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Sargachromanol G inhibits osteoclastogenesis by suppressing the activation NF-κB and MAPKs in RANKL-induced RAW 264.7 cells

Weon-Jong Yoon [a,d,1], Kil-Nam Kim [b,1], Soo-Jin Heo [c], Sang-Chul Han [a], Jihyeon Kim [d], Yeong-Jong Ko [d], Hee-Kyoung Kang [a], Eun-Sook Yoo [a,*]

[a] School of Medicine, Jeju National University, Jeju 690-756, Republic of Korea
[b] Marine Bio Research Team, Korea Basic Science Institute (KBSI), Jeju 690-756, Republic of Korea
[c] Global Bioresources Research Center, Korea Institute of Ocean Science & Technology, Ansan 426-744, Republic of Korea
[d] Jeju Biodiversity Research Institute (JBRI), Jeju TECHNOPARK (JTP), Jeju 699-943, Republic of Korea

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ABSTRACT

Inflammatory cytokines play a major role in osteoclastogenesis, leading to the bone resorption that is frequently associated with osteoporosis. Sargachromanol G (SG), isolated from the brown alga *Sargassum siliquastrum*, inhibits the production of inflammatory cytokines. In the present study, we determined the effect of SG on receptor activator of NF-κB ligand (RANKL)-induced osteoclast formation. SG inhibited RANKL-induced osteoclast differentiation from RAW264.7 cells without signs of cytotoxicity. Additionally, the expression of osteoclastic marker genes, such as tartrate-resistant acid phosphatase (*TRAP*), cathepsin K (*CTSK*), matrix metalloproteinase 9 (*MMP9*), and calcitonin receptor (*CTR*), was strongly inhibited. SG inhibited RANKL-induced activation of NF-κB by suppressing RANKL-mediated IκB-α degradation. Furthermore, SG inhibited RANKL-induced phosphorylation of mitogen activated protein kinases (p38, JNK, and ERK). This study identified SG as an inhibitor for osteoclast formation and provided evidence that natural compounds, such as SG, are alternative medicines for preventing and treating osteolysis.

1. Introduction

Bone remodeling is a physiological process that involves the resorption of bone by osteoclasts and the synthesis of bone matrix by osteoblasts. Osteoclasts are unique multinucleated cells that are responsible for bone resorption; these cells are derived from hematopoietic stem cells [1,2]. Excessive bone resorption by osteoclasts leads to an imbalance in bone remodeling and causes bone lytic diseases such as osteoporosis, periodontitis, rheumatoid arthritis, Paget's disease, hypercalcemia, and cancer metastasis to the bone [3,4].

Osteoclasts are formed by the fusion of monocyte/macrophage precursors [1]. The receptor activator of nuclear factor-kappa B ligand (RANKL) produced by osteoblasts plays a key role in osteoclast differentiation and activation [5]. RANKL-induced activation of RANK on osteoclast progenitor cells leads to the stimulation of TNF receptor-associated factors (TRAFs) and the subsequent activation of several downstream signaling molecules, including nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPKs), activating protein 1 (AP-1), nuclear factor of activated T cells (NFATc1), and phosphatidylinositol 3-kinase, resulting in the differentiation of osteoclast progenitor cells into cells that finally fuse to form multinucleated bone-resorbing osteoclasts [1,6].

Sargassum, a genus of brown seaweed (Phaeophyceae) in the Sargassaceae family, contains approximately 400 species [7]. *Sargassum* is found throughout all oceans and is consumed as food and medicines in many cultures. Bioactive compounds (currently approximately 200) such as meroterpenoids, phlorotannins, fucoidans, sterols, and glycolipids have been identified from this genus [8]. Meroterpenoids are structurally related to vitamin E and have biological activities similar to those of vitamin E, such as antioxidant and anticancer activities. The meroterpenoids have also been found to have *in vitro* bioactivity such as neuroprotective effects and as antimalarial and antiviral agents [8]. Among the meroterpenoids, sargachromanols have been reported to possess several biological activities, including anticancer [9], antioxidant [10], and anti-inflammatory [11] effects.

