Involvement of PGE_2, IL-1β, and TNF-α in RANKL-Dependent Osteoclastogenesis Induced by *Porphyromonas gingivalis*, *Treponema denticola* and *Treponema socranskii*

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감사의 말씀

본 논문이 완성되기 까지 세심한 지도와 배려로 연구할 수 있게 도와 주시고 가르침을 주신 유윤정 선생님께 감사 드립니다. 많은 충고와 조언으로 좋은 논문이 되도록 힘써주신 최봉규 선생님, 차정헌 선생님께 진심으로 감사를 드립니다. 실험에 많은 도움을 주신 강정화 선생님, 문선영 선생님께도 감사의 마음을 전하고 싶습니다.

여기에 오기까지 항상 묵묵히 지켜봐 주시고, 사랑으로 응원해 주신 아버지 어머니께 감사 드립니다.

또한, 저에게 학문의 길을 항상 권유해 주시고 도와 주신 김명래 선생님께도 감사를 드립니다.

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Table of contents

List of Figures

List of Table

Abstract

Periodontitis is an inflammatory disease that often leads to destruction of alveolar bone. Multiple species of bacteria in subgingival plaque are associated with bone destruction in periodontitis. In this kind of bone destruction, the osteoclast is known to play a key role. To determine the mediators which are involved in osteoclastogenesis by periodontopathogens, we studied the effect of three periodontopathogens, *Porphyromonas gingivalis*, *Treponema denticola*, and *Treponema socranskii* on osteoclastogenesis in coculture system of mouse calvaria derived osteoblastic cells and bone marrow cells. The expression of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, receptor activator of NF-κB ligand (RANKL) and prostaglandin E_2 (PGE_2) in mouse calvaria cells was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) or immunoassay. Sonicates of three bacteria induced osteoclast formation in coculture systems and the mRNA expression of IL-1β, TNF-α, and RANKL in osteoblastic cells. The production of PGE_2 was increased by three bacteria sonicates. Addition of osteoprotegerin (OPG), which is an inhibitor of RANKL, in the cocultures resulted in the complete suppression of the induction of the osteoclast formation. Anti-IL-1β antibody (Ab), anti-TNF-α Ab and indomethacin, which is an inhibitor of PGE_2, partially inhibited the induction of the osteoclast formation by each bacteria. In addition, indomethacin, anti-IL-1β Ab, or anti-TNF-α Ab decreased RANKL expression of osteoblastic cells treated with bacterial sonicates of *P. gingivalis*, *T. denticola*, and *T. socranskii.* These findings suggest that increased RANKL expression of osteoblastic cells may play an important role in the osteoclast formation induced by *P. gingivalis*, *T. denticola*, and *T. socranskii* and that PGE_2, IL-1β, and TNF-α are mediators for the induction of RANKL expression by these bacteria.

Key Words: *Porphyromonas gingivalis*, *Treponema denticola*, *Treponema socranskii.* osteoclast, interleukin-1β, tumor necrosis factor-α, receptor activator of NF-κB ligand, prostaglandin E_2

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Introduction

Since the multinucleated osteoclast plays a crucial role in bone resorption, the research on the osteoclast formation has been taken an important place in bone biology. Osteoclast is derived from colony forming unit–macrophage (CFU-M) through multiple steps including the expression of tartrate resistant acid phosphatase (TRAP), fusion of cells, and the formation of clear zone and ruffled border. Osteoclast differentiation requires for the osteoblasts or bone marrow stromal cells.[1,2] One of the osteoclastogenesis factors expressed by osteoblasts or bone marrow stromal cells is receptor activator of nuclear factor *k*B ligand (RANKL, also known as osteoclast differentiation factor). RANKL plays an essential role on the osteoclast differentiation. This factor interacts with the receptor called RANK on osteoclast surface or on its

precursor for differentiation or activation of osteoclast.[1,3,4,] There are multiple factors that lead to bone destruction and it has been suggested that these multiple factors increase RANKL expression of osteoblasts or stromal cells. RANKL expression was up-regulated, when the osteoblasts or stromal cells were stimulated by parathyroid hormon (PTH), 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), prostaglandin E_2 (PGE_2), interleukin-1 (IL-1), and tumor necrosis factor (TNF).[5,6,7,8] However, TNF-α treatment of RANK knockout mice induced TRAP-positive-cells at the site of injection.[9] This result suggests that the RANKL-RANK interaction is not the only way to induce the osteoclast differentiation.

Alveolar bone resorption followed by loss of teeth is clinically the most important issue in periodontitis. Bacteria harboured in periodontal pockets play a major role in bone destruction. As periodontitis progresses, the microbial flora of dental plaque has changes on its dominant bacteria and anaerobic gram negative bacteria establish late in the development of dental plaque. *Porphyromonas gingivalis* has been considered as one of the most important pathogenic microorganism associated with periodontal disease.[10,11] Some previous studies showed that cell wall components of *P. gingivalis*, such as fimbriae, lipopolysaccharide (LPS), and other surface-associated materials were potent stimulator of osteoclast differentiation and bone resorption via IL-1, IL-6, TNF, and PGE_2. [12,13,14]

Spirochetes can represent up to 50% of the detectable bacteria in subgingival plaque from patients with chronic adult periodontitis.[15] *Treponema denticola*, which is one of the oral spirochetes, has been most intensively studied in terms of its virulence factors. This bacterium is able to destruct host tissue by its proteolytic activity and cytotoxicity.[16,17] Expecially, LPS-like substance existing in the outer membrane of *T. denticola* increased calcium released from calvaria bone.[18] Also, *T. denticola* increased the number of osteoclast formation and up-regulation of the RANKL expression via PGE_2-dependant machanism was involved in this process.[19] These findings suggest that *T. denticola* may contribute to the alveolar bone resorption. *Treponema socranskii*, which is another specie of treponeme, predominates in deep periodontal pocket. *T socranskii* has a protease activity[20], viscosity-dependant locomotion, laminin[21] and collagen binding protein[22]. However, the relationship between *T. socranskii* and bone destruction is little known.

