Involvement of the mitogen-activated protein kinase signaling pathway in osteoprotegerin-induced inhibition of osteoclast differentiation and maturation

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Abstract. The present study aimed to determine whether the mitogen-activated protein kinase (MAPK) signaling pathway is involved in the osteoprotegerin (OPG)-mediated inhibition of osteoclast differentiation and maturation. RAW264.7 cells were incubated with macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) to stimulate osteoclastogenesis and treated with various concentrations of OPG, an inhibitor of osteoclast differentiation. The differentiation and activation of osteoclasts were monitored by tartrate-resistant acid phosphatase staining and bone resorption assays. The phosphorylation levels of p38-MAPK, c-Jun N-terminal kinase (JNK)-MAPK and extracellular signal-regulated kinase (ERK)-MAPK in the different treatment groups were determined by western blot analysis. The results confirmed that M-CSF + RANKL stimulated the differentiation and activation of osteoclasts as well as the phosphorylation of p38-MAPK, JNK-MAPK and ERK-MAPK in osteoclasts, which was attenuated by OPG treatment. These findings indicated that the MAPK signaling pathway is involved in the regulation of osteoclastogenesis and in the OPG-mediated inhibition of osteoclast differentiation and activation.

Introduction

Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases. The conserved MAPK signaling pathway exists in all eukaryotes and is considered a central hub in the regulation of cellular processes, including growth, proliferation, division, cell cycle progression, apoptosis, necrosis and cell-cell interactions (1-3). The signaling cascade includes no less than three enzymes that are activated in sequence: An MAPK kinase kinase, an MAPK kinase and an MAPK (1). These enzymes all possess 11 conserved sub-domains and are activated via phosphorylation of amino acid residues (4). Rossomando *et al* (5) identified the extracellular signal-regulated kinase (ERK)-MAPK in mammalian cells in 1991, while the stress-activated protein kinase (SAPK)-MAPK and p38-MAPK were discovered subsequently (6,7). Together, these three enzymes are the main members of the MAPK family.

Osteoclasts are specialized multinucleated cells that execute the catabolic phase of bone remodeling (8,9). Bone remodeling is a persistent physiological process in healthy humans and animals that is initiated by osteoclasts and is critical for bone mass homeostasis (10,11). Osteoclasts are responsible for bone resorption, while osteoblasts are responsible for bone matrix generation and mineralization. Together, these processes lead to whole bone remodeling (12). In instances where these processes become unbalanced, bone mass may increase or decrease abnormally and cause diseases including osteopetrosis, osteoporosis, chondropathy and rheumatoid arthritis (13). Receptor activator of nuclear factor κB ligand (RANKL) is a member of the tumor necrosis factor (TNF) ligand superfamily and is an essential cytokine for osteoclastogenesis (14). RANKL is produced by osteoblasts/stromal cells and combines with its receptor, RANK, which is expressed on the surface of osteoclast precursors (14). RANKL/RANK association triggers the signaling cascades involved in differentiation and activation of osteoclasts (9). Osteoprotegerin (OPG), a protein generated by osteoblast/stromal cells, acts as a decoy receptor for RANKL and inhibits RANKL/RANK association, inhibiting the development of osteoclasts (15). The RANK/RANKL/OPG axis regulates bone metabolism and osteoclastogenesis (14).

Previous studies have demonstrated that ERK-MAPK, c-Jun N-terminal kinase (JNK)-MAPK and p38-MAPK

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are involved in the RANKL/RANK signaling pathway, which regulates osteoclast differentiation, maturation and survival (16-19). It is, however, unknown whether the MAPK signaling pathway is involved in OPG-induced inhibition of osteoclast development. The present study attempted to examine the involvement of the MAPK signaling pathway by utilizing specific inhibitors of the MAPK pathway in order to test whether OPG or specific kinase inhibitors may have potential use in the treatment of bone loss-associated diseases resulting from dynamic bone resorption of osteoclasts.

