

Increased OPG/RANKL ratio in the conditioned medium of soybean-treated osteoblasts suppresses RANKL-induced osteoclast differentiation

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Received August 6, 2013; Accepted November 6, 2013

DOI: 10.3892/ijmm.2013.1557

Abstract. Soybean is a major dietary source of isoflavones, particularly daidzein and genistein, which stimulate osteoblastic functions that are initiated by binding to estrogen receptor (ER)- α and ER- β found on osteoblasts. However, coupled with a low expression of ER- α and ER- β in osteoclasts, the inhibitory effects of soy isoflavones on osteoclast differentiation is likely mediated through paracrine factors produced by osteoblasts. Therefore, in this study, we investigated whether soybean can indirectly inhibit osteoclast differentiation through the modulation of osteoclastic factors produced by osteoblasts. Treatment with soybean extracts increased the levels of osteoprotegerin (OPG) and decreased those of receptor activator of nuclear factor- κ B ligand (RANKL) in the conditioned medium (CM) of MC3T3-E1 osteoblasts. Subsequently, the RANKL-induced RAW264.7 osteoclast formation was markedly inhibited by treatment with CM collected from MC3T3-E1 osteoblasts incubated with soybean extracts (S-CM). Similarly, S-CM significantly attenuated the RANKL-induced increase in the mRNA and protein levels of matrix metalloproteinase-9

(MMP-9), a potential biomarker gene of osteoclast differentiation, through the suppression of nuclear factor of activated T cells c1 (NFATc1) activation. Of note, a soybean concentration of 0.001 mg/ml further increased the OPG/RANKL ratio compared to treatment with a 0.1 mg/ml soybean concentration and was overall, more effective at inhibiting RANKL-induced osteoclast formation and MMP-9 expression. Taken together, our data demonstrate that treatment with soybean extracts stimulates the secretion of OPG and inhibits that of RANKL, thus inhibiting RANKL-induced osteoclast differentiation through the suppression of NFATc1 activation.

Introduction

Estrogen has been shown to play critical role in bone homeostasis that is maintained by the coupled actions of two main bone cell types, bone-forming osteoblasts and bone-resorbing osteoclasts (1). The biological functions of estrogen are mediated by its binding to estrogen receptor (ER)- α and ER- β , which are widely expressed in a variety of tissues and cell types, e.g., liver tissue (2), heart tissue (3), breast epithelial cells (4), ovarian cells (5) and endothelial cells (6). As osteoblasts also express ER- α and ER- β , estrogen can directly bind to its receptors and initiate signals to regulate a number of osteoblast cellular responses, such as proliferation (7), differentiation (8), calcified matrix formation (9) and the secretion of osteoclastic mediators (1). Importantly, previous studies have demonstrated that estrogen regulates the production of two osteoclast differentiation-related mediators, receptor activator of nuclear factor- κ B ligand (RANKL) and osteoprotegerin (OPG), in osteoblasts (10). However, as the levels of ER- α and ER- β in osteoclasts are very low (11,12), the direct effects of estrogen on osteoclast functions through their binding to ERs remain unclear.

It is well known that estrogen replacement therapy (ERT) is a common treatment for women who have declining estrogen

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Key words: osteoclast differentiation, osteoblast-conditioned medium, soybean, osteoprotegerin, receptor activator of nuclear factor- κ B ligand

levels due to natural- or surgical-induced menopause that lead to a reduction in bone mass (13). Although ERT has proven to be effective for the prevention of menopausal-mediated bone loss, prolonged estrogen treatment increases undesirable systemic hormonal side-effects, such as the development of breast cancer, heart attack, strokes and dementia (13,14). In this regard, dietary phytoestrogens, which are naturally occurring plant-derived non-steroidal polyphenolic compounds, e.g., isoflavonoids, coumestans, lignans and stilbenes, are of particular interest as they are considered safe for human consumption and have estrogenic potency (15,16). Of note, as soy isoflavones, particularly genistein (4',5,7-trihydroxyisoflavone) and daidzein (4',7-dihydroxyisoflavone) have a similar chemical structure to mammalian estrogen, they can bind to ER- α and ER- β , and may thus mediate an increase in osteoblastic proliferation and differentiation via the canonical ER-dependent signaling pathway (16). Moreover, in our previous study, we demonstrated that both soybean and black soybean (*Rhynchosia volubilis*), which contain significant concentrations of isoflavones, stimulate osteoblast functions, such as cell proliferation and differentiation through their binding to ERs (17).

