Parthenolide inhibits osteoclast differentiation and bone resorbing activity by down-regulation of NFATc1 induction and c-Fos stability, during RANKL-mediated osteoclastogenesis

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INTRODUCTION

Osteoporosis is a fatal problem that is characterized by fragile bone and impaired bone quality. Bone fracture results in increased mortality, and poor quality of life in elderly people (1, 2). As the population of elderly people increases, the incidence of osteoporosis, is expected to rise, leading to increased risk of fracture. Therefore, in patients with diseases related to excessive bone loss, the key for sustaining the quality life is the regulation of bone resorption.

Osteoclasts are bone resorbing multinucleated giant cells that are differentiated from hematopoietic stem cells, upon stimulation by two essential cytokines, receptor activator of NF-κB ligand (RANKL), and macrophage-colony stimulating factor (M-CSF) (3). They play a central role in bone destructive disorders: RANKL promotes osteoclast formation from osteoclast precursors, while M-CSF supports the proliferation and survival of precursor cells during osteoclast differentiation (4). For successful osteoclast differentiation, gene expression controlled by multiple signaling pathways is required, including NF-κB, MAP kinases, Akt, and two major transcription factors, c-Fos and NFATc1, are required (5-9).

Numerous natural products have been widely used as traditional medicine, and as therapeutic agents. Since they have fewer side effects, and are more suitable for long-term use than synthetic drugs, several natural products have long been used to prevent and treat osteoporosis. These natural products contain numerous chemical compounds that have been reported to suppress osteoclast differentiation and activity, leading to the prevention of in vivo bone loss (10, 11).

Sesquiterpene lactones, found in Asteraceae and Compositae species, have been used as folk remedies for inflammation, arthritis, and tumors. Parthenolide, one of the major sesquiterpene lactones found in the medicinal plant Feverfew, is an active compound with anti-inflammatory properties. Parthenolide has various effects, such as protection from septic shock, proteinuria, and inflammatory renal diseases. In particular, parthenolide blocks LPS-mediated osteolysis, through suppression of NF-κB activation, suggesting that it may be useful for the treatment of infection-mediated bone loss disorders (12). However, the effects of parthenolide on the mechanism of RANKL-induced osteoclast differentiation and activity have not been thoroughly studied. In the present study, we investigated...
the effects of parthenolide on the signaling pathways that are involved in osteoclast differentiation and activation.

RESULTS
Parthenolide suppresses RANKL-mediated osteoclast differentiation in BMMs
Fig. 1A shows the structure of parthenolide used in this study. Primary bone marrow macrophages (BMMs) in the presence of RANKL and M-CSF were treated with, or without, various concentrations of parthenolide, to identify the efficacy of parthenolide on RANKL-mediated osteoclastogenesis. While RANKL differentiated the BMMs of the control group into TRAP-positive immature mono-nucleated and mature multi-nucleated osteoclasts, parthenolide dose-dependently decreased the formation of both TRAP-positive mono-nucleated (early stage of osteoclastogenesis) and multi-nucleated (late stage of osteoclastogenesis) osteoclasts (Figs. 1B-D). Next, we conducted the XTT assay, to exclude the possibility that the inhibitory effect of parthenolide on osteoclastogenesis was due to reduced viability and/or proliferation of the osteoclast precursor cells. Parthenolide had no cytotoxic effects, and did not affect cell proliferation, at doses that effectively inhibited osteoclast differentiation (Fig. 1E).

Parthenolide inhibits RANKL-mediated phosphorylation of p38, ERK, and IkB, as well as degradation of IkB in BMMs
RANKL activates several signal transducers involved in osteoclastogenesis, including p38, JNK, ERK and Akt, as well as NF-kB, which is recognized as a key transcriptional factor for osteoclast differentiation. The effects of parthenolide on RANKL-induced signaling pathways were investigated, to understand the inhibitory mechanism of parthenolide, during RANKL-induced osteoclast differentiation. After the osteoclast precursors were pretreated with parthenolide for 1 h, and stimulated with RANKL at the indicated time, we examined several signaling pathways, and found that phosphorylation of p38, ERK, and IkB were significantly inhibited by parthenolide, as well as degradation of IkB by RANKL (Fig. 2).