Periodontitis is a polymicrobial infection. Therefore it is important to clarify whether there is a common pathway of bone destruction induced by periodontopathogens. There were two groups tried to compare the machanism of bone resorption caused by each periotontopathic bacteria. LPS from *P. gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* promoted osteoclastic cell formation in bone marrow cell cultures.[23] Bone resorption in mouse calvaria was caused by three periodontal pathogens, *P.*

gingivalis, *Campylobacter rectus*, and *Fusobacterium nucleatum,* and it was mediated in part by prostaglandin.[24] Although these results suggest that periodontopathic bacteria may stimulate bone destruction in similar mechanism, the more precise mechanism such as involvement of cytokines and RANKL has not been yet discovered.

In clinical study, coinfection of *P. gingivalis* and *T. denticola* was frequently observed in periodontitis.[25] *P. gingivalis* and *T. denticola* coaggregated strongly[26] and showed a nutritional relationship.[27] Recently, it was reported that *P. gingivalis*, *T. denticola*, and *T. socranskii* were associated with severity of periodontal tissue destruction.[28] Therefore, to identify whether there is a common pathway of bone destruction by periodontopathogens, we compared the mechanisms of the osteoclastogenesis induced by these bacterial sonicates *P. gingivalis*, *T. denticola*, and *T. socranskii.* We found that three periodontopathic bacteria induce osteoclast formation via RANKL-dependent pathway and the RANKL expression is mediated by PGE_2, IL-1β, and TNF-α.

Materials and Method

Materials

The mice (ICR strain) were obtained from Bio Korea Co. (Seoul, Korea). The α-minimum essential medium (α-MEM) and heat-inactivated fetal bovine serum (FBS) were purchased from GIBCO BRL (Grand Island, NY). Indomethacin and tartrate-resistant acid phosphatase (TRAP; a marker of osteoclast) staining kit were obtained from Sigma (St. Louis, MO). OPG, anti-IL-1β antibody (IL-1β Ab), and anti-TNF-α Ab were purchased from R&D systems (Minneapolis, USA)

Method

Preparation of bacteria sonicates. *P. gingivalis* (ATCC 33277) were cultured anaerobically in barin-heart infusion medium containing hemin (5 μg/ml) and menadione (0.5 μg/ml) for 2 days. *T. denticola* (ATCC 33521) *and T. socrnaskii* (ATCC 33536) were cultured anaerobically in an OMIZ-PAT broth for 3-5 days, as described previously.[46] Bacterial cells were harvested by centrifuging at 5,000 x g for 10 min at 4℃. The cells were then washed 3 times with phosphate buffered saline (PBS). The bacterial cells were then disrupted for 5 min using an ultrasonic processor (Sonic Dismembrator, Fisher

Scientific, Pittsburgh, PA) at an output power of 8 watts with 20 sec intervals. The cell debris was removed after centrifuging at 15,000 × g 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).

Preparation of primary calvaria and bone marrow cells. The osteoblastic cells were isolated from the calvariae of 1-2-day-old ICR mice as previously described.[5] The calvariae were digested in 10 ㎖ of α-MEM containing 0.2% collagenase (Wako Pure Chemicals, Osaka, Japan) and 0.1% dispase (GIBCO BRL, Grand Island, NY) for 20 min at 37℃ with vigorous shaking, and then centrifuged at 1,500 ×g for 5 min. The first supernatant was discarded and another 10 ㎖ of the collagenase/dispase enzyme solution was added and incubated for 20 min. The digestion was repeated 4 times and the cells isolated by the last three digestions were combined as an osteoblastic cells. They were cultured in α-MEM containing 10% FBS, antibiotics solution (100 U/㎖ of penicillin, 100 ㎍/㎖ of streptomycin, 25 ㎍/㎖ of amphotericin B) and used for the coculture 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 at one end using a 25 gauge needle. The marrow cells were washed and used for the coculture.

Osteoclast formation assay. The isolated calvaria cells were seeded at a concentration of 10^6 cells in a 10 cm culture dish and grown to confluence. The cells were then detached from the culture dishes by trypsin-EDTA (GIBCO BRL, Grand Island, NY). Subsequently, the cells ($1x10^4$ cells/well) were cocultured with the bone marrow cells ($1 x10^5$ cells/well) in α-MEM containing 10% FBS in 48 well plates (Corning Inc., NY). The culture volume was made up to 200 μℓ per well with α-MEM medium containing 10% FBS. The bacteria sonicates was added to the coculture with or without OPG, indomethacin, anti-IL-1β Ab, or anti-TNF-α Ab after exchanging the medium on day 3. The coculture was then maintained for an additional 4 days. Osteoclast differentiation was monitored using a TRAP staining kit according to the manufacturer's instruction. The TRAP-positive multinucleated cells showing more than 3 nuclei per well were counted as an osteoclast.