More recently, in a previous study, we isolated sargachromanol G (SG) from *Sargassum siliquastrum* and evaluated its potential to inhibit the production of osteoclatogenic factors in human osteoblast-like MG-63 cells. However, the molecular mechanism

* Corresponding author. Department of pharmacology, College of Medicine, Jeju National University, Ara 1-dong, Jeju 690-756, Republic of Korea. Fax: +82 64 702 2687.
E-mail address: eunsyoo@jeju.ac.kr (E.-S. Yoo).
[1] These authors contributed equally to this work.

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Table 1
Sequences of primers and product lengths of the genes in RT-PCR analysis.

Gene		Primer sequences	Fragment(size (bp)
TRAP	F	5′-AAATCACTCTTTAAGACCAG-3′	317
	R	5′-TTATTGAATAGCAGTGACAG-3′	
CTSK	F	5′-CCTCTCTTGGTGTCCATACA-3′	490
	R	5′-ATCTCTCTGTACCCTCTGCA-3′	
MMP-9	F	5′-CTGTCCAGACCAAGGGTACAGCCT-3′	383
	R	5′-GTGGTATAGTGGGACACATAGTGG-3′	
CTR	F	5′-ACCGACGAGCAACGCCTACGC-3′	272
	R	5′-GCCTTCACAGCCTTCAGGTAC-3′	
β-actin	F	5′-GTGGGCCGCCCTAGGCACCAG-3′	603
	R	5′-GGAGGAAGAGGATGCGGCAGT-3′	

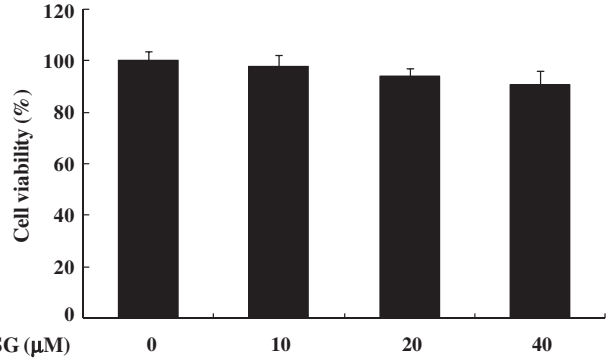

Fig. 1. Effect of SG on the cell viability. An MTT assay was performed after incubation of RAW 264.7 cells with SG (10, 20 and 40 μM) for 24 h at 37 °C in a 5% CO_2 atmosphere. Values are expressed as means ± S.D. of triplicate experiments.

underlying the role of SG in osteoclast formation has not yet been reported.

In this study, we aimed to clarify whether and how SG affects RANKL-induced osteoclast formation in RAW264.7 macrophages. Herein, we report a putative inhibitory mechanism for RANKL-induced osteoclast formation by SG.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY, USA). NF-κB (anti-p50, anti-p65, and anti-IκB-α) and MAPKs (anti-ERK1/2, anti-JNK, and anti-p38) mouse or rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). PD98059 (a specific inhibitor of ERK1/2), SB203580 (a specific inhibitor of p38), SP600125 (a specific inhibitor of JNK), and PDTC (a specific inhibitor of NF-κB) and all other reagents were purchased from Sigma–Aldrich Chemical Co. (St Louis, MO, USA).

2.2. Isolation of SG

SG was isolated as previously described [12]. Briefly, the dried *S. siliquastrum* was extract three times with 80% methanol and filtered. The filtrate was evaporated at 40 °C to obtain the methanol extract, which was suspended in distilled water and partitioned with dichloromethane. The dichloromethane fraction was subjected to silica gel and Sephadex-LH 20 column chromatography. The SG was finally purified by high-performance liquid chromatography, and the structure of the SG was identified by comparing the NMR spectral data with those in existing literature.

