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Mitogen-activated protein kinases mediate interleukin-1β-induced receptor activator of nuclear factor-κB ligand expression in human periodontal ligament cells

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Background and Objective: Interleukin-1β-stimulated receptor activator of nuclear factor-κB ligand (RANKL) expression in human periodontal ligament cells is partially mediated by endogenous prostaglandin E_2, whereas mitogen-activated protein kinases (MAPKs) are implicated in regulating various interleukin-1-responsive genes. We investigated herein the involvement of MAPKs in interleukin-1β-stimulated RANKL expression in human periodontal ligament cells.

Material and Methods: Human periodontal ligament cells were pretreated separately with specific inhibitors of MAPKs, including extracellular signal-regulated kinase, p38 MAPK and c-Jun N-terminal kinase, and subsequently treated with interleukin-1β. Following each treatment, the phosphorylation of each MAPK, the expression of RANKL, and the production of prostaglandin E_2 were determined. RANKL activity was evaluated using an assay to determine the survival of prefusion osteoclasts.

Results: Interleukin-1β induced RANKL expression at the mRNA and protein levels, as well as RANKL activity in human periodontal ligament cells. Interleukin-1β also activated extracellular signal-regulated kinase, p38 MAPK, and c-Jun N-terminal kinase. Pretreatment with each MAPK inhibitor partially, but significantly, suppressed interleukin-1β-induced RANKL expression and its activity, as well as prostaglandin E_2 production.

Conclusion: In human periodontal ligament cells, three types of MAPK inhibitor may abrogate RANKL expression and activity induced by interleukin-1β, directly or indirectly through partial suppression of prostaglandin E_2 synthesis. In addition, extracellular signal-regulated kinase, p38 MAPK, and c-Jun N-terminal kinase signals may co-operatively mediate interleukin-1β-stimulated RANKL expression and its activity in those cells.

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Receptor activator of nuclear factor-κB ligand (RANKL) is essential for the differentiation of osteoclast precursors to mature osteoclasts in the presence of macrophage colony-stimulating factor (1,2), as well as for the survival and activation of mature osteoclasts (3,4). RANKL also mediates bone resorption induced by osteotropic factors, such as interleukin-1, prostaglandin E_2, 1,25-dihydroxyvitamin D3, and parathyroid hormone, all of which have been implicated in metabolic and inflammatory bone diseases (5).

Of those osteotropic factors, interleukin-1 has exhibited potent bone-resorptive activity *in vivo* (6) and *in vitro* (7,8). Although interleukin-1 induces RANKL expression in various human osteoblasts/stromal cells (1,9,10), it also stimulates the survival, multinucleation and activation of osteoclasts in the absence of osteoblasts/stromal cells (4,11). Furthermore, interleukin-1 participates in the pathogenesis of bone resorption associated with multiple myeloma, rheumatoid arthritis, and osteoporosis (12). Periodontitis is also characterized by the progressive destruction of periodontal connective tissues, including alveolar bone. In addition, previous clinical observations have suggested that interleukin-1 plays an important role in the pathogenesis of periodontal disease (13,14). Therefore, interleukin-1 may be a key mediator in alveolar bone loss related to periodontitis.

Human periodontal ligament cells, which exhibit phenotypes of osteoblasts (15–17), show stimulated production of bone-resorptive factors, such as interleukin-6 (18,19) and prostaglandin E_2 (20), upon treatment with interleukin-1. In addition, human periodontal ligament cells treated with 1,25-dihydroxyvitamin D3 (21) or parathyroid hormone-related protein directly support osteoclastogenesis through cell-to-cell contact, which is the result of increased levels of RANKL produced by the cells (22). Interleukin-1 also stimulates RANKL expression, which is partially mediated by endogenous prostaglandin E_2 in human periodontal ligament cells (23,24). Therefore, human periodontal ligament cells activated by interleukin-1 may contribute to osteoclastogenesis by mediating RANKL as well as soluble bone-resorptive factors. In addition, RANKL mRNA levels were reported to be increased in patients with advanced periodontitis (25). These findings suggest an important role of interleukin-1-induced RANKL in human periodontal ligament cells in progressive alveolar bone loss associated with periodontitis.

Cellular responses to interleukin-1, such as the expression of various inflammatory and catabolic factors, may be mediated by cascades of intracellular signaling events, including activation of mitogen-activated protein kinases (MAPKs). As interleukin-1 actually activates three MAPK family members – extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun N-terminal kinase – in responsive cell types (26–28), those MAPK pathways are thought to be important signaling mechanisms associated with the inflammatory process. A recent study demonstrated that interleukin-1 stimulates RANKL expression in human periodontal ligament cells via ERK-dependent prostaglandin E_2 production (24). However, the participation of other MAPKs with interleukin-1-induced RANKL expression in human periodontal ligament cells has not been clarified. In the present study, we investigated the ability of each of the three MAPKs to mediate RANKL expression in human periodontal ligament cells stimulated with interleukin-1β.