Materials and methods

Cells and reagents. The murine monocyte/macrophage cell line RAW264.7 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The primary rabbit anti-phospho p38-MAPK polyclonal antibody (cat. no. SAB4504095), Acid Phosphatase kit 387-A [tartrate-resistant acid phosphatase (TRAP) staining kit] and the specific inhibitors U0126, SB202190, SP600125 were obtained from Sigma-Aldrich (St. Louis, MO, USA). The primary rabbit anti-ERK1/2 polyclonal antibody (cat. no. 06-642) was purchased from Millipore Corporation (Billerica, MA, USA). The primary anti-p38-MAPK polyclonal (cat. no. 9212S), rabbit anti-phospho SAPK/JNK monoclonal (cat. no. 4671S), rabbit anti-phospho ERK1/2 monoclonal (cat. no. 9101S), rabbit anti β-actin polyclonal (cat. no. 4970S) and rabbit anti-SAPK/JNK polyclonal (cat. no. 9258S) antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The rabbit anti-sheep immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated secondary antibody (cat. no. sc-2770) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Macrophage colony-stimulating factor (M-CSF), RANKL and OPG were purchased from PeproTech Inc. (Rocky Hill, NJ, USA). Bovine cortical bone was purchased from a slaughterhouse (Yangzhou, China) and was sawed into slices by a saw microtome (SP1600; Leica Microsystems, Wetzlar, Germany) at Shanghai Ninth People's Hospital Affiliated to Shanghai JiaoTong University School of Medicine (Shanghai, China).

TRAP-positive cell staining and bone resorption activity assay. RAW264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Gibco-BRL, Carlsbad, CA, USA), 2 mM/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. For studies on osteoclast differentiation, cells were adjusted to a concentration of 1.5×10^4 cells/ml in α-Minimum Essential Medum (α-MEM) containing 10% FBS, 2 mM/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 96-well and 48-well plates with bovine cortical bone slices. After a 24 h of incubation, the medium was replaced with serum-free α-MEM containing M-CSF (25 ng/ml) and RANKL (30 ng/ml), and the cells were cultured for another 48 h. At that time-point, in the presence of M-CSF + RANKL, 0, 10, 20, 50 or 100 ng/ml OPG was added and cells were incubated for an additional three days.

At the end of the incubation, TRAP staining of osteoclasts and bone resorption activity assays was performed according to the manufacturer's instructions. The number of TRAP-positive cells in each group was counted and compared. Briefly, TRAP-positive cells in ten randomized visual fields from three random wells were counted using an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). At the same time, bone resorption by differentiated osteoclasts in bovine cortical bone slices was calculated in the different groups. The bone slices co-cultured with differentiated osteoclasts were removed from the plates. Any remaining cells on bone slices were removed by ultrasonic cleaning. The resorption lacunae on bone slices were observed using an environmental scanning electron microscope (XL30-ESEM; Philips, Eindhoven, The Netherlands). The volume of resorption lacunae was determined by professional image analysis software (version 1.0; JEDA Technologies, Nanjing, China).

Western blot analysis. RAW264.7 cells were cultured in α-MEM containing 10% FBS, 2 mM/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in six-well plates for 24 h at a concentration of 1.5×10^4 cells/ml. Medium was then replaced with serum-free α-MEM with M-CSF (25 ng/ml) + RANKL (30 ng/ml) and the cells were cultured for a further 48 h. Subsequent treatment was dependent on the assay type. For time-course studies, 100 ng/ml OPG was added in the presence of M-CSF + RANKL and cells incubated for 15, 30, 60 or 120 min. For concentration gradient studies, 0, 10, 20, 50 or 100 ng/ml OPG were added in the presence of M-CSF + RANKL and cells were incubated for 30 min. For studies on inhibition of the MAPK signaling pathway, complete medium was removed and replaced with serum-free medium. The cells were pre-treated with 100 ng/ml OPG and either 0.2 μM U0126 (a specific inhibitor of the ERK-MAPK signaling pathway), 10 μM SB202190 (a specific inhibitor of the p38-MAPK signaling pathway) or 10 μM SP600125 (a specific inhibitor of the JNK-MAPK signaling pathway) for 30 min. M-CSF + RANKL were then added for an additional 30 min.

