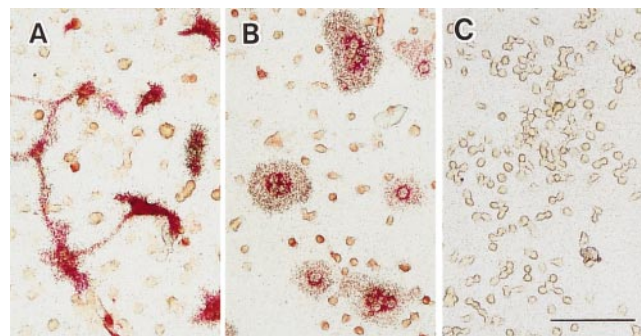


Figure 6. Autoradiography for [¹²⁵I]-calcitonin binding in M-BMM ϕ cultures. Bone marrow cells of ddY mice were first cultured for 3 d on LAB-Tek chamber slides in the presence of M-CSF (100 ng/ml). (A) M-BMM ϕ formed on the chamber slides were further cultured for 3 d with mouse TNF- α (20 ng/ml) plus M-CSF (100 ng/ml) in the presence of OCIF/OPG (100 ng/ml). (B and C) M-BMM ϕ were further cultured for 3 d with sODF/sRANKL (100 ng/ml) plus M-CSF (100 ng/ml) in the absence (B) and presence (C) of OCIF/OPG (100 ng/ml). Cells were then treated with 0.2 nM [¹²⁵I]-human calcitonin, stained for TRAP, and processed for autoradiography. Similar results were obtained in two independent experiments. Bar, 100 μ m.

We previously reported that IL-1 α , M-CSF, and sODF/sRANKL similarly stimulated the survival of purified osteoclasts via the respective receptors (9, 21). Using purified osteoclasts, we next examined whether TNF- α supports the survival of osteoclasts (Fig. 7). Most of the osteoclasts died within 36 h after removal of osteoblasts in the presence and absence of OCIF/OPG. Treatment of purified osteoclast preparations with mouse TNF- α stimulated the survival of osteoclasts. OCIF/OPG showed no inhibitory effects on the survival of osteoclasts supported by mouse TNF- α (Fig. 7). In agreement with previous finding (9), sODF/sRANKL and IL-1 α stimulated the survival of purified osteoclasts. OCIF/OPG strongly inhibited the survival of osteoclasts supported by sODF/sRANKL but not by IL-1 α (Fig. 7).

We previously reported that purified osteoclasts rapidly died and did not form resorption pits on dentine slices (19, 20). The pit-forming activity of purified osteoclasts was greatly recovered by adding either osteoblasts/stromal cells, IL-1 α , or sODF/sRANKL (9, 19, 21). When M-BMM ϕ were prepared on dentine slices, and cultured with mouse TNF- α , IL-1 α , or mouse TNF- α plus IL-1 α in the presence of M-CSF and OCIF/OPG, a similar number of TRAP-positive cells appeared on dentine slices in response to mouse TNF- α irrespective of the presence and absence of IL-1 α (Fig. 8, A and B). However, resorption pits on the

