Inhibitory effects of *Artemisia asiatica* on osteoclast formation induced by periodontopathogens

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감사의 글

연구 시작단계부터 본 논문의 완성까지 항상 자상하게 지도해 주시고 너무나 많은 배려를 해 주신 유윤정 교수님께 진심으로 깊은 감사를 드리며, 논문실험 시작부터 과정 내내 깊은 관심과 조언을 아끼지 않으신 최성호 교수님께도 깊은 감사의 말씀을 드립니다. 그리고 많은 조언과 논문의 작성에서 논리적 사고와 과학적 서술의 중요성을 일깨워주신 차정헌 교수님께 감사드립니다. 실험에 많은 도움을 주시고 항상 친절히 질문에 답해주신 조교선생님들께도 감사의 마음을 전합니다.

항상 애정을 갖고 지켜봐주시는 김종관 교수님을 비롯한 모든 치주과 교수님, 구강생물학교실 이승일 교수님께 진심으로 감사드립니다.

한결 같은 사랑으로 보살펴주신 양가 부모님, 친척, 친지 마지막으로 예쁘지만 너무도 별난 경빈, 경원과 매일 씨름하는 아내 지연과 함께 이 기쁨을 나누고 싶습니다.

도움을 주신 모든 분들께 감사드립니다.

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Abstract

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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 system of mouse osteoblasts and bone marrow cells. As previously reported, bacterial sonicates of *Porphyromonas gingivalis* and *Treponema denticola* induced osteoclastogenesis. *A. asiatica* ethanol extract abolished the bacteria-induced osteoclastogenesis. To determine inhibitory mechanism of *A. asiatica* against osteoclastogenesis, effects of *A. asiatica* on expressions of osteoclastogenesis-inducing factors, receptor activator of NF-κB ligand (RANKL), prostaglandin E_2 (PGE_2), interleukin (IL)-1β, 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 bacterial sonicate. These results

suggest inhibitory action of *A. asiatica* against the bacteria-induced 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

Inhibitory effects of *Artemisia asiatica* on osteoclast formation induced by periodontopathogens

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I. 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 (Takahashi et al., 1999; Hofbauer et al., 2000). Osteoblasts express the receptor activator of nuclear factor-κB (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 (Hsu et al., 1999). Prostaglandin E_2 (PGE_2), interleukin (IL)-1β, and tumor necrosis factor (TNF)-α up-regulate RANKL expression in osteoblasts and induce osteoclastogenesis (Yasuda et al., 1998; Tsukii et al., 1998; Hofbauer et al., 1999; Walsh et al., 2003).

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 (Schwartz et al., 1997). 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 (Kigure et al., 1995; Holt et al., 1999; Sela et al., 2001). RANKL-positive osteoblastic cells were reported in mice injected with *P. gingivalis* (Jiang et al., 2002). In osteoclast formation induced by *T. denticola*, PGE_2 was associated with an increase in RANKL expression by lipooligosaccharide of *T. denticola* (Choi et al., 2003). A study on mice injected with *P. gingivalis* demonstrated that *P. gingivalis*–induced bone resorption was partially mediated by prostaglandin (Zubery et al., 1998). In addition, *P. gingivalis* LPS–induced bone resorption was significantly reduced in mice lacking IL-1 and TNF receptor (Chiang et al., 1999). 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 chemical-induced liver damage (Ryu et al., 1998) and hepatic fibrosis (Cheong et al., 2002). 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* (Heo et al., 2001). Antimicrobial properties of *A. asiatica* were demonstrated against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* (Kalemba et al., 2002). 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 (Seo et al., 2002). 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*.

II. Materials and Methods

1. 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).

2. Preparation of bacteria sonicates

P. gingivalis (ATCC 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 (Wyss et al.,1996). 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℃, and the supernatant was collected. The protein concentrations were determined using a Coomassie brilliant protein assay reagent (Pierce, Rockford, IL, USA).

3. Preparation of primary osteoblasts and bone marrow cells

The osteoblasts were isolated from the calvaria of 1or 2-day-old ICR mice (Bio Korea Co., Seoul, Korea) as previously described (Choi et al., 2001). The calvaria

was 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℃ 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.

4. 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℃. 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.