2.3. Cell culture

The murine macrophage cell line RAW 264.7 was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). The medium used for routine subcultivation was DMEM, supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL). These cells were maintained at subconfluence in 95% air-5% CO_2 humidified atmosphere at 37 °C and were subcultured every 3 days. Cells were counted with a hemocytometer and the number of viable cells was determined through trypan blue dye exclusion assay.

2.4. Cell viability

Cell viability was measured by conventional MTT assay. RAW 264.7 cells was seeded on 96-well plates and cultured as described above for 24 h. The medium was then replaced with medium containing the horse placenta at various concentrations. After incubation for 24 h at 37 °C in a 95% air and 5% CO_2 atmosphere incubator, MTT solution (10 mg/mL in phosphate buffered-saline, pH 7.4) was added at 50 uL per well (0.5 mg/ml final concentration). The plates were incubated for 4 h at 37 °C to completely dissolve the formazan crystals. The incubation was stopped by addition of 15% sodium dodesyl sulfate into each well for solubilization of formazan and the optical density (OD) at 540 nm was measured with a microplate reader.

2.5. TRAP assay and staining

RAW 264.7 cells (1.0×10^5 cells/mL) were incubated for 18 h in 48-well plates with the same conditions. RANKL (100 ng/mL) and SG were then added to the cultured cells and incubated for 72 h. The cell culture medium from this treatment was subsequently used to measure TRAP activity using a TRAP assay kit with an ELISA plate reader at 450 nm. In a separate experiment to evaluate the TRAP activity via immunostaining, RAW 264.7 cells were plated at 2.0×10^4 cells/well in a 48-well tissue culture plate, in the presence of RANKL (100 ng/mL); the incubation period was 96 h. Cells were then fixed in 3.7% formalin for 10 min. Fixed cells were washed with PBS twice, and permeabilized with 0.1% Triton X-100 for 1 min. Following fixation, the cells were stained for TRAP activity using a kit (Sigma–Aldrich Chemical Co., St Louis, MO, USA), according to the manufacturer's instructions. After incubation at 37 °C in a humid and light-protected incubator for 1 h, cells were washed with distilled water 3 times. TRAP-positive cells appeared dark red, and those containing 3 or more nuclei were classified as osteoclasts.

2.6. RT-PCR analysis

Total RNA from cells was prepared by adding TRIzol Reagent (Gibco BRL), according to the manufacturer's protocol and stored at −70 °C until use. Semiquantitative RT reactions were performed with MuLV reverse transcriptase. Total RNAs (1 μg) were incubated with oligo-dT15 at 70 °C for 5 min and mixed with 5x first strand buffer, 10 mM dNTP, and 0.1 M DTT at 37 °C for 5 min, and for 60 min after the addition of MuLV reverse transcriptase (2 U). The reactions were terminated at 70 °C for 10 min and RNA was depleted by adding RNase H. The PCR mixture [2 μL cDNA, 4 μM 5′ and 3′ primers, 10x buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100), 250 μM dNTP, 25 mM $MgCl_2$, and 1 unit Taq polymerase (Promega, USA)] was run with a 30 s denaturation time at 94 °C, an annealing time of 60 s at 55 to 62 °C, an extension

