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Receptor activator of NF-κB ligand induces the expression of carbonic anhydrase II, cathepsin K, and matrix metalloproteinase-9 in osteoclast precursor RAW264.7 cells

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Abstract

Interleukin-1 (IL-1) is a proinflammatory cytokine that is a potent stimulator of bone resorption and an inhibitor of bone formation, whereas macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB (RANK) ligand (RANKL) are essential and sufficient for osteoclast differentiation. Recently, we showed that IL-1α affects mineralized nodule formation *in vitro* and halts bone matrix turnover. We also showed that IL-1α stimulates osteoclast formation via the interaction of RANKL with RANK by increasing M-CSF and prostaglandin E_2 and decreasing osteoprotegerin. Here, we examined the effects of IL-1α or RANKL and/or M-CSF in the presence of IL-1α on the expression of carbonic anhydrase II (CAII), cathepsin K, matrix metalloproteinase-9 (MMP-9), RANK, M-CSF receptor (c-*fms*), and c-*fos* transcription factor using RAW264.7 cells as osteoclast precursors. Cells were cultured for up to 14 days in 0 or 100 U/ml IL-1α and either 50 ng/ml RANKL, 10 ng/ml M-CSF, or 50 ng/ml RANKL + 10 ng/ml M-CSF in the presence of 100 U/ml IL-1α. The formation of osteoclast-like cells was estimated using tartrate-resistant acid phosphatase staining. Expression of the genes coding for the six proteins of interest was determined using real-time PCR, and the expression of the three enzymes was examined using Western blotting or ELISA. In the presence of IL-1α, expression of CAII, cathepsin K, and MMP-9 was markedly increased in cells cultured with RANKL or M-CSF + RANKL, whereas expression was difficult to detect in cells cultured with IL-1α alone and M-CSF. RANK and c-*fos* expression was also increased in cells cultured with RANKL or M-CSF + RANKL in the presence of IL-1α, whereas c-*fms* expression did not change. These results indicate that the expression of CAII, cathepsin K, and MMP-9 in RAW264.7 cells is not induced by M-CSF, but by RANKL in the presence of IL-1α.

Keywords: c-*fos*; Carbonic anhydrase II; Cathepsin K; MMP-9; RANKL

Introduction

Bone is a dynamic tissue that is constantly renewed through a series of highly regulated steps that depend on the interaction of two cell lineages, osteoclasts and osteoblasts (Robling et al., 2006). Osteoclasts are multinucleated cells belonging to the monocyte/macrophage lineage that form by fusion of mononuclear precursors. This multistep differentiation process is under

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the control of the bone microenvironment, which includes stromal cells, osteoblasts, and local factors (Boyle et al., 2003). Osteoblasts express two cytokines that are essential for osteoclast differentiation: receptor activator of NF-κB (RANK) ligand (RANKL; Anderson et al., 1997) and macrophage colony-stimulating factor (M-CSF; Jimi et al., 1995; Tanabe et al., 2005; Tanaka et al., 2006). RANKL and M-CSF promote osteoclast differentiation through interaction with their cognate signaling receptors RANK and c-*fms*, respectively (Lacey et al., 1998; Yasuda et al., 1998). The expression of RANKL in osteoblasts is up-regulated by several osteotropic factors such as 1α, 25-dihydroxyvitamin D_3, parathormone, prostaglandin E_2 (PGE_2), interleukin (IL)-11, and IL-1 (Feldmann et al., 1996; Suda et al., 1999, 2004; Hofbauer et al., 2000). The expression of M-CSF is up-regulated by such osteotropic factors as IL-1 and lipopolysaccharide (Tanabe et al., 2005; Tanaka et al., 2006; Shoji et al., 2006).

IL-1 is a proinflammatory cytokine that is a potent stimulator of bone resorption and an inhibitor of bone formation (Dinarello, 1994). On the other hand, osteoclast precursors differentiate into mature osteoclasts in the presence of RANKL and M-CSF (Lacey et al., 1998; Yasuda et al., 1998). Mature osteoclasts secrete hydrogen ions (H^+) and proteases such as cathepsin K and matrix metalloproteinase (MMP)-9 from the ruffled border; these dissolve the inorganic and organic components of bone, respectively. H^+ are produced via carbonic anhydrase II (CAII) from CO_2 and H_2O in the cytoplasm and are secreted extracellularly by H^+-ATPase. We previously reported that IL-1α inhibits bone mineralized nodule formation (Tanabe et al., 2004) and halts bone matrix turnover in osteoblastic ROS17/2.8 cells (Fujisaki et al., 2006). In addition, we reported that IL-1α stimulates the formation of osteoclast-like cells from RAW264.7 osteoclast precursor cells by increasing M-CSF and PGE_2 production and decreasing osteoprotegerin (OPG) production in ROS17/2.8 cells (Tanabe et al., 2005). Recently, it was reported that IL-1α enhances the formation of osteoclast-like cells in murine bone marrow monocyte cultures treated with M-CSF and RANKL, and also that RANKL-stimulated formation of osteoclast-like cells *in vitro* is partially dependent on endogenous IL-1 production (Lee et al., 2006). However, it is uncertain whether IL-1α, RANKL, or M-CSF affects the expression of enzymes necessary to induce bone resorption. In bone tissue with an inflammation, coexistence of a large amount of IL-1α and a small amount of RANKL and M-CSF is assumed. We hoped to accomplish this research under a situation near *in vivo* as much as possible. Thus, we examined the effects of IL-1α or RANKL and/or M-CSF in the presence of IL-1α on the expression of CAII, cathepsin K, MMP-9, RANK, c-*fms*, and c-*fos* transcription factors using RAW264.7 cells as osteoclast precursors.

