Selaginella tamariscina water extract inhibits receptor activator for the nuclear factor-κB ligand-induced osteoclast differentiation by blocking mitogen-activated protein kinase and NF-κB signaling

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Submitted: 29-09-2011 Revised: 26-10-2011 Published: 02-08-12

ABSTRACT

Background: *Selaginella tamariscina* has been traditionally used in Korea for treating hematochezia, hematuria, and prolapse of the anus. The aim of this study was to evaluate the inhibitory effect of *Selaginella tamariscina* water extract (ST-WE) on osteoclast differentiation, and to determine the underlying molecular mechanism. **Materials and Methods**: RAW264.7 cells were used as a model to examine receptor activator for the nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation. Expression of osteoclastic genes and transcription factors was evaluated by real-time quantitative polymerase chain reaction (QPCR). Activation of the mitogen-activated protein kinases, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, and NF-κB were determined by Western blot analysis. **Results**: ST-WE significantly inhibited RANKL-induced tartrate-resistant acid phosphatase (TRAP) activity and formation of multinucleated osteoclasts in RAW264.7 cells. ST-WE also significantly inhibited the RANKL-induced mRNA expression of TRAP, cathepsin K, and the d2 isoform of vacuolar ATPase V(0) domain (ATPv0d2) gene. In addition, ST-WE inhibited the RANKL-induced phosphorylation of ERK, JNK, and p38, phosphorylation of I-κB$_\alpha$ and NF-κB p65, and the expression of transcription factors c-fos, Fra-2, and nuclear factor of activated T cells 1. Furthermore, ST inhibited the bone resorptive activity of osteoclasts. **Conclusion**: ST-WE might have beneficial effects on bonedisease by inhibiting osteoclastogenesis and osteoclastic activity.

Key words: Osteoclast differentiation, RAW264.7 cells, receptor activator for nuclear factor-κB ligand, *Selaginella tamariscina*

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.99282

Quick Response Code:

INTRODUCTION

Bone remodeling is a homeostatic process that replaces the degradation of old bone with the formation of new bone. This is necessary for the maintenance of bone microstructure and mineral homeostasis in the body.[1] Defects in osteoclastogenesis, increased bone resorption, or the absence of functional osteoclasts causes an imbalance in bone remodeling that might result in various bone diseases, including osteoporosis, osteopetrosis, and rheumatoid arthritis.[2] Therefore, in bone research, it has been suggested that targeting the regulation of osteoclast differentiation and/or osteoclast activation could be a useful approach for developing therapeutic drugs for bone diseases.[3]

Osteoclasts are exclusively bone-resorbing multinuclear cells. They differentiate from the monocyte/macrophage lineage of hematopoietic stem cells in the presence of receptor activator for nuclear factor-κB (NF-κB) ligand (RANKL).[4] RANKL plays a key role in the efficiency of osteoclast differentiation and activation. An interaction between RANKL and its receptor, RANK, triggers the recruitment of tumor necrosis factor receptor-associated

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factor (TRAF) family proteins. Subsequently, TRAF proteins activate downstream transcription factors, including activation protein-1 (AP-1) and NF-κB, through the mitogen-activated protein (MAP) kinases (ERK [extracellular signal-regulated kinase], JNK [c-Jun N-terminal kinase], and p38) signaling pathway.[1] These RANKL-induced signaling pathways also induce the expression and activation of nuclear factor of activated T cells (NFATc1), which is known to be an essential transcription factor that regulates osteoclast differentiation.

Natural products have been used as sources for the active ingredients in new drug development for treating bone diseases. They have shown a protective effect on bone disease models by inhibiting osteoclastogenesis.[5] *Selaginella tamariscina* (ST) is medicinal plant traditionally used for treating the symptoms of blood in the excrement, hematuria, and traumatic bleeding in Korea.[6] Several studies have reported diverse pharmacological effects of ST, including anti-acne, anti-inflammation, and anti-tumor activity.[7-9] To date, no study has evaluated the inhibitory effect of ST on osteoclast differentiation to examine the potential of ST for treating bone diseases.