Osteoblastic cell cultures. Osteoblastic cells isolated from mouse calvariae were seeded in 48-well plate at a density of $1.3×10^4$ cells/dish in ml α-MEM containing 10% FBS. When the cells grew to 80% confluence, the medium was exchanged with α-MEM containing 10%FBS and cells were exposed to bacteria sonicates alone or in combination with anti-IL-1β Ab, anti-TNF-α Ab for indicated times. To determine mRNA expression of RANKL and

cytokines, mRNA was isolated from the cultured osteoblastic cells by TRIzol reagent according to the manufacturer's protocol (Life Technologies, Inc., Grand Island, NY). The concentration of PGE_2 and RANKL in culture media was measured by enzyme immunoassay.

Reverse transcriptase-polymerase chain reaction (RT-PCR). RANKL, IL-1β, and TNF-α mRNA expression was determined by RT-PCR. The total RNA (1 μg) from the non-treated and treated osteoblastic cells was used as a template for cDNA synthesis in a 20 μℓ of reaction volume using an RT kit (CLONTECH, Palo Alto, CA) according to the manufacturer's instructions. The RNA (1 μg) and oligo$(dT)_{18}$ primer (1 mM) were denatured at 70℃ for 5 min and incubated 1-2 min on ice. The denatured RNA and oligo$(dT)_{18}$ primers were added to the reaction mixture (1 unit/μℓ MMLV reverse transcriptase; 1× reaction buffer; 500 μM of each dATP, dCTP, dGTP and dTTP; 20 units of recombinant RNase inhibitor), and incubated at 42℃ for 60 min followed by 94℃ for 5 min.

The cDNA (4 μℓ) was amplified by PCR in a 50 μℓ reaction volume containing the 1 x PCR reaction buffer, 200 μM dNTPs, 200 pM of the forward and reverse primers, and 0.5 units of 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 and primer sequences and 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. The relative intensity of the gel bands was measured using an image-analyzing program (TINA 2.0e, Neuro-Image analysis Centre, Oxford, UK). In order to exclude DNA contamination in the isolated RNA, the RNA was subjected to PCR without cDNA synthesis. In all preparations, no band was detected after PCR.

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′		

Immunoassay for RANKL and PGE_2 The level of PGE_2 and RANKL in culture media of osteoblasts were determined using a PGE_2 enzyme immunoassay kit (Amersham Bioscience, Uppsala, Sweden) and RANKL enzyme immunoassay kit (R&D systems, Minneapolis, USA) according to the

manufacture's instruction.

Statistical analyses. The statistical differences were determined by Mann-Whitney U test. A p value < 0.05 was considered significant.

Results

Osteoclast formation induced by sonicates of periodontopathogens.

The osteoclast forming activity of *P. gingivalis, T. denticola*, and *T. socranskii* was determined in coculture system of mouse calvaria-derived osteoblastic cells and bone marrow cells (Fig. 1). TRAP-positive multinucleated cells which had 3 or more nuclei were defined as osteoclasts and counted. In untreated cultures, the number of TRAP-positive multinucleated cells were less than 50. Addition of sonicates from *P. gingivalis, T. denticola,* or *T. socranskii* increased the number of TRAP-positive multinucleated cells. Maximum effect of *P. gingivalis* sonicates was observed at 0.01-0.1 μg/ml. *T. denticola* sonicates increased the number of TRAP-positive cells in a dose dependent manner in the range of 0.1-1 μg/ml, and the number of TRAP-positive cells was decreased at 10 μg/ml when compared with 1 μg/ml sonicates-treated cells. In cells treated with *T. soncranskii* sonicates, maxiumum effect was maintained at the range of 0.1-10 μg/ml.

Effect of bacteria sonicates on expression of RANKL, cytokines, and PGE_2

To elucidate the effect of periodontopathogens on expression of RANKL, IL-1β, and TNF-α which are involved in osteoclastogenensis, The mRNA level of RANKL, IL-1β, and TNF-α was observed in osteoblastic cells