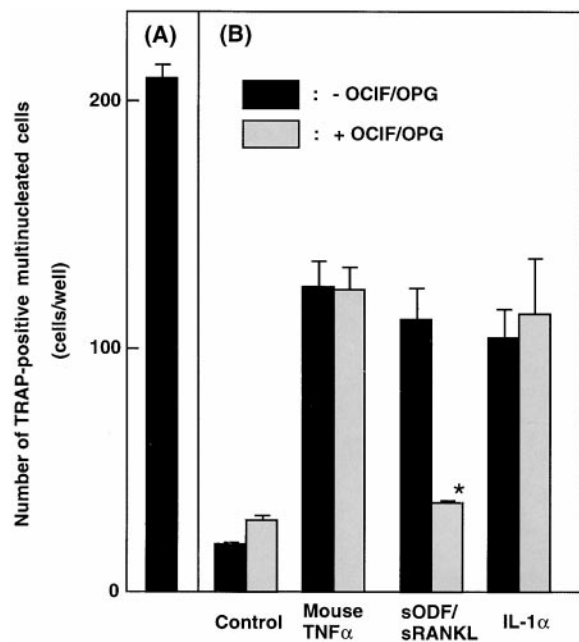


Figure 7. Effects of mouse TNF- α on the survival of purified osteoclasts. Purified osteoclasts of ddY mouse origin were prepared as described in Materials and Methods. Purified osteoclasts were incubated with mouse TNF- α (20 ng/ml), sODF/sRANKL (100 ng/ml), IL-1 α (10 ng/ml), or vehicle (control) in the presence or absence of OCIF/OPG (100 ng/ml). Before (A) and after (B) incubation of purified osteoclasts for 36 h, TRAP-positive osteoclasts containing more than three nuclei were counted. Results were expressed as the means \pm SEM of three cultures. Similar results were obtained from three independent experiments. *Significantly different from the culture treated without OCIF/OPG; $P < 0.01$.

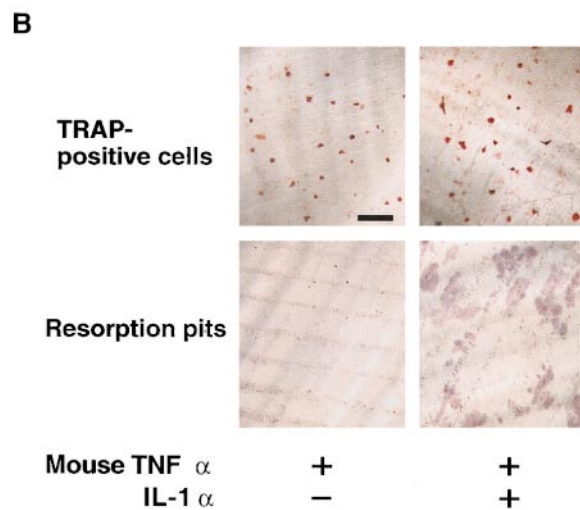
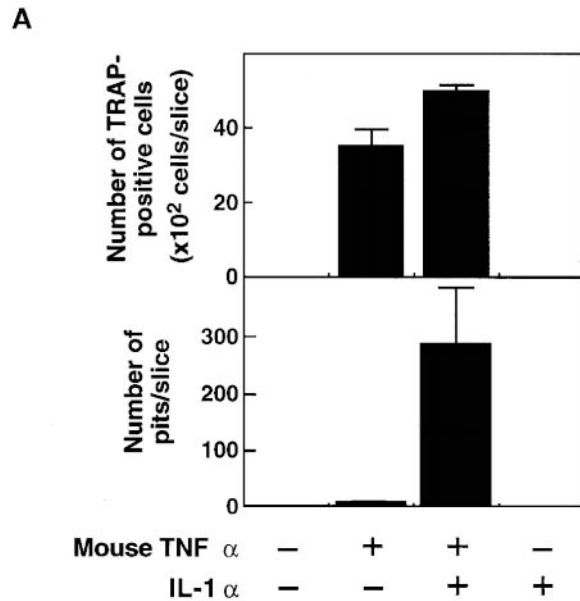


Figure 8. Pit-forming activity of TRAP-positive cells formed in M-BMM ϕ cultures. (A) Mouse bone marrow cells were cultured on dentine slices for 3 d in the presence of M-CSF (100 ng/ml). M-BMM ϕ formed on the slices were further cultured with mouse TNF- α (20 ng/ml), IL-1 α (10 ng/ml), or mouse TNF- α (20 ng/ml) plus IL-1 α (10 ng/ml) in the presence of M-CSF (100 ng/ml) and OCIF/OPG (200 ng/ml). After culturing for an additional 3 d, some dentine slices were stained for TRAP. The remaining dentine slices were cleaned by ultrasonication to remove adherent cells, and stained with Mayer's hematoxylin to visualize resorption pits. The number of TRAP-positive cells and that of resorption pits on dentine slices were counted. Results were expressed as the means \pm SEM of three cultures. (B) TRAP staining (top) and resorption pits (bottom) on dentine slices obtained from cultures treated with mouse TNF- α (left) and from cultures treated with mouse TNF- α plus IL-1 α (right). Similar results were obtained in three sets of independent experiments. Bar, 100 μ m.