5. 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 x 10^4 cells/well) were co-cultured with the bone marrow cells (1 x 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, and *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 manufacturer's instructions. The TRAP-positive multinucleated cells showing more than three nuclei per well were considered to be osteoclasts.

6. Osteoblast cultures

Osteoblasts isolated from mouse calvaria were seeded in 48-well plate at 1.3 X 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.

7. 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 manufacturer's instructions. cDNA (4 μL) was amplified by PCR in 50 μL reaction volume containing the 1 x 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.

8. Statistical analyses

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

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		

III. Results

1. 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 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.

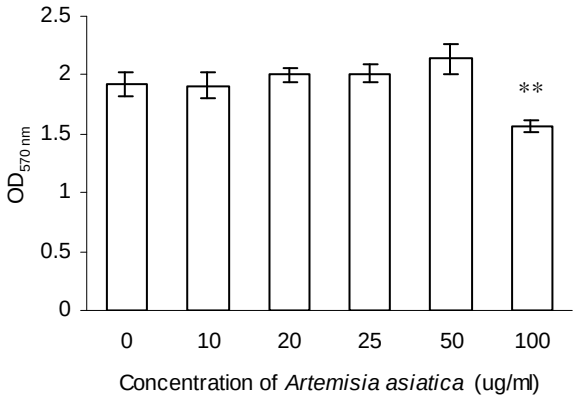

Fig. 1. Cytotoxic effects of *A. asiatica* on mouse calvaria-derived osteoblasts and bone marrow cells. The calvaria-derived osteoblasts and bone marrow cells were co-cultured in the absence or presence of *A. asiatica* extract (10-100 μg/mL). After 3 days, the viability of cells was determined by MTT 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.

2. Effect of *A. asiatica* on osteoclastogenensis

To determine the effect of *A. asiatica* on osteoclast formation, a co-culture system of osteoblasts from mouse calvaria, and mouse bone marrow cells containing osteoclast progenitors was used in this study (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.

3. Effect of *A. asiatica* on expression of RANKL

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). When osteoblasts were treated with sonicate of *P. gingivalis* (0.1 μg/mL) or *T. denticola* (1 μg/mL), they expressed a higher level of RANKL mRNA compared with non-treated osteoblasts. *A. asiatica* completely blocked the elevation of RANKL mRNA expression by each bacterial sonicate.

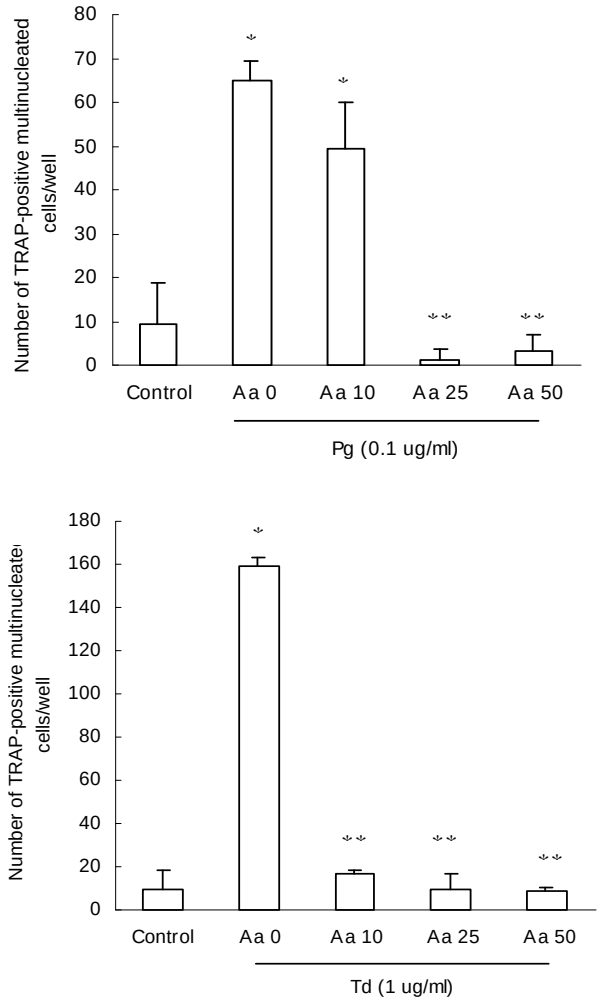

Fig. 2. Effects of *A. asiatica* on osteoclast formation induced by sonicates of *P. gingivalis* or *T. denticola*. Mouse bone marrow and calvaria-derived osteoblasts were co-cultured to be confluent and were treated with either *P. gingivalis* (Pg, 0.1 μg/mL) or *T. denticola* (Td, 1 μg/mL) in the absence 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.