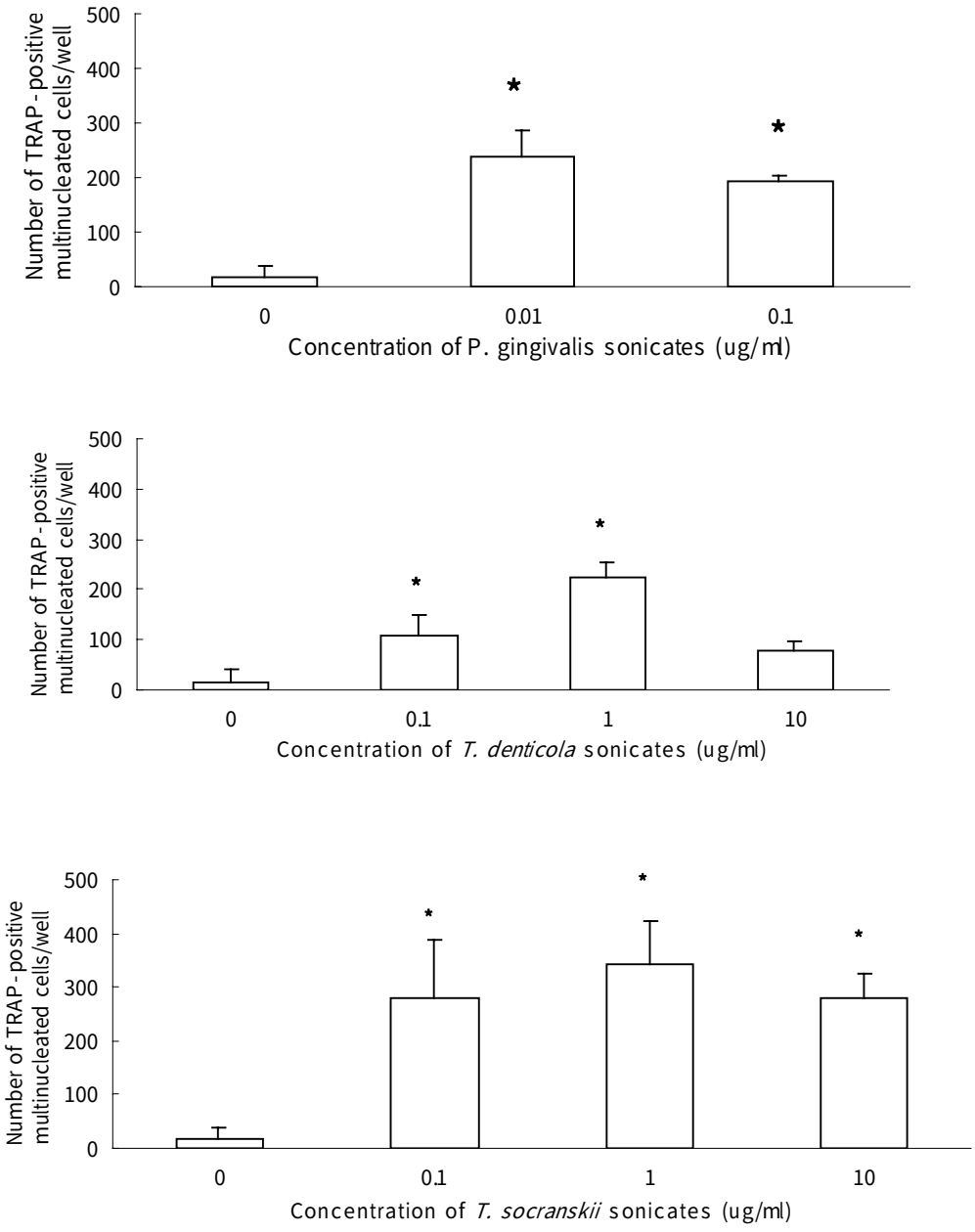

Figure 1. Osteoclastogenesis induced by sonicates of *Porphyromonas gingivalis*, *Treponema denticola*, and *Treponema socranskii*. Mouse bone marrow and calvarial cells were cocultured to confluence and treated with sonicates of *P. gingivalis* (0.01-

0.1 μg/ml), *T. denticola* (0.1-10 μg/ml) or *T. socranskii* (0.1-10 μg/ml) for additional 4 days. The cells were then stained for TRAP. The TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. The data are the means ± standard errors for four cultures. * P <0.05 for a comparison with the results for the nontreated cultures.

treated with sonicates of *P. gingivalis* (0.1 μg/ml), *T. denticola* (1 μg/ml) or *T. socranskii* (1μg/ml) (Fig. 2). Untreated osteoblastic cells expressed low level of RANKL, IL-1β, and TNF-α mRNA. When osteoblastic cells were treated with *P. gingivalis* sonicates, the mRNA level of RANKL, IL-1β, and TNF-α was increased. Sonicates of *T. denticola* and *T. socranskii* also increased the expression of RANKL, IL-1β, and TNF-α mRNA. Immunoassay for PGE_2 revealed a significant increase in the amount of PGE_2 in the culture supernatants of osteoblastic cells treated with sonicates of each bacteria (Fig 3). Indomethacin, which is inhibitor of PGE_2 synthesis, completely suppressed PGE_2 production of treated cells.

Involvement of RANKL, PGE_2, IL-1β, and TNF-α in osteoclastogenesis induced by three periodontopathogens.

To confirm the involvement of RANKL, PGE_2, IL-1β, and TNF-α in periodontopathogen–inudced osteoclastogenesis, cocultures was treated with sonicates of *P. gingivalis* (0.1 μg/ml), *T denticola* (1 μg/ml) or *T. socranskii* (1 μg/ml) in the presence or absence of OPG, indomethacin, anti

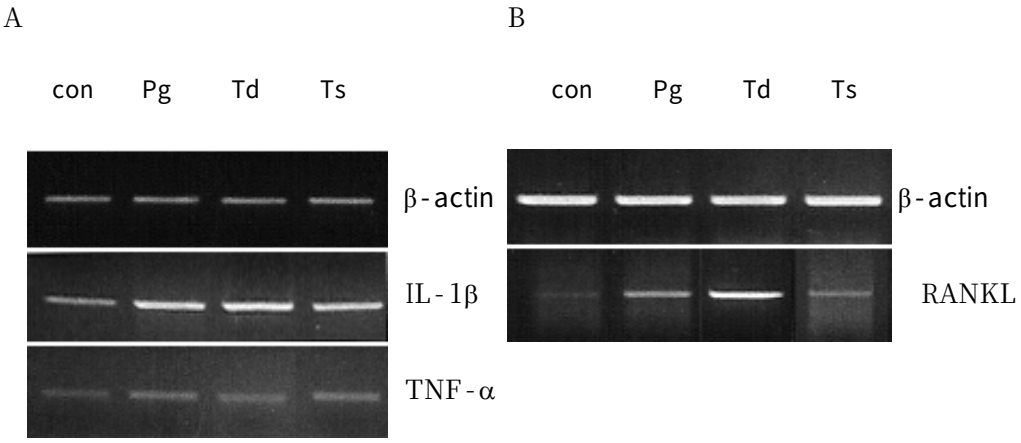

Figure 2. mRNA expression of RANKL, IL-1β, and TNF-α in osteoblastic cells treated with sonicates of *Porphyromonas gingivalis*, *Treponema denticola*, or *Treponema socranskii*. A, Osteoblastic cells isolated form mouse calvaria were treated with sonicates of *P. gingivalis* (Pg, 0.1 μg/ml*), T. denticola* (Td, 1 μg/ml) or *T. socranskii* (Ts, 1 μg/ml) for 8 h. The RNA was isolated from the treated cells. The mRNA level of IL-1β, TNF-α, and β-actin was analyzed by RT-PCR. B, Mouse calvaria-derived osteoblastic cells were cultured in the presence of sonicates of *P. gingivalis* (0.1 μg/ml), *T. denticola* (1 μg/ml) or *T. socranskii* (1 μg/ml) for 72 h and, then, the RNA was isolated from cultured cells. The mRNA level of RANKL was determined by RT-PCR.