slices were detected only in the presence of both mouse TNF- α and IL-1 α (Fig. 8, A and B). These results suggest that TNF- α stimulates the differentiation and survival of osteoclasts, but not the function of osteoclasts.

Discussion

Since the discovery of the ODF/RANKL–RANK signal transduction, it has been believed that ODF/RANKL is the sole factor responsible for inducing osteoclast differentiation. This notion was supported by the finding that the targeted disruption of the gene encoding OPGL (ODF/RANKL) in mice developed severe osteopetrosis with complete absence of TRAP-positive cells in bone tissues (40). However, the present study clearly shows that TNF- α together with M-CSF induces TRAP-positive cell formation in M-BMM ϕ cultures without any help of osteoblasts/stromal cells. Alkaline phosphatase-positive cells were seldom detected in the M-BMM ϕ preparation even after culture for an additional 3 d. No TRAP-positive cells were formed in the cultures of the M-BMM ϕ preparation treated with $1\alpha,25(\text{OH})_2\text{D}_3$ in the presence or absence of M-CSF. A recent study on vitamin D receptor knockout mice proved that the target cells of $1\alpha,25(\text{OH})_2\text{D}_3$ in inducing osteoclast formation are osteoblasts/stromal cells, but not osteoclast progenitors (41). These results suggest that even if a small number of stromal cells were present in the M-BMM ϕ preparation, they could not support osteoclast formation.

Like authentic osteoclasts, TRAP-positive cells formed in response to TNF- α and M-CSF expressed calcitonin receptors. TNF- α -induced TRAP-positive cells formed resorption pits only in the presence of IL-1 α . This property of TRAP-positive cells induced by TNF- α is recognized in ODF/RANKL-induced osteoclasts as well (19, 21). M-CSF was essentially required for osteoclast formation induced by TNF- α as in sODF/sRANKL-induced osteoclast formation. These results indicate that TNF- α -induced TRAP-positive cells in M-BMM ϕ cultures satisfy major criteria for osteoclasts. OCIF/OPG and the Fab fragment of anti-RANK antibody did not inhibit the TNF- α -induced TRAP-positive and calcitonin receptor-positive osteoclast formation. Using RT-PCR, we found that M-BMM ϕ expressed ODF/RANKL mRNA, but its level was very low and was not up-regulated by adding mouse TNF- α (data not shown). These results further confirm that ODF/RANKL is not involved in the TNF- α -induced osteoclast formation in M-BMM ϕ cultures. In our preliminary experiments, human TNF- α stimulated the formation of TRAP- and vitronectin receptor (23C6)-positive osteoclast-like cells in cultures of human peripheral blood mononuclear cells even in the presence of OCIF/OPG (data not shown). These results suggest that the ODF/RANKL–RANK signaling system is not the sole pathway for inducing osteoclast differentiation in vitro (Fig. 9).

M-BMM ϕ expressed both TNFR1 and TNFR2. Activation of NF- κ B in M-BMM ϕ was induced not only by mouse TNF- α , but also human TNF- α . However, the potency of human TNF- α to induce TRAP-positive osteoclast formation in M-BMM ϕ cultures was much weaker than that of mouse TNF- α . Anti-TNFR1 antibody completely inhibited osteoclast formation induced by mouse TNF- α , but not osteoclast formation by sODF/sRANKL. M-BMM ϕ prepared from TNFR1(-/-) mice did not differentiate into TRAP-positive cells in response to mouse