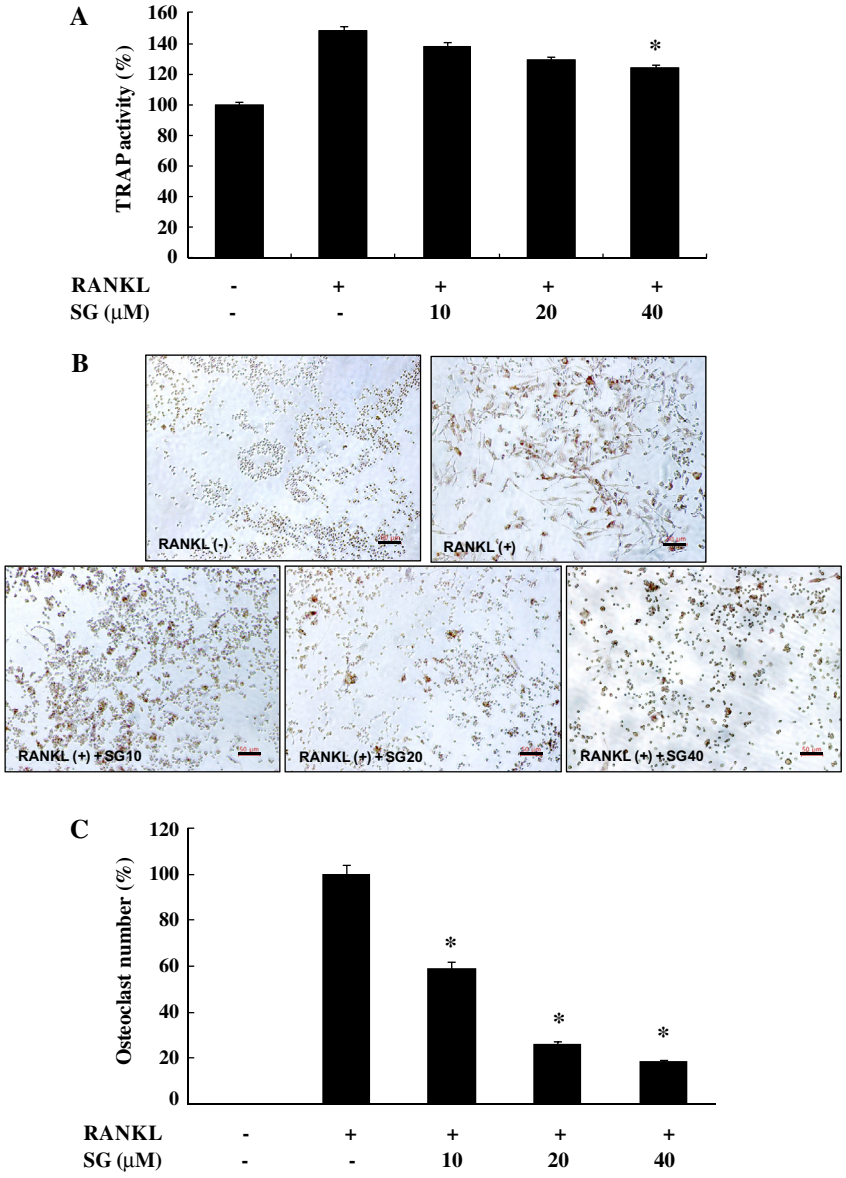

Fig. 2. Effect of SG on osteoclast differentiation in RANKL-stimulated RAW 264.7 cells. RAW 264.7 cells (1.0×10^5 cells/mL) were stimulated with RANKL (100 ng/mL) for 96 h in the presence of SG (10, 20 and 40 μM). (A) Supernatants were collected, and the TRAP concentration in the supernatants was determined in 540 nm by ELISA reader. Cells were fixed with 3.7% formalin, permeabilized with 0.1% Triton X-100, and stained with TRAP solution. (B) TRAP activity and TRAP-positive cells were identified with microscope (× 200, scale bar = 50 μM) (C) Multinucleated osteoclasts were counted. Values are expressed as means ± S.D. of triplicate experiments. $*P < 0.05$ indicates significant differences from the RANKL-stimulated group.

time of 60 s at 72 °C, and final extension of 7 min at 72 °C at the end of 30 cycles. Primers (Bioneer, Seoul, Korea) are shown in Table 1.