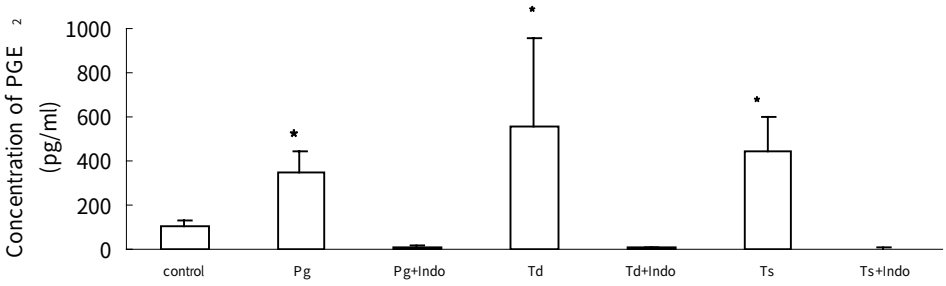

Figure 3. Production of PGE_2 in calvaria-derived osteoblastic cells treated with sonicates of *Porphyromonas gingivalis*, *Treponema denticola*, and *Treponema socranskii*. Calvaria-derived osteoblastic cells were treated with sonicates of *P. gingivalis* (Pg, 0.1 μg/ml), *Treponema denticola* (Td, 1 μg/ml), or

Treponema socranskii (Ts, 1 μg/ml) for 72h in the absence or presence of indomethacin (1 mM). The PGE_2 concentration was determined by using a PGE_2 enzyme immunoassay kit. The data are the means ± standard errors for three cultures. * P <0.05 for a comparison with the results for the nontreated cells. ** P <0.05 for a comparison with the results for sonicates treated cells

IL-1β Ab or anti-TNF-α Ab for 4 days. OPG is a decoy receptor for RANKL and bind to RANKL to inhibit its activity.[4] Indomethacin is an inhibitor of PGE_2 synthesis. OPG, indomethacin, anti-IL-1β Ab, and anti-TNF-α Ab decreased formation of TRAP-positive multinucleated cells when compared with each sonicate treated cells (Fig. 4). Addition of OPG in cocultures completely reduced the number of TRAP-positive cells to the level of non treated cells. Indomethacin,

anti-IL-1β Ab, and anti-TNF-α Ab partially decreased the number of TRAP-positive multinucleated cells induced by *P. gingivalis*, *T. denticola* or *T. socranskii* sonicates. OPG, indomethacin, anti-IL-1β Ab or anti-TNF-α Ab did not induce the formation of TRAP-positive multinucleated cells.

Involvement of PGE_2, IL-1β, and TNF-α on expression of RANKL induced by periodontopathogens.