Osteoblasts interact closely with osteoclasts, and paracrine factors that are released from osteoblasts mediate osteoclast functions, including osteoclast differentiation (18). The osteoclast differentiation factor, RANKL, binds to its receptor, RANK, that is expressed in pre-osteoclasts, initiating osteoclast differentiation through previously identified mechanism(s), i.e., membrane adaptor protein tumor necrosis factor (TNF) receptor-associated factors (TRAFs) signal to increase the expression/activity of MAP kinases that transactivate multiple osteoclastic transcription factors, including nuclear factor of activated T cells c1 (NFATc1) (19,20). However, considering that osteoclasts express low levels of ERs, the anti-differentiation effects of estrogen and phytoestrogen on these cells may mainly be regulated by the modulation of various osteoclastic mediators produced by osteoblasts. Therefore, in this study, we investigated the indirect effects of soybean, which contains high concentrations of isoflavones, on the inhibition of osteoclast differentiation. Our data demonstrate that the increased secretion of OPG as opposed to that of RANKL in the conditioned medium (CM) of MC3T3-E1 osteoblasts incubated with soybean extracts, inhibits RANKL-induced osteoclast differentiation through the suppression of NFATc1 activation. The data presented in this study elucidate the indirect role of soybean extracts in inhibiting osteoclast differentiation.

Materials and methods

Preparation of soybean extracts. Soybean extracts were prepared from the seeds of soybean (product of Yangjoo-si, Gyunggi-do, Korea), as previously described (17). Briefly, soybean (100 g) was extracted under reflux with 70% methanol (100 ml, 5 times). The solvents were evaporated and freeze-dried for 72 h to yield 13.6 g of soybean extracts. The extracts were dissolved in dimethyl sulfoxide (DMSO), which was subjected to membrane (0.45 μ m) filtration (EMD Millipore, Billerica, MA, USA).

Cell culture. Murine MC3T3-E1 subclone 14 pre-osteoblastic cells [American Type Culture Collection (ATCC), Manassas,

VA, USA] were maintained in ascorbic acid-free α -minimum essential medium (α -MEM) containing 10% fetal bovine serum (FBS) (both from Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO₂. To induce the differentiation of pre-osteoblasts into mature osteoblasts, MC3T3-E1 pre-osteoblasts were incubated with 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate (both from Sigma-Aldrich, St. Louis, MO, USA) for 3 days, followed by treatment with soybean extracts or 17 β -estradiol (E2) or a combination of daidzein and genistein (D/G) (positive controls) for 6 days.

In addition to osteoblasts, murine RAW264.7 pre-osteoclasts (ATCC) were maintained in α -MEM (Invitrogen) supplemented with 10% FBS. To induce differentiation into mature osteoclasts, the cells were treated with 30 ng/ml of soluble RANKL (PeproTech, Rocky Hill, NJ, USA), followed by treatment with the mixed medium of CM collected from MC3T3-E1 osteoblasts treated with 0.001-0.1 mg/ml soybean extracts and osteoclast culture medium (1:1, v/v) for 3 days, as previously described (18).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). qRT-PCR was performed using cDNA prepared from total RNA fractions of cell lysates, as previously described (21). The following primer sets were used: matrix metalloproteinase-9 (MMP-9) forward, 5'-CTGGACAGCCAGACAC TAAAG-3' and reverse, 5'-CTCGCGGCAAGTCTTCAGAG-3'; and mouse glyceraldehyde 3'-phosphate dehydrogenase (GAPDH) forward, 5'-TTGTCAAGTCCATTTCCCTGGT ATG-3' and reverse, 5'-GCCATGTAGGCCATGAGGTC-3'. mRNA expression was normalized to the levels of GAPDH.