Material and methods

Isolation of human periodontal ligament cells

The protocol for this study was approved by the ethics committee of Showa University (Tokyo, Japan). Isolation of human periodontal ligament cells was performed according to a method described in our previous study (23). Briefly, human periodontal ligament cells were obtained by culturing explants of healthy human periodontal ligament tissues collected from the mid-third portion of premolar roots extracted from three young adult patients (18, 20, and 22 years of age; one male and two female subjects), after receiving informed consent. Typical human periodontal ligament cells were obtained from each of the three patients and designated human periodontal ligament-1, -2, and -3 cells, respectively. Each cell population (1×10^5 cells) in 25-cm^2 culture flasks (Corning Inc., New York, NY, USA) was subcultured in alpha minimal essential medium (Flow Laboratories, McLean, VA, USA), supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotics. Cells in the present study were used after four or five passages.

Cell treatment

The human periodontal ligament cell populations were seeded in 96-, 24- and 6-well flat-bottomed tissue culture plates (Corning) at a density of 1×10^4, 1×10^5 and 1×10^6 cells/well, respectively, then cultured for 3 d in 0.3, 1 or 3 mL of alpha minimal essential medium, containing 10% fetal bovine serum and antibiotics, until confluence. SaOS-4/3 cells (29), a subclone of the human osteosarcoma cell line, SaOS-2, and established by transfecting human parathyroid hormone/parathyroid hormone-related protein receptor cDNA, were cultured in six-well plates at a density of 1×10^6 cells/well for 3 d in 1 mL of alpha minimal essential medium containing 10% fetal bovine serum and human 1–34 parathyroid hormone (10^{-8} M; Sigma Chemical Co., St Louis, MO, USA) until confluence. Confluent human periodontal ligament cells were washed and preincubated for 12 h in alpha minimal essential medium containing 1% fetal bovine serum, then treated with interleukin-1β (2.5 ng/mL; GT, Boston, MA, USA) for 10, 15, 30, 60 or 120 min in 0.1, 0.5 or 1 mL of alpha minimal essential medium containing 1% fetal bovine serum. In another experiment, confluent human periodontal ligament cells were pretreated for 30 min with PD098059 (1 μM; Sigma Chemical Co.), U0126 (1 μM; Promega Co., Madison, WI, USA), SB203580 (1 μM; Sigma Chemical Co.) or SP600125 (1 μM;

Calbiochem, San Diego, CA, USA), then subsequently treated with interleukin-1β (2.5 ng/mL) for 12 or 48 h.

Reverse transcription-polymerase chain reaction (RT-PCR) and Southern hybridization

The expression of RANKL mRNA was evaluated by RT-PCR, followed by Southern hybridization. After each treatment, total RNA was extracted from human periodontal ligament cells in 24-well plates using a reagent (Isogen; Nippon Gene Co., Tokyo, Japan). Each 2 μg of total RNA was reverse transcribed using reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) and a random primer (Takara Shuzo Co., Shiga, Japan). The RT tubes were incubated at 42°C for 60 min, and the reaction was terminated by heating the tubes at 95°C for 3 min. One microliter of aliquot was taken from each tube and subjected to a PCR assay for human RANKL or glyceraldehyde-3-phosphate dehydrogenase. The primers used for PCR were as follows: RANKL, 5′-AGCAGAGAAAGCGATGGT-3′ (sense) and 5′-GGGTATGAGAACTTGGGATT-3′ (antisense) (21,23), with the expected length of the PCR products 341 bp; glyceraldehyde-3-phosphate dehydrogenase, 5′-TCATCTCTGCCCCCTCTGCTG-3′ (sense) and 5′-GCCTGCTTCACCACCTTCTTG-3′ (antisense), with the expected length of the PCR products 436 bp. To perform PCR, 1 μL of RT products, 17 μL of PCR buffer (Platinum PCR Super Mix; Invitrogen), and the specific sense and antisense primers at 1 μL each, were added to the tube to achieve a final volume of 20 μL used for the PCR reaction. The program used for PCR for RANKL was 35 cycles at 94°C for 30 s, 58°C for 45 s, and 72°C for 30 s, whereas that for glyceraldehyde-3-phosphate dehydrogenase was 35 cycles at 94°C for 30 s, 60°C for 45 s and 72°C for 30 s.

