Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 772–779

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2010.01298.x

Td92, an outer membrane protein of *Treponema denticola*, induces osteoclastogenesis via prostaglandin E_2-mediated RANKL/osteoprotegerin regulation

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Kim M, Jun H-K, Choi B-K, Cha J-H, Yoo Y-J. Td92, an outer membrane protein of Treponema denticola*, induces osteoclastogenesis via prostaglandin E_2-mediated RANKL/osteoprotegerin regulation. J Periodont Res 2010; 45: 772–779.*

Background and Objective: Periodontitis is a chronic inflammatory disease of the periodontium that causes significant alveolar bone loss. Osteoclasts are bone-resorbing multinucleated cells. Osteoblasts regulate osteoclast differentiation by expression of RANKL and osteoprotegerin (OPG). Td92 is a surface-exposed outer membrane protein of *Treponema denticola*, a periodontopathogen. Although it has been demonstrated that Td92 acts as a stimulator of various proinflammatory mediators, the role of Td92 in alveolar bone resorption remains unclear. Therefore, in this study, we investigated the role of Td92 in bone resorption.

Material and Methods: Mouse bone marrow cells were co-cultured with calvariae-derived osteoblasts in the presence or absence of Td92. Osteoclast formation was assessed by TRAP staining. Expressions of RANKL, osteoprotegerin (OPG) and prostaglandin E_2 (PGE_2) in osteoblasts were estimated by ELISA.

Results: Td92 induced osteoclast formation in the co-cultures. In the osteoblasts, RANKL and PGE_2 expressions were up-regulated, whereas OPG expression was down-regulated by Td92. The addition of OPG inhibited Td92-induced osteoclast formation. The prostaglandin synthesis inhibitors NS398 and indomethacin were also shown to inhibit Td92-induced osteoclast formation. The effects of Td92 on the expressions of RANKL, OPG and PGE_2 in osteoblasts were blocked by NS398 or indomethacin.

Conclusion: These results suggest that Td92 promotes osteoclast formation through the regulation of RANKL and OPG production via a PGE_2-dependent mechanism.

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Key words: *Treponema denticola*; Td92; osteoclast formation; periodontitis

Accepted for publication April 24, 2010

Osteoclasts are TRAP-positive multinucleated cells with bone-resorption activity. The formation of osteoclasts is induced by the RANKL and macrophage colony-stimulating factor (M-CSF) expressed by osteoblast/stromal cells (1,2). RANKL binds to the RANK, a receptor of RANKL, expressed on osteoclast precursors and the RANK–RANKL interaction causes osteoclast precursors to differentiate into osteoclasts in the presence of M-CSF (2). Osteoprotegerin (OPG), a soluble protein secreted by osteoblasts, blocks osteoclastogenesis by interfering with the RANKL–RANK interaction (2). The expressions of RANKL and OPG are regulated by several bone-resorption inducing factors, including 1α,25-dihydroxyvitamin D_3 [1α,25 $(OH)_2D_3$], parathyroid hormone (PTH), prostaglandin E_2(PGE_2) and lipopolysaccharide (LPS) (1–3). It has been suggested that osteoclastogenesis is principally determined by the ratio of RANKL to OPG in the bone microenvironment (2,4).

Periodontitis is a chronic inflammatory disease of bacterial etiology that arises in the periodontal tissue. The alveolar bone resorption observed in periodontitis is an important characteristic of this disease, which is irreversible and induced by an increase in osteoclast formation. Unlike other infectious diseases, periodontitis appears to be caused by a group of bacteria, not by a single bacterium (5,6). Although various bacteria have been proposed to be involved in the tissue destruction in periodontitis, the underlying mechanisms responsible for the resorption of alveolar bone in such a pathogenic microenvironment have yet to be clearly elucidated. It was reported that some periodontal pathogens, such as *Porphyromonas gingivalis*, *Prevotella nigrescens* and *Aggregatibacter actinomycetemcomitans*, have bone resorption activities. The LPS of *P. gingivalis* or *P. nigrescens* has been implicated as an inducer of osteoclast formation in periodontitis (5,7,8). *A. actinomycetemcomitans* also has several osteoclastogenic factors, including LPS, capsular polysaccharide or homolog of the chaperone GroEL (9–13). These studies indicate that various components of bacteria, including LPS, appear to be potent activators of osteoclastogenesis in periodontitis.

