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Inhibitory Effects of *Artemisia asiatica* on Osteoclast Formation Induced by Periodontopathogens

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Abstract Bone resorption surrounding tooth root causes tooth loss in periodontitis patients. Osteoclast has bone resorption activity. Effects of *Artemisia asiatica* on bone resorption induced by periodontopathogens, *Porphyromonas gingivalis* and *Treponema denticola*, were examined using co-culture systems of mouse osteoblasts and bone marrow cells. Addition of *A. asiatica* ethanol extract to bacterial sonicate abolished bacteria-induced osteoclastogenesis. To determine inhibitory mechanism of *A. asiatica* against osteoclastogenesis, effects of *A. asiatica* on expressions of osteoclastogenesis-inducing factors such as receptor activator of NF-κB ligand (RANKL), prostaglandin E_2 (PGE_2), interleukin (IL)-1, and tumor necrosis factor (TNF)-α, in osteoblasts were examined. *A. asiatica* suppressed expressions of RANKL, PGE_2, IL-1β, and TNF-α increased by each bacterial sonicate. These results suggest inhibitory action of *A. asiatica* against osteoclastogenesis is associated with down-regulations of RANKL, PGE_2, IL-1β, and TNF-α expressions.

Keywords: *Artemisia asiatica*, osteoclast formation, *Porphyromonas gingivalis*, *Treponema denticola*, RANKL

Introduction

Osteoclasts are tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells with bone-resorbing activity. They are derived from hematopoietic cells through multiple steps including TRAP expression and cell fusion (1, 2). Osteoblasts express the receptor activator of nuclear factor-B (RANK) ligand (RANKL, also known as an osteoclast differentiation factor and an osteoprotegerin ligand). The osteoclast precursors express RANK, which interacts with RANKL, and differentiate into osteoclasts (3). Prostaglandin E_2 (PGE_2), interleukin (IL)-1, and tumor necrosis factor (TNF)-α up-regulate RANKL expression in osteoblasts and induce osteoclastogenesis (4-7).

Periodontitis is an inflammatory disease in the supporting tissue of the teeth including alveolar bone. Irreversible alveolar bone resorption is clinically one of the significant characteristics in periodontitis, causing tooth loss (8). Bacteria harbored in subgingival plaque are causative agents of periodontitis, and these bacteria play a major role in alveolar bone resorption. *Porphyromonas gingivalis* and *Treponema denticola* are representative pathogens of periodontitis (9-11). RANKL-positive osteoblastic cells were reported in mice injected with *P. gingivalis* (12). In osteoclast formation induced by *T. denticola*, PGE_2 was associated with an increase in RANKL expression by lipooligosaccharide of *T. denticola* (13). A study on mice injected with *P. gingivalis* demonstrated that *P. gingivalis* – induced bone resorption was partially mediated by prostaglandin (14). In addition, *P. gingivalis* LPS induced bone resorption was significantly reduced in mice lacking IL-1 and TNF receptor (15). Taken together, these studies indicate that RANKL, PGE_2, IL-1, and TNF are important mediators in bone resorption induced by periodontopathogens.

Artemisia asiatica, used in traditional Asian medicine, was found to have protective activity against the chemically-induced liver damage (16) and hepatic fibrosis (17). Amyloid beta protein (Abeta)-induced free radical-mediated neurotoxicity, hypothesized to be the leading cause of Alzheimer's disease, was found to be inhibited by *A. asiatica* (18). Antimicrobial properties of *A. asiatica* were demonstrated against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* (19). Cyclooxygenase (COX)-2 and inducible nitric oxide synthetase (iNOS) play crucial roles in mediating inflammatory responses and are implicated in the pathophysiology of tumorigenesis. *A. asiatica* showed inhibitory effects on tumor formation, COX-2 expression, and iNOS expression in mouse skin (20). These results suggest that *A. asiatica* has protective activity against liver damage, neurotoxicity, infection, tumor, and inflammation. Because *A. asiatica* has antimicrobial and anti-inflammatory activities, it would be of interest to examine the inhibitory effect of *A. asiatica* on periodontopathic bacteria-induced bone resorption. Thus, in the present study, we determined the effect of *A. asiatica* on osteoclastogenesis and expression of RANKL, IL-1β, TNF-α, and PGE_2 induced by *P. gingivalis* and *T. denticola*.