Following incubation, cells from each experimental group were collected and lysed in 180 μl radioimmunoprecipitation assay (RIPA) buffer with 1% (v/v) PMSF for 30 min with intermittent vibration. After sonication, the solution of lysed cells was centrifuged (12,000 x g for 10 min at 4°C) and the supernatants were extracted. The total protein concentration was 6-15 mg/ml. The protein was loaded (60-150 μg per lane) and were separated, transferred, blocked and incubated overnight at 4°C with primary anti-phospho p38-MAPK, anti-phospho JNK-MAPK, anti-phospho ERK-MAPK, anti-p38-MAPK, anti-JNK-MAPK, anti-ERK-MAPK or anti-β-actin antibodies diluted in 5% BSA-Tris-buffered saline with Tween-20 (TBST). Following incubation with the primary antibodies, the samples were incubated with anti-sheep IgG HRP secondary antibody diluted in 5% BSA-TBST for 90 min at room temperature. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) using ECL-plus detection reagents (Thermo Fisher Scientific, Waltham, MA, USA). The experiments were repeated three times.

Results

OPG inhibits the differentiation and bone resorption activities of osteoclasts. Multinucleated TRAP-positive cells were
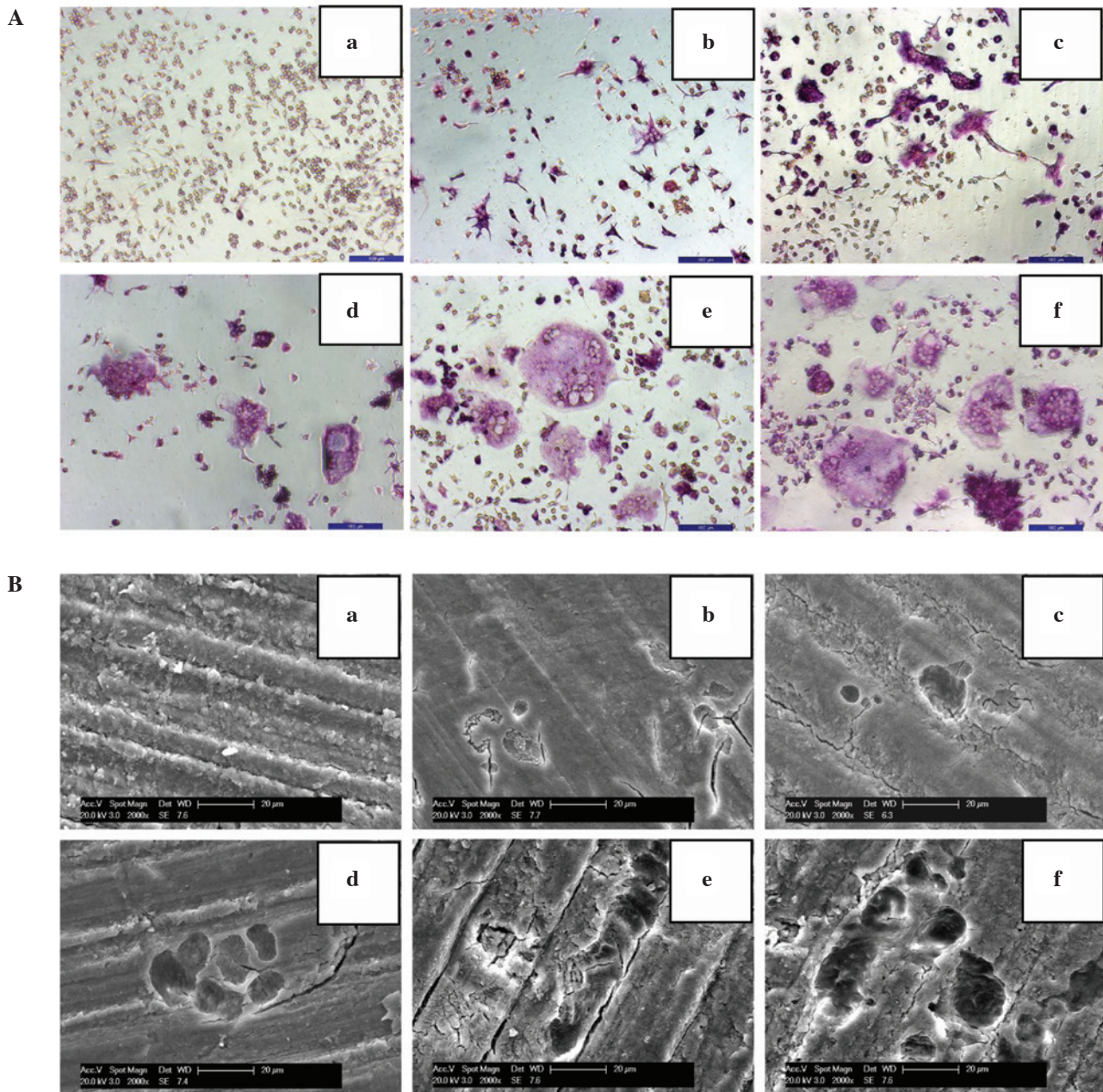
Figure 1. OPG inhibits differentiation and bone resorption activity of osteoclasts. (A) Light microscopic images showing the inhibition of tartrate-resistant acid phosphatase-positive multinucleated osteoclast differentiation in M-CSF + RANKL-induced RAW264.7 cells by OPG (original magnification, x200; scale bar, 100 μm). (B) Electron micrographs showing the inhibitory effects of OPG on osteoclast bone resorption activity (original magnification, x2,000; scale bar, 20 μm). Ten visual fields were selected randomly and counted in each well for each group. (a) RAW264.7 cells (control); (b-f) M-CSF + RANKL-induced RAW264.7 cells treated with (b) 100 ng/ml OPG; (c) 50 ng/ml OPG; (d) 20 ng/ml OPG; (e) 10 ng/ml OPG; (f) No OPG. OPG, steoprotegerin; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor-κB ligand.