Next, the PCR products were identified by Southern hybridization. First, 20-μL samples were taken from each tube containing PCR products and subjected to electrophoresis on 2% agarose gels, then samples of human RANKL and glyceraldehyde-3-phosphate dehydrogenase were analyzed by Southern hybridization. The oligoprobes used for Southern hybridization were designed from two PCR primers for each RANKL gene. The probe for RANKL was a 60-mer oligonucleotide (5′-ATCCCATCTGGTTCCCATAAAGTGAGTCTGTCCTCTTGGTACCATGATCGGGGTTGGGCC-3′), which corresponded to bases 651–710 of human RANKL cDNA. The probe for glyceraldehyde-3-phosphate dehydrogenase was a 60-mer oligonucleotide (5′-CCCTCCGGGAAACTGTGGCGTGATGGCCGCGGGGCTCTCCAGAACATCATCCCTGCCTCT-3′), which corresponded to bases 631–690 of glyceraldehyde-3-phosphate dehydrogenase cDNA. The probes were 5′ end-labeled with γ-^{32}P ATP using an end-labeling kit (Takara). Hybridization was performed using a nylon membrane (Hybond-N$^+$ Amersham Pharmacia Biotech, Bucks., UK) that was prehybridized at 42°C for 2 h in 5 × Saline Sodium Chloride (Sodium Chloride 3 mol, Sodium Citrate 0.3 mol), 5 × Denhardt's solution (Sigma Chemical Co.), 50 μM Tris-HCl, 0.25 mg/mL of salmon sperm DNA (Sigma Chemical Co.), 0.1% sodium dodecyl sulfate, and 50% (v/v) formamide. Hybridization was carried out at 42°C for 16 h with γ-^{32}P ATP-labeled specific oligoprobes in the prehybridization buffer. The membrane was then washed twice in 6 × Saline Sodium Chloride (Sodium Chloride 3 mol, Sodium Citrate 0.3 mol) at 37°C for 15 min and three times in 2 × Saline Sodium Chloride (Sodium Chloride 3 mol, Sodium Citrate 0.3 mol) at 37°C for 15 min, and exposed to X-ray film (Hyper film MP, Amersham Pharmacia Biotech). Autoradiographic signals were quantitatively analyzed using a laser image analyzer (BAS 2000; Fuji Film, Tokyo, Japan).

Western blot analysis

Treated human periodontal ligament cells and SaOS-4/3 cells in six-well plates were washed with cold phosphate-buffered saline and lysed in RIPA buffer (1 × phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing 100 ng/mL of phenylmethanesulfonyl fluoride. The lysates were immediately scraped, collected into microcentrifuge tubes, and boiled in sodium dodecyl sulfate sample buffer containing 5% 2-mercaptoethanol. The protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA), then protein samples (20 μg per lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel). The separated proteins were then electrotransferred to poly(vinylidene difluoride) membranes (Hybond-P; Amersham Pharmacia Biotech) using a semidry blotter (Milli Blot-SDE system; Millipore Co., Bedford, MA, USA). The membranes were washed once with TBS buffer [10 mM Tris-HCl (pH 7.2), 150 mM NaCl] containing 0.1% (v/v) Tween 20 (TBS-T) and then blocked for 2 h at room temperature in TBS-T containing 5% (w/v) skim milk. After washing, antibodies against human soluble RANKL (Pepro Tech EC, London, UK), ERK1/2 (Cell Signaling Technology Inc., Beverly, MA, USA), phosphorylated ERK1/2 (Cell Signaling), c-Jun N-terminal kinase 1/2 (Cell Signaling), phosphorylated c-Jun N-terminal kinase 1/2 (Cell Signaling), p38 MAPK (Cell Signaling), and phosphorylated p38 MAPK (Cell Signaling) were added separately at a dilution of 1 : 500 or 1 : 1000 in TBS-T containing 5% (w/v) skim milk or 5% bovine serum albumin, and incubated for 1 or 8 h at 4°C. The immunoreactive bands were visualized using a Phototope®-Horseradish peroxidase Western Blot Detection System (Cell Signaling). Briefly, the membrane was incubated for 1 h with horseradish peroxidase-conjugated antigoat immunoglobulin G (1 : 1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or horseradish peroxidase-conjugated antirabbit immunoglobulin G (1 : 2000; Cell Signaling) in TBS-T containing 5% (w/v) skim milk at room temperature. After washing, the membrane was incubated with Lumi-GLO reagent for 1 min at room temperature. Quantitative analysis was

performed using an image analyzer (Fluoro Image Analyzer FLA-2000F; Fuji Film).

Quantification of prostaglandin E_2

The amount of prostaglandin E_2 secreted into the culture supernatant was determined using enzyme immunoassay kits for human prostaglandin E_2 (Amersham Pharmacia Biotech).

Measurement of DNA content

Following each treatment, the cell layers were washed four times with Dulbecco's modified Eagle's minimal essential medium. The DNA content in the cell layer was measured using the method of Labarca & Paigen (30), with calf thymus DNA as the standard.

