Osteoclast differentiation induced by salicylates via MAPK signaling

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Salicylates such as sodium salicylate and aspirin (acetylsalicylic acid) have been widely used for various inflammatory diseases including rheumatoid arthritis and periodontitis. However, the effects of salicylates on bone metabolism remain unclear. We investigated whether salicylates affect differentiation of mouse monocyctic RAW264 cells into osteoclast-like cells. Both sodium salicylate and aspirin slightly induced differentiation of RAW264 cells into tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts, and enhanced osteoclast differentiation induced by a low concentration of receptor activator of nuclear factor-κB ligand (RANKL). Other nonsteroidal anti-inflammatory drugs (NSAIDs) without salicylic structure including ibuprofen, meloxicam, and celecoxib did not affect osteoclast differentiation. Since it has been reported that mitogen-activated protein kinase (MAPK) signaling plays a crucial role in RANKL-induced osteoclast differentiation, we examined the effects of salicylates on extracellular signal-regulated kinase (ERK) and p38 MAPK. Although phosphorylation of p38 was augmented by both aspirin and sodium salicylate, ERK phosphorylation was suppressed. Furthermore, osteoclast differentiation induced by RANKL and salicylates was suppressed by SB 203580, a specific inhibitor of p38 MAPK. These results were consistent with the previous report which found that although RANKL-induced osteoclast differentiation was suppressed by inhibition of p38, it was enhanced by ERK inhibition. This suggests that salicylates induce osteoclast differentiation via MAPK signaling. (J Osaka Dent Univ 2009; 43: 149-156)

Key words: RANKL; Osteoclast differentiation; Salicylates; Aspirin; ERK; p38 MAPK

INTRODUCTION

Osteoclasts are large multinuclear cells formed from mononuclear macrophage-like cells by stimulation with receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) and macrophage colony stimulating factor (M-CSF). In differentiation into osteoclast-like cells, RANKL activates the signal transduction pathway known as the mitogen-activated protein kinase (MAPK) cascade including extracellular signal-regulated kinase (ERK) and p38 MAPK. It has been reported that activation of p38 MAPK is required for formation of osteoclast-like cells. SB 203580, a specific inhibitor of p38 MAPK, prevents osteoclast differentiation. On the other hand, PD 98059 and U-0126, both of which inhibit the activation of ERK, enhance osteoclast differentiation. These results suggest that the activation of p38 MAPK and the inhibition of ERK promote differentiation of RAW264 into osteoclast-like cells.

Salicylates such as sodium salicylate and aspirin (acetylsalicylic acid) are widely used as nonsteroidal anti-inflammatory drugs (NSAIDs). However, the effects of salicylates on bone metabolism remain unclear. Bauer et al. reported that regular use of aspirin or other NSAIDs has a modest beneficial effect on bone mineral density (BMD) in postmenopausal women, but that there was no clinically significant protective effect on the risk of fractures. Carbone et al. reported that significantly elevated
BMD was found in users of relative cyclooxygenase (COX)-2 selective NSAIDs with aspirin compared with nonusers. In vitro, sodium salicylate and indomethacin inhibited osteoclast formation in mouse bone marrow culture. A recent study demonstrated involvement of COX-2 in RANKL-induced osteoclastogenesis.

The mechanism by which salicylates reduce inflammation has been extensively investigated. Most of NSAIDs, including aspirin, inhibit COX, which is required for prostaglandin synthesis. However, since sodium salicylate lacks an acetyl group, it is much less potent than aspirin at inhibiting COX. Moreover, much higher doses of aspirin are required to treat inflammatory diseases than those required to inhibit COX. Therefore, salicylates must have other abilities to reduce inflammation. It has been reported that salicylates inhibit NF-κB, an important transcription factor involved in various cellular responses including inflammation. Another report demonstrated that salicylates specifically inhibit IκB kinase-β, which phosphorylates IκB leading to its degradation and activation of NF-κB. Other studies have shown that salicylates affect mitogen-activated protein kinases (MAPKs) including ERK and p38 MAPK. MAPKs are involved in various cellular responses. In general, ERK is involved in proliferation whereas p38 is involved in differentiation and inflammation. It has been reported that salicylates inhibit ERK activation and promote p38 MAPK activation.