Materials and Methods

Preperation of ethanol extracts of *A. asiatica* The standardized ethanol extract of *A. asiatica*, prepared according to the published procedure (16), was supplied by Dong-A Pharmaceutical Co., Ltd. (Kyunggi-do, Korea).

Preparation of bacteria sonicates *P. gingivalis* (ATCC

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33277) was cultured anaerobically in brain-heart infusion medium containing hemin (5 μg/mL) and menadione (0.5 μg/mL) for 2 days. *T. denticola* (ATCC 33521) was cultured anaerobically in an OMIZ-PAT broth for 3-5 days as described previously (21). Bacterial cells were harvested and disrupted using an ultrasonic processor (Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA) for 5 min at an output power of 8 watts with 20-sec intervals. The cell debris was removed by centrifuging at 15,000xg for 5 min at 4°C, and the supernatant was collected. The protein concentrations were determined using a Coomassie brilliant protein assay reagent (Pierce, Rockford, IL, USA).

Preparation of primary osteoblasts and bone marrow cells The osteoblasts were isolated from the calvaria of 1 -2-day-old ICR mice (Bio Korea Co., Seoul, Korea) as previously described (22). The calvaria were digested in 10 mL α-MEM (GIBCO BRL., Grand Island, NY, USA) containing 0.2% collagenase (Wako Pure Chemicals, Osaka, Japan) and 0.1% dispase (GIBCO BRL, Grand Island, NY, USA) for 20 min at 37°C with vigorous shaking, and then centrifuged at 1,500xg for 5 min. The first supernatant was discarded, and additional 10 mL collagenase/dispase enzyme solution was added and incubated for 20 min. The digestion was repeated four times, and the cells isolated from the last three digestions, combined as an osteoblast, were cultured in α-MEM containing 10% FBS (GIBCO BRL., Grand Island, NY, USA) and antibiotics solution (GIBCO BRL, Grand Island, NY, USA; 100 U/mL penicillin, 100 μg/mL streptomycin, 25 μg/mL amphotericin B), and used for the co-culture system. The bone marrow cells were collected from 5-8-week-old mice. The ends of the tibiae and femurs were removed, and the marrow cavity was flushed by slowly injecting media through one end using a 25 gauge needle. The marrow cells were washed and used for the co-culture.

Cytotoxic assay To determine the cellular toxicity of *A. asiatica*, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) assay was performed. After bone marrow cells (5×10^3) were co-cultured with calvarial cells (5×10^4) in α-MEM containing 10% FBS in 96-well plates for 3 days, *A. asiatica* extract (10, 25, 50, and 100 μg/mL) was added to each well and cultured for additional 3 days. After culture, 50 μL MTT solution (5 mg/mL) was added to each well and incubated for 4 hr at 37°C. After removing the reaction solution, 50 μL dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals formed within cells, and the optical density of the formazan solution was read at 570 nm.

Osteoclast formation assay The isolated osteoblasts were seeded at 10^6 cells in a 10-cm culture dish and grown to confluence. The cells were then detached from the culture dishes using 0.5% trypsin-EDTA (GIBCO BRL, Grand Island, NY, USA). The cells (1×10^4 cells/well) were co-cultured with the bone marrow cells (1×10^5 cells/well) in α-MEM containing 10% FBS in 48-well plates (Corning Inc., Corning, NY, USA). The culture volume was made up to 400 μL per well with α-MEM medium containing 10% FBS. After 3 days, the medium was exchanged with α-MEM medium containing 10% FBS, indicated concentration of *A. asiatica* extract (10, 25, and 50 μg/mL), and each bacterial sonicate (*P. gingivalis* 0.1 μg/mL, *T. denticola* 1 μg/mL). The co-culture was then maintained for an additional 4 days. The extent of osteoclast differentiation was monitored using a TRAP staining kit (Sigma, St. Louis, MO, USA) according to the manufacturers instructions. The TRAP-positive multinucleated cells showing more than three nuclei per well were considered to be osteoclasts.