Development of prefusion osteoclasts

Prefusion osteoclasts were obtained by coculturing mouse bone marrow cells with mouse osteoblasts, as described previously (31). Briefly, 3- to 5-wk-old female ddY strain mice (SEASCO™; Saitama, Kitakatsusika-gun, Japan) were used. After dissecting the femora and tibiae, mouse bone marrow cells were flushed out. Mouse osteoblasts were obtained from the calvariae of 1-d-old newborn ddY mice (SEASCO™) using a collagenase digestion method (0.1% collagenase, 0.05% trypsin, 4 mM 2NaEDTA). The collected bone marrow cells and osteoblasts were then cocultured in alpha minimal essential medium containing 1,25-dihydroxyvitamin D3 (10^{-8} M; Sigma Chemical Co.), 10% fetal bovine serum, 2.0 g/L of sodium bicarbonate, and antibiotics. Half of the medium was changed gently every 2 d. After culturing for 6 d, mononuclear cells attached to the osteoblastic cell layer were harvested by gentle pipetting with alpha minimal essential medium and centrifuged at 150 ***g*** for 5 min. The collected mononuclear cells were washed and fixed in 10% formalin for 5 min, followed by ethanol/acetone (1 : 1) for 1 min. The cells were then stained for tartrate-resistant acid phosphatase (TRAP) using a commercially available kit (Sigma Chemical Co.). TRAP-positive [TRAP(+)] mononuclear cells were counted as prefusion osteoclasts. The ratio of TRAP(+) cells in the collected mononuclear cells was approximately 60%, whereas no osteoblasts were detected after staining the fixed cells with alkaline phosphatase.

Assay for survival of prefusion osteoclasts

Following treatment of the human periodontal ligament cells in 96-well plates, the cell layers were gently washed with phosphate-buffered saline several times, fixed with 1% paraformaldehyde for 15 min, and washed again. To remove paraformaldehyde completely, fixed human periodontal ligament cells were incubated with serum-free alpha minimal essential medium for 24 h. Mononuclear cells (1×10^4 cells/well) were collected and prepared as described above, then cultured, for 24 h, on the fixed human periodontal ligament cells in 100 μL of alpha minimal essential medium containing 10% fetal bovine serum. At the start and end of culture, the mononuclear cells were washed with phosphate-buffered saline, then fixed and stained for TRAP. TRAP(+) mononuclear cells, as prefusion osteoclasts, were counted under a light microscope. Prefusion osteoclast survival was evaluated by determining the number of TRAP(+) mononuclear cells.

Statistical analysis

All experiments were performed three times. For assessing prostaglandin E_2 production and the survival of prefusion osteoclasts, the experiments were performed three times for each population of human periodontal ligament cells, with each experiment conducted in triplicate, and the mean and standard deviation were calculated. Any statistical significance of differences among the groups was examined by one-way analysis of variance and a post hoc *t*-test, which was performed when the analysis of variance test indicated significance at a level of $p < 0.05$.

Results

Interleukin-1β stimulates phosphorylation of ERK, p38, and c-Jun N-terminal kinase

Phosphorylation of the three types of MAPKs in human periodontal ligament cells treated with 2.5 ng/mL of interleukin-1β was determined using western blotting. Expression of the phosphorylated forms of ERK1/2, p38 MAPK, and c-Jun N-terminal kinase 1/2 in human periodontal ligament-1 cells was stimulated between 10 and 60 min after starting treatment (Fig. 1). Interleukin-1β-induced activation of the MAPKs was also seen in human periodontal ligament-2 and -3 cells (data not shown).

MAPK inhibitors inhibit interleukin-1β-induced RANKL expression

A PCR product (341 bp), corresponding to the size estimated from the DNA sequence of human RANKL, was obtained from all three populations of human periodontal ligament cells (data not shown). Furthermore, the DNA sequence of the PCR product was completely identical to that of human RANKL.

Similarly to results of our previous study (23), treatment with interleukin-1β (2.5 ng/mL) for 12 h markedly stimulated RANKL mRNA expression in the three human periodontal ligament cell populations (mean 3.7-fold increase compared with the vehicle) (Fig. 2A). To investigate the participation of each MAPK with the interleukin-1β-stimulated RANKL mRNA expression in human periodontal ligament cells, the three human periodontal ligament cell populations were pretreated for 30 min with each specific MAPK inhibitor [U0126 (ERK1/2 inhibitor; 1 μM), PD098059 (ERK1/2 inhibitor; 1 μM), SB203580 (p38 MAPK inhibitor; 1μM) or SP600125 (c-Jun N-terminal kinase 1/2 inhibitor; 1μM)] for 30 min and subsequently treated with interleukin-1β (2.5 ng/mL) for 12 h. The interleukin-1β-stimulated RANKL mRNA expression was partially, but significantly, inhibited by pretreatment with each MAPK inhibitor (Fig. 2A).