Parthenolide inhibits osteoclast differentiation through down-regulation of RANKL-induced NFATc1 induction and c-Fos stability, and ectopic overexpression of c-Fos or NFATc1 reverses the parthenolide-mediated inhibition of osteoclast differentiation
The c-Fos and NFATc1 play an essential role in osteoclast differentiation, and regulate the expression of osteoclast-related genes. To assess whether parthenolide has any effects on the induction of c-Fos and NFATc1, the expression of c-Fos and NFATc1 by RANKL was examined. Osteoclast precursors were treated with parthenolide, and further stimulated with RANKL at the indicated time points. We found that in response to RANKL, mRNA and protein levels of c-Fos and NFATc1 increased. Increased protein and mRNA expression of NFATc1 were significantly inhibited by parthenolide (Figs. 3A, B). Parthenolide decreased the increased amount of c-Fos protein by RANKL, without any marked changes in c-Fos mRNA expression (Figs. 3A, B). Therefore, we evaluated whether parthenolide enhances the degradation of c-Fos protein. After 48 h post-transfection with c-Fos, treatment with CHX, an inhibitor of protein synthesis, decreased the amount of c-Fos protein, compared to control (Fig. 3C); and additional parthenolide
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Fig. 3. Parthenolide inhibits osteoclast differentiation through suppression of RANKL-induced NFATc1 induction and c-Fos stability, and overexpression of c-Fos or NFATc1 rescues osteoclast differentiation, despite the presence of parthenolide. (A) BMMs were stimulated with RANKL and M-CSF in the presence, or absence, of parthenolide (1 μM), for the indicated times. The mRNA expression levels were evaluated by RT-PCR. (B) Effects of parthenolide on protein expression levels of c-Fos and NFATc1 were evaluated by western blot analysis. (C) Plat E cells were transfected with c-Fos (2 μg), and then treated with DMSO or parthenolide (1 μM). After 48 h of transfection, 5 μM MG132 or 20 μM ALLN, and 2 μg/ml CHX were added to the cultures for 4 h, before harvest. c-Fos protein levels were detected by western blot analysis. (D, E) BMMs were infected with retroviruses expressing pMX-IRES-EGFP, pMX-c-Fos-EGFP, and pMX-CA-NFATc1-EGFP. (D) Infected BMMs were cultured for 12 h or 48 h, and western blot analysis was performed for c-Fos or NFATc1 expression levels, respectively. (E) Infected cells were cultured with or without parthenolide in the presence of M-CSF and RANKL, for 4 days. After culturing, the cells were stained for TRAP. (F) TRAP-positive multinucleated cells (TRAP⁺ MNCs) were counted.

Parthenolide inhibits mRNA expression of OSCAR, TRAP, DC-STAMP, and cathepsin K by RANKL, and inhibits the bone resorbing activity of mature osteoclasts

During osteoclastogenesis, the expression of OSCAR, TRAP, DC-STAMP, and cathepsin K is regulated by NFATc1. We examined whether parthenolide regulates the expression of OSCAR, TRAP, DC-STAMP, and cathepsin K, which play essential roles during RANKL-mediated osteoclast differentiation. Parthenolide suppressed mRNA expression of OSCAR, TRAP, DC-STAMP, and cathepsin K by RANKL (Fig. 4A). These data suggest that, through suppression of NFATc1, parthenolide may inhibit RANKL-induced mRNA expression of OSCAR, TRAP, DC-STAMP, and cathepsin K. Next, we examined whether parthenolide has the potential to inhibit the bone resorbing activity of mature osteoclasts. After mature osteoclasts were cultured on hydroxyapatite-coated plates for 24 h, we observed the resolved area of the plate under a microscope.