This study aimed to investigate *Selaginella tamariscina* water extract (ST-WE) as an inhibitor of osteoclastogenesis. We had previously used the murine monocyte RAW264.7 cells to screen a library of extracts from traditional medicinal plant. RAW264.7 cells are widely used as an *in vitro* model for studying osteoclast differentiation.[10] On this preliminary screening, we found that ST-WE strongly inhibited RANKL-induced tartrate-resistant acid phosphatase (TRAP) activity in osteoclasts. In this study, we confirmed the inhibitory effects of ST-WE on RANKL-induced osteoclast differentiation in RAW264.7 cells, and we targeted signaling pathways and relevant transcription factors to elucidate the molecular mechanism underlying ST-WE inhibitory activity.

MATERIALS AND METHODS

Plant material

ST was commercially available and purchased from Baekje herb (Daejeon, Chungnam, Republic of Korea). The voucher specimen (No. G18) was deposited in the herbal bank of the Center for Herbal Medicine Improvement Research, Korea Institute of Oriental Medicine. ST (50.06 g) was placed in 1 l of distilled water and then extracted by heating for 3 hours. After extraction, the ST-WE was filtered out using standard testing sieves (106 μm) (Retsch, Haan, Germany), lyophilized overnight, and stored at 4°C before use. The extraction yield of ST-WE was approximately 5.62% (w/w).

Cell culture and induction of multinucleated osteoclasts

All materials for cell culture were purchased from Gibco (Invitrogen Inc., Carlsbad, CA, USA). RAW264.7 cells (TIB-71) were purchased from ATCC (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS), 100 U/ ml penicillin, and 100 mg/ml streptomycin in 5% CO_2 at 37°C. The medium was changed every 3 days. For osteoclast differentiation, RAW264.7 cells (1×0^3 cells/well) were cultured in a-minimal essential medium (a-MEM) supplemented with 10% FBS and 100 ng/ml RANKL (RandD Systems Inc., Minneapolis, MN, USA). After 3 days, multinucleated osteoclasts were observed.

Cell viability assay

RAW264.7 cells (1×10^3 cells/well) were plated in a 96-well plate in a-MEM containing 10% FBS. After 24 hours, ST-WE was serially diluted and incubated for 3 days. A Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA) was used to examine cell viability according to the manufacturer's protocol. Data represented the mean ± SD of triplicate.

Tartrate-resistant acid phosphatase staining and activity assay

Multinucleated osteoclasts were fixed in 10% formalin for 10 minutes and ethanol/acetone (1 : 1) for 1 minute, then stained using a Leukocyte Acid Phosphatase Kit 387-A (Sigma, St. Louis, MO, USA). The images of TRAP-positive multinucleated cells were captured using a microscope with a DIXI eXcope 5.0 (DIXI Optics Co. Ltd., Daejeon, Republic of Korea). Micrographs of multinucleated osteoclasts were observed at a magnification of 100×. To measure TRAP activity, multinucleated osteoclasts were fixed in 10% formalin for 10 minutes and 95% ethanol for 1 minute, and then incubated with 100 μl of citrate buffer (50 mM, pH 4.6) containing 10 mM sodium tartrate and 5 mM *p*-nitrophenylphosphate (Sigma, St. Louis, MO, USA). After incubation for 1 hour at 37°C, the enzyme reaction mixtures were transferred into new plates containing an equal volume of 0.1N NaOH. Absorbance was measured at 410 nm, and TRAP activity was presented as a percentage (%) of the control. The experiment was performed in triplicate.