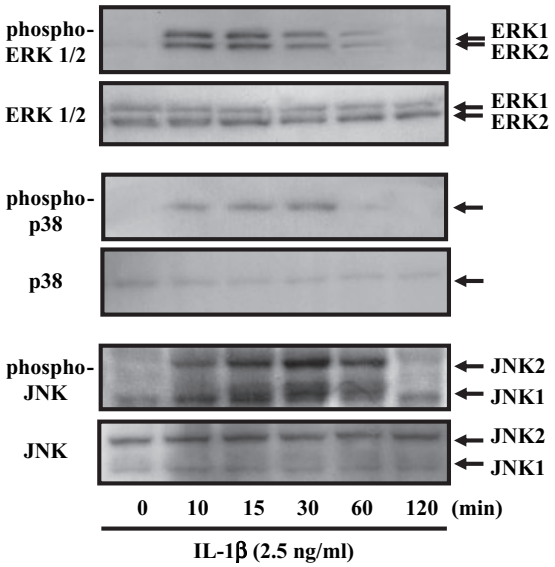

Fig. 1. Effects of interleukin-1β on the phosphorylation of mitogen-activated protein kinases (MAPKs). Human periodontal ligament-1 cells at confluence were treated with interleukin-1β (2.5 ng/mL) for the indicated periods of time. Following each treatment, the phosphorylation of MAPKs (extracellular signal-regulated kinase 1/2, p38 MAPK and c-Jun N-terminal kinase 1/2) was determined by western blotting, as described in the Material and methods. Each photograph is representative of the results of three separate experiments. Similar results were obtained with human periodontal ligament-2 and -3 cells (data not shown). ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IL, interleukin; phospho, phosphorylated.

As shown in Fig. 2B, parathyroid hormone-treated SaOS-4/3 cells, used as a positive control, expressed RANKL protein at a molecular weight of 40 kDa. When the three populations of human periodontal ligament cells were left untreated for 48 h, each expressed undetectable levels of RANKL protein, whereas those levels were remarkably increased by treatment with interleukin-1β (2.5 ng/mL) for 48 h (mean 4.5-fold increase compared with vehicle). Furthermore, pretreatment with each MAPK inhibitor for 30 min also partially, but significantly, inhibited the interleukin-1β-induced expression of RANKL protein in the cells (Fig. 2B).

MAPK inhibitors partially suppress interleukin-1β-stimulated prostaglandin E_2 production

We previously demonstrated that endogenous prostaglandin E_2 partially mediates interleukin-1-stimulated RANKL expression in human periodontal ligament cells (23). In the present study, we examined the involvement of each MAPK in prostaglandin E_2 production by interleukin-1β-treated cells. Treatment with interleukin-1β (2.5 ng/mL) for 48 h resulted in a marked stimulation of prostaglandin E_2 production in the three cell populations (Fig. 3). Next, we treated human periodontal ligament cells with each MAPK inhibitor for 1 h, followed by interleukin-1β for 48 h. As shown in Fig. 3, each of the MAPK inhibitors partially, but significantly, suppressed interleukin-1-stimulated prostaglandin E_2 production (U0126, 53%; PD098059, 60%; SB203580, 59%; SP600125, 67%; of the levels obtained by interleukin-1β treatment alone).

MAPK inhibitors abrogate interleukin-1β-induced RANKL activity

RANKL activity expressed by human periodontal ligament cells was determined using an assay to determine prefusion osteoclast survival. Mononuclear cells (1×10^4 cells per well) were cultured on the three populations of fixed human periodontal ligament cells for 24 h. The ratio of prefusion osteoclasts [TRAP(+) cells] among the mononuclear cells was approximately 60%. As shown in Fig. 4A,B, after 24 h of culture, the numbers of prefusion osteoclasts on the fixed cell layers of untreated human periodontal ligament cells showed a marked reduction. When the mononuclear cells were cultured on fixed human periodontal ligament cells treated with interleukin-1β for 48 h, the numbers of prefusion osteoclasts were increased [3.7-fold increase compared with untreated human periodontal ligament cells (vehicle)]. Furthermore, the enhanced prefusion osteoclast survival was inhibited by adding 100 ng/mL of osteoprotegerin (Fig. 4), as well as by contact inhibition (data not shown), until nearly the level of the vehicle. We also attempted to determine if the MAPK inhibitors could suppress RANKL activity induced by interleukin-1β in human periodontal ligament cells. Augmentation of prefusion osteoclast survival observed in the interleukin-1β-treated human periodontal ligament cells was partially, but significantly, inhibited by pretreatment of the human periodontal ligament cells for 30 min with each MAPK inhibitor to 20% (PD098059), 26% (SB203580), and 35% (SP600125) of the levels increased by interleukin-1β. Therefore, we considered that the inhibitory effects of the MAPK inhibitors observed were probably a result of the inhibition of interleukin-1β-induced RANKL expression.

Discussion

Previous studies have demonstrated that interleukin-1 stimulates the expression of RANKL mRNA in human periodontal ligament cells (22,23), as well as in various human osteoblasts/stromal cells (10), human microvascular endothelial cells (32), and human mesenchymal stem cells (33), whereas RANKL mediates bone resorption induced by interleukin-1α (5). In the present study, interleukin-1β stimulated RANKL expression at the mRNA and protein levels in human periodontal ligament cells.