Materials and methods

Cell culture

We used the murine monocyte/macrophage cell line RAW264.7 (Denlinger et al., 1996), obtained from a commercial source (Dainippon Pharmaceutical, Osaka, Japan), as osteoclast precursors. The cells differentiate into osteoclast-like cells in the presence of RANKL and M-CSF (Hotokezaka et al., 2002). The cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Rockville, MD, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), 1% (v/v) penicillin–streptomycin solution (Sigma Chemical, St. Louis, MO, USA), 1 mM sodium pyruvate (Gibco), 2 mM L-glutamine (Gibco), and 0.1 mM non-essential amino acids (Gibco) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

For treatment with IL-1α (Genzyme/Techne, Minneapolis, MN, USA), RANKL (Wako Pure Chemical, Osaka, Japan), and M-CSF (Wako), the cells were seeded onto 100-mm tissue culture plates at a density of 5×10^6 cells/cm^2. After overnight incubation, the cells were cultured for up to 14 days with DMEM containing 10% FBS, 0 or 100 U/ml IL-1α, and 50 ng/ml RANKL, 10 ng/ml M-CSF, or 50 ng/ml RANKL+10 ng/ml M-CSF in the presence of 100 U/ml IL-1α. The cells were observed daily using light microscopy. The concentrations of IL-1α and RANKL were identical to those used in our previous studies (Tanabe et al., 2004, 2005; Fujisaki et al., 2006). The concentration of M-CSF used was previously reported to increase osteoclast formation by IL-1α in bone marrow monocytes treated with M-CSF and RANKL (Lee et al., 2006).

Real-time polymerase chain reaction (PCR)

RAW264.7 cells were plated on six-well microplates at a density of 5×10^6 cells/cm^2 and cultured for up to 14 days in DMEM containing 10% FBS with 0 or 100 U/ml IL-1α, and 10 ng/ml RANKL, 50 ng/ml M-CSF, or 50 ng/ml RANKL+

Table 1
PCR primers used in the experiments

Target	Primers	bp	GenBank Accession
CAII	5′-CATTACTGTCAGCAGCGAGCA-3′ 5′-GACGCCAGTTGTCCACCATC-3′	95	NM_009801
MMP-9	5′-GCCCTGGAACTCACACGACA-3′ 5′-TTGGAAACTCACACGCCAGAAG-3′	85	NM_013599
Cathepsin K	5′-CAGCAGAACGGAGGCATTGA-3′ 5′-CCTTTGCCGTGGCGTTATAC-3′	85	NM_007802
RANK	5′-CCAGGACAGGGCTGATGAGAA-3′ 5′-TGGCTGACATACACCACGATGA-3′	162	NM_009399
c-*fos*	5′-CGGGTTTCAACGCCGACTAC-3′ 5′-AAAGTTGGCACTAGAGACGGACAGA-3′	170	NM_010234
c-*fms*	5′-GAGAGCATCTTTGACTGCGTCATC-3′ 5′-ACAGGCTGGGCCATTTGGTA-3′	164	NM_007779
GAPDH	5′-AAATGGTGAAGGTCGGTGTG-3′ 5′-TGAAGGGGTCGTTGATGG-3	108	NM_001001303