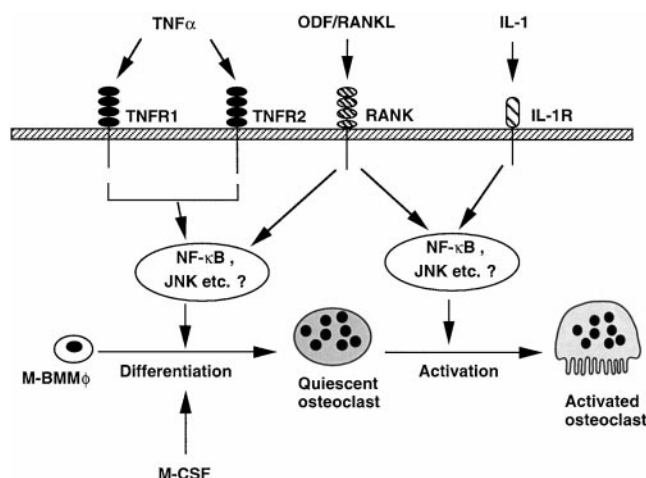


Figure 9. Schematic representation of ligand-receptor systems in osteoclast differentiation and function regulated by TNF- α , ODF/RANKL, and IL-1 α . TNF- α and ODF/RANKL independently stimulate osteoclast differentiation. Osteoclast differentiation induced by TNF- α occurs via TNFR1 and TNFR2 expressed by osteoclast precursors. ODF/RANKL induces osteoclast differentiation through RANK-mediated signals. M-CSF is a common factor required for both TNF- α - and ODF/RANKL-induced osteoclast differentiation. Activation of osteoclasts is induced by ODF/RANKL and IL-1 α through RANK and type 1 IL-1 receptor, respectively. Common signaling cascades such as NF- κ B and JNK activation may be involved in the differentiation and activation of osteoclasts induced by TNF- α , ODF/RANKL, and IL-1 α .

TNF- α . These results suggest that TNFR1-mediated signals are essential for TRAP-positive cell formation induced by TNF- α . Furthermore, addition of anti-TNFR2 antibody to M-BMM ϕ cultures significantly inhibited TRAP-positive osteoclast formation induced by mouse TNF- α . In addition, TRAP-positive osteoclast formation induced by mouse TNF- α was markedly reduced in TNFR2(-/-) M-BMM ϕ cultures. These results suggest that TNFR2-mediated signals play important roles in osteoclast differentiation. Collectively, both signals mediated by TNFR1 and TNFR2 appear to be required for inducing osteoclast formation at the level similar to ODF/RANKL-induced osteoclast formation (Fig. 9). Using the anti-TNFR1 and -TNFR2 antibody, it was clearly shown that only TNFR1-mediated signals led to the endotoxin-induced shock and protective response against *Listeria monocytogenes* infection, and both TNFR1- and TNFR2-mediated signals were required for the development of TNF- α -induced skin necrosis in mice (42). Abu-Amer et al. (27) reported that osteoclastogenesis induced by lipopolysaccharides is mediated by TNF- α via TNFR1. They also showed that human as well as mouse TNF- α stimulated the expression of c-Src, a marker protein of osteoclasts, in mouse bone marrow macrophages (27). These findings support the conclusion that TNFR1-mediated signals are essential for osteoclast differentiation.

We previously reported that purified osteoclasts rapidly underwent apoptosis and did not form resorption pits on dentine slices (19). IL-1 α as well as sODF/sRANKL induced

the survival and pit-forming activity of purified osteoclasts (9, 21). The present study showed that the survival of purified osteoclasts was stimulated by human as well as mouse TNF- α . However, resorption pits were not formed on dentine slices in M-BMM ϕ cultures treated with mouse TNF- α and M-CSF. Resorption pits were observed on dentine slices in the M-BMM ϕ culture further treated with IL-1 α . These results suggest that TNF- α is capable of stimulating the differentiation and survival of osteoclasts, but not the pit-forming activity of osteoclasts. On the other hand, ODF/RANKL supports all the processes of differentiation, survival, and activation of osteoclasts (Fig. 9).