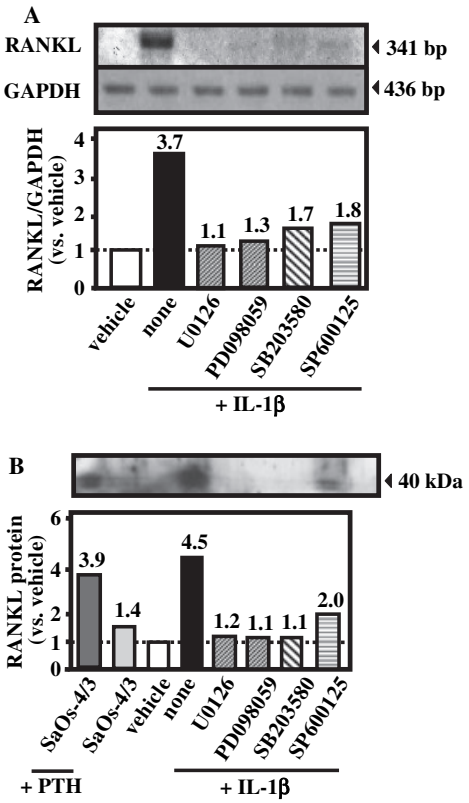

Fig. 2. Involvement of mitogen-activated protein kinases (MAPKs) on interleukin-1β-induced receptor activator of nuclear factor-κB ligand (RANKL) expression. Three cell populations of human periodontal ligament cells (human periodontal ligament-1, -2, -3 cells) at confluence were pretreated for 30 min with each specific MAPK inhibitor [U0126 (1 μM), PD098059 (1 μM), SB203580 (1 μM) or SP600125 (1 μM)] and subsequently treated with interleukin-1β (2.5 ng/mL) for 12 h (A) or 48 h (B). As a positive control, SaOS-4/3 cells were cultured for 72 h in the presence of human 1–34 parathyroid hormone (10^{-8} M) until reaching confluence. Following each treatment, the expression of RANKL at mRNA (A) and protein (B) levels was determined using reverse transcription-polymerase chain reaction/Southern hybridization and western blotting, respectively. Each photograph is representative of the results of three separate experiments in human periodontal ligament-1 cells. The graphs demonstrate the ratio of RANKL to glyceraldehyde-3-phosphate dehydrogenase as a multiple of the ratio of vehicle to glyceraldehyde-3-phosphate dehydrogenase (A) and the ratio of RANKL to the vehicle as a multiple of the value in vehicle (B). Each value shown represents the mean of values obtained in three separate experiments for each of the three human periodontal ligament cell populations ($n = 9$). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; PTH, parathyroid hormone.

RANKL expressed by osteoblasts/stromal cells is responsible for the survival of mature osteoclasts as well as for the differentiation of osteoclast precursors to mature osteoclasts (1–4). An important survival factor in osteoclasts, RANKL has also been reported to activate survival signaling pathways, such as Akt and phosphatidylinositol 3-kinase (34), and mTOR/S6 kinase (35). RANKL has also been shown to reduce the levels of Fas expression and Fas-mediated apoptosis in mature osteoclasts (36). We assessed the activity of RANKL expressed by interleukin-1β-treated human periodontal ligament cells using an assay that determined the survival of prefusion osteoclasts. When cultured on fixed cell layers of interleukin-1β-treated human periodontal ligament cells, the number of prefusion osteoclasts was increased compared with those cultured on fixed untreated human periodontal ligament cells. Furthermore, the enhanced prefusion osteoclast survival was inhibited by adding osteoprotegerin or by contact inhibition. These results suggest that stimulation of the survival of prefusion osteoclasts cultured on fixed interleukin-1β-treated human periodontal ligament cells is predominantly mediated by RANKL and that expressed on the cell surface stimulates prefusion osteoclast survival by cell-to-cell contact. These results also indicate that interleukin-1β stimulates RANKL activity in human periodontal ligament cells. Although the present results do not provide evidence of the participation of RANKL expressed by interleukin-1β-treated human periodontal ligament cells with all the processes of osteoclastogenesis, they demonstrate, at least, that those cells stimulate the survival of osteoclasts via RANKL.

MAPKs may mediate interleukin-1-induced cellular responses such as the expression of various inflammatory and catabolic factors. It has been shown that interleukin-1 activates three MAPK family members – ERK, p38 MAPK, and c-Jun N-terminal kinase – in responsive cell types (26–28,33,37). In the present study, interleukin-1β stimulated the phosphorylation of all three MAPK types in human periodontal ligament cells. Of those, the role of p38 MAPK in RANKL expression, as well as bone resorption induced by pro-inflammatory cytokines, has been most clearly characterized. Zwerina *et al.* (38) found that p38 MAPK specific inhibitors decreased the severity of synovial inflammation and prevented bone destruction in tumor necrosis factor-transgenic mice, and that those inhibitory effects were associated with suppressed expression of cytokines, such as interleukin-1 and RANKL, in the synovial membrane and also resulted in reduced osteoclastogenesis in synovial tissue. Kumar *et al.* (39) also showed that the inhibition of p38 MAPK activity effectively prevents interleukin-1- and tumor necrosis factor-induced bone resorption *in vitro*. In another report, interleukin-1 mediated tumor necrosis factor-induced osteoclastogenesis *in vitro* and the interleukin-1-mediated effect was shown to be associated with RANKL induction in stromal cells (33). In