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Parthenolide decreased the area of resolved pits by mature osteoclasts, while the vehicle could not suppress the increase in the area and number of resolved pits by mature osteoclasts (Fig. 4B), suggesting that parthenolide inhibits the bone resorbing activity of mature osteoclasts.

**DISCUSSION**

Bone fractures by osteoporosis can result in significant problems, including high mortality, and economic burden in the elderly population. To improve the quality of life in patients with excessive bone-resolving disorders, prevention and treatment of bone loss and fracture are critically important. In recent years, the major remedies used for osteoporosis have included estrogen replacement therapy, along with bisphosphonates, selective estrogen receptor modulators (SERM), and calcitonin. However, such therapies are related to severe side effects, including hyperlipidemia, hypertension, endometrial cancer, and gastrointestinal problems (13, 14). Thus, efforts have been made to find candidates with fewer side effects, and excellent efficacies for the prevention and/or treatment of bone resorbing disorders. Natural products have received recent attention as potential therapeutic and preventative drugs for human diseases, because they have historically yielded a variety of therapeutic agents. In the present study, we have shown that parthenolides derived from Feverfew effectively inhibited osteoclast differentiation, without any cytotoxicity, at both early and late stages of RANKL-mediated osteoclastogenesis (Fig. 1).

Excessive enhanced RANKL signaling causes bones to turn very fragile. This condition is related to several pathologic disorders, including osteoporosis, periodontitis, and rheumatoid arthritis; thus, down-regulation of RANKL signaling may be a key factor, in the treatment of excessive bone resorbing diseases. Binding of RANKL to its receptor RANK activates various downstream signals, including NF-κB, p38, ERK, JNK, and Akt, to induce the expression of critical genes for osteoclast differentiation. NF-κB p50 and p 52 double knockout mice display defects of osteoclastogenesis and severe osteopetrosis, indicating that NF-κB is an essential factor in osteoclast differentiation (15, 16). We found that by suppressing RANKL-mediated iκB α phosphorylation and degradation, parthenolide inhibited activation of NF-κB. Our data is consistent with the previous report that, through down-regulation of NF-κB signaling, parthenolide inhibits LPS-mediated infectious osteolysis. In addition, parthenolide inhibited the RANKL-induced phosphorylation of p38 and ERK (Fig. 2). Since MAP kinases consisting of p38, ERK, and JNK translocate key transcription factors into the nucleus, activation of MAP kinases is essential for osteoclast differentiation. In particular, the importance of p38 signaling in RANKL-induced osteoclast differentiation has been suggested in several reports, and it is considered to be a potential therapeutic agent for bone resorption disorders (6, 17). Therefore, our data suggest that through suppression of NF-κB, p38, and ERK, parthenolide inhibits RANKL-mediated osteoclastogenesis.

NFATc1 acts as an essential modulator of RANKL-mediated osteoclast differentiation. Ectopic expression of NFATc1 eliminates the requirement of RANKL for mature osteoclast differentiation, even in the absence of RANKL. Furthermore, NFATc1β embryonic stem cells do not successfully differentiate into osteoclasts in response to RANKL (9), suggesting that NFATc1 plays an essential role in RANKL-induced osteoclastogenesis. In addition, c-Fos is a critical element for the induction of NFATc1 by RANKL (8). Therefore, we determined the effects of parthenolide on RANKL-induced mRNA expression and protein levels of c-Fos and NFATc1. In our study, parthenolide down-regulated RANKL-induced protein level and mRNA induction of NFATc1 in a time-dependent manner, while in c-Fos mRNA induction, only the protein level of c-Fos was decreased, without any marked changes. In addition, our protein stability assay showed that down-regulation of c-Fos stability by parthenolide was reversed by treatment with MG132 or ALLN proteasome inhibitors (Figs. 3A-C). These data raise the possibility that parthenolide has dual roles: suppression of NFATc1 induction, and down-regulation of c-Fos stability during RANKL-mediated osteoclast differentiation. Furthermore, ectopic expression of c-Fos or CA-NFATc1 reversed the parthenolide-induced inhibition of osteoclast differentiation, suggesting that down-regulation of c-Fos and NFATc1 is responsible for the inhibitory effects of parthenolide (Figs. 3D, E).