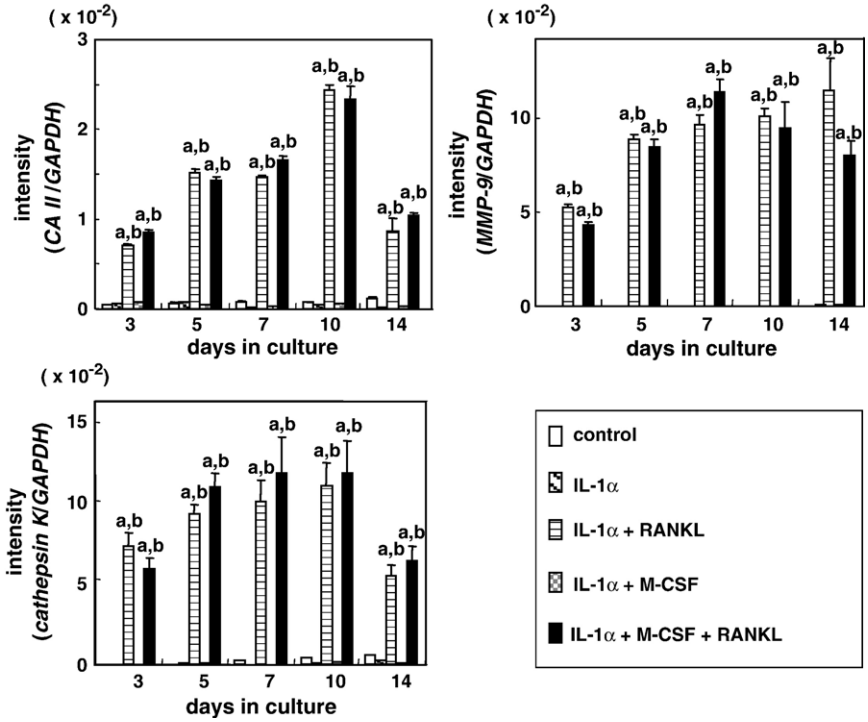

Fig. 1. Effect of IL-1α, RANKL, and M-CSF on the expression of *CAII*, *MMP-9*, and *cathepsin K genes*. RAW264.7 cells were cultured with or without IL-1α or with RANKL and/or M-CSF in the presence of IL-1α for up to 14 days. Expression of the *CAII*, *cathepsin K*, and *MMP-9* was detected by real-time PCR on days 3, 5, 7, 10, and 14 of culture. Data are shown as means±SDs for three separate experiments; [a]$p<0.01$, IL-1α+RANKL or IL-1α+M-CSF+RANKL treatments versus control; [b]$p<0.01$, IL-1α+RANKL or IL-1α+M-CSF+RANKL treatments versus IL-1α treatment.

10 ng/ml M-CSF in the presence of 100 U/ml IL-1α. Total RNA was isolated from the cultured cells on days 3, 5, 7, 10, and 14 using an RNeasy mini kit (Qiagen, Valencia, CA, USA). The mRNA was reverse transcribed into complementary DNA (cDNA) using an RNA PCR kit (GeneAmp, Perkin-Elmer, Branchburg, NJ, USA), and the resulting cDNA mixture was diluted fivefold in sterile distilled water.

Two microliters of the diluted cDNA was subjected to real-time PCR using SYBR Green I dye. The reactions were performed in 25 μl of a SYBR® premixed *Ex Taq*™ solution (Takara Shuzo, Osaka, Japan) containing 20 μM sense and anti-sense primers (Table 1). The primers were designed using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The PCR assays were performed on a Smart Cycler (Cepheid, Sunnyvale, CA, USA) and were analyzed using Smart Cycler software (version 1.2d). The PCR protocol for CAII, cathepsin K, MMP-9, RANK, c-*fms*, and c-*fos* consisted of 35 cycles of 95 °C for 3 s and 60 °C for 20 s. All real-time PCR reactions were performed in triplicate, and the specificity of the PCR products was verified by melting curve analysis. Gene expression levels were normalized by dividing the resulting mRNA values by the value for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA isolated at the same time.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

The cells were plated on six-well microplates at a density of 5×10^6 cells/cm^2 and cultured for up to 7 days with DMEM containing 10% FBS, 0 or 100 U/ml IL-1α, and 50 ng/ml RANKL, 10 ng/ml M-CSF, or 50 ng/ml RANKL+10 ng/ml M-CSF in the presence of 100 U/ml IL-1α. The culture medium was changed to serum-free DMEM on day 7 of culture, and the cells were then cultured for 24 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was collected, dialyzed using a PD-10 column (Amersham Biosciences, Piscataway, NJ, USA), and subjected to SDS-PAGE on 12.5% polyacrylamide gels (8.3 cm×6.5 cm×0.75 mm) with a discontinuous Tris–glycine buffer system (Laemmli, 1970). Media samples containing 20 μg of extracellular protein were dissolved in 10 μl of sample buffer containing 1% SDS, 2 M urea, 15 mg/ml dithiothreitol, and bromophenol blue, and heated at 95 °C for 5 min before loading. Gels were run at 150 V for 60 min.