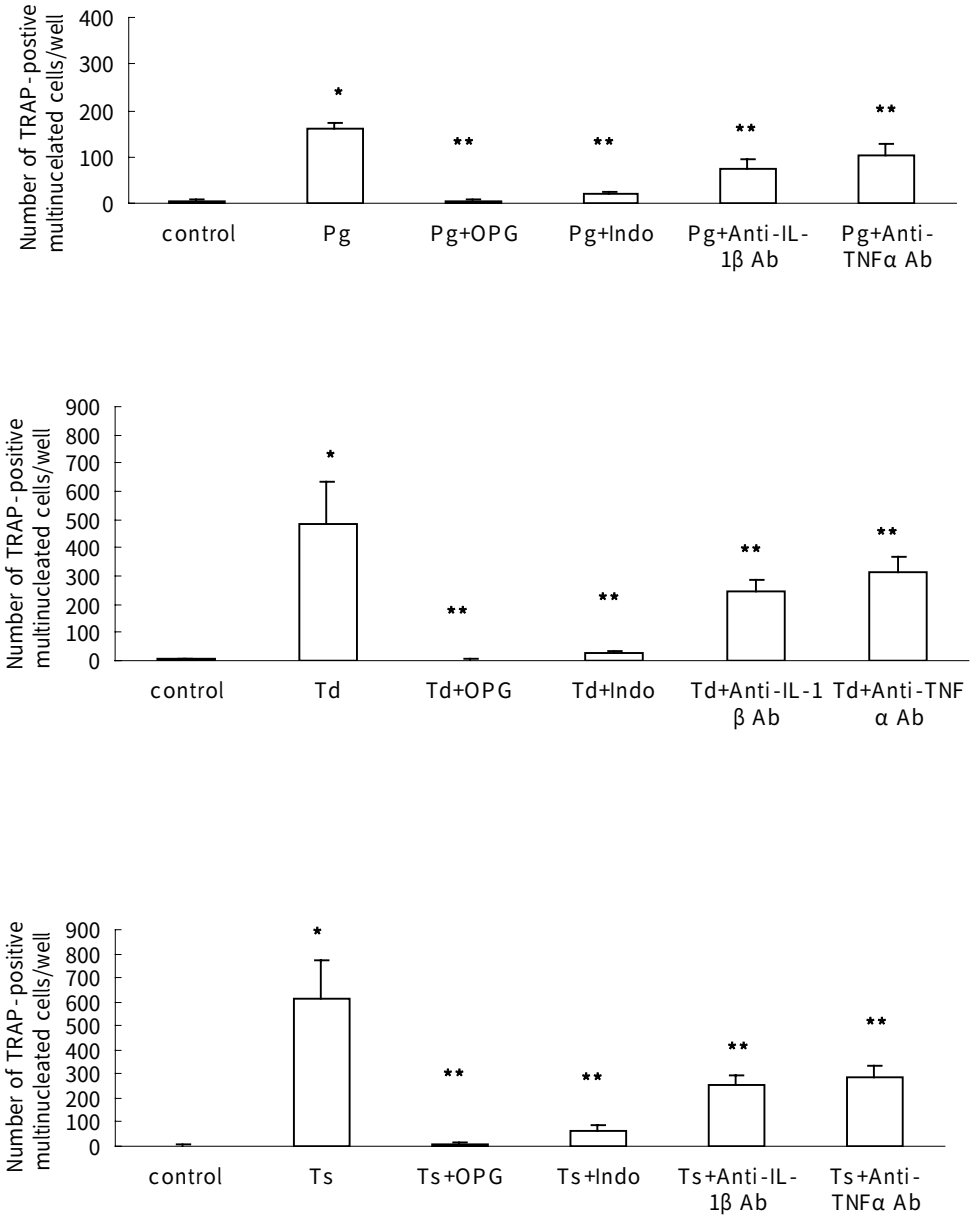

Figure 4. The effect of OPG, indomethacin, anti-IL-1β Ab, and anti-TNF-α Ab on osteoclastogenesis induced by sonicates of *Porphyromonas gingivalis*, *Treponema denticola*, or *Treponema socranskii*. Cocultures were treated with sonicates of *P. gingivalis* (Pg, 0.1 μg/ml), *T. denticola* (Td, 1 μg/ml), and *T. socranskii* (Ts, 1 μg/ml) in the absence or presence of OPG (100 ng/ml), indometahcin (1 mM), anti-IL-1β Ab (20

μg/ml), or anti-TNF-α Ab (2 μg/ml) for 4 days. The cells were then stained for TRAP to count the number of osteoclasts. The data are the means ± standard errors for three cultures. * P <0.05 for a comparison with the results for the nontreated cells. ** P <0.05 for a comparison with the results for the sonicates treated cells

To determine the mediators which stimulate RANKL expression of osteoblastic cells treated with P. gingivalis, T. denticola or T. socranskii, indomethacin, anti-IL-1β Ab or anti-TNF-α Ab was added to culture of osteoblastic cells in combination with sonicates of P. gingivalis (0.1 μg/ml) T. denticola (1 μg/ml), or T. socranskii (1 μg/ml) and, after 3 days culture, RANKL production was determined by immunoassay (Fig. 5). Addition of these inhibitors resulted in a reduction of RANKL production stimulated by sonicates of P. gingivalis, T. denticola, or T. socranskii. Inhibitory effect of indomethacin was stronger than that of anti-IL-1β Ab and anti-TNF-α Ab. Indomethacin ,anti-IL-1β Ab or anti-TNF-α Ab did not affect on RANKL expression of cells. inhibitors resulted in a reduction of RANKL production stimulated by sonicates of P. gingivalis, T. denticola, or T. socranskii. Inhibitory effect of indomethacin was stronger than that of anti-IL-1β Ab and anti-TNF-α Ab. Indomethacin ,anti-IL-1β Ab or anti-TNF-α Ab did not affect on RANKL expression of cells.