Primer design and real-time quantitative polymerase chain reaction

Primers were designed using an online primer design program, Primer3.[11] Primer nucleotide sequences used in this study were shown in Table 1. For osteoclast differentiation-related gene expression, RAW264.7 cells (2×10^5 cells/well in a 6-well plate) were treated with RANKL for 3 days, ST-WE was added 1 day after RANKL treatment began, and mRNA expression of

Table 1: Primer sequence used in this study

Target gene	Forward (5'-3')	Reverse (5'-3')
c-fos	CCAGTCAAGAGCATCAGCAA	AAGTAGTGCAGCCCGGAGTA
Fra-1	CAGCCTCATTTCCTGGGACC	CCTTTCTTCGGTTTCTGCACT
Fra-2	ATCCACGCTCACATCCCTAC	GTTTCTCTCCCTCCGGATTC
NFATc1	GGGTCAGTGTGACCGAAGAT	GGAAGTCAGAAGTGGGTGGA
TRAP	ACACAGTGATGCTGTGTGGCAACTC	CCAGAGGCTTCCACATATATGATGG
c-Src	CCAGGCTGAGGAGTGGTACT	CAGCTTGCGGATCTTGTAGT
Cathepsin K	GGCCAACTCAAGAAGAAAAC	GTGCTTGCTTCCCTTCTGG
ATP6v0d2	AGACCACGGACTATGGCAAC	CAGTGGGTGACACTTGGCTA
MMP-9	GCCCACCGTCCTTTCTTGTT	CGGTGAAGTGCCTGTCACAA
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

osteoclast differentiation-related genes were evaluated after 3 days of RANKL treatment. For transcription factor gene expression, cells (2×10^5 cells/well in a 6-well plate) were pre-treated with or without ST-WE for 2 hours, and then stimulated with RANKL (100 ng/ ml) for 1 day. Total RNA was isolated with RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized with 2 μg total RNA, 1 μM of oligo-dT_{18} primer, 10 units of the RNase inhibitor RNasin (Promega, Fitchburg, WI, USA), and Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. Subsequently, SYBR green–based quantitative polymerase chanin reaction (QPCR) amplification was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the Applied Biosystems 7500 Real-Time PCR System, with first-strand cDNA diluted to 1 : 50 and 20 pmol of primers, according to the manufacturer's protocol. The PCR reaction consisted of three segments. The first segment was conducted for polymerase activation at 95°C for 10 minutes. The second segment consisted of a three-step cycle run for 40 cycles at 94°C for 40 seconds, 55°C for 40 seconds, and 72°C for 1 minute. The third segment, for the generation of PCR product temperature dissociation curves (melting curves), was run at 95°C for 1 minute, 55°C for 30 seconds, and 95°C for 30 seconds. All reactions were run in triplicate, and data were analyzed using the $2^{-\Delta\Delta CT}$ method described previously.[12] Glyceraldehde-3-phosphate dehydrogenase (GAPDH) was used as the internal standard.

Western blot analysis

RAW264.7 cells (2×10^5 cells/well) were plated in a 6-well plate and pre-treated with or without ST-WE for 2 hours, and then stimulated with RANKL (100 ng/ml) for 0, 5, 15, and 30 minutes. Cells were homogenized in an ice-cold protein extraction buffer consisting of 50 mM Tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, one protease inhibitor cocktail tablet, and one phosphatase inhibitor cocktail tablet (Roche, Mannheim, Germany). Cell lysates were centrifuged at 10 000 × g for 15 minutes at 4°C. Protein concentration was determined with BCA protein assay kit (Pierce, Rockford, IL, USA). Protein samples (10 mg) were mixed with sample buffer (100 mM Tris-HCl, 2% SDS, 1% 2-mercaptoethanol, 2% glycerol, and 0.01% bromophenol blue, pH 7.6), incubated at 95°C for 5 minutes, and loaded onto 10% polyacrylamide gels. Electrophoresis was performed using a Mini protean 3 Cell (Bio-Rad, Hercules, CA, USA). Proteins separated on the gels were transferred onto a nitrocellulose membrane (Whatman, Dassel, Germany). The membrane was incubated in blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and 3% non-fat dry milk). The membrane was then incubated for 2 hours at room temperature with 1 : 1 000 diluted primary antibodies (Cell Signaling Technology Inc., Danvers, MA, USA). Antibodies specific for phosphor-ERK1/2 (thr202/Tyr204), ERK, phosphor-JNK1/2 (Thr183/Tyr185), JNK, phosphor-p38 (Thr180/Tyr182), p38, phosphor-I-κBα (Ser32/36), I-κBα, phosphor-NF-κB p65 (Ser536), NF-κB p65 were from Cell singling Technology (Danvers, MA, USA). After washing with a washing buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) three times for 10 minutes each, the membrane was probed with 1 : 2 000 diluted secondary antibodies (Cell Signaling Technology Inc., Danvers, MA, USA) for 1 hour. The membrane was then washed with a washing buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) three times for 10 minutes each and developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA). Chemiluminescent signal was detected with a LAS-3000 Luminescent image analyzer (Fuji Photo Film Co., Japan). Band densities were measured with Multi Gauge software version 3.0 (Fuji Photo Film Co., Japan).