Gel-separated proteins were transferred to a membrane using a semi-dry electrotransfer unit with a continuous buffer

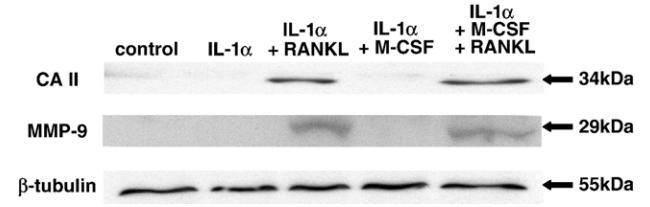

Fig. 2. Effect of IL-1α, RANKL, and M-CSF on CAII and MMP-9 protein levels. RAW264.7 cells were cultured with or without IL-1α or with RANKL and/or M-CSF in the presence of IL-1α for up to 7 days. The levels of CAII and MMP-9 were examined by Western blotting on day 7 of culture.

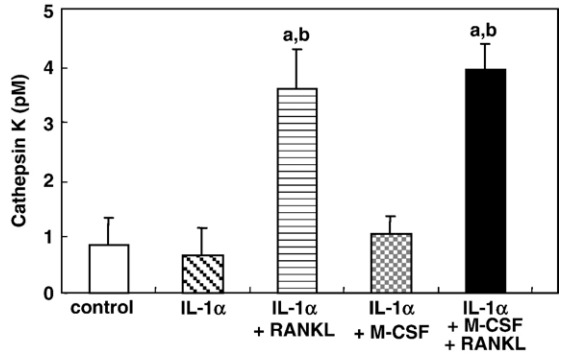

Fig. 3. Effect of IL-1α, RANKL, and M-CSF on the cathepsin K protein level. RAW264.7 cells were cultured with or without IL-1α or with RANKL and/or M-CSF in the presence of IL-1α for up to 7 days. Expression of cathepsin K was examined by ELISA on day 7 of culture. Data are shown as means±SDs for three separate experiments; [a]$p<0.01$, IL-1α+RANKL or IL-1α+M-CSF+RANKL treatments versus control; [b]$p<0.01$, IL-1α+RANKL or IL-1α+M-CSF+RANKL treatments versus IL-1α treatment.

system consisting of 39 mM glycine, 48 mM Tris, 0.0375% SDS, and 20% (v/v) methanol at 0.8 mA/cm^2 (constant amperage) for 60–90 min. On completion of the transfer, the membrane was treated with 25% (v/v) blocking reagent in Tris-buffered saline (TBS: 10 mM Tris, 145 mM NaCl, pH 7.4) at 4 °C for 18 h. The sheet was washed in TBS containing Tween-20 (TBS-Tween) and then incubated at room temperature for 90 min with polyclonal IgG antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) against rabbit anti-CAII, goat anti-MMP-9, or mouse anti-β-tubulin (internal standard) that were diluted 1:500 in 10% (v/v) blocking reagent. The membranes were washed in TBS-Tween and incubated at room temperature for 60 min with appropriate biotin-conjugated secondary antibodies that were diluted 1:10,000 in 10% blocking agent; goat anti-rabbit IgG (American Qualex, San Clemente, CA, USA), donkey anti-goat IgG (Chemicon International, Temecula, CA, USA), or goat anti-mouse IgG (Zymed, San Francisco, CA, USA). The membranes were then washed in TBS-Tween and phosphate-buffered saline (PBS, Nissui Pharmaceutical, Tokyo, Japan) and incubated for 30 min at room temperature with horseradish peroxidase-conjugated streptavidin diluted with PBS. Immunoreactive proteins were visualized using a commercial chemiluminescence kit (Amersham Life Science, Buckinghamshire, UK) and autoradiography with X-ray film (Eastman Kodak, New Haven, CT, USA). As a control, membranes were exposed to diluted normal rabbit serum; the dilution factor was the same as that used for the primary antibodies. Prestained molecular weight standards run on the same gel were used to estimate protein size.

Enzyme-linked immunosorbent assay (ELISA)

The cells were plated on six-well microplates at a density of 5×10^6 cells/cm^2 and cultured for up to 7 days with DMEM containing 10% FBS with 0 or 100 U/ml IL-1α, and 50 ng/ml