Since inhibition of ERK and activation of p38 MAPK augmented RANKL-induced osteoclast differentiation, we investigated whether salicylates could induce differentiation of RAW264 into osteoclast-like cells. We found that salicylates alone slightly induced osteoclast differentiation. Moreover, RANKL-induced osteoclast differentiation was augmented by salicylates. However, other NSAIDs such as ibuprofen, meloxicam and celecoxib did not promote osteoclast differentiation. Western blot analysis revealed that although phosphorylation of p38 MAPK was augmented by salicylates, phosphorylation of ERK was suppressed. However, phosphorylation of ERK was not suppressed by other NSAIDs. SB 203580, suppressed osteoclast differentiation induced by RANKL and salicylates. These results suggest that salicylates promote osteoclast differentiation via MAPK signaling in accordance with RANKL-induced osteoclast differentiation.

MATERIALS AND METHODS

Materials

RAW264 murine monocytic cell line was used as the osteoclast precursor. Mouse recombinant soluble RANKL was purchased from Wako Pure Chemical Industries, Osaka, Japan. SB 203580 was purchased from Calbiochem, San Diego, CA, USA. Aspirin and sodium salicylate were purchased from Nacalai Tesque, Kyoto, Japan. Meloxicam and ibuprofen sodium salt were purchased from Sigma-Aldrich, St. Louis, MO, USA. Celecoxib was provided by Pfizer, New York, NY, USA. Mouse monoclonal anti-phospho-p 44/42 MAPK (ERK 1/2) (Thr 202/Tyr 204) (E 10) antibody and mouse monoclonal anti-phospho-p38 MAPK (Thr 180/Tyr 182) (28 B 10) antibody were purchased from Cell Signaling Technology, Danvers, MA, USA. Horseradish peroxidase-conjugated goat anti-mouse IgG was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Cell culture

RAW264 cells were cultured in MEMα with L-glutamine (Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum (Biosource, Bethesda, MD, USA) and 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) at 37 °C in a humidified 5% CO2 incubator. The cells were detached from the culture flask with trypsin-EDTA (Immuno-Biological Laboratories, Takasaki, Gunma, Japan).

Osteoclast differentiation

RAW264 cells were seeded at 3×104 cells/well in 24 well plates for TRAP staining, or at 5×103 cells/well in 96 well plates for TRAP quantitative assay. After 4 h incubation at 37°C, the medium was removed. Either 5 ng/mL mouse recombinant soluble
RANKL or other reagents as indicated below was added to new medium (200 μL for each well in 96 well plates and 2 mL for each well in 24 well plates). The cells were incubated at 37°C for 4 days to induce osteoclast differentiation.

**Tartrate-resistant acid phosphatase (TRAP) staining**

After 4 days incubation at 37°C, the medium was removed from each well in the 24 well plates, and the cells were washed with PBS twice. Then 500 μL of 10% formalin solution (Nacalai Tesque, Kyoto, Japan) diluted in PBS was added to each well. After incubation for 10 min at room temperature, formalin solution was removed and the cells were washed with PBS twice. Then 500 μL of acetone/ethanol (50:50) was added to each well. After incubation at room temperature for 1 min, acetone/ethanol was removed, and the cells were dried. The TRAP staining solution was 5 mg of naphthol AS-MX phosphate (Sigma-Aldrich) dissolved in 0.5 mL N, N-dimethylformamide (Katayama Chemical Industries, Osaka, Japan) which was mixed with 50 mL of 0.1 M sodium acetate (Nacalai Tesque) containing 50 mM L(+)-tartaric acid (pH 5.0) and 60 mg fast red violet LB salt (Sigma-Aldrich). One hundred microliters of this TRAP staining solution were added to each well. After incubation at room temperature for 30 min, TRAP-positive osteoclast-like cells were observed under light microscope.