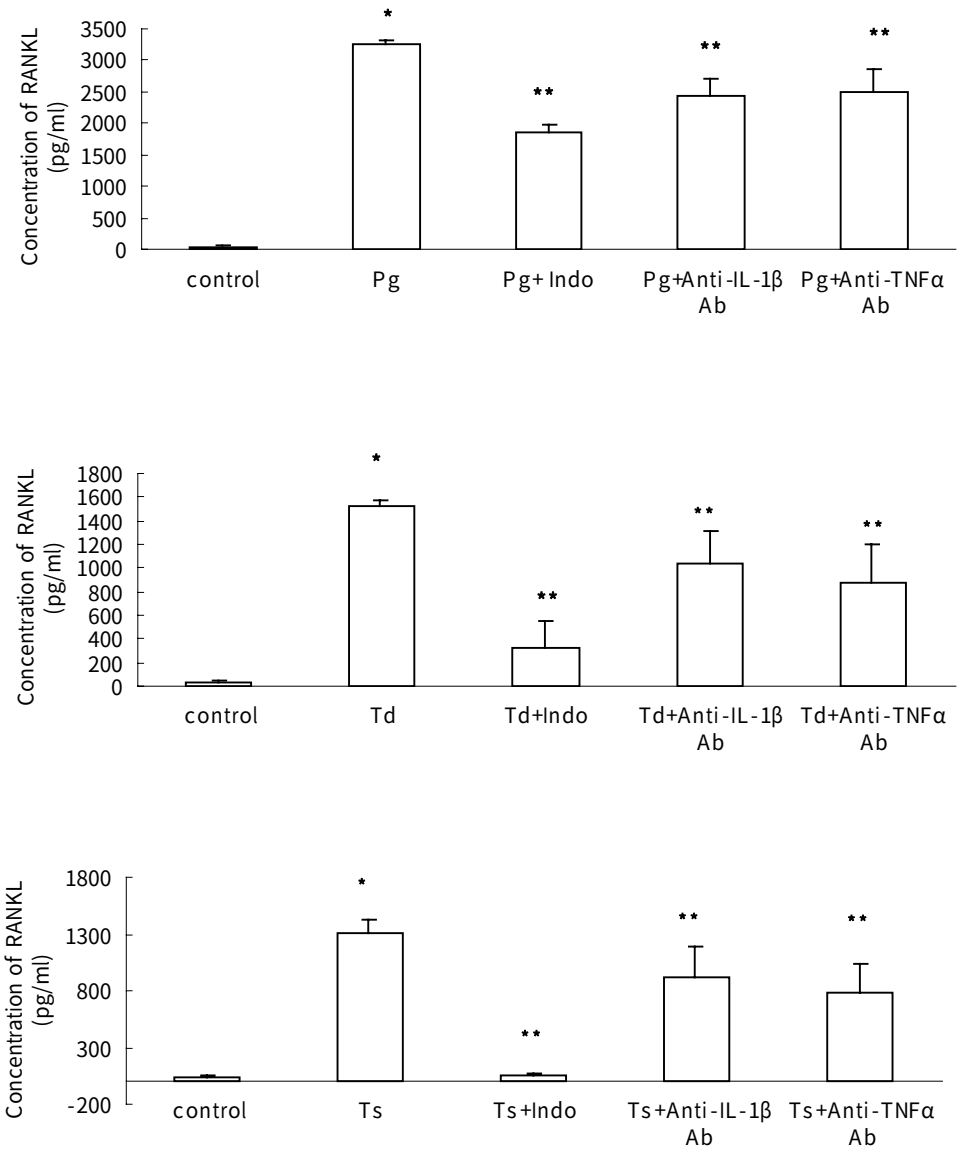

Figure 5. The effect of indomethacin, anti-IL-1β Ab, and anti-TNF-α Ab on RANKL expression of osteoblastic cells by sonicates of of *Porphyromonas gingivalis*, *Treponema denticola*, and *Treponema socranskii*. Osteoblastic cells isolated form mouse calvaria were treated with sonicates of *P. gingivalis* (Pg, 0.1 μg/ml), *T. denticola* (Td, 1 μg/ml) or *T. socranskii* (Ts, 1 μg/ml) in the absence or presence of indomethacin

(1 mM), anti-IL-1β Ab (20 μg/ml) or anti-TNF-α Ab (2μg/ml) for 72h. The concentration of RANKL was determined by RANKL ELISA kit. The data are the means ± standard errors for three cultures. * P <0.05 for a comparison with the results for the nontreated cells. ** P <0.05 for a comparison with the results for bacteria sonicates treated cells

Discussion

The results of the present study show that osteoclastogenesis is stimulated by *P. gingivalis, T. denticola,* and *T. socranskii*. We analyzed the ability of three periodontopathogens, *P. gingivalis, T. denticola,* and *T socranskii,* to form osteoclast. All bacteria enhanced osteoclastogenesis in coculture system. Both of *T. denticola* and *T. socrankii* showed maximum effect on osteoclastogensis at 1 μg/ml, but *P. gingivalis* sonicates showed cytotoxic effect on cells at 1 μg/ml. One of the potentially significant virulence characteristics of *P. gingivalis* is that this bacterium produces a large number of hydrolytic, proteolytic and lipolytic enzymes compared with other bacteria and many of these enzyme are either exposed at outer membrane, within the periplasmic space or in outer membrane vesicles.[29] This suggest that cytotoxic effect at 1 μg/ml of *P. gingivalis* sonicates might be due to various enzymes which are contained in bacterial sonicates.

Osteoclastogenesis is controlled by various factors including cytokine, PGE_2, and hormones. RANKL, produced by osteoblasts, is an essential factor that plays a key role in osteoclastogenesis.[1] Also, it has been reported that prostaglandins[30,31,32], TNF-α[33], IL-1β[34] increase bone resorption and osteoclast formation. It has been reported that *P. gingivalis* can induce the up-regulation of IL-1, TNF, and PGE_2.[35] *T. denticola* also can induce the up-

regulation of IL-1 and TNF-α.[36] In present study, sonicates of three bacteria stimulated expression of RANKL, PGE_2, IL-1β, and TNF-α in calvaria derived osteobasltic cells. In addition, the stimulated osteoclastogenesis was significantly inhibited by treatment with antibodies against IL-1β, and TNF-α and inhibitors against RANKL and PGE_2. This means that the bacteria sonicate-stimulated osteoclastogenesis in our assay was mediated by endogenous RANKL, PGE_2, IL-1β, and TNF-α activity. Interestingly, the bacteria sonicates-induced osteoclastogenesis was strongly suppressed to basal level by OPG, which is RANKL inhibitor, while partially suppressed by indomethacin, antibody of IL-1β and antibody of TNF-α. These results suggested that osteoclastogenesis induced by *P. gingivalis, T. denticola*, and *T. socranskii* is mainly regulated via RANKL.