Observation of an elevated level of *Treponema* species in the subgingival plaque of periodontitis sites supports the hypothesis that *Treponema* species play an important role in periodontitis. Thus far, 10 species of oral spirochetes have been cultivated, and *Treponema denticola* is the most intensively studied species among them. *T. denticola* has been shown to be involved in early-onset periodontitis, necrotizing ulcerative gingivitis and acute pericoronitis (14). Several studies have suggested that *T. denticola* forms and releases a variety of virulence factors, such as the proteolytic enzymes and cytolytic factors involved in the pathogenesis of periodontitis (15–17). Tp92 is a surface antigen of *Treponema pallidum*, the causative agent of syphilis, with a molecular size of 92 kDa. It has been reported that Tp92 possesses immunoprotective capabilities based on the induction of opsonization and phagocytosis, and thus may be useful in the development of a vaccine for syphilis (18). Tp92 homologs (88–92 kDa) were recently identified as highly conserved surface proteins of four representative oral spirochetes (*T. denticola*, *T. lecithinolyticum*, *T. maltophilum* and *T. socranskii* subsp. s*ocranskii*), and they were demonstrated to have amino-acid sequence identities of 37.9–49.3% and similarities of 54.5–66.9% compared with Tp92 (19). Tp92 homologs including Td92, a Tp92 homolog of *T. denticola*, have been shown to bind to epithelial cells and to induce proinflammatory factors such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8 and PGE_2 in THP-1 human monocytic cell line and periodontal ligament cells, suggesting their pathological effects in periodontitis (19). However, the role of Tp92 homologs in bone resorption has yet to be determined. Therefore, we investigated the effects of Td92, a representative Tp92 homolog, on osteoclast formation. Td92 induced osteoclast formation in co-cultures of osteoblasts and bone marrow cells. Td92 was shown to up-regulate the expression of RANKL in osteoblasts, but down-regulated the expression of OPG. The Td92-induced regulation of RANKL/OPG expression in osteoblasts was shown to be mediated by PGE_2.

Material and methods

Mice and reagents

Newborn and 6-wk-old mice (ddY strain) were obtained from Sankyo Laboratory Animal Center (Tokyo, Japan). Animal studies were conducted according to the experimental protocols approved by the animal ethics committee of the Yonsei University College of Dentistry. Lipopolysaccharide (*Escherichia coli* O26:B6), indomethacin, Cel-Lytic™ M and protease inhibitor cocktail were purchased from Sigma (St Louis, MO, USA). NS398 was purchased from Calbiochem (San Diego, CA, USA). Human recombinant M-CSF and human recombinant OPG were obtained from Peprotech (Rocky Hill, NJ, USA). Collagenase was purchased from Wako Pure Chemicals (Osaka, Japan). α-Minimal essential medium (α-MEM), dispase, fetal bovine serum (FBS), phosphate-buffered saline (PBS), Dulbecco's phosphate-buffered saline (DPBS), antibiotic–antimycotic (10000 units/mL penicillin, 10000 μg/mL streptomycin and 25 μg/mL amphotericin B) and 25% trypsin–EDTA were purchased from Gibco BRL (Grand Island, NY, USA). The chemicals used for TRAP staining were as follows: sodium acetate trihydrate, fast red violet LB salt and naphthol AS-MX phosphate, all of which were purchased from Sigma; sodium (+)-tartrate dehydrate, which was purchased from Wako Pure Chemicals; and acetic acid, which was purchased from Junsei Chemicals (Tokyo, Japan).

Preparation of recombinant Td92

T. denticola ATCC 33521 was cultured using OMIZ (Oral Microbiology and Immunology, Zürich)-Pat medium as described previously in an anaerobic atmosphere (10% CO_2, 5% H_2 and 85% N_2) (20). Recombinant Td92 was prepared as previously described (19), and the major experimental procedures

were as follows. The *tp92* gene homolog of *T. denticola* was amplified from genomic DNA and cloned in *E. coli* using the pQE-30 expression vector. Td92 expression in *E. coli* was confirmed by immunoblotting using a monoclonal mouse antihistidine antibody (Qiagen, Alencia, CA, USA) and rabbit anti-Td92 antibody. The expected molecular size of the prepared Td92 was approximately 92 kDa. The recombinant Td92 was purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) followed by the decontamination of endotoxin using polymyxin B agarose (Sigma). Endotoxin decontamination of the recombinant Td92 was confirmed using CHO/CD14/TLR4 cells that expressed CD25 via Toll-like receptor 4 (TLR4)-dependent nuclear factor κB (NF-κB) activation as previously described (19).