Osteoblast cultures Osteoblasts isolated from mouse calvaria were seeded in 48-well plate at 1.3×10^4 cells/well in 400 μL α-MEM containing 10% FBS. When the cells reached 80% confluence, the medium was exchanged with α-MEM containing 10% FBS, and the cells were exposed to bacterial sonicate (*P. gingivalis* 0.1 μg/mL, *T. denticola* 1 μg/mL) with or without *A. asiatica* extract (50 μg/mL) for indicated time. The levels of RANKL, IL-1β, and TNF-α mRNAs were measured through RT-PCR, and the levels of PGE_2 in culture media were determined using enzyme immunoassay kits for PGE_2 (Amersham Bioscience, Uppsala, Sweden) according to the manufacture's instructions.

Reverse transcriptase-polymerase chain reaction (RT-PCR) mRNA expressions of RANKL, IL-1β, and TNF-α were determined by RT-PCR. Total RNA (1 μg) from the non-treated and treated osteoblasts were used as templates for cDNA synthesis in 20 μL reaction volume using an RT kit (CLONTECH, Palo Alto, CA, USA) according to the manufacturers instructions. cDNA (4 μL) was amplified by PCR in 50 μL reaction volume containing the 1 × PCR reaction buffer, 200 μM dNTPs, 200 pM each forward and reverse primers, and 0.5 units Taq DNA polymerase (Amersham Pharmacia Biotech., Little Chalfont, Buckinghamshire, UK) in a DNA thermal cycler (Biometra, Goettingen, Germany). The amplification reaction was performed for 35 cycles. The primer sequences and the annealing temperatures are presented in Table 1. The PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. To exclude DNA contamination in the isolated RNA, the RNA was subjected to PCR without cDNA synthesis as a negative control. In all preparations, no band was detected after PCR.

Statistical analyses Statistical differences were determined using a Kruskal-Wallis test. A p value<0.05 was considered significant.

Results and Discussion

Effect of *A. asiatica* on cell viability of mouse calvaria-derived osteblasts and bone marrow cells To examine the cytotoxicity of *A. asiatica*, osteoblasts and bone marrow cells were co-cultured in the absence and presence of *A. asiatica* extract (10-100 μg/mL) for 3 days, and the viability of cells was determined by MTT assay. At 100 μg/mL, *A. asiatica* showed 10% reduction in cell viability when compared with that of non-treated cells. Because no

Table 1. Sequences of primers for RANKL, IL-1β, TNF-α, and β-actin

Molecule	Direction	Primer sequence	Annealing Temp (°C)	Product size (bp)
RANKL	Forward	5-ATCAGAAGACAGCACTCACT-3	45.3	750
	Reverse	5-ATCTAGGACATCCATGCTAATGTTC-3		
TNF-α	Forward	5-TTCTGTCTACTGAACTTCGGGGTGATCGGTCC-3	60	354
	Reverse	5-GTATGAGATAGCAAATCGGCTGACGGTGTGGG-3		
IL-1β	Forward	5-ATGGCAACTGTTCCTGAACTCAAGT-3	50	563
	Reverse	5-CAGGACAGGTATAGATTCTTTCCTTT-3		
β-actin	Forward	5-GGACTCCTATGGTGGGTGACGAGG-3	58	366
	Reverse	5-GGGAGAGCATAGCCCTCGTAGAT-3		

cytotoxic effects were observed in cells at less than 50 μg/mL (Fig. 1), concentration less than 50 μg/mL was used in this study.