Western blot analysis. Western blot analysis was performed as previously described (21). Cell lysates, prepared in radio-immunoprecipitation assay (RIPA) buffer, were resolved by electrophoresis on a 10-12% SDS-PAGE gel. The resultant bands were blotted onto nitrocellulose membranes, probed with anti-murine β -actin, MMP-9 and NFATc1 (Abcam, Cambridge, MA, USA), and detected by enhanced chemiluminescence reagent (Thermo Scientific, Waltham, MA, USA).

Quantification of OPG and RANKL levels. OPG and RANKL levels were quantified using the OPG and RANKL murine ELISA kits (BD Biosciences, San Jose, CA, USA) in accordance with the manufacturer's instructions.

Tartrate-resistant acid phosphatase (TRAP) staining. TRAP staining was performed using the leukocyte acid phosphatase staining kit 387-A (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, the fixed cells were treated with fast garnet GBC base/sodium nitrite (1:1, v/v) at room temperature for 3 min; subsequently, substrate solution (2.5 mM naphthol-ASBI phosphate, 100 mM acetate solution, pH 5.0 and 50 mM tartrate solution) was added followed by incubation for 30 min at 37°C in the dark. TRAP-stained giant multinucleated cells were observed under a light microscope.

siRNA and transfection. RAW264.7 cells were transfected with 20 nM siRNA for NFATc1, or non-targeting, control siRNA, using Lipofectamine RNAiMAX (all from Invitrogen), as previously described (21).

Statistical analyses. Statistical comparisons were performed using one-way ANOVA coupled with the Duncan's multiple range test. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Treatment with soybean extracts increases the secretion of OPG as opposed to that of RANKL from MC3T3-E1 osteoblasts. We wished to investigate whether treatment with soybean extracts can modulate the secretion of RANKL and OPG by osteoblasts, which are critical paracrine factors for osteoclast differentiation (10,22). MC3T3-E1 pre-osteoblasts were differentiated into mature osteoblasts by treatment with ascorbic acid (50 $\mu\text{g/ml}$) and β -glycerophosphate (10 mM) for 3 days, followed by incubation in soybean extracts [0.001 mg/ml, low concentration of soybean extracts (LS); 0.1 mg/ml, high concentration of soybean extracts (HS)], E2 (10^{-9} M), or a combination of daidzein and genistein (D/G, 0.1×10^{-8} M/each) for 6 days. The concentration of E2 was based on the normal plasma estrogen concentration (50-500 pg/ml: 0.18 - 1.8×10^{-9} M) in healthy adult women (23). In addition, as in our previous study, we demonstrated that the potent estrogenic effects of soybean on osteoblastic function are mediated by the synergism of the combination of daidzein and genistein at 0.1×10^{-8} M/each (17), we employed these isoflavones at a concentration of 0.1×10^{-8} M/each as the controls in all subsequent experiments. ELISA revealed that the secretion of RANKL and OPG increased when the cells differentiated into osteoblasts following treatment with ascorbic acid and β -glycerophosphate, while RANKL and OPG were detected at minimal levels (or not detected) in the CM of undifferentiated cells (Table I). Moreover, we observed a significant increase in the secretion of OPG from osteoblasts that were incubated with E2 [182% vs. differentiated control (+C)], D/G (142% vs. +C), LS (188% vs. +C) or HS (194% vs. +C). Conversely, the secretion of RANKL was attenuated in the CM of cells in response to all the compounds ($\sim 51\%$ vs. +C), apart from HS (114% vs. +C), which induced a modest increase.

As the OPG/RANKL ratio is considered an important parameter in the regulation of osteoclast differentiation (24), we then analyzed the relative abundance of OPG and RANKL (Table I). The secreted OPG/RANKL ratio significantly increased following treatment with E2, D/G, LS or HS. Of note, LS (288.7% vs. +C) increased the ratio to a greater extent than HS (170.1% vs. +C). These results suggest that a lower concentration of soybean extracts (0.001 vs. 0.1 mg/ml) is more effective at stimulating the secretion of OPG as opposed to RANKL, thus exerting inhibitory effects on osteoclast differentiation.