Pit formation assay

RAW264.7 cells (1×10^3 cells/well) were seeded into calcium phosphate apatite-coated plates (BioCoat

Osteologic multitest slides, BD Biosciences, MA, USA) with a-MEM containing 10% FBS and 100 ng/ml RANKL. ST-WE was treated with RANKL every 3 days from the differentiation day 6 at which the mature osteoclasts were almost functionally activated. After 10 days incubation, the slides were washed with 6% sodium hypochlorite solution to remove cells. The resorbed areas on the slides were observed under microscope.

Statistical analysis

SPSS software (SPSS Inc., Chicago, IL, USA) was used to perform all statistical analyses. The significance of TRAP activity was determined using a Student's *t*-test. The significance of the mRNA expression levels of osteoclast differentiation-related genes was determined with a Student's *t*-test using GAPDH-normalized $2^{-\Delta\Delta CT}$ values. A *P* value less than 0.05 was considered to be significant.

RESULTS

Selaginella tamariscina water extract inhibited RANKL-induced osteoclast formation in RAW264.7 cells

We first evaluated the effect of ST-WE on RAW264.7 cell viability to exclude any potential cytotoxic effect. No ST-WE concentration used adversely affected cell proliferation [Figure 1a]. Next, we investigated the effect of ST-WE on RANKL-induced osteoclast differentiation in RAW264.7 cells. After 3 days of ST-WE treatment, TRAP activity and multinuclear osteoclast formation were examined. RANKL treatment alone induced formation of TRAP-positive multinuclear osteoclasts in RAW264.7 cells. ST-WE at 200 and 400 μg/ml significantly reduced both RANKL-induced TRAP activity, $P<0.01$, Figure 1b and multinuclear osteoclast formation [Figure 1c].

Selaginella tamariscina water extract inhibited RANKL-induced mRNA expression of osteoclast differentiation-related genes

RANKL-induced osteoclastogenesis is associated with increased expression of osteoclast differentiation-related genes, including TRAP, c-Src, cathepsin K, the d2 isoform of vacuolar ATPase V(0) domain (ATP6v0d2), and matrix metalloproteinase (MMP)-9. Therefore, we investigated whether ST-WE inhibited the mRNA expression of these five genes by real-time QPCR. RANKL significantly upregulated the mRNA expression of TRAP, c-Src, cathepsin K, ATP6v0d2, and MMP-9 [$P<0.05$, Table 2]. However, at 400 μg/ml, ST-WE significantly inhibited RANKL-induced expression of TRAP, cathepsin K, and ATP6v0d2 mRNA ($P<0.05$).

Selaginella tamariscina water extract inhibited RANKL-induced mRNA expression of activation protein-1 and nuclear factor of activated T cells 1

AP-1 and NFATc1 are essential transcription factors that regulate RANKL-induced osteoclastogenic gene expression. Therefore, we explored whether ST-WE inhibited the mRNA expression of AP-1 family members (c-fos, Fra-1, and Fra-2) and NFATc1 by real-time QPCR. The mRNA expression was significantly increased upon RANKL stimulation, but 50 μg/ml of ST-WE significantly decreased RANKL-induced expression of c-fos and NFATc1 mRNA [$P<0.05$, Table 3]. In addition, ST-WE at 400 μg/ml significantly inhibited the expression of c-fos, Fra-2, and NFATc1 mRNA ($P<0.05$).