Preparation of primary osteoblasts

Mouse osteoblasts were isolated and cultured as described in previous studies (21). Between 25 and 30 newborn ddY mice (1 d old) were used for one preparation of osteoblasts. The mice were killed with CO_2, and the calvariae, including the frontal and parietal bones, were anatomically detached. The calvariae were then collected in α-MEM and briefly washed in 10 mL of α-MEM containing 0.2% collagenase and 0.1% dispase in order to remove debris and blood cells. The calvariae were incubated in 10 mL of collagenase–dispase solution for 15 min at 37°C, in a 250*g* shaking water bath. The first supernatant was discarded, and 10 mL of fresh collagenase–dispase solution was added and incubated for 15 min at 37°C in a 250*g* shaking water bath. The supernatant was collected, and further incubation with fresh solution was repeated four times. The last four supernatants were collected as a primary osteoblast population. Primary osteoblasts were cultured for 3 d in α-MEM supplemented with 10% FBS and 1% antibiotic–antimycotic. The cells were detached using trypsin–EDTA, centrifuged at 135g for 5 min, suspended in 90% FBS and 10% dimethyl sulfoxide, and stored at −80°C.

Osteoclast formation assay

Primary osteoblasts stored at −80°C were cultured in α-MEM supplemented with 10% FBS and 1% antibiotic-antimycotic for 2 d prior to co-cultures. The bone marrow cells were obtained from the tibiae of 6-wk-old male ddY mice killed with CO_2 and cultured for 16 h in the presence of M-CSF (50 ng/mL) prior to co-cultures. Primary osteoblasts (8×10^3 cells) were co-cultured for 6 d with bone marrow cells (8×10^4 cells) in 200 μL of α-MEM containing 10% FBS and 1% antibiotic–antimycotic in 96-well plates (NUNC, Roskilde, Denmark). The co-cultures were treated with 1–10 μg/mL of Td92 or 0.001–1 μg/mL of LPS in the absence or presence of OPG (100 ng/mL), NS398 (1 μM) or indomethacin (1 μM). The medium was refreshed on day 3. Osteoclast formation was evaluated by staining with TRAP, a marker enzyme of osteoclasts. The TRAP staining was conducted as previously described (22). The cells were fixed with 10% formaldehyde in PBS and with ethanol–acetone (1:1) solution. One hundred microliters of TRAP staining solution was added to each well and stained for 5 min. TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. All assays were conducted in triplicate in three experimental runs.

ELISA for cytokines and PGE_2

Effects of Td92 on RANKL and OPG expression were determined at the protein level by ELISA. Primary osteoblasts (2×10^4 cells) were cultured in α-MEM containing 10% FBS and 1% antibiotic–antimycotic in 48-well culture plates until confluence. The cells were then refreshed with fresh α-MEM containing 10% FBS and 1% antibiotic–antimycotic and incubated in the presence or absence of Td92 (10 μg/mL) or LPS (0.01 μg/mL) for 3 d. Some cultures were treated in combination with NS398 (1 μM) or indomethacin (1 μM). The cells were harvested in order to determine the concentration of RANKL, and the culture supernatants were used to determine the concentrations of OPG and PGE_2. For the preparation of the cell lysates, the cells were washed in DPBS and lysed by 15 min of treatment with CelLytic™ M. After 15 min of centrifugation at 16,000*g* to remove the cell debris, the lysates were collected and then used to determine the concentration of RANKL after the protein contents were determined using a Bio-Rad Protein Assay kit. The levels of RANKL, OPG and PGE_2 were determined using ELISA kits (R&D Systems, Minneapolis, MN, USA). All assays were conducted in duplicate in three experimental runs.

Statistical analyses

Statistical analysis was conducted by a Student's unpaired *t*-test with the Sigma Plot 8.0 program (Systat software Inc., San Jose, CA, USA). A p value of < 0.05 was considered statistically significant.