2.7. Immunoblotting

The cells were lysed in lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 g/mL leupeptin) and kept on ice for 30 min. The cell lysates were centrifuged at 12,000*g* at 4 °C for 15 min and the supernatants were stored at −70 °C until use. Protein concentrations were measured using the Bradford method. Aliquots of the lysates (30–40 μg of protein) were separated on an 8–12% SDS–polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC, USA) with a glycine transfer buffer (192 mM glycine, 25 mM Tris–HCl [pH 8.8], 20% MeOH [v/v]). After blocking the nonspecific sites with 5% non-fat dried milk, the membrane was incubated with primary antibody (1:1000) at 4 °C overnight. The membrane was further incubated for 60 min with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:5000; Amersham Pharmacia Biotech, Little Chalfont, UK). The proteins were detected using an enhanced chemiluminescence (ECL) Western Blotting detection kit (Amersham Pharmacia Biotech., NY, USA).

2.8. Statistical analyses

The student's t-test and one-way ANOVA were used to determine the statistical significance of the differences between values for different experimental and control groups. Data are expressed as mean ± standard deviation (SD) values of at least 3 independent experiments performed in triplicate. A value of $*P < 0.05$ was considered to be statistically significant.

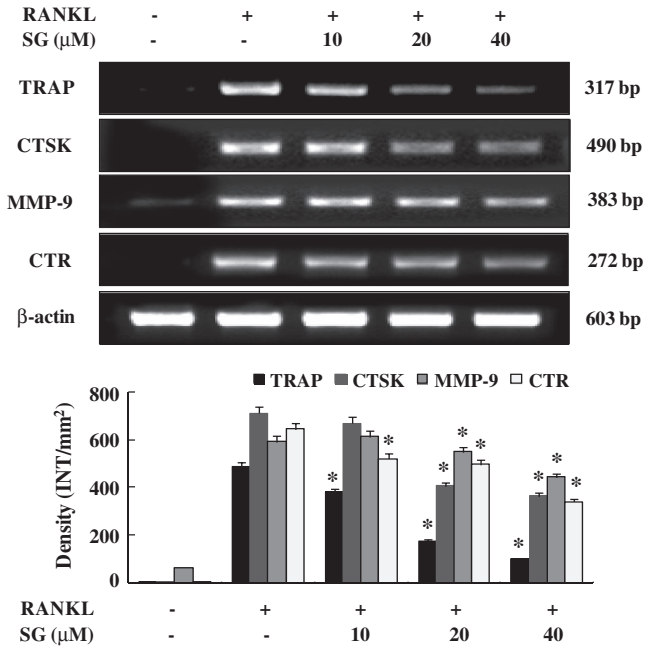

Fig. 3. Effect of SG on mRNA expression of osteoclastic marker genes in RANKL-stimulated RAW 264.7 cells. RAW 264.7 cells (1.0×10^5 cells/mL) were pre-incubated for 18 h, and the cells were stimulated with RANKL (100 ng/mL) in the presence of SG (10, 20 and 40 μM) for 96 h. mRNA expressions of osteoclastogenic marker genes were determined using RT-PCR. Values are expressed as means ± SD of triplicate experiments. $^*P < 0.05$ indicates significant differences from the RANKL-stimulated group.

3. Results

3.1. Effects of SG on cell viability

RAW 264.7 cells were treated with various concentrations of SG for 24 h, and cell viability was assessed using an MTT assay. SG (40 μM) exhibited no cytotoxic effects in cells, as compared to the control cells that had received no treatment. The highest concentration of SG in solvent that did not cause more than 10% loss in cell viability was 40 μM (Fig. 1). Therefore, 40 μM SG was chosen for subsequent studies.

3.2. Effect of SG on osteoclast differentiation in RANKL-stimulated RAW264.7 cells

We first examined the effect of SG on osteoclast differentiation from RAW264.7 cells in the presence of RANKL by using TRAP staining (Fig. 2). At first, we examined whether SG has any effect on TRAP activity. SG dose-dependently inhibited TRAP activity in RANKL-stimulated RAW264.7 cells (Fig. 2A). Next, we examined whether SG has any effect on multinucleated osteoclast-like cell formation in RANKL-stimulated RAW264.7 cells. SG markedly inhibited the RANKL-stimulated osteoclast-like cell formation in RAW264.7 cells in a dose-dependent manner (Fig. 2B, C).