Determination of signals involved in osteoclast differentiation is an important issue in the research on osteoclast biology. At present, two signals, NF- κ B and JNK, have been proposed to be involved in osteoclast differentiation. Hsu et al. (11) reported that ODF/RANKL stimulated JNK activity of murine myeloid RAW-264.7 cells and induced differentiation of these cells into osteoclasts. Activation of NF- κ B in RAW-264.7 cells was not induced by ODF/RANKL. This indicates that the signals mediated by JNK rather than NF- κ B are important for ODF/RANKL-induced osteoclast differentiation in RAW-264.7 cells. In our experiments, IL-1 activated NF- κ B in M-BMM ϕ (data not shown), but failed to induce osteoclast differentiation in M-BMM ϕ cultures. These results suggest that some other signals than NF- κ B may play an important role in osteoclast differentiation. RAW-264.7 cells differentiated into TRAP-positive cells in response to mouse TNF- α as well as ODF/RANKL (data not shown). Possible involvement of JNK and NF- κ B in osteoclastogenesis is currently under investigation in our laboratories.

Mounting evidence has demonstrated that TNF receptor family members interact with TNF receptor-associated factors (TRAFs) to modulate JNK and NF- κ B activity as well as apoptosis in the target cells (43, 44). TRAF2 has been shown to be an adaptor protein for TNFR1- and TNFR2-mediated signals in the target cells (43, 44). Recent studies have demonstrated that the cytoplasmic tail of RANK interacts with TRAF1, TRAF2, TRAF3, TRAF5, and TRAF6 (45–48). Recently, it was reported that TRAF6 knockout mice developed severe osteopetrosis (49, 50). Similar numbers of TRAP-positive osteoclasts were observed in TRAF6 knockout mice and normal mice, but osteoclasts in TRAF6-deficient mice did not form ruffled borders, structures necessary for bone resorption (49). IL-1 α receptors have been shown to use TRAF6 as a signal-transducing molecule in the target cells (51). We previously showed that IL-1 α activated NF- κ B in purified osteoclasts (39). IL-1 α also induced pit-forming activity of osteoclasts placed on dentine slices (21). However, IL-1 α failed to induce osteoclast differentiation in M-BMM ϕ cultures. These results suggest that TRAF6 is a common signaling molecule responsible for osteoclast activation induced by ODF/RANKL and IL-1 α . TRAF2 and/or its related TRAFs may be involved in the differentiation of osteoclasts. Thus, ODF/RANKL-RANK interaction induces signals necessary for both differentiation and activation of osteoclasts (Fig. 9).

TNF- α stimulates osteoclastogenesis in the absence of ODF/RANKL in vitro. However, this is not consistent with the finding that the disruption of the ODF/RANKL gene in mice showed a complete lack of osteoclasts in bone (40). Recently, it was reported that TNF- α - and IL-1 α -induced bone resorption in mice was inhibited by concomitant treatment with OCIF/OPG (52). Our preliminary experiments showed that TNF- α as well as IL-1 α upregulated expression of ODF/RANKL mRNA in osteoblasts/stromal cells (data not shown), suggesting that a part of bone resorption induced by TNF- α and IL-1 α could be inhibited by OCIF/OPG. In fact, we reported that bone resorption induced by parathyroid hormone and 1 α ,25(OH) $_2$ D $_3$ was completely inhibited by adding OCIF/OPG in an organ culture system, but the inhibition by OCIF/OPG of IL-1 α -induced bone resorption was only partial (53). Ammann et al. (54) also reported that transgenic mice insensitive to TNF due to the overexpression of soluble TNF receptors are protected against bone loss due to estrogen deficiency. These results suggest that TNF- α and IL-1 α are directly involved in pathologically occurring bone resorption observed in inflammatory and metabolic bone diseases such as rheumatoid arthritis, periodontitis, and/or postmenopausal osteoporosis (24–27). Further studies will elucidate the biological significance of osteoclast differentiation and function stimulated by a combination of TNF- α and IL-1 α in pathological bone resorption.

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