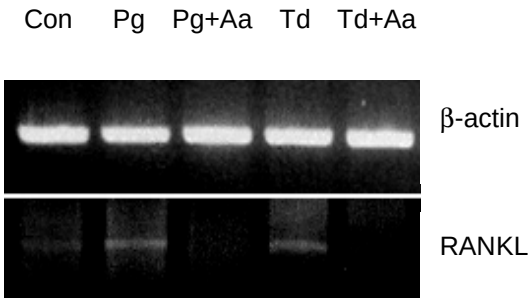

Fig. 3. The effects of *A. asiatica* on mRNA expression of RANKL in osteoblasts stimulated by sonicates of *P. gingivalis* or *T. denticola*. Osteoblasts were treated with sonicates of *P. gingivalis* (Pg, 0.1 μg/mL) or *T. denticola* (Td, 1 μg/mL) in the absence or presence of *A. asiatica* (Aa, 50 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.

4. Effect of *A. asiatica* on expression of PGE_2, IL-1β, and TNF-α

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). Sonicates from *P. gingivalis* (0.1 μg/mL) and *T. denticola* (1 μg/mL) up-regulated mRNA expression of PGE_2, IL-1β, and TNF-α in osteoblasts. *A. asiatica* suppressed the up-regulation of PGE_2 production, and IL-1β and TNF-α mRNA expressions by each bacterial sonicate.

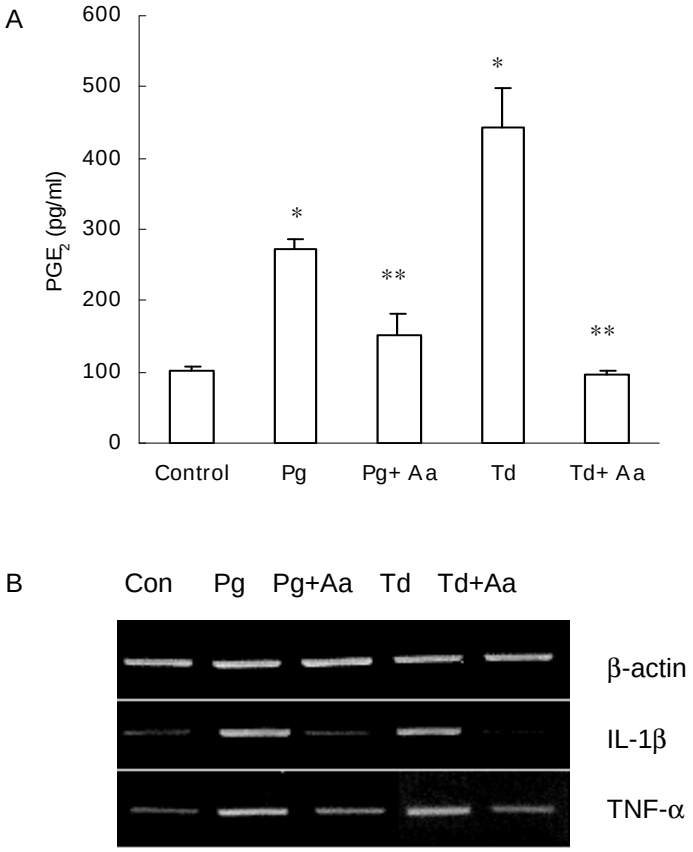

Fig. 4. The effects of *A. asiatica* on production of PGE_2 and expressions of IL-1β and TNF-α mRNA in osteoblasts stimulated by sonicates of *P. gingivalis* or *T. denticola*. Osteoblasts were treated with sonicates of *P. gingivalis* (Pg, 0.1 μg/mL) or *T. denticola* (Td, 1 μg/mL) in the absence or 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.