CM of soybean-treated osteoblasts attenuates RANKL-induced osteoclast differentiation. To investigate the indirect inhibitory effects of soybean on osteoclast differentiation, RAW264.7 pre-osteoclasts treated with or without soluble RANKL (30 ng/ml), an inducer of osteoclast differentiation, were exposed to CM collected from MC3T3-E1 osteoblasts treated with either E2, D/G, LS or HS. As expected, the number of TRAP-positive giant multinucleated osteoclasts significantly increased in the cells following treatment with RANKL (Fig. 1A). However, the RANKL-induced osteoclast formation was significantly attenu-

Table I. Levels of OPG and RANKL and the OPG/RANKL ratio in the conditioned medium of MC3T3-E1 osteoblasts in response to treatment with soybean extracts, estrogen or a combination of isoflavones.

Treatment	OPG (ng/ml)	RANKL (ng/ml)	OPG/RANKL ratio (% of +C)
-C	1.7 \pm 1.3 ^d	ND ¹	NA ²
+C	32.4 \pm 2.6 ^e	4.3 \pm 0.3 ^a	100 \pm 8.7 ^d
E2	59.0 \pm 12.8 ^a	3.3 \pm 0.4 ^b	237.3 \pm 32.0 ^b
D/G	46.1 \pm 5.7 ^b	2.2 \pm 0.4 ^c	278.1 \pm 14.3 ^a
LS	60.9 \pm 2.0 ^a	2.8 \pm 0.3 ^{b,c}	288.7 \pm 6.7 ^a
HS	62.8 \pm 4.3 ^a	4.9 \pm 0.7 ^a	170.1 \pm 6.1 ^c

All values are the means \pm SD. Different letters in same columns indicate significant difference at $p < 0.05$ ($n=3$). -C, control cells that were not differentiated into osteoblasts; +C, control cells that were differentiated into osteoblasts; E2, 17 β -estradiol (10^{-9} M); D/G, combination of daidzein and genistein (0.1×10^{-8} M/each); LS, soybean extracts at 0.001 mg/ml; HS, soybean extracts at 0.1 mg/ml; ND¹, not detected; NA², not applicable; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor- κ B ligand.

ated by treatment with CM of MC3T3-E1 osteoblasts incubated with E2 (E-CM), or D/G (D/G-CM) (Fig. 1A). Similarly, CM of MC3T3-E1 osteoblasts following treatment with soybean extracts (S-CM) also attenuated the effects of RANKL, while S-CM-mediated decreases in osteoclast formation were evident in the cells treated with CM of a lower concentration of soybean extracts (LS-CM) compared with that of a high concentration of soybean extracts (HS-CM).

To further delineate the effects of S-CM-mediated decreases in osteoclast differentiation, we determined the expression levels of MMP-9, a potential biomarker of osteoclast differentiation (25). Similar to the changes observed with TRAP staining, treatment with RANKL significantly increased MMP-9 mRNA and protein levels (Fig. 1B and C). However, the RANKL-induced increase in MMP-9 expression was attenuated in the cells exposed to all compounds tested. Again, LS-CM was more effective at suppressing MMP-9 expression than HS-CM. Taken together, these results suggest that osteoclast differentiation is indirectly regulated by the key paracrine factors, OPG and RANKL, that are produced and secreted by osteoblasts.

Recombinant OPG peptides attenuate RANKL-induced osteoclast differentiation. As previous studies have demonstrated that OPG, a decoy receptor of RANKL that binds to RANK, decreases the binding ability of RANKL to RANK and subsequently inhibits osteoclast differentiation (22,24), we then investigated whether exogenous OPG, and at what concentrations, inhibits osteoclast formation. TRAP staining revealed that treatment with OPG peptides significantly decreased osteoclast formation induced by RANKL in a dose-dependent manner (Fig. 2A). Consistent with the changes observed with TRAP staining, the RANKL-induced increase in MMP-9 protein expression was markedly attenuated by treatment with OPG peptides at 60 ng/ml (Fig. 2B), while the