Results

Osteoclast formation induced by Td92

Recombinant Td92, which was expressed in *E. coli* and purified with Ni-NTA-agarose, showed the expected molecular size of about 92 kDa and did not activate CHO/CD14/TLR4 cells (data not shown), thereby indicating that the Td92 was endotoxin free. In order to determine the effects of Td92 on osteoclast formation, co-cultures composed of osteoblasts and bone marrow cells were treated with Td92 (1–10 μg/mL), and the numbers of TRAP-positive multinucleated cells per well were counted. Td92 stimulated the formation of TRAP-positive osteoclasts, and the maximal number of osteoclasts was noted at 5–10 μg/mL (Fig. 1A,B). Lipopolysaccharide, a positive control, exhibited peak osteoclast formation effects at a concentration of 0.01–0.1 μg/mL (Fig. 1A,C). Td92 (10 μg/mL) heated for 30 min at 99°C did not induce osteoclast formation, whereas heated LPS still induced osteoclast formation to a similar extent as unheated LPS (Fig. 1D), suggesting that Td92 is a heat-labile stimulator of osteoclast formation.

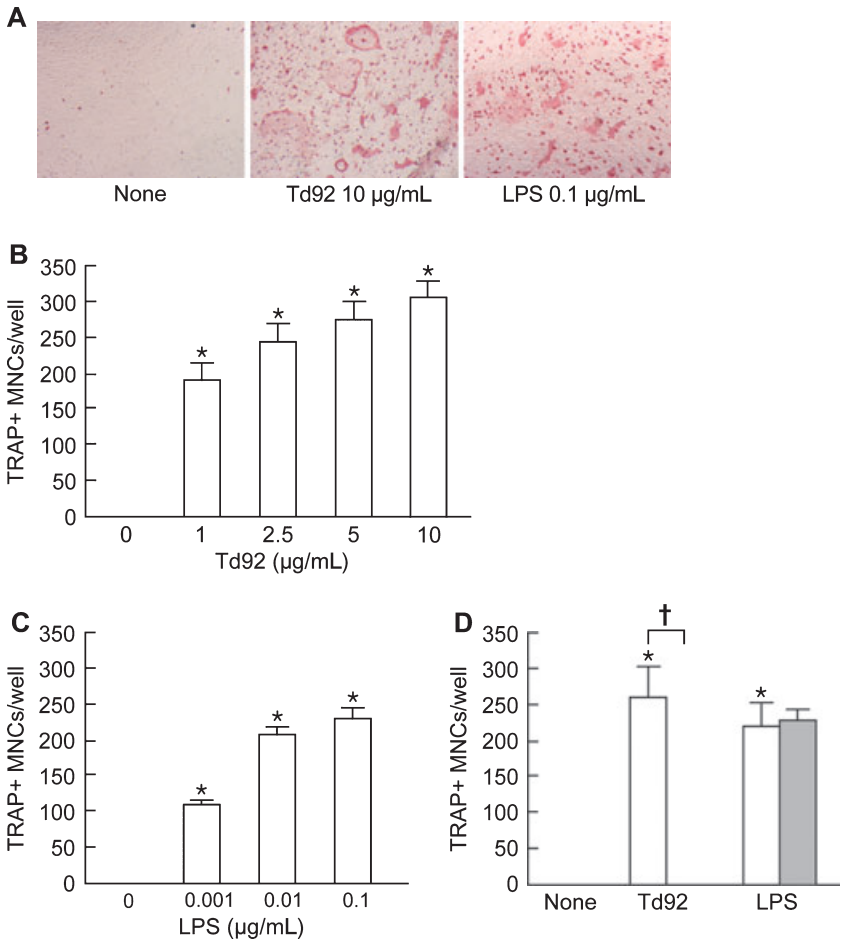

Fig. 1. Effect of Td92 on osteoclast formation. (A) Mouse calvariae-derived osteoblasts and bone marrow cells were co-cultured in the absence or presence of Td92 (1–10 μg/mL) or lipopolysaccaride (LPS;(0.001–0.1 μg/mL) for 6 d, and the cells were stained for TRAP. (B,C) TRAP-positive multinucleated cells (MNCs) containing more than three nuclei were counted as osteoclasts. (D) Co-cultures were treated with Td92 (10 μg/mL; open bar), heated Td92 (10 μg/mL; bar too small to be drawn), LPS (0.1 μg/mL; open bar) or heated LPS (0.1 μg/mL; grey bar) for 6 d. TRAP-positive MNCs containing more than three nuclei were counted as osteoclasts. *Significant difference ($p < 0.05$) compared with the untreated cells. †Significant difference ($p < 0.05$) compared with unheated Td92- or LPS-treated cells.