Selaginella tamariscina water extract inhibition of RANKL-induced mitogen-activated protein kinases and NF-κB activation

MAP kinases and NF-κB are the key signaling pathways activated during RANKL-stimulated osteoclastogenesis. To

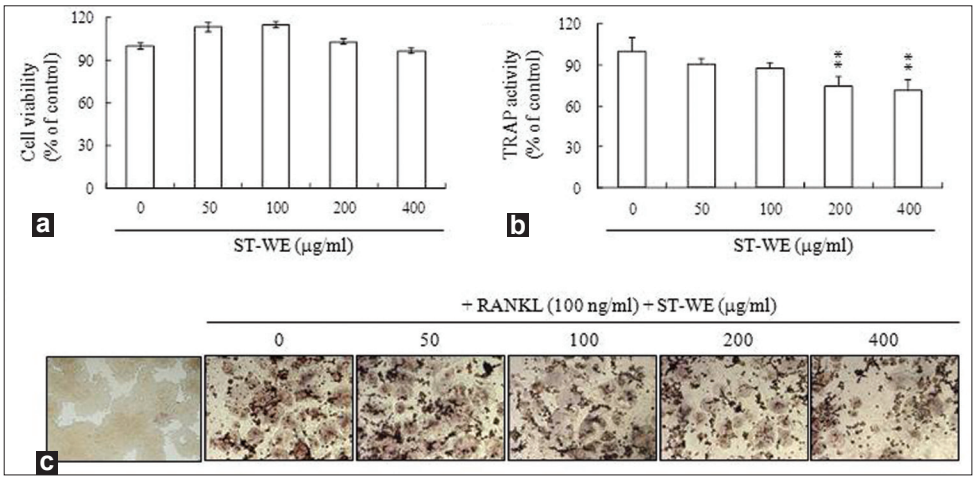

Figure 1: Effects of ST-WE on (a) cell growth, (b) RANKL-induced TRAP activity, and (c) RANKL-induced formation of TRAP-positive multinucleated osteoclasts. RAW264.7 cells (1×10^3 cells/well in a 96-well plate) were incubated with RANKL (100 ng/ml) and the indicated concentrations of ST-WE for 3 days. Micrographs of multinucleated osteoclasts were shown at a magnification of 100´. Data represented the mean ± SD of triplicate, $^{**}P<0.01$

Table 2: Effects of ST-WE on receptor activator for the nuclear factor-κB ligand-induced mRNA expression of osteoclast differentiation-related genes

	TRAP	c-Src	Cathepsin K	ATP6v0d2	MMP-9
Without RANKL	1.01 ± 0.18	1.05 ± 0.35	1.01 ± 0.19	1.02 ± 0.27	1.08 ± 0.53
With RANKL	1249.28 ± 127.76[a]	41.37 ± 11.81[a]	2426.69 ± 157.49[a]	112.57 ± 4.35[a]	1520.06 ± 337.93[a]
RANKL+ST-WE (50 μg/ml)	1238.83 ± 100.58[a]	35.69 ± 2.12[a]	2461.01 ± 103.42[a]	87.55 ± 13.79[a]	1288.12 ± 113.19[a]
RANKL+ST-WE (400 μg/ml)	749.41 ± 110.73[ab]	39.66 ± 4.42[a]	1590.04 ± 333.98[ab]	85.97 ± 10.15[ab]	1306.62 ± 217.23[a]

ST-WE, *Selaginella tamariscina* water extract.All mRNA expression was analyzed by quantitativePCR. For osteoclast differentiation-related gene expression, cells were treated with RANKL for 3days, ST-WE was added 1 day after RANKL treatment began, and mRNA expression of osteoclastdifferentiation-related genes were evaluated after 3 days of RANKL treatment. Data represented themean ± SD of triplicate. [a]$p < 0.05$, compared to group without RANKL; [b]$p < 0.05$, compared togroup with RANKL.