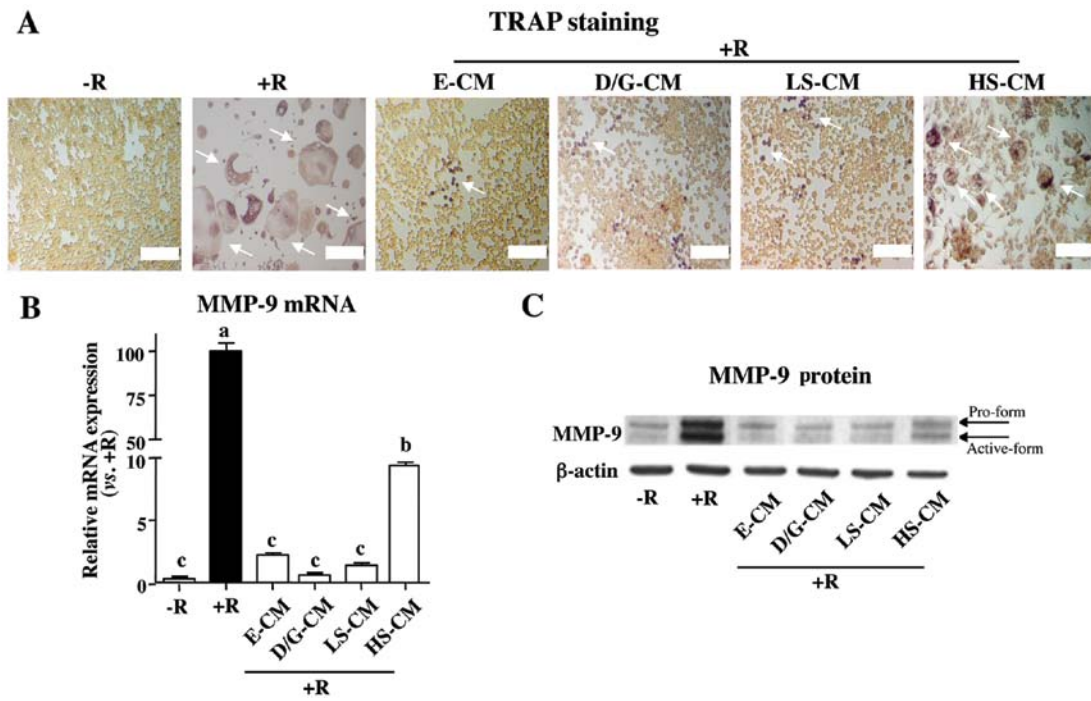


Figure 1. Conditioned medium (CM) of soybean-treated MC3T3-E1 osteoblasts suppresses receptor activator of nuclear factor- κ B ligand (RANKL)-induced osteoclast differentiation. RAW264.7 pre-osteoclasts were incubated with or without RANKL (30 ng/ml), followed by treatment with CM collected from MC3T3-E1 osteoblasts incubated with soybean extracts (0.001 mg/ml, LS; 0.1 mg/ml, HS), estrogen (E, 10^{-9} M), or a combination of isoflavones (0.1×10^{-8} M/each, D/G). (A) Osteoclast formation was assessed by tartate-resistant acid phosphatase (TRAP) staining. The number of TRAP-positive giant multinucleated osteoclasts (indicated by arrows) significantly increased in the cells following treatment with RANKL. The number of these cells decreased following treatment with E, D/G, LS and HS. However, LS was more effective than HS. Matrix metalloproteinase-9 (MMP-9) mRNA and protein levels were quantified by (B) qRT-PCR and (C) western blot analysis, respectively. Different letters indicate significant difference at $p < 0.05$. Similar results were obtained when the experiment was repeated (in triplicate) using different cell preparations. Scale bar, 100 μ m. R, RANKL; D/G, daidzein and genistein.