3.3. Effects of SG on expression of osteoclastic marker gene in RANKL-stimulated RAW264.7 cells

To further elucidate the role of SG in osteoclast differentiation, we examined its effect on the expression of osteoclastic marker genes during osteoclastogenesis. The osteoclastic markers, including tartrate-resistant acid phosphatase (*TRAP*), cathepsin K (*CTSK*), matrix metalloproteinase 9 (*MMP9*), and calcitonin receptor (*CTR*), were significantly upregulated upon treatment with RANKL. However, the upregulation of osteoclastic marker genes was attenuated by addition of SG (Fig. 3).

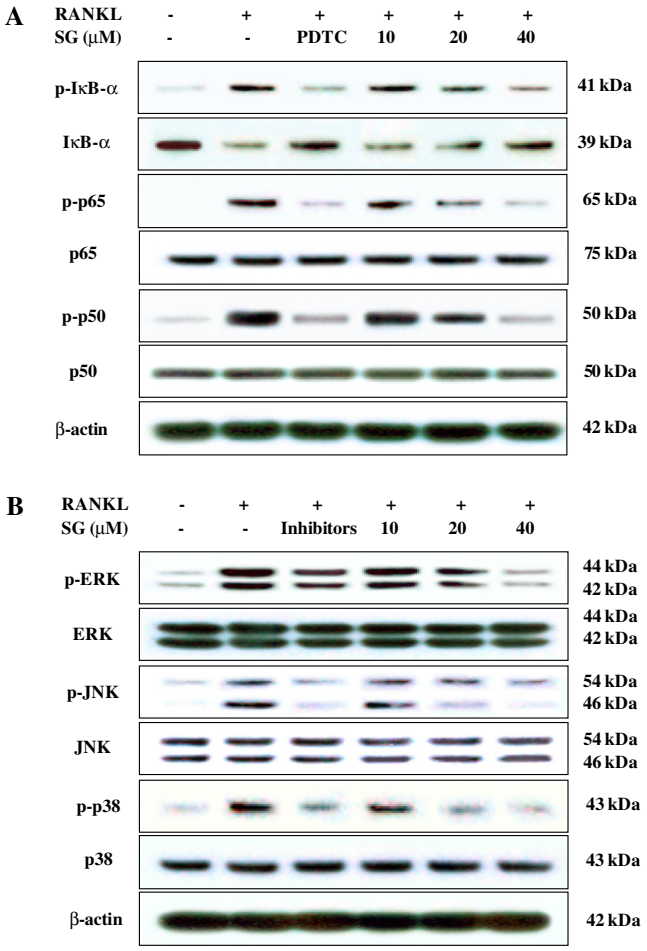

Fig. 4. Effect of SG on (A) NF-κB and (B) MAPKs protein level in RANKL-stimulated RAW 264.7 cells. RAW 264.7 cells (1.0×10^6 cells/mL) were cultured for 18 h, pre-incubated with SG (10, 20 and 40 μM), PDTC (10 μM) or inhibitors (PD : PD98059 (40 μM), SB: SB203580 (40 μM) and SP: SP600125 (20 μM)) at indicated concentrations for 30 min, and then stimulated with RANKL (100 ng/mL) for 10 or 20 min. The protein levels were determined using immunoblotting method.

3.4. Effect of SG on NF-κB activation in RANKL-stimulated RAW264.7 cells

The activation of NF-κB is critical for RANKL-induced osteoclastogenesis [13]. NF-κB is inactive in the cytosol because it is bound to IκB, and becomes active after IκB has been phosphorylated and subsequently degraded [14] Thus, we investigated whether SG inhibits phosphorylation and degradation of IκB. Accordingly, RAW 264.7 cells were pretreated for 30 min with SG, and IκB-α protein levels were determined after 10 min of further exposure to RANKL (100 ng/ml). SG was found to significantly suppress the RANKL-induced phosphorylation and degradation of IκB-α (Fig. 4A). Furthermore, phosphorylation of p50 and p65, which enhances NF-κB transcriptional potential [15], was investigated. SG also markedly reduced RANKL-induced phosphorylation of p50 and p65 (Fig. 4A).