RANKL is produced from osteoblasts and its production is controlled by various bone resorption-inducing factors. IL-1 increase RANKL production in stromal /osteoblastic cells.[37] Like IL-1, TNF–stimulated induction of osteoclastogenesis in bone marrow culture[38] was mediated by increase in RANKL expression.[39] PGE_2 stimulate osteoclast formation in marrow cultures.[40] Induction of RANKL has been shown to be essential for bone resorption via PGE_2.[41] Taken together with these studies and our results, the possibility that PGE_2, IL-1β, and TNF-α may relate to RANKL expression of osteoblatic cells by periodontopathogens. In our study, osteoblastic cells

treated with each bacterial sonicates showed increased the production of PGE_2 and the mRNA expression of IL-1β and TNF-α. Inhibitors of these factors including indomethacin, anti IL-1β Ab, and anti TNF-α Ab reduced not only osteoclatogensis but also RANKL production of osteobasltic cells by bacteria sonicates. These results strongly suggest the mediation of PGE_2, IL-1β and TNF-α in the RANKL expression stimulated by three periodontopathogens.

It has been reported that *P. gingivalis* can contribute to bone resorption. Previously, it was reported that *P. gingivalis* sonicates enhanced the mineral resorption via osteogenic cells.[42] Live and heat killed *P. gingivalis* stimulated bone resorption of mouse calvaria. Treatment of mice concomitantly with indometahcin reduced bone resorption by this bacteria, suggesting that their effects were mediated by prostaglandins somehow.[43] This bacteria elicited mRNA expression of IL-1β and IL-6, potent bone resorbing cytokines, in vivo calvarial model. In murine calvaria injected with live *P. gingivalis*, IL-1 and TNF mRNA was elicited.[44] LPS of *P. gingivalis* significantly increased the number of osteoclast-like cells in bone marrow culture.[23] *P. gingivalis* LPS stimulated expression of IL-1β and IL-6 genes in calvarial cells and LPS-stimulated bone resorption was markedly inhibited by antibodies against IL-1β and IL-6.[45] IL-1 was markedly expressed in the *P. gingivalis* fimbriae-treated calvarial bone cells and fimbriae stimulated bone resorption was abolished

significantly by antisera against IL-1.[46] These reports suggest that *P. gingivalis* induce the bone resorption via IL-1β, TNF, and PGE_2. In this study, we found that P. gingivalis stimulated osteoclastogenesis via RANKL and RANKL expression was mediated by IL-1β, TNF-α, and PGE_2. In addition, *T. denticola* and *T. socranskii* enhanced osteoclast formation via the similar mechanism to *P. gingivalis.* These suggest that three periodontopathogens appear to stimulate osteocalstogenesis in the same manner. It has been reported that IL-1 and TNF-α induce PGE_2.[47] Therefore, the IL-1β and TNF-α may contribute to PGE_2 induction in osteoblast by three periodontopathogens used in this study and further studies are required to clarify this question.

In summary, the present study provides evidence for the involvement of RANKL in osteoclastogenesis induced by three periodontopathogens such as *P. gingivalis*, *T. denticola,* and *T. socranskii,* and for the induction of RANKL via PGE_2, IL-1β, and TNF-α. It is important clinically to develop drugs that can prevent the alveolar bone loss associated with periodontal disease. In this consideration, inhibitor of PGE_2, IL-1β, and TNF-α will be good therapeutic agent and, especially, inhibitor of RANKL such as OPG will be the most effective on prevention of bone destruction by periodontopathogens

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국문 요약

*Porphyromonas gingivalis, Treponema denticola, Treponema socranskii*에 의하여 유도된 RANKL 의존 파골세포 형성에 있어서 PGE_2, IL-1β, TNF-α의 매개

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치주염은 치조골의 흡수를 동반하는 염증성 질환으로 치은연하 치태내의 여러 종류의 미생물이 치조골 흡수에 영향을 준다. 이러한 여러 종류의 치주병인체로 인하여 발생하는 여러 파골세포형성 인자 중에서 공통된 인자를 찾기 위해, 마우스 두개골 기원의 조골세포와 골수 세포의 혼합배양에서 세 종류의 치주병인체, 즉, *Porphyromonas gingivalis, Treponema denticola* 및 *Treponema socranskii*에 의한 파골세포형성 기전을 관찰하였다. 조골세포의 interleukin (IL)-1β, tumor necrosis factor (TNF)-α, receptor activator of NF-κB ligand (RANKL) 및 prostaglandin E2 (PGE2)의 발현은 reverse transcriptase polymerase chain reaction (RT-PCR) 및 면역분석법을 통하여 관찰하였다. 세 종의 세균 분쇄액은 혼합배양에서 파골세포형성을 촉진하였고, 조골세포의 RANKL, IL-1β 및 TNF-α의 mRNA 발현 그리고 PGE2 의 생성을 증가시켰다. RANKL의 억제 인자인 osteopotegerin (OPG)는 각 세균 분쇄액에 의한 파골세포형성을 완전히 억제하였

다. 또한 PGE_2 형성 억제 인자인 indomethacin, anti-IL-1β antibody (Ab), 및 anti-TNF-α Ab가 더해졌을 때 각각의 미세균 분쇄액의 파골세포 형성능이 저하되었다. 또한, indomethacin, anti-IL-1β Ab, 또는 anti-TNF-α Ab는 *P. gingivalis, T. denticola,* 및 *T. socranskii* 분쇄액 으로 처리된 조골 세포내에서 RANKL 발현을 저하시켰다. 이는 *P. gingivalis, T. denticola,* 및 *T. socranskii*가 조골세포의 RANKL의 발현을 증가시켜 파골세포 형성을 촉진하며, 이러한 세 종의 미생물에 의한 RANKL 발현에 IL-1β, TNF-α 및 PGE_2 가 중간 매개체로 작용한다는 것을 보여주고 있다.