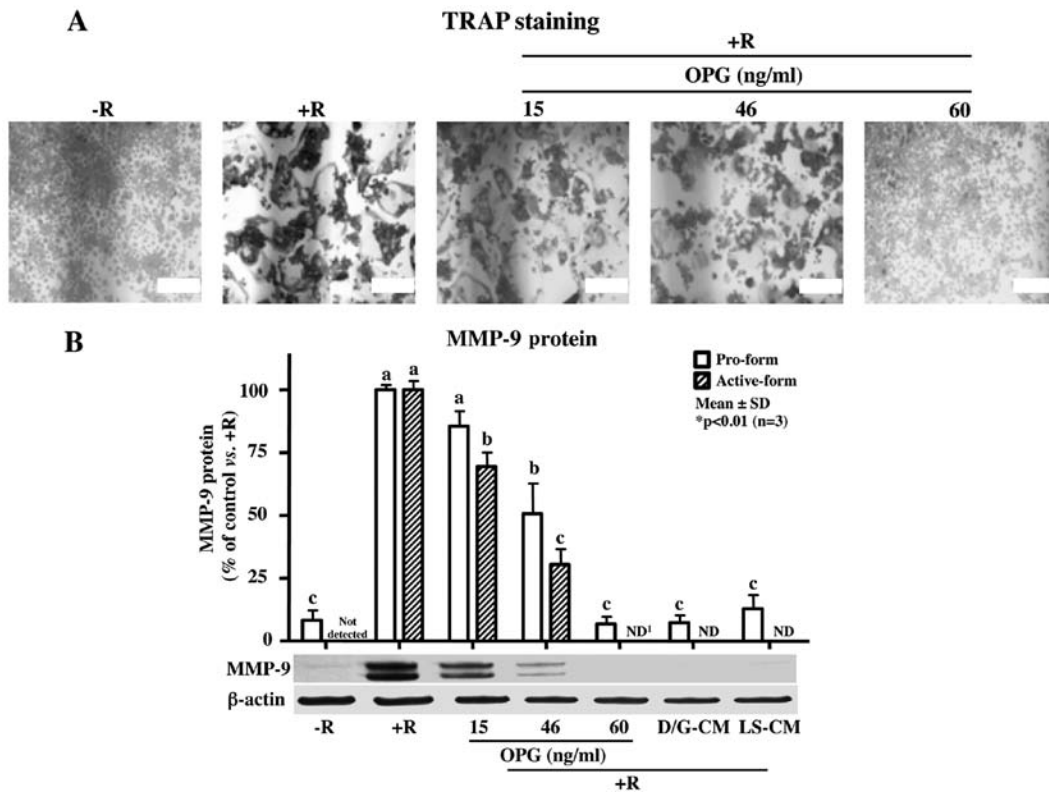


Figure 2. Recombinant osteoprotegerin (OPG) peptides inhibit receptor activator of nuclear factor- κ B ligand (RANKL)-induced osteoclast differentiation and formation. RAW264.7 pre-osteoclasts were incubated with or without RANKL (30 ng/ml), followed by treatment with exogenous OPG peptides or conditioned medium collected from MC3T3-E1 osteoblasts incubated with soybean extracts (0.001 mg/ml, LS) or a combination of isoflavones (0.1×10^{-8} M/each, D/G). (A) Osteoclast formation was assessed by tartate-resistant acid phosphatase (TRAP) staining. (B) Matrix metalloproteinase-9 (MMP-9) protein levels were quantified by western blot analysis. Different letters indicate significant difference at $p < 0.05$. Similar results were obtained when the experiment was repeated (in duplicate) using different cell preparations. ND, not detected. Scale bar, 100 μ m. R, RANKL; D/G, daidzein and genistein.