NFATc1 plays an essential role in the regulation of genes in the middle or late stages of osteoclast differentiation mediated by RANKL, and it subsequently induces the expression of osteoclast-specific genes, including OSCAR, TRAP, cathepsin K, and DC-STAMP (18-20). In this study, parthenolide suppressed the induction of OSCAR, TRAP, cathepsin K, and DC-STAMP (Fig. 4A). Bone resorption is a critical event, which leads to bone destruction and fragility. Osteoclasts perform bone resorption, by resorbing components of the bone matrix. Cathepsin K is highly expressed in osteoclasts, and is a well-known proteolytic enzyme that degrades the bone matrix (21). Induction of cathepsin K by NFATc1 is responsible for the degradation of collagen matrix by osteoclasts. In this study, parthenolide inhibited the bone-resorption activity of mature osteoclasts (Fig. 4B); thus, we suggest that parthenolide inhibits the bone-resorbing activity of mature osteoclasts, through down- regulation of NFATc1.

In conclusion, parthenolide prevents differentiation and bone-resorbing activity of osteoclasts, via down-regulation of p38, ERK, and NF-κB, as well as NFATc1 signaling molecules, and suppression of c-Fos stability. These results suggest that parthenolide has potential therapeutic value for bone destructive disorders that are associated with increased bone resorption.

**MATERIALS AND METHODS**

**Mice and reagents**

Male, 5-week-old ICR mice were purchased from Damul Science (Daejeon, Korea). All experiments in this study were performed in accordance with the animal experiment guidelines of the Institute Committee of Wonkwang University.
Recombinant soluble human M-CSF and human RANKL were obtained from PeproTech EC Ltd. (London, UK). Parthenolide, N-acetyl-leu-leu-norleucinal (ALLN), and monoclonal β-actin antibody were obtained from Sigma (St. Louis, MO, USA). Anti-JNK, anti-phospho-JNK, anti-ERK 1/2, anti-phospho-ERK 1/2, anti-p38, and anti-phospho-p38 antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-c-Fos and anti-NFATc1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Isolated Total RNA was isolated with QIAzol reagent (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Isolated RNA was reverse-transcribed to cDNA, using SuperScript II RT-PCR was conducted in a 20 μl reaction mixture, containing 10 μl SYBR Green Premix (Bioneer Co., Daejeon, Korea), 10 pmol forward primer, 10 pmol reverse primer, and 1 μg cDNA. The mouse GAPDH gene was used as the internal control. The amplification parameters consisted of initial denaturation at 95°C for 5 min, and 40 cycles of 3-step PCR (denaturation at 95°C for 1 min, annealing at 60°C for 30 sec, and extension at 72°C for 1 min). The data was normalized to GAPDH, and presented as the mean fold change, as compared to controls.

Mouse bone marrow macrophage preparation and osteoclast differentiation

BMMs were obtained from 5-week-old male ICR mice, by flushing the femurs and tibias with α-MEM supplemented with 10% FBS and 1% antibiotics. To obtain BMMs, the BMMs were cultured in α-MEM supplemented with 10% FBS and M-CSF (10 ng/ml) for 1 day. Non-adherent cells were transferred to 10 cm Petri dishes, and further cultured in the presence of M-CSF (30 ng/ml), for 3 days. After the non-adherent cells were removed, adherent cells were used as BMMs. To generate osteoclasts from the BMMs culture system, BMMs (3.5 × 10⁴ cells/well) were cultured in complete medium containing M-CSF (30 ng/ml) and RANKL (100 ng/ml) for 4 days with or, without, parthenolide. The cells were fixed in 3.7% formalin for 10 min, permeabilized with 0.1% Triton X-100, and then stained with TRAP (Sigma).