observed in M-CSF + RANKL-treated RAW264.7 cells after four days of incubation. The differentiated cells showed characteristic bone resorption activity, as indicated by the observation that cells corroded the bone slices to produce cavities. OPG treatment reduced the number of multinucleated TRAP-positive cells and inhibited the bone resorption activity of differentiated cells in a concentration-dependent manner as compared to those in the control group (Fig. 1A and B).

OPG affects the MAPK signaling pathway in a time-dependent manner. All three branches of the MAPK signaling pathway, namely p38-MAPK, JNK-MAPK and ERK-MAPK, were

activated in the M-CSF + RANKL-treated RAW264.7 cells within 15 min of incubation. Phosphorylation levels of p38-MAPK peaked within 15 min and subsequently declined. Phosphorylation levels of JNK-MAPK and ERK-MAPK peaked within 30 min and declined afterwards. The addition of OPG decreased the phosphorylation levels of p38-MAPK, JNK-MAPK and ERK-MAPK in a time-dependent manner (Fig. 2).

OPG inhibits the MAPK signaling pathway in a concentration-dependent manner. Compared to those in the RAW264.7 control group, the phosphorylation levels of
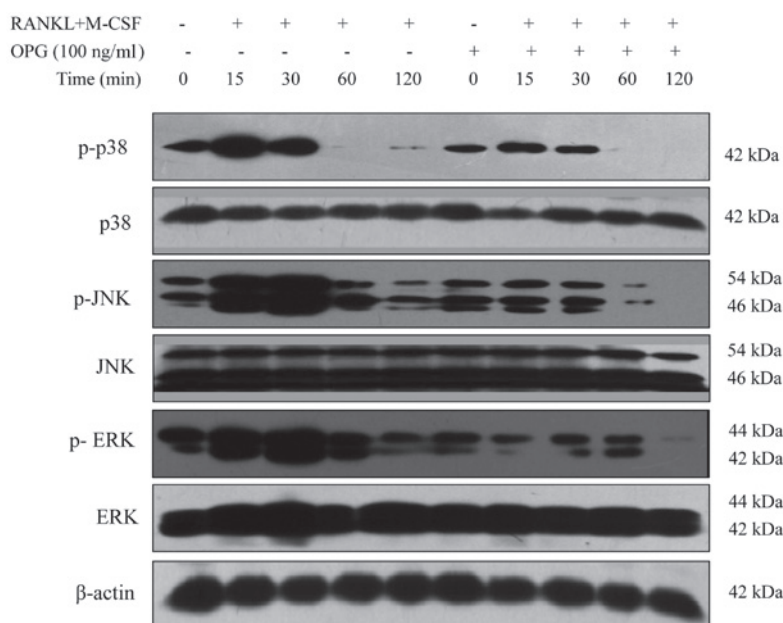
Figure 2. Effects of OPG on phosphorylation levels of proteins involved in the mitogen-activated protein kinase signaling pathway. 100 ng/ml OPG were added in the presence of M-CSF + RANKL and treatment was sustained for 15, 30, 60 or 120 min. Following incubation, cells were collected and total protein extracted and analyzed by western blotting. Phosphorylation levels of the signaling proteins are shown. Each experiment was repeated three times. OPG, steoprotegerin; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor-κB ligand; ERK, extracellular signal-regulated kinase; p-JNK, phosphorylated c-Jun N-terminal kinase.
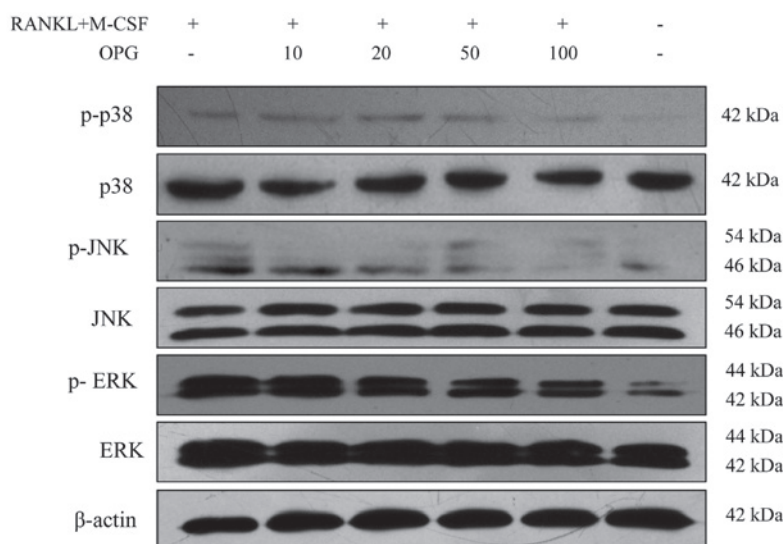

Figure 3. Effects of OPG concentration on the phosphorylation of mitogen-activated protein kinase signaling pathway proteins. 0, 10, 20, 50 or 100 ng/ml OPG were added in the presence of M-CSF + RANKL and treatment was sustained for 30 min. Following incubation, cells were collected and total protein was extracted and analyzed by western blotting. Phosphorylation levels of the signaling proteins are shown. Each experiment was repeated three times. OPG, steoprotegerin; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor-κB ligand; ERK, extracellular signal-regulated kinase; p-JNK, phosphorylated c-Jun N-terminal kinase.

p38-MAPK, JNK-MAPK and ERK-MAPK increased with M-CSF + RANKL treatment. Of note, OPG decreased the phosphorylation levels of p38-MAPK, JNK-MAPK and ERK-MAPK in a concentration-dependent manner (Fig. 3).