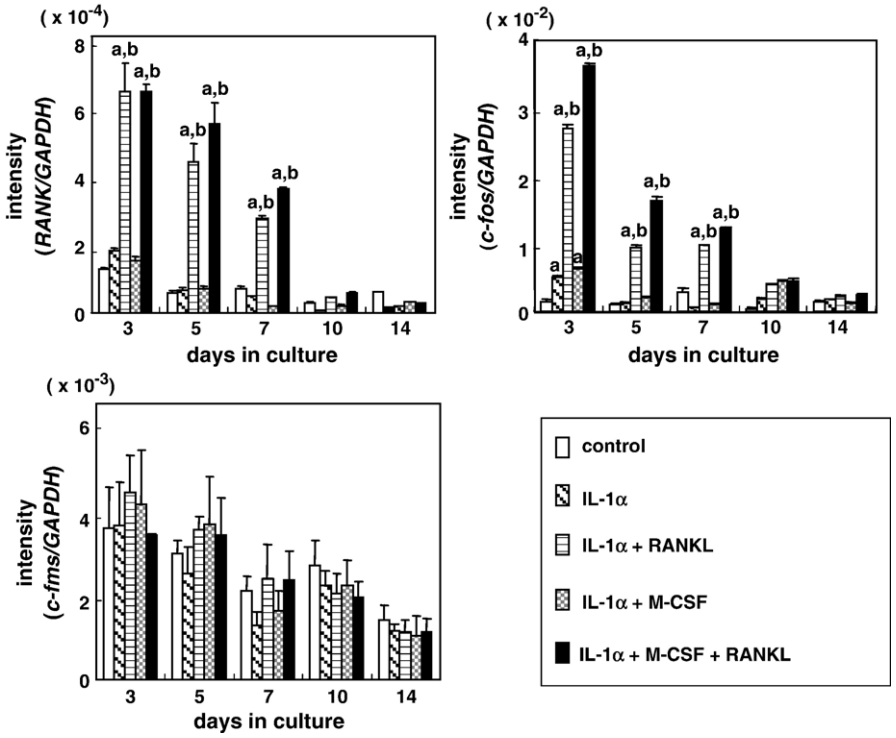

Fig. 4. Effect of IL-1α, RANKL, and M-CSF on expression of the *RANK*, c-*fos*, and c-*fms* genes. RAW264.7 cells were cultured with or without IL-1α or with RANKL and/or M-CSF in the presence of IL-1α for up to 14 days. Expression of *RANK*, c-*fos*, and c-*fms* was determined by real-time PCR on days 3, 5, 7, 10, and 14 of culture. Data are shown as means±SDs for three separate experiments; [a]$p<0.01$, IL-1α+RANKL or IL-1α+M-CSF+RANKL treatments versus control; [b]$p<0.01$, IL-1α+RANKL or IL-1α+M-CSF+RANKL treatments versus IL-1α treatment.

RANKL, 10 ng/ml M-CSF, or 50 ng/ml RANKL + 10 ng/ml M-CSF in the presence of 100 U/ml IL-1α. The culture medium was changed to serum-free DMEM on day 7 of culture, and the cells were then cultured for 24 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

The amounts of cathepsin K in the culture medium were determined using a "Cathepsin K" measuring kit (Biomedica, Wien, Germany). Three assays were performed on each sample, and the absorbance at 492 nm was recorded.

Tartrate-resistant acid phosphatase (TRAP) staining

The cells were plated on six-well microplates at a density of 5×10^6 cells/cm^2 and cultured for up to 14 days in DMEM containing 10% FBS with 0 or 100 U/ml IL-1α, and 50 ng/ml RANKL, 10 ng/ml M-CSF, or 50 ng/ml RANKL + 10 ng/ml M-CSF in the presence of 100 U/ml IL-1α. Cells cultured under the five different conditions were fixed and stained using a TRAP staining kit (Cellgarage, Tokyo, Japan) according to the manufacturer's protocol on days 3, 5, 7, 10, and 14 of culture. TRAP-positive cells with more than three nuclei were considered osteoclast-like cells. The number of osteoclast-like cells was counted under light microscope.

Statistical analysis

All experiments were performed in triplicate. Each value represents the means ± standard deviation of triplicate experiments. Significant differences ($p < 0.05$) were determined using the Bonferroni's modification of the Student's *t*-test.

Results

Effect of IL-1α, RANKL, and M-CSF on CAII, MMP-9, and cathepsin K gene expression

Expression of the *CAII*, *MMP-9*, and *cathepsin K* genes was determined by real-time PCR using RAW264.7 cells cultured for up to 14 days with or without IL-1α, or RANKL and/or M-CSF in the presence of IL-1α. Expression of *CAII*, *MMP-9*, and *cathepsin K* was markedly increased in cells cultured with RANKL or M-CSF + RANKL in the presence of IL-1α compared with control cells and cells treated with IL-1α alone or with M-CSF in the presence of IL-1α. In addition, the expression of each gene increased gradually through day 10, and expression of *CAII* and *cathepsin K* decreased on day 14 (Fig. 1).