3.5. Effects of SG on phosphorylation of MAPKs in RANKL-stimulated RAW264.7 cells

RANKL is known to activate MAPKs, which play important roles in the differentiation and formation of osteoclasts. To investigate whether the inhibition of NF-κB activation by SG was mediated through the MAPK pathway, we examined the effects of SG on RANKL-induced phosphorylation of ERK, JNK, and P38 in RAW

264.7 cells by immunoblotting. We found that these proteins are phosphorylated following stimulation with RANKL (Fig. 4B). SG (10, 20, and 40 μM) markedly inhibited ERK, p38, and JNK MAPK activation in a dose-dependent manner (Fig. 4B). However, the amount of unphosphorylated ERK, p38, and JNK was unaffected by RANKL and/or SG treatment.

4. Discussion

Excessive bone resorption plays a central role on the development of age-related osteoporosis [16]. As such, inhibiting osteoclast formation should be a valuable treatment for postmenopausal osteoporosis. In this study, we determined the effect of SG on the formation of osteoclasts from RAW 264.7 cells. Multinucleated osteoclasts are generated from precursors by the activation of RANKL [5,17]. The results of our study showed that SG could inhibit RANKL-induced formation of osteoclasts from precursor cells without cytotoxicity.

Osteoclasts are known to be formed by the fusion of hematopoietic cells of the monocyte-macrophage lineage during the early stage of the differentiation process [18]. Terminal differentiation in this lineage is characterized by the acquisition of mature phenotypic markers, such as the expression of *TRAP*, *CTR*, *MMP9*, and *CTSK*, as well as morphological conversion into large multinucleated cells and the capability to form resorption lacunae on the bone [19–22]. SG reduced the RANKL-induced production of *TRAP*, *CTR*, *MMP-9*, and *CTSK* gene in a dose-dependent manner.

Activation of the NF-κB pathway is a key factor in RANKL-induced osteoclast differentiation [23]. The classical NF-κB signaling pathway involves activation of the IκB kinase (IKK) complex, which phosphorylates IκBα and targets it for ubiquitin-dependent degradation [24,25]. In the alternative IκB-independent pathway, direct phosphorylation of the NF-κB subunits p50 and p65 by IKK also modulates NF-κB transcription activity [26,27]. Our results showed that SG inhibited cytoplasmic degradation of IκBα and the nuclear translocation of p50 and p65 proteins, resulting in reduced levels of NF-κB transactivation. The results indicated that inhibition of the NF-κB-dependent pathway is one of the mechanisms involved in the anti-osteoclastogenic effect of SG.

The MAPKs, JNK, ERK and p38, have been reported to be activated by RANKL stimulation and to be associated with osteoclastogenesis [1,28]. p38 is important in the early stage of osteoclast generation because it regulates the microphthalmia-associated transcription factor [29], while dominant-negative JNK prevents RANKL-induced osteoclastogenesis [30]. In comparison, ERK is known to induce c-Fos for osteoclastogenesis [31], and inhibition of ERK has been shown to decrease osteoclast formation [32,33]. In this study, we evaluated the effects of SG on the activation of these MAPKs and found that SG dose-dependently inhibited the phosphorylation of MAPKs. These results demonstrated that phosphorylation of MAPKs may contribute to the anti-osteoclastogenic effect of SG in RANKL-stimulated RAW264.7 cells.

In summary, the present study demonstrated that SG inhibits osteoclastogenesis from macrophages *in vitro*. SG also reduced the RANKL-induced expression of osteoclastic marker genes. In addition, SG attenuated RANKL-induced p38, JNK, and NF-κB activation. Although additional experiments are needed to confirm the efficacy of SG in treating disease conditions *in vivo*, our results indicate that it may have potential in the development of a therapeutic drug for disorders associated with bone loss.

Acknowledgments

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