Effect of *A. asiatica* on osteoclastogenensis Osteoblasts are required for the differentiation of osteoclast progenitors (23, 24). Osteoclasts are TRAP-positive multinucleated cells derived from hematopoietic cells, which are present in bone marrow and spleen. Accordingly, a co-culture system of osteoblasts from mouse calvaria, and mouse bone marrow cells containing osteoclast progenitors were used in this study to determine the effect of *A. asiatica* on osteoclastogenesis (Fig. 2). TRAP-positive multinucleated cells containing more than three nuclei were defined as osteoclasts. Addition of sonicates from *P. gingivalis* (0.1 μg/mL) or *T. denticola* (1 μg/mL) to the co-cultures increased the number of osteoclast compared with that of non-treated co-cultures. In the co-culture treated with *P. gingivalis* and *A. asiatica* (25 and 50 μg/mL), the number of osteoclast decreased to that of the non-treated cultures. In the case of *T. denticola, A. asiatica* suppressed the osteoclast formation at concentrations higher than 10 μg/mL. These results suggest that *A. asiatica* inhibited up-regulation of osteoclast formation by *P. gingivalis* and *T. denticola.*

Effect of *A. asiatica* on expression of RANKL RANKL is an osteoclast formation inducing factor expressed in osteoblasts (1). When osteoblasts were treated with sonicate of *P. gingivalis* (0.1 μg/mL) or *T. denticola* (1 μg/mL), they

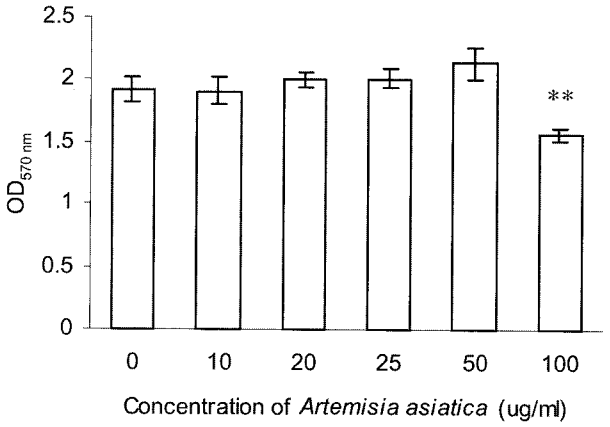

Fig. 1. Cytotoxic effects of *A. asiatica* on mouse calvaria-derived osteoblasts and bone marrow cells. The calvaria-derived osteoblasts and bone marrow cells were co-cultured in the absence or presence of *A. asiatica* extract (10-100 μg/mL). After 3 days, the viability of cells was determined by MTT assay. The data is represented as a mean±standard error for three cultures. $^{**}P<0.05$ for a comparison with the results for the nontreated cultures.