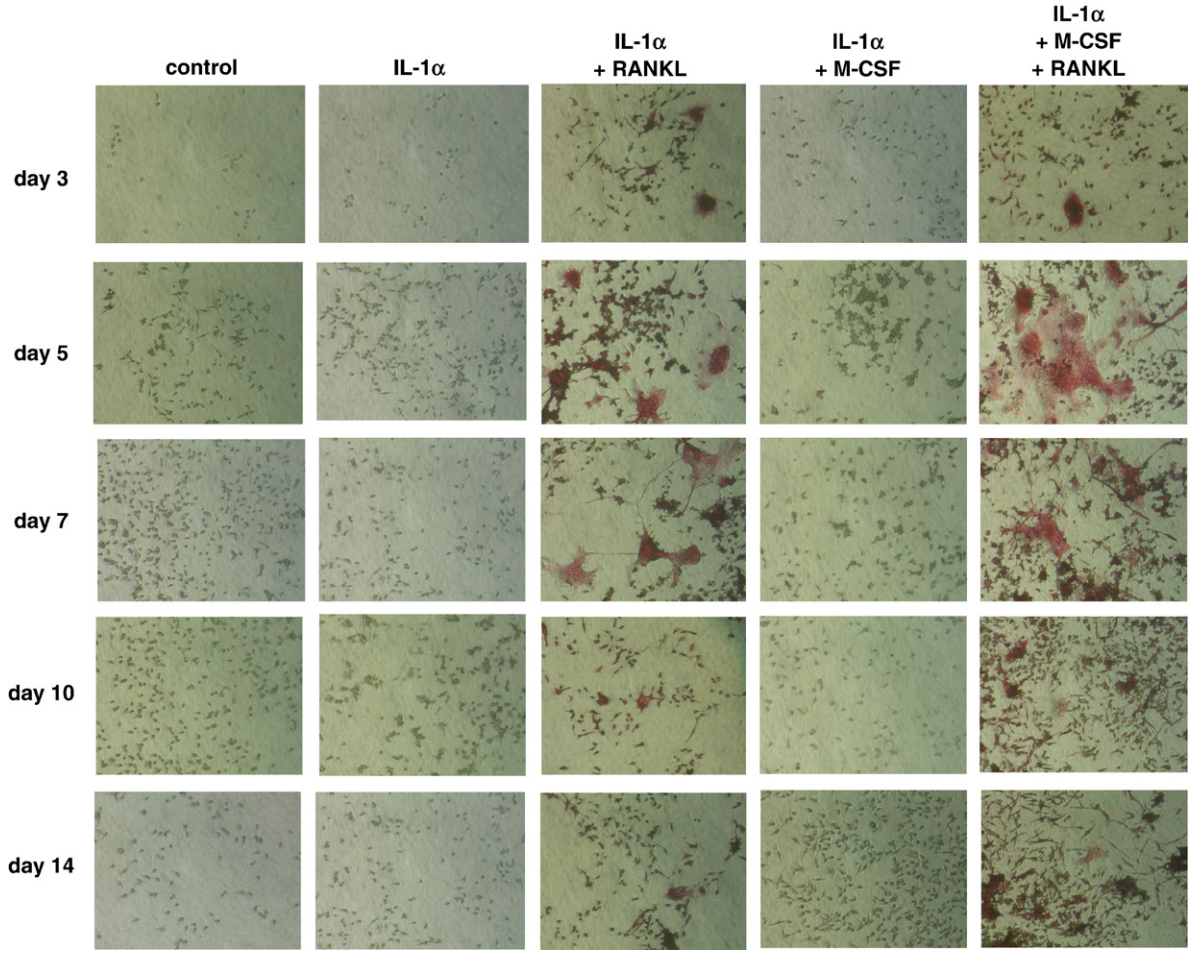

Fig. 5. Effect of IL-1α, RANKL, and M-CSF on TRAP staining of osteoclast-like cells. RAW264.7 cells were cultured with or without IL-1α or with RANKL and/or M-CSF in the presence of IL-1α for up to 14 days. The cells were stained using a TRAP staining kit on days 3, 5, 7, 10, and 14 of culture (original magnification ×200).

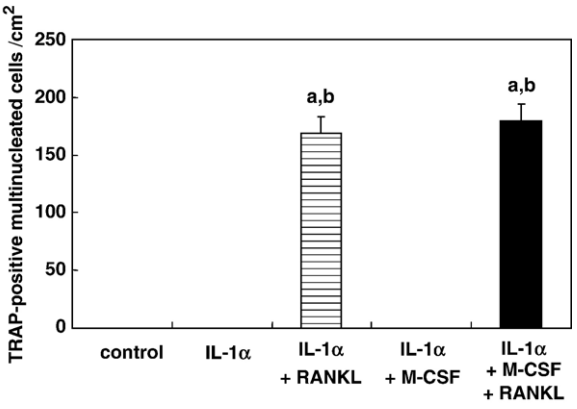

Fig. 6. The number of TRAP-positive multinucleated cells. RAW264.7 cells were cultured with or without IL-1α or with RANKL and/or M-CSF in the presence of IL-1α for up to 7 days. The number of TRAP-positive cells with more than three nuclei was counted under light microscope on day 7 of culture (original magnification ×200). Data are shown as means ± SDs for three separate experiments; [a]$p<0.01$, IL-1α + RANKL or IL-1α + M-CSF + RANKL treatments versus control; [b]$p<0.01$, IL-1α + RANKL or IL-1α + M-CSF + RANKL treatments versus IL-1α treatment.