**TRAP quantitative assay**

TRAP quantitative assay was performed as previously described.21 After 4 days incubation at 37°C, the medium was removed from each well in 96 well plates, and the cells were washed with PBS twice. Then 50 μL of 10% formalin solution diluted in PBS was added to each well. After incubation for 10 min at room temperature, formalin solution was removed from each well and the cells were washed with PBS twice. Then 100 μL of 95% ethanol was added to each well. After incubation at room temperature for 1 min, ethanol was removed, and the cells were dried. Then 100 μL TRAP reaction mixture (13.6 mg p-nitrophenylphosphoric acid disodium salt (Nacalai Tesque) in 10 mL of 50 mM citric acid (Nacalai Tesque) containing 10 mM L(+) tartaric acid (pH 4.6)) were added to each well. After incubation at room temperature for 30 min, the TRAP reaction mixture was transferred to another 96 well plate, and 100 μL of 0.1 N sodium hydroxide (Wako Pure Chemical Industries) was added to each well. Optical density was measured at 405 nm using Wellreader SME 3400 (Iwaki-Asahi Techno Glass Co., Funabashi, Chiba, Japan).

**Western Blot Analysis**

RAW264 cells were seeded at 5 x 10^6 cells/well in 24 well plates with 500 μL of culture medium as described above. After 4 h incubation at 37°C, 500 μL of the medium containing a double concentration of the various reagents to induce or inhibit osteoclast differentiation were added to each well, and the cells were incubated at 37°C for the indicated times. The plates were then kept on ice, and the cells were washed twice with cold PBS, and lysed with 50 μL of lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% sodium dodecyl sulfate (Wako Pure Chemical Industries), 10% glycerol (Wako Pure Chemical Industries), 5% 2-mercaptoethanol (Wako Pure Chemical Industries), 20 mM EGTA, 50 mM β-sodium glycerophosphate, 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), 1 μg/mL leupeptin (Peptide Institute, Osaka, Japan), 5 μg/mL aprotinin (Sigma-Aldrich), 1 mM sodium orthovanadate (Sigma-Aldrich), and 5% bromophenol blue (Katayama Chemical Industries). The cell lysate was collected in 1.5 mL tubes, and was homogenized with a sonicator. The cell lysate was then boiled at 100°C for 2 min. The lysate was separated in 10% SDS-PAGE at 20 mA for 80 min. The protein was transferred to Immobilon-P transfer membrane (Millipore Corporation, Billerica, MA, USA) at 150 mA for 90 min. The membrane was blocked with 20% Blocking One (Nacalai Tesque) diluted with TBS containing 0.1% Tween-20 (TBS-T) at 4°C overnight. The membrane was then incubated with the primary antibody diluted at 1:1000 with TBS-T at room temperature for 3 h. After washing with TBS-T for 5 min three times, the
membrane was incubated with the secondary antibody conjugated with horseradish peroxidase diluted at 1:4000 in TBS-T at room temperature for 45 min. After washing with TBS-T for 5 min three times, the membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer’s protocol. The image was detected with VersaDoc 5000 (Bio-Rad Laboratories, Hercules, CA, USA).

RESULTS

Differentiation of RAW264 cells into osteoclasts by aspirin or sodium salicylate

In this study, we used murine monocytic cell line RAW264, which was previously shown to differentiate into TRAP-positive osteoclast-like multinuclear cells on stimulation with RANKL.[22]

To investigate the effects of salicylates on differentiation of RAW264 cells into TRAP-positive osteoclast-like cells, various concentrations of aspirin or sodium salicylate were added to cells in the presence or absence of a low concentration (5 ng/mL) of RANKL. TRAP activity was quantified by measuring absorbance at 405 nm as previously described.[23] As shown in Fig. 1, aspirin or sodium salicylate alone slightly induced osteoclast differentiation in a dose-dependent manner up to 5 mM. In the presence of 5 ng/mL RANKL, TRAP activity was further increased by aspirin or sodium salicylate dose-dependently up to 5 mM (Fig. 1). Although most of the TRAP-positive cells remained mononuclear when treated with either aspirin or sodium salicylate alone, many multinuclear giant cells similar to mature osteoclasts were observed in the presence of RANKL and either aspirin or sodium salicylate. TRAP activity was decreased at 10 mM of either aspirin or sodium salicylate due to reduction of the number of viable cells.