Regulation of RANKL and OPG expression by Td92 in osteoblasts

In an effort to evaluate the effects of Td92 on RANKL and OPG expression, osteoblasts were treated with Td92 or heated Td92, and the protein levels of RANKL in cell lysates and OPG in culture supernatants were assayed by ELISA. Td92 increased RANKL expression to a similar extent to that observed with LPS, and its expression was 4.7-fold higher than that of the untreated group (Fig. 2A). Heated Td92 did not increase RANKL expression, but heat treatment of LPS did not result in the blockage of LPS-induced RANKL expression (Fig. 2A). Td92 reduced OPG expression to a similar extent to that observed with LPS, and its expression was 2.4-fold lower than that of the untreated group (Fig. 2B). Heat treatment of Td92 resulted in significant increase in the level of OPG. In contrast to Td92, heat treatment of LPS did not result in a recovery of LPS-inhibited OPG expression (Fig. 2B).

Inhibition of Td92-induced osteoclast formation by OPG

In order to verify the involvement of RANKL in Td92-induced osteoclast formation, co-cultures were treated with Td92 in the presence or absence of OPG. The osteoclast formation stimulated by Td92 was completely inhibited by the addition of OPG (Fig. 3). The osteoclast formation induced by LPS was similarly inhibited as the result of the addition of OPG. These results indicate that the expression level of RANKL/OPG in osteoblasts is primarily involved in Td92-stimulated osteoclast formation.

Induction of PGE_2 expression by Td92 in osteoblasts

In order to evaluate the involvement of PGE_2 in Td92-induced osteoclast formation, the osteoblasts were stimulated with Td92, and the level of PGE_2 expression was measured by ELISA. Osteoblasts treated with Td92 or LPS showed an increased level of PGE_2 expression (Fig. 4). This increased expression of PGE_2 was completely inhibited by the addition of NS398 or indomethacin, inhibitors of prostaglandin synthesis (Fig. 4). The effect of NS398 or indomethacin on the cell viability of osteoblasts was evaluated by 3-(4,5-dimethylthiazol-2-yle)2,5-diphenyltetrazolium bromide (MTT) assay. Neither NS398 nor indomethacin affected cell viability (data not shown).

Inhibition of Td92-regulated RANKL/OPG expression by NS398 and indomethacin

In order to determine whether PGE_2 is involved in the regulatory activity of Td92 on RANKL and OPG expression, osteoblasts were treated with Td92 in the presence or absence of NS398 or indomethacin. The levels of RANKL and OPG were measured by ELISA. The RANKL expression induced by Td92 or LPS was down-regulated by NS398 or indomethacin (Fig. 5A). Down-regulated OPG expression induced by Td92 or LPS was recovered by NS398 or indomethacin (Fig. 5B). These results show that the regulation of RANKL and OPG by Tp92 in osteoblasts is closely related to PGE_2 production.

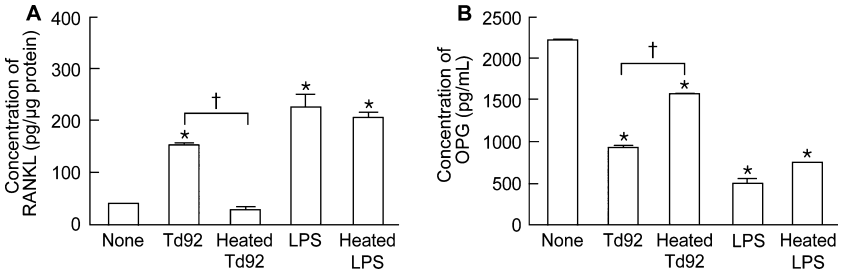

Fig. 2. Effect of Td92 on RANKL and osteoprotegerin (OPG) expression in osteoblasts. Calvariae-derived osteoblasts were cultured with Td92 (10 μg/mL), heated Td92 (10 μg/mL), LPS (0.1 μg/mL) or heated LPS (0.1 μg/mL) for 3 d. The concentration of RANKL in cell lysates (A) and OPG in culture supernatants (B) were determined by ELISA. *Significant difference ($p < 0.05$) compared with the untreated cells. †Significant difference ($p < 0.05$) compared with unheated Td92- or LPS-treated cells.