Effect of IL-1α, RANKL, and M-CSF on CAII, MMP-9, and cathepsin K protein expression

CAII and MMP-9 protein levels were examined by Western blotting using RAW264.7 cells cultured for up to 7 days with or without IL-1α, or RANKL and/or M-CSF in the presence of IL-1α. Protein expression of CAII and MMP-9 was markedly increased in cells cultured with RANKL or M-CSF + RANKL in the presence of IL-1α; however, the proteins were difficult to detect in control cells and cells treated with IL-1α alone or with M-CSF in the presence of IL-1α (Fig. 2).

Protein expression of cathepsin K was determined by ELISA using RAW264.7 cells cultured for up to 7 days with or without IL-1α or RANKL and/or M-CSF in the presence of IL-1α. Protein expression of cathepsin K was markedly increased in cells cultured with RANKL or M-CSF + RANKL in the presence of IL-1α compared with control cells and cells treated with IL-1α alone or with M-CSF in the presence of IL-1α (Fig. 3).

Effect of IL-1α, RANKL, and M-CSF on the gene expression of RANK, c-fos, and c-fms

Expression of the *RANK*, c-*fos*, and c-*fms* genes was determined by real-time PCR using RAW264.7 cells cultured for up to 14 days with or without IL-1α or RANKL and/or M-CSF in the presence of IL-1α. On days 3, 5, and 7, expression of *RANK* and c-*fos* was markedly increased in cells cultured with RANKL or M-CSF + RANKL in the presence of IL-1α compared with control cells and cells cultured with IL-1α alone or with M-CSF in the presence of IL-1α. On the other hand, c-*fms* expression was detected under all conditions and was not affected by the addition of IL-1α or RANKL and/or M-CSF in the presence of IL-1α. In addition, the expression levels for all three genes decreased gradually through day 14 of culture (Fig. 4).

Effect of IL-1α, RANKL, and M-CSF on TRAP staining of osteoclast-like cells

RAW264.7 cells were cultured with or without IL-1α or RANKL and/or M-CSF in the presence of IL-1α, and stained on days 3, 5, 7, 10, and 14 using a TRAP staining kit prior to observation under light microscopy. TRAP-positive multinucleated cells were observed in cells cultured with RANKL or M-CSF + RANKL in the presence of IL-1α; staining of M-CSF + RANKL-treated cells was slightly stronger than RANKL alone-treated cells. On the other hand, TRAP-positive multinucleated cells were not observed in cells cultured with IL-1α alone, M-CSF in the presence of IL-1α, or control cells (Fig. 5).

The number of TRAP-positive cells with more than three nuclei was counted under light microscope. TRAP-positive multinucleated cells of 170–180 cells/cm^2 were counted in cells cultured with RANKL or M-CSF + RANKL in the presence of IL-1α. On the other hand, TRAP-positive multinucleated cells were not counted in other conditions (Fig. 6).

Discussion

Our results show that RANKL may induce bone resorption by osteoclasts via increased CAII, cathepsin K, and MMP-9 production.

Osteoclasts are multinucleated cells responsible for resorbing bone, and are derived from mononuclear precursors of the monocyte/macrophage lineage. Differentiation of the precursor cells into osteoclasts during normal bone development is affected by cells of the osteoblast lineage. Critically, membrane-bound RANKL and M-CSF, which are expressed in osteoblastic cells, promote osteoclast differentiation through interaction with their cognate signaling receptors RANK and c-*fms*, respectively (Lacey et al., 1998; Yasuda et al., 1998). On the other hand, IL-1 is one of the most potent factors involved in bone loss associated with inflammation (Masi and Brandi, 2001) and it also inhibits bone and cartilage formation (Ellies and Aubin, 1990; Aida et al., 2004; Tanabe et al., 2004).

This study is part of a long-term *in vitro* approach to clarify the molecular mechanisms of expansion in dental radicular cysts following IL-1 stimulation. Previously, Fujisaki et al. (2006) reported that IL-1α may stimulate bone matrix turnover by increasing the production of MMPs, tissue-type plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA), and decreasing the production of plasminogen activator inhibitor-1 (PAI-1) in ROS17/2.8 cells. Based on these results, it was concluded that the degradation of bone matrix proteins by MMPs, tPA, and uPA may promote the expansion of dental radicular cysts. On the other hand, Tanabe et al. (2004) reported that IL-1α inhibits mineralized nodule formation by ROS17/2.8 cells. In addition, Tanabe et al. (2005) reported that IL-1α increases M-CSF and PGE_2 production and decreases OPG production in ROS17/2.8 cells, and that conditioned medium from IL-1α-treated ROS17/2.8 cells containing M-CSF and soluble RANKL stimulates the formation of osteoclasts from RAW264.7 cells, and thus may facilitate the extension of dental

radicular cysts. Even so, the effects of IL-1α, RANKL, and M-CSF on the proteolytic mechanisms responsible for the degradation of bone matrix by osteoclasts were not clear. Therefore, we examined the effects of IL-1α alone or RANKL and/or M-CSF in the presence of IL-1α on the expression of CAII, cathepsin K, MMP-9, RANK, c-*fms*, and c-*fos* in RAW264.7 cells. As in our previous studies, the experimental period lasted 14 days (Fujisaki et al., 2006; Tanabe et al., 2004, 2005).