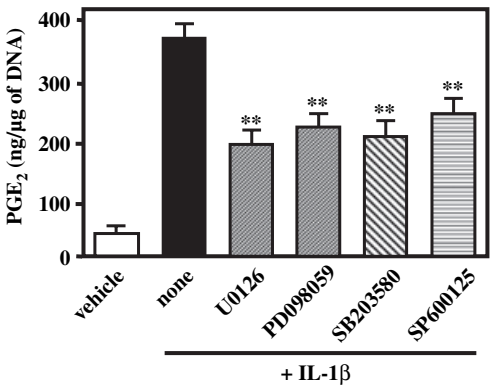

Fig. 3. Effects of mitogen-activated protein kinase (MAPK) inhibitors on interleukin-1β-induced prostaglandin E_2 production. The three cell populations of human periodontal ligament cells at confluence were pretreated for 30 min with each MAPK inhibitor (1 μM) and subsequently treated with interleukin-1β (2.5 ng/mL) for 48 h. After each treatment, the amount of prostaglandin E_2 secreted into the culture supernatant was measured using an enzyme immunoassay kit for human prostaglandin E_2. Each value (ng/μg of DNA) shown represents the mean ± standard deviation of three separate experiments, each conducted in triplicate, for each of the three human periodontal ligament cell populations ($n = 27$). **Significant difference from the value of cells treated with interleukin-1β alone ($p < 0.01$). IL, interleukin; PGE_2, prostaglandin E_2.

addition, p38 MAPK mediates tumor necrosis factor- and interleukin-1-induced RANKL expression in human mesenchymal stem cells (33). In the present study, pretreatment with SB203580, a p38 MAPK specific inhibitor, partially, but significantly, inhibited the subsequent interleukin-1β-induced RANKL expression at mRNA and protein levels in human periodontal ligament cells. We also attempted to determine whether the inhibitor can suppress the activity of RANKL induced by interleukin-1β in human periodontal ligament cells. As expected, the enhancement of pre-fusion osteoclast survival in interleukin-1β-treated human periodontal ligament cells was also partially, but significantly, inhibited when the human periodontal ligament cells were pretreated with the inhibitor. These results suggest an important role of p38 MAPK in interleukin-1β-stimulated RANKL expression and activity in human periodontal ligament cells.

Few studies concerning the involvement of ERK and c-Jun N-terminal kinase in RANKL induction by pro-inflammatory cytokines have been reported, although Kim *et al.* (40) demonstrated that the pathway employing ERK, but not p38 MAPK, is involved in RANKL expression induced by a high level of extracellular calcium in osteoblasts. Furthermore, RANKL mRNA induction by lipopolysaccharide and synthetic lipid A was shown to be dependent on ERK activation in osteoblasts (41), whereas Fukushima *et al.* (22) showed that PD98059, a specific ERK inhibitor, inhibited the up-regulation of RANKL expression induced by interleukin-1α in human periodontal ligament cells. In the present study, pretreatment with PD98059 partially, but significantly, inhibited interleukin-1β-induced RANKL expression and activity. In addition, those inhibitory effects were observed following pretreatment with SP600125, a specific inhibitor of c-Jun N-terminal kinase, whereas its inhibitory effects were relatively weak compared with those of SB203580 and PD98059. These findings suggest that the activation of ERK and c-Jun N-terminal kinase, as well as p38 MAPK, constitute important signaling pathways in interleukin-1β-induced RANKL expression in human periodontal ligament cells, and that those three pathways play a distinct role in the induction of RANKL by interleukin-1β.