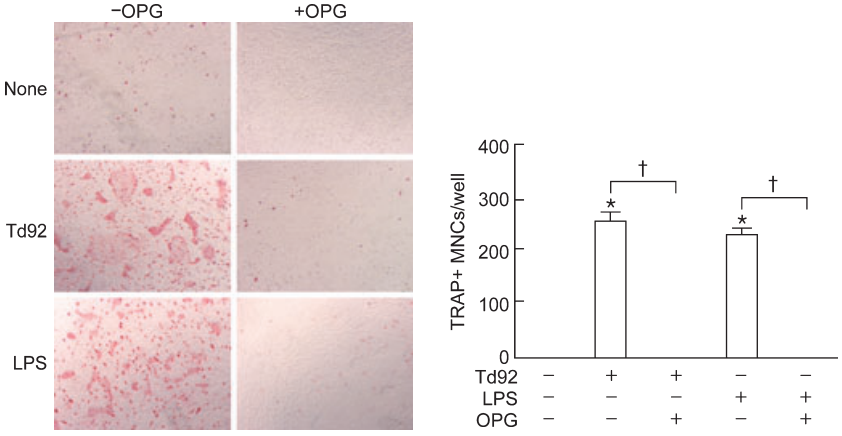

Fig. 3. Effect of OPG on Td92-induced osteoclast formation. Osteoblasts and bone marrow cells were co-cultured with Td92 (10 μg/mL) or LPS (0.1 μg/mL) in the absence or presence of OPG (100 ng/mL) for 6 d. TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. *Significant difference ($p < 0.05$) compared with the untreated cells. †Significant difference ($p < 0.05$) compared with Td92- or LPS-treated cells.

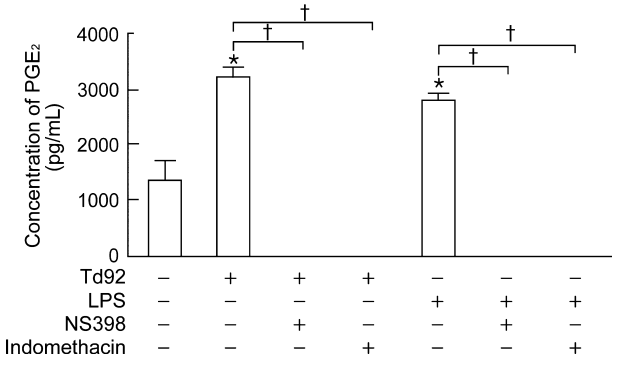

Fig. 4. Effect of Td92 on PGE_2 production in osteoblasts. Osteoblasts were cultured with Td92 (10 μg/mL) or LPS (0.1 μg/mL) in the absence or presence of NS398 (1 μM) or indomethacin (1 μM). The cultures were incubated for 3 d, and the concentration of PGE_2 was determined by ELISA. *Significant difference ($p < 0.05$) compared with the untreated cells. †Significant difference ($p < 0.05$) compared with Td92- or LPS-treated cells.

Inhibition of Td92-induced osteoclast formation by NS398 or indomethacin

To confirm the involvement of PGE_2 in Td92-induced osteoclast formation, co-cultures were treated with Td92 in the presence or absence of NS398 or indomethacin. The osteoclast formation induced by Td92 was inhibited by the addition of NS398 or indomethacin (Fig. 6). These results indicate that PGE_2 is critically involved in Td92-induced osteoclastogenesis.

Discussion

Td92 has been proposed to contribute to inflammation and osteoclastogenesis by inducing the production of TNF-α, IL-1β, IL-6, IL-8 and PGE_2 in monocytes and periodontal ligament cells (19). Although this observation suggested that Td92 may have osteoclastogenic ability by inducing osteoclastogenic factors, the precise role of Td92 in bone cells with regard to the regulation of RANKL and OPG expression has until now remained unclear. For the first time, the results of our study have demonstrated that Td92 exerts a stimulatory effect on osteoclastogenesis via the regulation of RANKL/OPG/PGE_2 in osteoblasts.