Bone consists largely of type-I collagen (>90%) and non-collagenous proteins containing a mineral phase of substituted hydroxylapatite. Dissolution of the inorganic phase of bone precedes matrix degradation. CAII and vacuolar H^+-ATPases are involved in the extracellular acidification caused by osteoclasts. CAII generates H^+ and HCO_3^- by the hydration of CO_2, and the H^+ are transported through the apical ruffled border of the osteoclasts to the resorption zone by a vacuolar H^+-ATPase (Teitelbaum, 2000). The result is secretion of HCl into the resorptive microenvironment, producing a pH of ~4.5 (Mundlos et al., 1997). This acidic milieu first mobilizes bone mineral; subsequently, the demineralized organic component of bone is degraded by a lysosomal protease, cathepsin K, and MMP-9 (Teitelbaum, 2000; Andersen et al., 2004). Cathepsin K and MMP-9 are efficient collagenases that cleave both collagen types I and II (Liu et al., 2003).

In light of these findings, we examined the effect of IL-1α alone or RANKL and/or M-CSF in the presence of IL-1α on the expression of *CAII*, *cathepsin K*, and *MMP-9* in RAW264.7 cells cultured for 14 days. In the presence of IL-1α, expression of *CAII*, *cathepsin K*, and *MMP-9* was markedly increased in cells cultured with RANKL or with M-CSF+RANKL. On the other hand, the expression levels were difficult to detect in cells cultured with IL-1α alone or with M-CSF in the presence of IL-1α. In addition, the expression levels gradually increased through day 10 of culture. These results suggest that the expression of *CAII*, *cathepsin K*, and *MMP-9* is induced not by IL-1α and M-CSF, but by RANKL, and that their expression levels are increased according to the maturity and differentiation of the RAW264.7 cells. In terms of the receptors, *RANK* expression increased markedly in cells cultured with RANKL or with M-CSF+RANKL in the presence of IL-1α, whereas c-*fms* expression was not affected by the addition of IL-1α, RANKL, or M-CSF. In addition, the expression of *RANK* and c-*fms* gradually decreased through day 14 of culture. These results suggest that RANKL induces RANK expression, but is not related to c-*fms* expression, and that the RANKL–RANK interaction is important at the early stages of RAW264.7 cell differentiation.

Osteoclasts belong to the monocyte/macrophage lineage and arise from multipotent hematopoietic stem cells through complex, finely regulated differentiation steps. Many studies have focused on the final determinants of the commitment process that separates osteoclasts from macrophages (Roodman, 1996). Among such factors is c-*fos*, an immediate early gene that belongs to the AP-1 transcription factor family. Deletion of c-*fos* in mice resulted in a complete lack of mature osteoclasts and in osteoporosis, although early osteoclast progenitors and macrophages were still present (Teitelbaum, 2000).

Given these findings, we examined the effect of IL-1α and RANKL and/or M-CSF in the presence of IL-1α on the expression of c-*fos* in RAW264.7 cells cultured for 14 days. In the presence of IL-1α, c-*fos* expression was markedly increased in cells cultured with RANKL or with M-CSF+RANKL. On the other hand, the expression level was difficult to detect by treatment with IL-1α alone or M-CSF in the presence of IL-1α. In addition, the expression level gradually decreased through day 14 of culture. These results suggest that RANKL induces c-*fos* expression, and that expression occurs at an early stage of RAW264.7 cell differentiation, similar to *RANK* and c-*fms*.

TRAP staining is a general method to examine osteoclast formation, and TRAP-positive multinucleated cells include osteoclasts or osteoclast-like cells (Tanabe et al., 2005; Tanaka et al., 2006). In this study, TRAP-positive multinucleated cells were observed in cells cultured with RANKL or M-CSF+RANKL in the presence of IL-1α, whereas multinucleated cells were not observed in cells cultured with IL-1α alone or with M-CSF in the presence of IL-1α. These results suggest that RANKL plays an important role in osteoclast formation.

In conclusion, our results indicate that the production of CAII, cathepsin K, and MMP-9 for bone resorption by osteoclasts is not induced by M-CSF, but by RANKL in the presence of IL-1α. In addition, the increase in bone resorption by osteoclasts via RANKL may promote the expansion of dental radicular cysts.

In this research, the effects of RANKL and/or M-CSF are examined in the presence of IL-1α. It will be necessary to examine the effects of RANKL or M-CSF alone in the absence of IL-1α in the future.

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