In a previous study, we demonstrated that interleukin-1β-induced prostaglandin E_2 generation in human periodontal ligament cells was primarily dependent on the *de novo* induction of cyclooxygenase-2 and cytosolic phospholipase A2 (20). Furthermore, endogenous prostaglandin E_2 partially mediated interleukin-1β-induced RANKL mRNA expression by human periodontal ligament cells (22,23). In the present study, we examined the effects of the three MAPK inhibitors on interleukin-1β-induced prostaglandin E_2 generation in human periodontal ligament cells. The results of a number of previous *in vitro* studies which used various cell types suggest that prostaglandin E_2 production and upstream cytosolic phospholipase A2/cyclooxygenase-2 expression induced by interleukin-1β are mediated through the activation of ERK, p38 MAPK, and/or c-Jun N-terminal kinase signaling pathways (42–49). Furthermore, p38 MAPK (50,51) or ERK (45) act to stabilize cyclooxygenase-2 mRNA induced by interleukin-1β. We found that all three MAPK inhibitors partially, but significantly, reduced prostaglandin E_2 production by interleukin-1β-treated human periodontal ligament cells, suggesting that activation of each MAPK pathway, at least in part, plays a role in the signaling cascades that mediate the up-regulation of prostaglandin E_2 production in cells exposed to interleukin-1β. However, the inhibitory effects of the MAPK inhibitors on interleukin-1β-stimulated RANKL expression were greater than the effects on prostaglandin E_2 production induced by interleukin-1β. On the other hand, prostaglandin E_2-induced RANKL expression is mediated by cAMP/protein kinase A in human periodontal ligament cells (22,23), but not by p38 MAPK in osteoblasts (52). Therefore, these inhibitors may act on interleukin-1β-stimulated RANKL expression, but not on endogenous prostaglandin E_2-dependent expression. Together, these results suggest that the inhibitors abrogate interleukin-1β-induced RANKL expression in human periodontal ligament cells, directly or indirectly, through the partial suppression of prostaglandin E_2 synthesis.

In the present study, we assessed the involvement of MAPKs in interleukin-1β-induced RANKL expression and

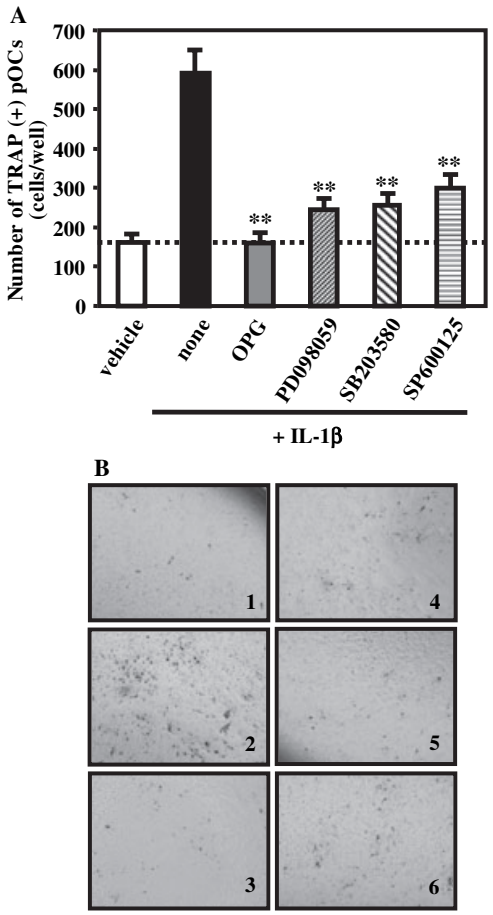

Fig. 4. Effects of mitogen-activated protein kinase (MAPK) inhibitors on receptor activator of nuclear factor-κB ligand (RANKL) activity induced by interleukin-1β. The three cell populations of human periodontal ligament cells at confluence were pretreated for 30 min with each MAPK inhibitor and subsequently treated with interleukin-1β (2.5 ng/mL) for 48 h. Following each treatment, the cell layers were fixed with 1% paraformaldehyde. Mononuclear cells (1×10^4 cells/well) were collected and prepared as described in the Material and methods section, and cultured on the fixed human periodontal ligament cells in the presence or absence of osteoprotegerin (100 ng/mL) for 24 h. At the start and end of culture, the mononuclear cells were fixed and stained for tartrate-resistant acid phosphate (TRAP), and the number of TRAP(+) mononuclear cells, as prefusion osteoclasts, was counted. (A) Each value represents the mean ± standard deviation of values obtained in three separate experiments for each of the three human periodontal ligament cell populations ($n = 9$). **Significant difference from the value of human periodontal ligament cells treated with interleukin-1β alone ($p < 0.01$). (B) TRAP staining (×40). Panel 1, untreated human periodontal ligament cells (vehicle); panel 2, interleukin-1β-treated human periodontal ligament cells; panel 3, interleukin-1β-treated human periodontal ligament cells + osteoprotegerin; panel 4, PD098059 → interleukin-1β-treated human periodontal ligament cells; panel 5, SB203580 → interleukin-1β-treated human periodontal ligament cells; and panel 6, SP600125 → interleukin-1β-treated human periodontal ligament cells. IL, interleukin; OPG, osteoprotegerin; pOCs, prefusion osteoclasts.

studied its activity (such as stimulation of prefusion osteoclast survival) in human periodontal ligament cells. The results obtained suggest that each type of MAPK inhibitor inhibited RANKL expression and its activity induced by interleukin-1β, directly or indirectly, by partially suppressing prostaglandin E_2 synthesis. In addition, ERK, p38 MAPK, and c-Jun N-terminal kinase signaling may co-operatively mediate interleukin-1β-stimulated RANKL expression and activity in human periodontal ligament cells.

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