Table 3: Effects of ST-WE on receptor activator for the nuclear factor-κB ligand-induced mRNA expression of transcription factors

	c-fos	Fra-1	Fra-2	NFATc1
Without RANKL	1.00 ± 0.07	1.01 ± 0.15	1.00 ± 0.08	1.01 ± 0.10
With RANKL	1.83 ± 0.30[a]	2.06 ± 0.61[a]	7.42 ± 1.64[a]	9.78 ± 1.55[a]
RANKL+ST-WE (50 μg/ml)	0.80 ± 0.02[ab]	2.45 ± 0.35[a]	5.04 ± 0.41[a]	5.84 ± 0.78[ab]
RANKL+ST-WE (400 μg/ml)	1.09 ± 0.17[ab]	2.54 ± 0.29[a]	4.10 ± 0.58[ab]	3.21 ± 0.23[ab]

ST-WE, *Selaginella tamariscina* water extract, All mRNA expression was analyzed by quantitative PCR. For transcription factor gene expression,cells were treated with ST-WE 2 hours before RANKL treatment, and mRNA expression of transcription factors were evaluated 1 day after RANKL treatment. Data represented the mean ± SDof triplicate. [a]$p < 0.05$, compared to group without RANKL; [b]$p < 0.05$, compared to group withRANKL

determine whether ST-WE affected the activities of these pathways, we explored the activation of MAP kinases, I-κBα, and NF-κB p65 by measuring phosphorylation levels using Western blot analysis. Within only 15 minutes after RANKL treatment, the phosphorylation levels of three MAP kinases (ERK, JNK, and p38) had increased [Figure 2]. At later times, phosphorylation of ERK was sustained, but that of JNK and p38 decreased at 30 minutes. In contrast, ST-WE treatment inhibited RANKL-induced phosphorylation of MAP kinases at 15 minutes. In addition, RANKL-induced I-κBα phosphorylation was slightly increased at 5 minutes but markedly elevated at 30 minutes [Figure 3]. NF-κB p65 phosphorylation attained a maximal level at 5 minutes and decreased to the basal level at later times. However, ST-WE treatment completely inhibited increase in I-κBα phosphorylation to level that was below the basal level of ST-WE-free control at 30 minutes. In addition, ST-WE treatment also inhibited increase in NF-κB p65 phosphorylation to level that was half of maximal level of ST-WE-free control at 5 minutes.

Selaginella tamariscina water extract inhibited RANKL-induced pit formation

We used a pit formation assay to further explore the inhibitory effect of ST-WE on bone resorption by osteoclasts. RAW264.7 cells were incubated on calcium phosphate apatite-coated plates with RANKL for 6 days and next treated with ST-WE for a further 10 days. RANKL treatment increased the pit formation compared with that of cells cultured in the absence of RANKL. ST-WE markedly inhibited RANKL-induced pit formation [Figure 4].

DISCUSSION

RANKL binding to its receptor results in phosphorylation of MAP kinases, which are proline-directed serine/threonine kinases that transmit RANKL signals to the nucleus during osteoclast differentiation.[1] AP-1, composed of the Jun and Fos proteins, has been identified as a target of MAP kinase-signaling pathway. Each activated MAP kinase plays a distinct role in the osteoclastogenic signaling pathway. p38 stimulates osteoclast differentiation, which is reciprocally controlled by ERK.[13] In parallel, JNK activation is required to induce AP-1 expression and activation, particularly that of c-Jun and JunD, which in turn regulates NFATc1 expression.[14,15] After initial expression induced by RANKL stimulation, NFATc1 and AP-1 cooperatively trigger amplification of NFATc1.[4,16] Next, AP-1 and NFAT form transcription complex that bind to regulatory sites on the promoters of osteoclast differentiation-related genes, including TRAP, cathepsin K, and ATP6v0d2, to regulate gene expression.[17-20] Therefore, ST-WE inhibition of MAP kinases [Figure 2], followed by suppression of AP-1 (c-fos and Fra-2) and NFATc1 expression [Table 3], may inhibit osteoclastogenesis by blocking osteoclastogenic gene transcription.