OPG inhibits osteoclast differentiation via the MAPK signaling pathway. Specific inhibitors of p38-MAPK, JNK-MAPK and ERK-MAPK were employed in the present study in order to test the involvement of the MAPK signaling pathway in the effects of OPG on osteoclast differentiation. The results further

confirmed that the three signaling pathways are involved in the differentiation and activation of osteoclasts and that OPG affects the differentiation and activation of osteoclasts.

Discussion

In view of the fact that the amounts of purified primary osteo-clasts required for signal transduction studies were prohibitive, RAW264.7 cells were used to study the MAPK signaling pathway. These cells are considered optimal osteoclast
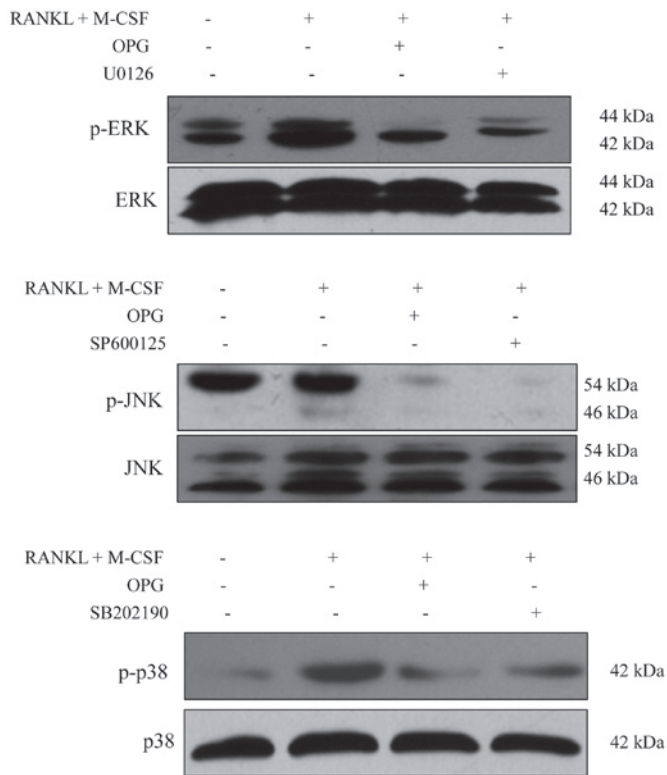
Figure 4. Effects of the signaling inhibitors on the MAPK signaling pathway in OPG-inhibited osteoclastogenesis. The cells were pre-treated with 100 ng/ml OPG and 0.2 μM U0126 (specific inhibitor of the ERK-MAPK signaling pathway), 10 μM SB202190 (specific inhibitor of the p38-MAPK signaling pathway) or 10 μM SP600125 (specific inhibitor of the JNK-MAPK signaling pathway) in the corresponding experimental groups for 30 min. M-CSF + RANKL were then added into the corresponding experimental groups for a further 30 min. The cells were then collected, total protein was extracted and changes in the levels of signaling proteins were detected by western blot analysis. Each experiment was repeated three times. OPG, steoprotegerin; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of nuclear factor-κB ligand; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; p-JNK, phosphorylated c-Jun N-terminal kinase.

precursors and have been extensively applied in *in vitro* studies on osteoclasts (20-22). A previous study by our group demonstrated that M-CSF + RANKL induces RAW264.7-cell differentiation into osteoclasts on a morphological as well as molecular basis; and showed that this process was inhibited by OPG (23). These results laid a foundation for the present study, which aimed to investigate the involvement of the MAPK signaling pathway in this process. Over the previous decades, multiple studies have demonstrated that OPG inhibits the differentiation and activation of osteoclasts (24-26). Few of these studies, however, have focused on the associated signal transduction mechanisms. The present study demonstrated that the MAPK signaling pathway is involved in the M-CSF + RANKL-induced differentiation and activation of osteoclasts, which can be modulated by OPG.