IV. Discussion

In present study, it was firstly demonstrated that *A. asiatica* possesses inhibitory activity against osteoclast formation induced by periodontopathogens, such as *P. gingivalis* and *T. denticola*. Osteoblasts are required for the differentiation of osteoclast progenitors (Takahashi et al., 1988; Suda et al., 1997). Therefore, to investigate the effect of *A. asiatica* on osteoclast formation, a co-culture system consisting of mouse calvarial cells which contained osteoblasts and bone marrow cells which contained osteoclast precursors was used. It was reported that lipopolysaccharide (LPS) and Fimbriae of *P. gingivalis* and LPS of *T. denticola* increased osteoclast formation (Choi et al., 2003; Hiramine et al., 2003; Miyata et al., 1997). In this study, sonicates from *P. gingivalis* and *T. denticola* induced osteoclast formation and *A. asiatica* inhibited the induction of the osteoclast formation by these bacterial sonicates. These results suggest that *A. asiatica* inhibited bone resorption induced by *P. gingivalis* and *T. denticola* via down-regulation of osteoclast formation.

RANKL is an osteoclast formation–inducing factor expressed in osteoblasts (Takahashi et al., 1999). LPS of *T. denticola* increased expression of RANKL in co-cultures of bone cells (Choi et al., 2003). *P. gingivalis* infection increased RANKL expression in culture of osteoblasts (Okahashi et al., 2004) and RANKL-positive osteoblasts were reported in mice injected with *P. gingivalis* (Jiang et al., 2002). In

this study, osteoblasts treated with sonicate of *P. gingivalis* or *T. denticola* expressed a higher level of RANKL mRNA. These results suggest that both *P. gingivalis* and *T. denticola* stimulate osteoclastogenesis by up-regulating RANKL expression in osteoblasts. *A. asiatica* completely blocked the elevation of RANKL mRNA expression by each bacterial sonicate. This means that the inhibitory action of *A. asiatica* on osteoclast formation is due to the suppression of RANKL expression in bacteria-treated osteoblasts.

RANKL expression is up-regulated by various factors including PGE_2, IL-1β, and TNF-α (Takahashi et al., 1999; Hofbauer et al., 1999). The activities of prostaglandin, TNF-α, and IL-1 account for the bone resorption caused by *P. gingivalis* (Zubery et al., 1998; Chiang et al., 1999). The PGE_2 is involved in osteoclastogenesis induced by *T. denticola* (Choi et al., 2003). We found up-regulation of PGE_2, IL-1β, and TNF-α expressions by *P. gingivalis* and *T. denticola* in osteoblasts. 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 these factors were examined in osteoblasts. *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.

Eupatilin, which is a component of ethanol extract, has an inhibitory effect on the expression of COX-2, an inducer of PGE_2 synthesis (Heo et al., 2001). 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 to determine the mechanism involved in the inhibition of osteoclast formation by *A. asiatica.*

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.

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국문요약

*A. asiatica*의 치주염관련세균에 의한 파골세포 형성 억제효과

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치주질환에서의 치조골 흡수는 치아상실의 주요한 원인이다. *Artemisia asiatica*의 골흡수 억제능을 평가하기 위하여 *A. asiatica*가 치주질환을 일으키는 원인균인 *Porphyromonas gingivalis*와 *Treponema denticola*에 의한 파골세포형성에 미치는 영향을 평가하였다. *A. asiatica*의 골흡수 억제능은 두개골과 골수세포를 사용한 혼합배양을 이용하여 평가하였다. *P. gingivalis*와 *T. denticola*분쇄액은 tartrate-resistant acid phosphatase (TRAP) 발현 다핵 세포의 수를 증가시켰다. *A. asiatica* 에탄올 추출물은 이들세균 분쇄액에 의한 파골세포형성을 억제하였다. *P. gingivalis*와 *T. denticola*의 균분쇄액은 receptor activator of NF-κB ligand (RANKL), prostaglandin E_2 (PGE_2), interleukin (IL)-1β, and tumor necrosis factor (TNF)-α의 발현을 증가시켰으며 *A. asiatica* 추출물은 이들 세균에 의한 파골세포형성

유도인자인 RANKL, PGE_2, IL-1β, and TNF-α의 발현을 저하시켰다. 이상의 결과는 *A. asiatica*의 파골세포형성 방해기전이 RANKL, PGE_2, IL-1β, and TNF-α의 발현감소와 연관되어 있음을 시사한다.

핵심단어: *Artemisia asiatica*, 파골세포, *Porphyromonas gingivalis*, *Treponema denticola*, RANKL