The transcription factor NF-κB is composed of homo- or hetero-dimers of NF-κB family members, Rel-A/

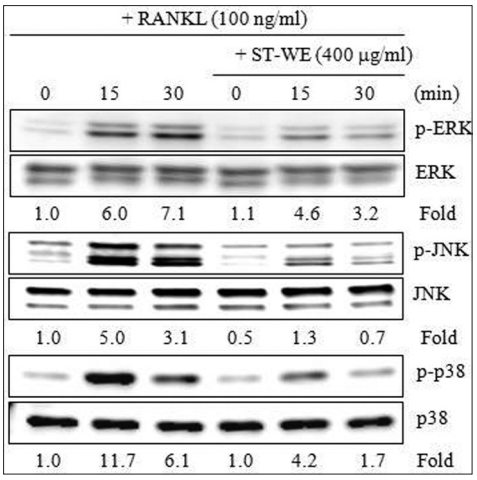

Figure 2: Effects of ST-WE on RANKL-induced MAP kinase activation. MAP kinase activation was represented by the levels of protein phosphorylation. RAW264.7 cells (2×10^5 cells/well in a 6-well plate) were pre-treated with or without ST-WE (400 μg/ml) for 2 hours and then stimulated with RANKL (100 ng/ml) for the time indicated. Western blot analysis was performed with whole cell lysates (10 mg). Blots were probed with antibodies specific for MAP kinases. The densities of phosphorylated protein (p-) levels (upper panels) were normalized to the density of total protein levels (lower panels)

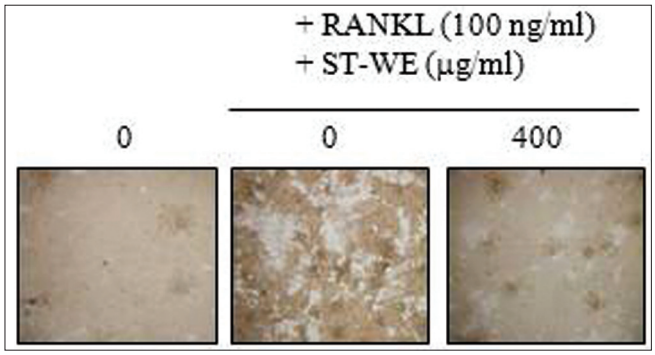

Figure 4: Effects of ST-WE on RANKL-induced pit formation. RAW264.7 cells (1×10^3 cells/well) were seeded onto calcium phosphate apatite-coated plates with a-MEM containing 10% FBS and 100 ng/ml RANKL. ST-WE was treated with RANKL every 3 days from differentiation day 6, at which time the mature osteoclasts were almost functionally activated. After 10 days of incubation, cells were lysed and images obtained with microscope at 100× magnification

p65, Rel-B, c-Rel, p50, and p52.[21] In the canonical NF-κB pathway, interaction between RANKL and RANK activates the I-κB kinase complex, which in turn phosphorylates NF-κB-associated I-κB, leading to ubiquitination and degradation of I-κB. The NF-κB dimers, mostly p50 and Rel-A, are sequentially released and translocated into nucleus to regulate gene transcription. Osteoclast precursor cells deficient in I-κBα phosphorylation fail to form multinuclear osteoclasts and decrease bone resorption activity.[22] In addition, osteoclast precursor cells from *RelA*$^{-/-}$*Tnfr1*$^{-/-}$ mice show an increased level of cell death that prevents