ERK-MAPK, JNK-MAPK and p38-MAPK in osteoclasts and osteoclast precursors are activated by the combination of RANKL and RANK (16-18,27). p38-MAPK is activated by MAPK kinase 6, which is activated by the combination of RANKL and RANK, and subsequently phosphorylates microphthalmia-associated transcription factor (MITF),

promoting the differentiation of osteoclasts (28). Lee *et al* (17) and Li *et al* (18) further confirmed that p38-MAPK is involved in the differentiation of osteoclasts through studies using specific signaling inhibitors (17,18). Previous studies suggested that ERK-MAPK was involved in survival of osteoclasts rather than bone resorption activities (29,30). Tumor necrosis factor receptor-associated factor (TRAF)-6 is an adapter protein that is the binding site of the RANK cytoplasmic motif PFQEP369-373 (31). RANK regulates the development of osteoclasts using TRAF-6 as an intermediary (32). Transforming growth factor beta-activated kinase 1/MAP3K7 binding protein 2 are proteins downstream of TRAF-6, which activate JNK-MAPK and p38-MAPK (33). Downstream transcription factors of ERKs and JNKs include activator protein (AP)-1, the Fos dipolymer family (c-Fos, FosB, Fra-1, and Fra-2), and the Jun family (c-Jun, JunB, and JunD) (25). These transcription factors are master regulators of osteoclast differentiation (25,34). ERK-MAPK activates c-Fos, while JNK-MAPK enhances the transcriptional activity of AP-1 through the phosphorylation of c-Jun (35). Furthermore, AP-1 triggers the gene-encoding matrix metalloproteinase and alkaline phosphatase, promoting the differentiation, survival and fusion of osteoclast precursors, and advancing the activation of mature osteoclasts as well (36,37). Activated ERK enters the nucleus of mature macrophages and sequentially activates the transcription factor Elk, associating with the cis-regulating element located in the promoter region of the c-Fos gene, and driving the differentiation of the mature macrophages into osteoclast precursors (38). M-CSF specifically stimulates the growth of macrophage colonies (39). M-CSF has an important role in the differentiation and survival of osteoclast precursors as well as the survival of mature osteoclasts (40,41). Weilbaecher *et al* (42) suggested that M-CSF impacts the development of osteoclasts through MAPK signaling pathway activation, further activating MITF and transcription factor binding to immunoglobulin heavy constant mu enhancer 3, and promoting the differentiation of osteoclasts and the formation of TRAP-positive multinucleated cells. In addition, the present study confirmed that ERK-MAPK, JNK-MAPK and p38-MAPK were phosphorylated during M-CSF + RANKL-induced osteoclastogenesis.

Simonet *et al* (24) reported that OPG is involved in the regulation of bone density by specifically inhibiting osteoclastogenesis. Subsequent studies suggested that the differentiation and activation of osteoclasts were blocked by OPG through direct and indirect effects. OPG binds to RANKL in a competitive manner with respect to RANK, impeding the RANKL-RANK signaling cascade (25), by an indirect mechanism. In addition, OPG can induce apoptosis in mature osteoclasts by damaging cellular structures such as the F-actin ring (43,44), via a direct mechanism. In the present study, OPG was added to differentiating osteoclast precursors rather than differentiated osteoclasts. It appears, therefore, that OPG interfered with the differentiation of osteoclasts via the indirect mechanism. Theoleyre *et al* (20) investigated the direct influences of OPG on osteoclasts and demonstrated that OPG induces the phosphorylation of ERK1/2 and p38 in differentiated osteoclasts. The study suggested that OPG had specific inhibitory activity on the development of osteoclasts via ERK1/2 phosphorylation (20). In the present study,

however, ERK1/2 phosphorylation levels were found to be decreased alongside the inhibition of osteoclastogenesis by OPG. A possible interpretation of this discrepancy is that ERK1/2 phosphorylation levels were increased during apoptosis of osteoclasts in the study by Theoleyre *et al* (20).

In conclusion, the findings of the present study indicated that the MAPK signaling pathway is involved in the regulation of osteoclastogenesis as well as in the OPG-mediated inhibition of osteoclast differentiation and activation.

Acknowledgements

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