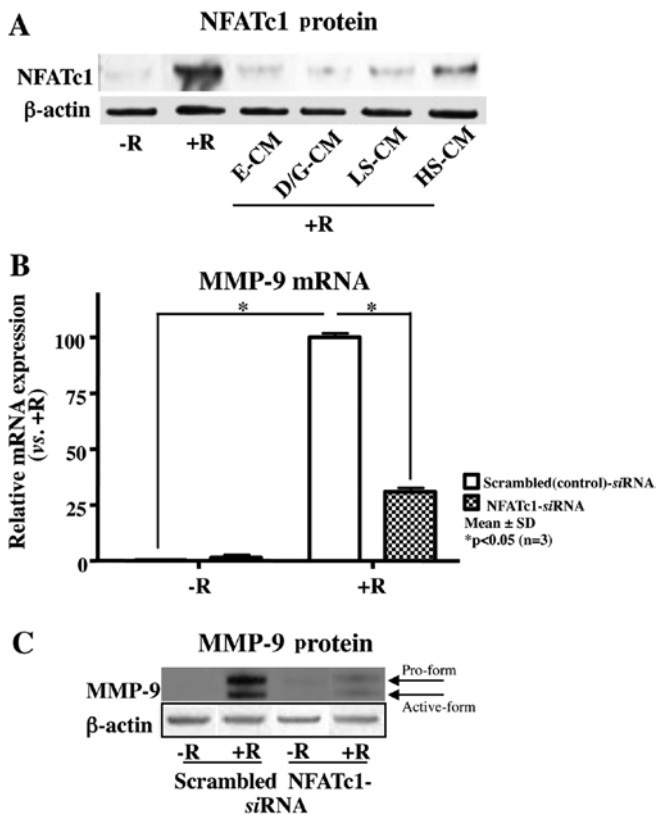


Figure 3. Conditioned medium of soybean-treated MC3T3-E1 osteoblasts inhibits osteoclast differentiation through the suppression of receptor activator of nuclear factor- κ B ligand (RANKL)-mediated nuclear factor of activated T cells c1 (NFATc1) activation. RAW264.7 pre-osteoclasts were incubated as in Fig. 1. (A) Protein levels of NFATc1 were determined by western blot analysis. To further ascertain the role of NFATc1 on RANKL-induced matrix metalloproteinase-9 (MMP-9) expression, cells were transfected with or without NFATc1-siRNA, followed by treatment with RANKL (30 ng/ml) for 3 days. MMP-9 mRNA and protein levels were quantified by (B) qRT-PCR and (C) western blot analysis, respectively. Similar results were obtained when the experiment was repeated (in triplicate) using different cell preparations. R, RANKL; CM, conditioned medium; E, estrogen; D/G, daidzein and genistein; LS, 0.001 mg/ml soybean extracts; HS, 0.1 mg/ml soybean extracts.

addition of LS-CM with OPG concentrations of >60 ng/ml, completely inhibited RANKL-induced TRAP-positive giant multinucleated osteoclasts and MMP-9 expression. Of note, although the OPG concentration of 46 ng/ml was not sufficient to suppress RANKL-induced osteoclast formation and MMP-9 expression, treatment with D/G-CM, which contains 46.1 ng/ml OPG, completely blocked both osteoclast formation and MMP-9 expression, suggesting that other regulators in addition to OPG, are involved in the regulation of osteoclast differentiation and formation.

CM of soybean-treated osteoblasts attenuates RANKL-induced osteoclast differentiation by suppressing the activation of the transcription factor, NFATc1. Since NFATc1 is a critical transcription factor that is responsible for osteoclast differentiation (20), we examined whether S-CM can alter RANKL-induced NFATc1 expression. Western blot analysis revealed that all the treatments, including S-CM, markedly attenuated the RANKL-induced increase in NFATc1 expression (Fig. 3). In a similar manner, LS-CM was more effective at decreasing the expression levels of NFATc1 than HS-CM (Fig. 3A). To further identify

NFATc1 as the mediator responsible for RANKL-induced osteoclast differentiation in our culture system, we transfected the cells with siRNA targeting NFATc1 or scrambled (control) siRNA, followed by treatment with RANKL. The silencing of NFATc1 markedly attenuated the RANKL-induced MMP-9 mRNA and protein expression (Fig. 3B). Taken together, these results suggest that CM collected from MC3T3-E1 osteoblasts incubated with a lower concentration of soybean extracts was more effective at inhibiting osteoclast differentiation by suppressing the activation of NFATc1 compared to incubation with a high concentration of soybean extracts.

Discussion

Soybean is a major dietary source of isoflavones, particularly daidzein and genistein that can bind to ER- α and ER- β , due to their structural similarity to estrogen (16). Accordingly, these isoflavones have been shown to inhibit osteoclast differentiation and bone resorption *in vitro* and *in vivo* (16,26-28). However, coupled with the low expression of ER- α and ER- β in osteoclasts (12), the inhibitory effects of isoflavones on osteoclast differentiation are likely regulated by various paracrine factors, including OPG and RANKL, released from osteoblasts. In this study, we demonstrate that the soybean-mediated increase in the levels of secreted OPG as opposed to RANKL in osteoblast-CM attenuates RANKL-induced osteoclast differentiation.