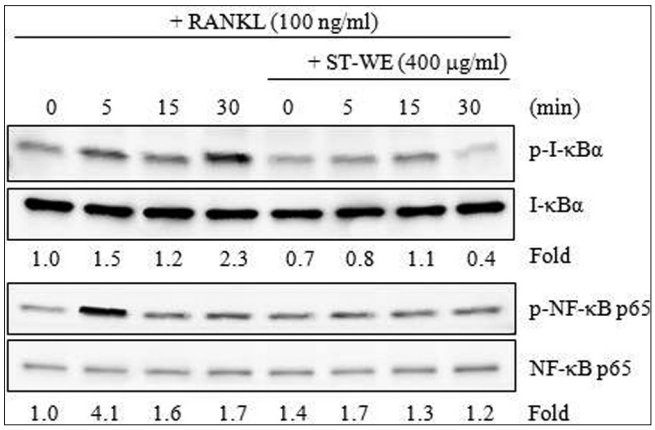

Figure 3: Effects of ST-WE on RANKL-induced I-κB and NF-κB activation. I-κB$_\alpha$ and NF-κB p65 activation were represented by the levels of protein phosphorylation. RAW264.7 cells (2×10^5 cells/well in a 6-well plate) were pre-treated with or without ST-WE (400 μg/ml) for 2 hours and then stimulated with RANKL (100 ng/ml) for the time indicated. Western blot analysis was performed with whole cell lysates (10 mg). Blots were probed with antibodies specific for I-κB$_\alpha$ or NF-κB p65. The densities of phosphorylated protein (p-) levels (upper panels) were normalized to the density of total protein levels (lower panels)

effective RANKL-induced osteoclastogenesis.[23] Interestingly, p38 mediates NFATc1 expression through phosphorylation of NF-κB p65 (Rel-A), which in turn increases the transactivation activity of NF-κB.[24] Cells from "knock-in" mice expressing the unphosphorylated form of NF-κB p65 show a dramatic reduction in NF-κB transcriptional activity. Phosphorylation of NF-κB p65 is also required for association of NF-κB with either CBP/p300 or HDAC, to mediate an efficient transcription.[25] Therefore, the inhibitory effect of ST-WE on RANKL-induced phosphorylation of I-κBα and NF-κB p65 [Figure 3] may inhibit the NF-κB signaling pathway, in turn inhibiting efficient transcription of genes involved in osteoclastogenesis.

RANKL signaling modulates the expression of osteoclast differentiation-related genes (TRAP, cathepsin K, and ATP6v0d2) through NFAT and AP-1 activity in their promoter regions.[19,20,26] As mRNA expression of TRAP, cathepsin K, and ATP6v0d2 increases during osteoclast differentiation, TRAP, cathepsin K, and ATP6v0d2 participate in the bone resorption function of osteoclasts.[27-29] We found that ST-WE inhibited both mRNA expression of these genes [Table 2] and bone resorption activity [Figure 4]. This suggests that ST-WE inhibition of the expression of osteoclast differentiation-related genes might block the bone resorption activity of osteoclasts.

ST contains a large number of active compounds, including a biflavonoid.[30] Although biflavonoids from *Cephalotaxus koreana* stimulate osteoblast differentiation, no study has

yet addressed the inhibitory effects of biflavonoids or ST-WE on osteoclast differentiation.[31] In the present study, we found that ST-WE inhibited RANKL-induced MAP kinases, NF-κB activation [Figures 2 and 3], and mRNA expression of transcription factors [Table 3] that are crucial for osteoclast differentiation. Further study is required to determine whether a biflavonoid may be the active ST-WE component that inhibits RANKL-induced osteoclastogenesis.

In conclusion, we showed that ST-WE significantly reduced RANKL-induced osteoclastogenesis by inhibiting the activation of signaling molecules and the expression of relevant transcription factors. These results suggest that ST-WE may have beneficial effects on bone-destructive disease by inhibiting osteoclastogenesis and the activity of osteoclasts. Further studies are required to identify the active components of ST-WE and to evaluate their efficacies *in vivo*.

ACKNOWLEDGMENTS

We thank Sung-Up Choi (Korea Institute of Oriental Medicine) for critical reading of the manuscript. This work was supported by a grant from the Korea Institute of Oriental Medicine (D10040), Ministry of Knowledge Economy, Republic of Korea.

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Cite this article as: Shim K, Kang J, Lee M, Ma JY. Selaginella tamariscina water extract inhibits receptor activator for the nuclear factor-κB ligand-induced osteoclast differentiation by blocking mitogen-activated protein kinase and NF-κB signaling. Phcog Mag 2012;8:184-91.

Source of Support: Grant from the Korea Institute of Oriental Medicine (D10040), Ministry of Knowledge Economy, Republic of Korea, **Conflict of Interest:** None declared.