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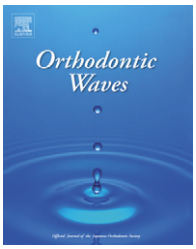

Research paper

Augmentation of TNF-induced osteoclast differentiation by inhibition of ERK and activation of p38: Similar intracellular signaling between RANKL- and TNF-induced osteoclast differentiation

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ABSTRACT

Research in osteoclast differentiation has been greatly advanced since the identification of receptor activator of nuclear factor-κB ligand (RANKL) as osteoclast differentiation factor. The mechanisms of RANKL-induced osteoclast differentiation have been extensively investigated. Mitogen-activated protein kinases (MAPKs) were shown to play crucial roles in RANKL-induced osteoclast differentiation. RANKL-induced osteoclast differentiation was enhanced by inhibition of extracellular signal-regulated kinase (ERK), whereas it was suppressed by inhibition of p38 MAPK. It was reported that tumor necrosis factor (TNF), a major proinflammatory cytokine, induced osteoclast differentiation independently of RANKL. A report showed that inhibition of p38 suppressed TNF-induced osteoclast differentiation, whereas inhibition of ERK did not augment TNF-induced osteoclast differentiation. In this study we reevaluated the roles for MAPKs in TNF-induced osteoclast differentiation. In contrast with the previous report, pretreatment of mouse monocytic RAW264 cells with MAPK/ERK kinase (MEK) inhibitors including PD98059 and U-0126 augmented TNF-induced osteoclast differentiation. Furthermore, we found that U-0126 was more effective in augmentation of osteoclast differentiation than PD98059. Western blot analysis showed that U-0126 inhibited ERK phosphorylation and enhanced p38 phosphorylation, whereas PD98059 inhibited both ERK and p38 phosphorylation. SB203580, a p38 inhibitor, suppressed TNF-induced osteoclast differentiation, and inhibited p38 phosphorylation whereas it augmented ERK phosphorylation. These results demonstrate that ERK inhibition and p38 activation play crucial roles in both RANKL- and TNF-induced osteoclast differentiation.

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1. Introduction

Bone marrow-derived cells of monocyte/macrophage lineage differentiate into mature multinucleated osteoclasts through several differentiation steps, and multiple pathways exist in the regulatory mechanism of osteoclast differentiation [1,2]. It would be quite important in the field of orthodontics to elucidate the intracellular signal transduction pathways in osteoclast differentiation for improvement of clinical course.

RANK, receptor activator of nuclear factor-κB (NF-κB), and its ligand RANKL play crucial roles in osteoclastogenesis [3–6]. It has been shown that mice deficient in RANK or RANKL present osteopetrotic phenotype due to loss of osteoclasts [7,8]. The mechanisms by which RANKL induces osteoclast differentiation have been profoundly investigated. As its name indicates, RANKL binds to RANK expressed on the cell surface and activates NF-κB [9]. It has also been reported that RANKL activates mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinase (ERK) and p38 MAPK [10,11]. Furthermore, it was shown that RANKL-induced osteoclast differentiation was enhanced by MAPK/ERK kinase (MEK) inhibitors including PD98059 and U-0126, whereas it was suppressed by a p38 MAPK inhibitor, SB203580 [10,11].

Tumor necrosis factor (TNF), a major proinflammatory cytokine, has been reported to induce differentiation of preosteoclasts into mature multinucleated osteoclasts independently of RANKL, suggesting that TNF may be involved in bone resorption under chronic inflammatory conditions such as rheumatic arthritis and periodontitis [12,13]. RANKL and TNF are both members of TNF family and have similar structure. A report showed that TNF-induced osteoclast differentiation was inhibited by SB203580 in accordance with RANKL-induced differentiation, whereas TNF-induced osteoclast differentiation was not augmented by PD98059 in contrast with RANKL-induced differentiation [14].

In this study we investigated the role for MAPKs in TNF-induced differentiation of mouse macrophage-like RAW264 cells into osteoclasts. We found that TNF-induced osteoclast differentiation was augmented by PD98059 or U-0126 whereas it was inhibited by SB203580, demonstrating that RANKL and TNF share similar intracellular signal transduction pathways in osteoclast differentiation.

2. Materials and methods

2.1. Cell culture

Mouse monocytic RAW264 cells were maintained in MEMα (Wako Pure Chemical Industries, Japan) containing 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma) at 37 °C in a humidified 5% CO_2 incubator. RAW264 cells at 5000 cells/well were incubated in 96-well plates overnight. Then, the cells were treated with RANKL or TNF in the presence or absence of PD98059, U-0126 (MEK inhibitors) or SB203580 (a p38 MAPK inhibitor) (Calbiochem) for 3 days, and TRAP staining and TRAP quantitative assay (by OD measurement) were performed as described below.

2.2. TRAP staining

The cells were fixed with 10% formalin solution in PBS for 10 min and acetone/ethanol (1:1) for 1 min. Then the cells were incubated for 1 h in mixture of 50 mM sodium acetate and 25 mM tartaric acid (pH 5.0) containing 1.2 mg/ml fast red violet LB salt (dissolved in *N*,*N*-dimethylformamide) and 0.1 mg/ml Naphthol AS-MX phosphate.

2.3. TRAP quantitative assay

TRAP activity was quantified as described [15]. The cells were fixed with 10% formalin solution in PBS for 10 min, 95% ethanol for 1 min, and incubated for 1 h in mixture of 50 mM sodium citrate and 10 mM tartaric acid (pH 4.6) containing 5 mM *p*-nitrophenyl phosphate. The solution was mixed with equal volume of 0.1N sodium hydroxide, and absorbance was measured at 405 nm with a 96-well plate reader. Statistical analysis was performed by Student's *t*-test.

2.4. Western blot analysis

The cells at 2.5×10^6 cells/well were incubated for 24 h in 24-well plates and stimulated with 10–50 ng/ml RANKL or TNF for the indicated times. Then the cells were lysed on ice in Laemmli's sample buffer containing 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 1 mM PMSF, 5 μg/ml aprotinin and 1 μg/ml leupeptin. The lysate was sonicated, and boiled for 2 min at 100 °C. Equal volume of samples was loaded in each lane, and 10% SDS–PAGE was performed, and the protein was transferred to PVDF membrane. The membrane was preincubated with 2% Blocking One (Nacalai Tesque) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h, and incubated with anti-phospho-p44/42 ERK or phospho-p38 antibody (Cell Signaling) for 2 h, washed with TBS-T for 15 and 5 min. The membrane was incubated with HRP-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology) for 1 h, washed with TBS-T for 5 min three times, and incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 2 min. The images were obtained using VersaDoc (Bio-Rad). Equal protein loading was confirmed by reprobing the membranes with anti-actin antibody (Santa Cruz Biotechnology).

3. Results

3.1. Induction of osteoclast differentiation of RAW264 cells by RANKL or TNF

As previously reported [14,16], mouse monocytic RAW264 cells were induced to differentiate into TRAP-positive multinucleated osteoclasts by RANKL or TNF (Fig. 1A–C). To quantitatively evaluate osteoclast differentiation, TRAP activity was measured as described in Section 2. TNF was less effective than RANKL in inducing differentiation of RAW264 cells into multinucleated osteoclasts when compared at the same concentrations (Fig. 1D). As reported [11], pretreatment with PD98059 or U-0126, MEK inhibitors, augmented RANKL-induced osteoclast differentiation. Moreover, it was observed