A number of studies have shown soy isoflavones to exert direct inhibitory effects on osteoclast differentiation via previously suggested cellular mechanisms (29,30): i) an increased extracellular calcium concentration that directly stimulates osteoclast apoptosis; ii) an inhibition of protein tyrosine kinase Src, that is a critical factor in inducing osteoclast differentiation; and iii) an activation of protein tyrosine phosphatase that is a negative regulator of osteoclast differentiation. However, our preliminary experiments revealed that direct treatment with estrogen and a combination of isoflavones failed to suppress RANKL-induced osteoclast formation (data not shown). In addition, the partial inhibition of osteoclast formation by treatment with soybean extracts may be due to other components of soybeans, individually or in conjunction with isoflavones, as the RANKL-induced osteoclast formation was not attenuated by the combination of daidzein and genistein, which is the concentration of these two isoflavones that is similar to soybean (data not shown). Instead, we demonstrated that osteoclast differentiation was significantly attenuated in cells following treatment with CM collected from osteoblasts incubated with soybean extracts, as well as estrogen or a combination of isoflavones, which were employed as the controls. These results demonstrate that although the direct inhibitory effects of soybean extracts on osteoclast formation remain feasible, the inhibitory effects of soybean extracts on osteoclast differentiation in our culture system are likely mediated indirectly through paracrine factors produced by osteoblasts. Of note, such an effect was evident following treatment with low concentrations of soybean extracts or the combination of isoflavones (daidzein and genistein) at 0.1×10^{-8} M/each, which is a concentration of these two isoflavones that is similar to soybean at 0.001 mg/ml, suggesting that the synergistic effects of daidzein and genistein and a low concentration of soybean likely led to an upregulation

of paracrine factors in osteoblasts, which in effect modulated an increase in the OPG/RANKL ratio.

As noted above, OPG is able to bind to RANKL as a decoy receptor, resulting in the inhibition of osteoclast differentiation (10,22,24). In the present study, we demonstrated that recombinant OPG at 60 ng/ml is sufficient to inhibit the RANKL-induced osteoclast differentiation, whereas osteoclast differentiation was not completely suppressed by OPG peptides at 46 ng/ml. These results are in agreement with those of previous studies, demonstrating that an exogenous OPG concentration up to 100 ng/ml inhibits RANKL-induced RAW264.7 osteoclast differentiation (31). Of note, however, the combination of isoflavones, which induced the secretion of OPG at ~46 ng/ml, was sufficient to inhibit the RANKL-induced osteoclast differentiation. Conversely, although a high concentration of soybean extracts increased the OPG secretion by >60 ng/ml, osteoclast differentiation was not completely suppressed as treatment also highly stimulated RANKL levels that induced a decrease in the OPG/RANKL ratio (278% D/G vs. 170% HS). Hence, our study further demonstrates that although a certain concentration of OPG has the ability to inhibit osteoclast differentiation, the OPG/RANKL ratio is a critical parameter in the inhibition of osteoclast differentiation.

Among diverse cellular mediators that are associated with RANKL-mediated osteoclast differentiation, the importance of the transcription factor, NFATc1, has been suggested by previous studies, demonstrating that NFATc1-deficient cells fail to differentiate from pre-osteoclasts into osteoclasts (32). The results from the present study further support the key role of NFATc1 in RANKL-induced MMP-9 expression and osteoclast differentiation.

In conclusion, the present study demonstrates the indirect inhibitory effects of soybean extracts on osteoclast differentiation. Although further studies are required to elucidate the direct and/or indirect inhibitory effects of other functional components of soybeans, e.g., lecithins, lectins, linoleic acid and saponins, on osteoclast differentiation, the indirect effects that we observed are likely caused by the synergistic action of daidzein and genistein. Furthermore, a low concentration of soybean extracts markedly mediated an increase in the OPG/RANKL ratio, which in effect inhibited osteoclast differentiation by suppressing NFATc1 activation.

Acknowledgements

The authors thank Dr Taj Mann for providing superb editorial assistance. This study was supported by a 2006 grant from Kyung Hee University (KHU-20060554) and by a grant from the Agenda Program (PJ008559), Rural Development Administration, Republic of Korea.

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