Effects of various NSAIDs on differentiation of RAW264 cells into osteoclasts

It is well known that aspirin inhibits COX activity by acetylation, whereas sodium salicylate is much less potent than aspirin at inhibiting COX since it lacks an acetyl group.[11,12] To investigate whether inhibition of COX is involved in induction of osteoclast differentiation, we examined the effects of various NSAIDs other than salicylates on differentiation of RAW264 cells into osteoclast-like cells. These NSAIDs including ibuprofen, meloxicam and celecoxib are without salicylic structure and inhibit COX; ibuprofen inhibits both COX-1 and -2, whereas meloxicam and celecoxib preferentially inhibit COX-2.[15,23] On the other hand, aspirin inhibits COX-1 more potently than COX-2.[12] As shown in Fig. 2, these three NSAIDs did not enhance osteoclast differentiation of RAW264 cells in the presence or absence of RANKL. The concentrations of the NSAIDs used in this study were far above the IC50 values and above the levels needed to inhibit growth (data not shown). These results suggest that inhibition of neither COX-1 nor -2 plays a major role in osteoclast differentiation and that salicylates stimulate osteoclast differentiation by mechanism(s) unrelated to inhibition of COX.

Salicylates inhibit ERK phosphorylation and augment p38 MAPK phosphorylation in RAW264 cells

It has been reported that MAPKs play a crucial role in RANKL-induced osteoclast differentiation, that is, inhibition of p38 MAPK suppresses RANKL-induced osteoclast differentiation whereas inhibition of ERK enhances it.[3,4] To investigate whether MAPKs were involved in salicylate-induced osteoclast differentiation, we performed Western blot analysis for phospho-ERK and phospho-p38 MAPK. As previously reported,[16-18] phosphorylation of p38 MAPK was enhanced by aspirin whereas phosphorylation of ERK was inhibited (Fig. 3). In the presence of RANKL, phosphorylation of p38 MAPK was further enhanced, whereas RANKL-induced phosphorylation of ERK was suppressed by aspirin. Similarly, sodium salicylate augmented p38 phosphorylation whereas it reduced ERK phosphorylation in both the presence of and the absence of RANKL (Fig. 4). On the other hand, NSAIDs other than salicylates did not augment p38 phosphorylation nor reduce ERK phosphorylation (Fig. 4). It seemed...
Salicylate-induced osteoclast differentiation

Fig. 1 Differentiation of RAW264 cells into osteoclasts by aspirin or sodium salicylate. (A) RAW264 cells were seeded at $3 \times 10^4$ cells/well in 24 well plates and treated with or without 5 ng/mL RANKL and either aspirin or sodium salicylate. After 4 days at 37°C, TRAP staining was performed. 1, control ($\times 40$); 2, RANKL 5 ng/mL ($\times 40$); 3, RANKL 5 ng/mL and aspirin 5 mM ($\times 40$); 4, RANKL 5 ng/mL and sodium salicylate 5 mM ($\times 40$); 5, RANKL 5 ng/mL and aspirin 5 mM ($\times 200$); 6, RANKL 5 ng/mL and sodium salicylate 5 mM ($\times 200$). (B) RAW264 cells were seeded at $5 \times 10^4$ cells/well in 96 well plates and treated with or without 5 ng/mL RANKL and the indicated concentrations of aspirin or sodium salicylate. After 4 days at 37°C, TRAP quantitative assay was performed. The data represent means plus standard deviations of three independent experiments. The values are shown as % compared with those treated with 5 ng/mL RANKL only.
that ibuprofen and meloxicam rather increased ERK phosphorylation. These results suggest that salicylates may enhance osteoclast differentiation through activation of p38 and inhibition of ERK, consistent with RANKL-induced osteoclast differentiation.