Cell viability assay

BMMs were seeded in 96-well plates (1 × 10⁴ cells/well), and cultured overnight, then treated with M-CSF (30 ng/ml), and various concentrations of parthenolide. After 3 days, 50 μl XTT reagent were added to each well, and then incubated for 4 h. The optical density of each well was then measured at 450 nm, using an ELISA reader.

Real-time RT-PCR

Total RNA was isolated with QIAzol reagent (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Isolated RNA was reverse-transcribed to cDNA, using SuperScript II Reverse Transcriptase (Invitrogen, San Diego, CA, USA). Real-time RT-PCR was conducted in a 20 μl reaction mixture, containing 10 μl SYBR Green Premix (Bioneer Co., Daejeon, Korea), 10 pmol forward primer, 10 pmol reverse primer, and 1 μg cDNA. The mouse GAPDH gene was used as the internal control. The amplification parameters consisted of initial denaturation at 95°C for 5 min, and 40 cycles of 3-step PCR (denaturation at 95°C for 1 min, annealing at 60°C for 30 sec, and extension at 72°C for 1 min). The data was normalized to GAPDH, and presented as the mean fold change, as compared to controls.

Western blot analysis

Cell lysates were prepared, using lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate, and protease inhibitors. The protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Equal amounts of proteins (30 μg) were run on 8-10% SDS-PAGE gels, and transferred by electroblotting onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat dry milk, and probed for 2 h with the primary antibodies. After washing with Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST), the membranes were incubated for 1 h with horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit immunoglobulin antibodies. After washing with TBST, the specific signals were detected, using an enhanced chemiluminescence detection system (Millipore).

Retrovirus preparation and infection

The retroviral vectors pMX-IRES-EGFP, pMX-cFos-IRES-EGFP, and pMX-constitutively active (CA)-NFATc1-IRES-EGFP packaging were performed by transient transfection of these pMX vectors into Plat-E retroviral packaging cells, using X-tremeGENE 9 (Roche, Nutley, NJ, USA), according to the manufacturer’s protocol. After incubation in fresh medium for 2 days, the culture supernatants of the retrovirus-producing cells were collected. For retroviral infection, nonadherent BMMs were cultured in M-CSF (30 ng/ml) for 2 days. The BMMs were incubated with viral supernatant pMX-IRES-EGFP, pMX-cFos-IRES-EGFP, and pMX-CA-NFATc1-IRES-EGFP virus-producing Plat-E cells, together with polybrene (10 μg/ml) and M-CSF (30 ng/ml), for 6 h. After infection, the BMMs were induced to differentiate, in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml), for 4 days.

Bone resorption assay

Mature osteoclasts were prepared from the BMC and primary osteoblast coculture, as previously described (22). BMC (1 × 10⁵ cells) and primary osteoblasts (1 × 10⁵ cells) were seeded on collagen gel-coated culture dishes, and cultured for 7 days, in the presence of 10⁻⁸ M 1,25-dihydroxyvitamin D₃ and 10⁻⁶ M prostaglandin E₂ (PGE₂). The cocultured cells were detached by 0.1% collagenase treatment at 37°C for 10 min, and were then replated on hydroxyapatite-coated plates (Corning, NY, USA). The cells were incubated on the plates with, or without, parthenolide. After 24 h, the cells were removed, and the total resorption pits were photographed and analyzed, using Image-Pro Plus version 4.0 (Media Cybernetics, Silver Spring, MD, USA).

Statistical analyses

Experiments were conducted separately at least 3 times, and all data are presented as the mean ± standard deviation (SD). All statistical analyses were performed using SPSS (Korean ver-
sion 14.0i. Student’s t-test was used to compare the parameters between 2 groups, while the analysis of variance (ANOVA) test, followed by the Tukey post-hoc test, were used to compare the parameters among the 3 groups. P < 0.05 was considered statistically significant.

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