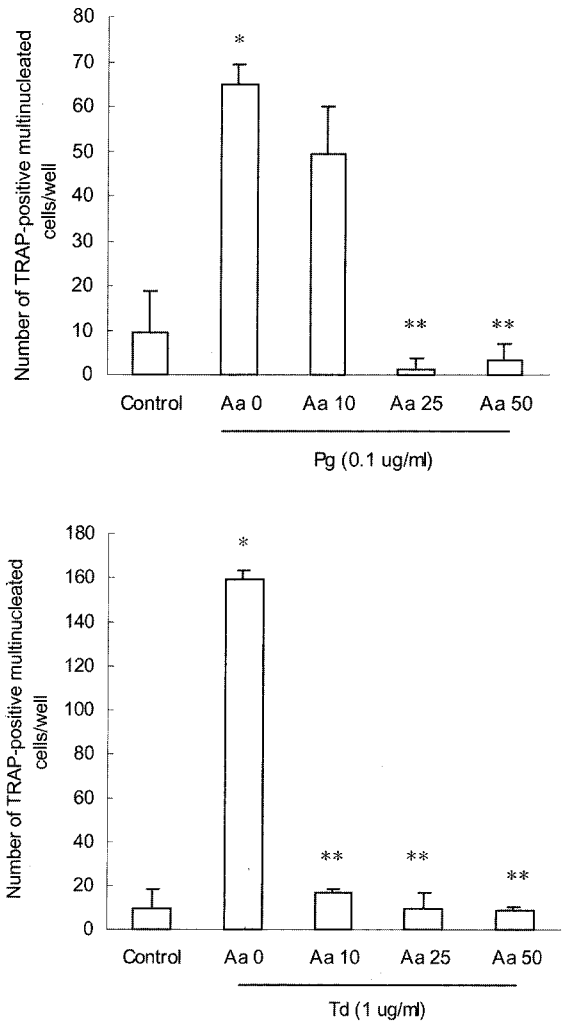

Fig. 2. Effects of *A. asiatica* on osteoclast formation induced by sonicates of *P. gingivalis* or *T. denticola*. Mouse bone marrow and calvaria-derived osteoblasts were co-cultured to be confluent and were treated with either *P. gingivalis* (Pg, 0.1 μg/mL) or *T. denticola* (Td, 1 μg/mL) in the absence or presence of ethanol extract of *A. asiatica* (Aa, 10-50 μg/mL) for additional 4 days. The cells were stained for TRAP. The TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. The data is represented as mean± standard error for three cultures. $^{*}P<0.05$ for a comparison with the results for the nontreated cultures and $^{**}P<0.05$ for a comparison with the results for the cultures treated with each bacterial sonicate.

expressed a higher level of RANKL mRNA compared with non-treated osteoblasts (Fig. 3). These results are consistent with the recent findings (12, 13), suggesting that both *P. gingivalis* and *T. denticola* stimulate osteoclastogenesis by up-regulating RANKL expression in osteoblasts.

To determine the mechanism involved in the inhibition of osteoclast formation by *A. asiatica*, the effects of *A. asiatica* (50 μg/mL) on the expression of RANKL in osteoblasts treated with sonicates of *P. gingivalis* (0.1 μg/mL) or *T. denticola* (1 μg/mL) were examined (Fig. 3). In both cases, *A. asiatica* completely blocked the elevation of RANKL mRNA expression by each bacterial sonicate. This suggests that the inhibitory action of *A. asiatica* on osteoclast formation is due to the suppression of RANKL expression in bacteria-treated osteoblasts.

Effect of *A. asiatica* on expression of PGE_2, IL-1β, and TNF-α RANKL expression is up-regulated by various factors including PGE_2, IL-1β, and TNF-α (1, 6). The activities of prostaglandin (14), TNF-α, and IL-1 (15) account for the bone resorption caused by *P. gingivalis*. The PGE_2 is involved in osteoclastogenesis induced by *T. denticola* (13). We found up-regulation of PGE_2, IL-1β, and TNF-α expressions by sonicates from *P. gingivalis* (0.1 μg/mL) and *T. denticola* (1 μg/mL) in osteoblasts (Fig. 4). These results indicate that PGE_2, IL-1β, and TNF-α are involved in the elevation of RANKL expressions by *P. gingivalis* and *T. denticola*.

To determine the inhibitory mechanism of RANKL expression by *A. asiatica*, the expression levels of PGE_2, IL-1β, and TNF-α were examined in osteoblasts treated with sonicates of *P. gingivalis* (0.1 μg/mL) and *T. denticola* (1 μg/mL) in the presence of *A. asiatica* (50 μg/mL) (Fig. 4). *A. asiatica* suppressed the up-regulation of PGE_2 production, and IL-1β and TNF-α mRNA expressions by each bacterial sonicate. These results indicate that inhibitory action of *A. asiatica* on RANKL expression may be mediated through the depression of PGE_2, IL-1β, and TNF-α expressions in bacteria-treated osteoblasts.

In summary, this study demonstrated that *A. asiatica* inhibits the osteoclast formation stimulated by *P. gingivalis* and *T. denticola*, and that the inhibition of osteoclastogenesis is mediated by down-regulation of RANKL, PGE_2, IL-1β, and TNF-α expressions. These results suggest that *A. asiatica* has anti-resorptive factors for the prevention of alveolar bone resorption in periodontitis patients.