### Effect of inhibition of p38 MAPK by SB 203580 on attenuation of osteoclast differentiation by RANKL and salicylates

To further investigate the role of p38 MAPK in salicylate-induced differentiation of RAW264 into osteoclast-like cells, SB 203580, a selective inhibitor of p38 MAPK, was used in the following experiment. When the cells were pretreated with SB 203580 and then treated with RANKL and either...
aspirin or sodium salicylate, TRAP activity was not increased, indicating that inhibition of p38 MAPK completely suppressed osteoclast differentiation induced by RANKL and salicylates (Fig. 5). These results demonstrate that activation of p38 MAPK plays a crucial role in osteoclast differentiation induced by salicylates as well as RANKL.

DISCUSSION

The mechanisms of RANKL-induced osteoclast differentiation have been extensively investigated and it has been reported that inhibition of ERK and activation of p38 MAPK augmented RANKL-induced osteoclast differentiation. Since salicylates were reported to inhibit ERK and activate p38 MAPK, we investigated whether salicylates could induce osteoclast differentiation. In this study, we found that salicylates alone slightly induced osteoclast differentiation. Moreover, salicylates further enhanced osteoclast differentiation in the presence of a low concentration of RANKL. NSAIDs other than salicylates did not enhance osteoclast differentiation in the presence or absence of RANKL, and they did not inhibit ERK nor activate p38 MAPK. SB 203580, a specific inhibitor of p38 MAPK, completely suppressed osteoclast differentiation induced by RANKL and salicylates. These results suggest that salicylates enhance osteoclast differentiation via inhibition of ERK and activation of p38 MAPK in accordance with RANKL-induced osteoclast differentiation.

We observed induction of osteoclast differentiation at 1 to 5 mM of salicylates. It has been reported that the plasma concentration of salicylates attained in treatment of inflammation is approximately 1 to 3 mM. However, the local conditions of the inflamed areas may favor accumulation of salicylates. Therefore, the concentrations of salicylates used in this study seem to be clinically relevant.

Although aspirin inhibits COX by acetylating it, sodium salicylate does not inhibit COX effectively due to lack of an acetyl group. NSAIDs other than salicylates (ibuprofen, meloxicam and celecoxib, which inhibit COX-1 and/or COX-2) did not affect osteoclast differentiation, confirming that COX does not play a major role in osteoclast differentiation induced by salicylates.

It has been reported that salicylates inhibit NF-κB by specifically inhibiting IκB kinase-β, which phosphorylates IκB leading to its degradation and activation of NF-κB. However, it has been shown that NF-κB is required for RANKL-induced osteoclast differentiation. Therefore, inhibition of NF-κB by salicylates would have a negative effect on osteoclast differentiation. It seems plausible that at high concentrations (such as 10 mM) salicylates attenuate osteoclast differentiation due to inhibition of NF-κB.

Since salicylates enhance osteoclast differentiation, they may stimulate bone resorption. However, salicylates may affect not only osteoclasts but also osteoblasts. Prostaglandins enhance RANKL expression on osteoblasts, thereby inducing osteoclast differentiation. Since aspirin and other NSAIDs (except for sodium salicylate) inhibit COX to attenuate prostaglandin production, they may suppress RANKL expression on osteoblasts and osteoclast differentiation. Thus, aspirin may have both stimulatory and inhibitory effects on osteoclast differentiation. On the other hand, sodium salicylate may augment bone resorption by inducing osteoclast differentiation without inhibiting COX and RANKL expression on osteoblasts. Further studies will be required to elucidate the effects of salicylates on bone metabolism in vivo.

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REFERENCES