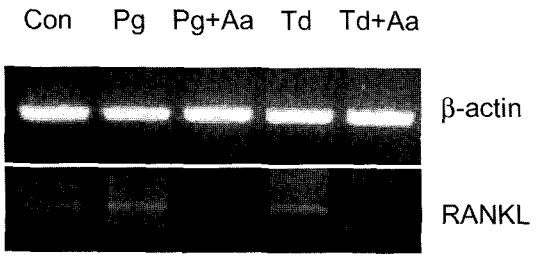

Fig. 3. The effects of *A. asiatica* on mRNA expression of RANKL in osteoblasts stimulated by sonicates of *P. gingivalis* or *T. denticola*. Osteoblasts were treated with sonicates of *P. gingivalis* (Pg, 0.1 μg/mL) or *T. denticola* (Td, 1 μg/mL) in the absence or presence of *A. asiatica* (Aa, 50 μg/mL) for 72 hr. The mRNA level of RANKL and β-actin were analyzed by RT-PCR. The data are representative of three cultures.

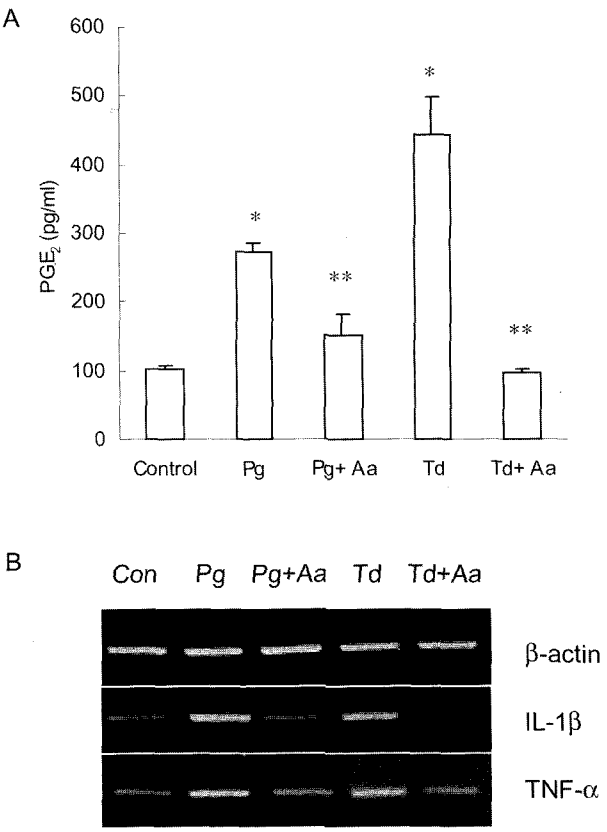

Fig. 4. The effects of *A. asiatica* on production of PGE_2 and expressions of IL-1β and TNF-α mRNA in osteoblasts stimulated by sonicates of *P. gingivalis* or *T. denticola*. Osteoblasts were treated with sonicates of *P. gingivalis* (Pg, 0.1 μg/mL) or *T. denticola* (Td, 1 μg/mL) in the absence or presence of *A. asiatica* (Aa, 50 μg/mL). A. After 72 hr of culture, the level of PGE_2 in culture media of osteoblast was determined by immunoassay. The data is represented as a mean ± standard error for three cultures. $*P<0.05$ for a comparison with the results for the nontreated cultures and $**P<0.05$ for a comparison with the results for the cultures treated with each bacterial sonicate. B. After 8 hr of culture, the mRNA levels of IL-1β, TNF-α, and β-actin were determined by RT-PCR. The data are representative for three cultures.

Eupatilin, which is a component of ethanol extract, has an inhibitory effect on the expression of COX-2, an inducer of PGE_2 synthesis (18). Because the inhibition of PGE_2 production by *A. asiatica* is suggested to play a role in the repression of bacteria-induced osteoclastogenesis, eupatilin may be one of the anti-bone resorptive factors. The precise involvement of eupatilin as well as other anti-resorptive factors are currently under investigation.

Acknowledgments

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