The outer membrane proteins of *T. denticola* include major surface protein (Msp), dentilisin, oligopeptide binding protein (Opp) A and hemin binding protein (Hbp) A/HbpB (23). The Msp exhibits pore-forming activity and adhesive activity (23,24). These adhesion and pore-forming properties of Msp have been suggested to contribute to the pathogenesis of periodontitis. Dentilisin, a chymotrypsin-like protease, is a major surface protease that functions as an adherent and cytotoxic molecule in periodontal ligament epithelial cells (25). OppA binds to soluble plasminogen and fibronectin, and thus plays an important role in spirochete–host interactions (26). HbpA/HbpB exhibit hemin-binding activity, thereby providing an essential nutrient for *T. denticola* (23). The results of these previous studies suggest that the outer membrane proteins of *T. denticola* are critical virulence factors with binding and cytotoxic activity during the host–

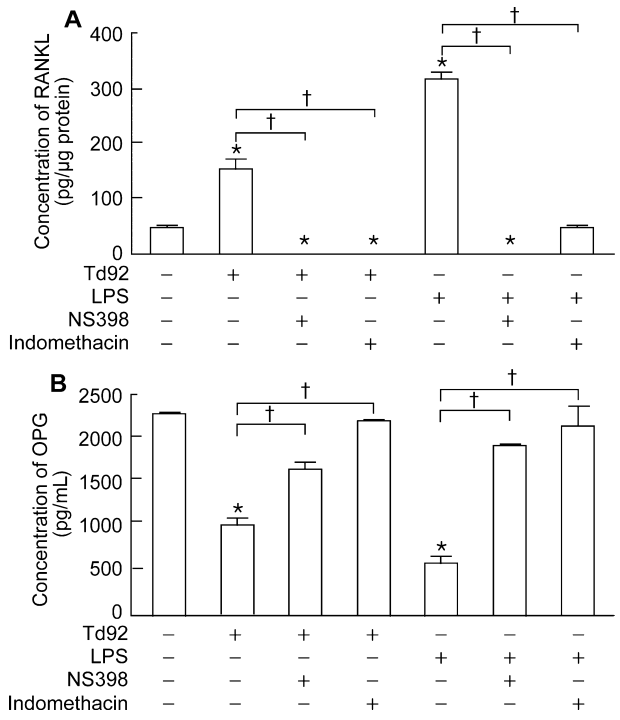

Fig. 5. Effect of NS398 and indomethacin on Td92-regulated RANKL (A) and OPG expression (B) in osteoblasts. Osteoblasts were cultured with Td92 (10 μg/mL) or LPS (0.1 μg/mL) in the absence or presence of NS398 (1 μM) or indomethacin (1 μM). The cultures were incubated for 3 d, and the concentrations of RANKL (A) and OPG (B) were determined by ELISA. *Significant difference ($p < 0.05$) compared with the untreated cells. †Significant difference ($p < 0.05$) compared with Td92- or LPS-treated cells.

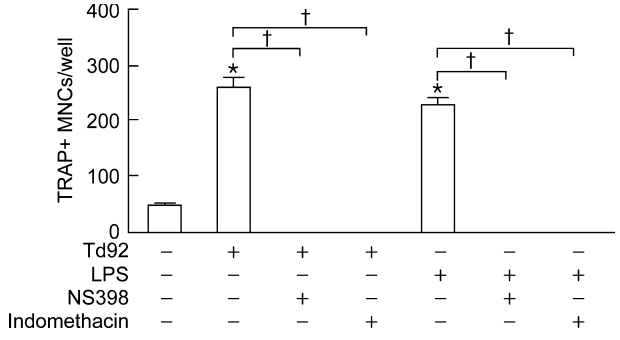

Fig. 6. Effect of NS398 and indomethacin on Td92-induced osteoclast formation. Osteoblasts and bone marrow cells were co-cultured for 6 d with Td92 (10 μg/mL) or LPS (0.1 μg/mL) in the absence or presence of NS398 (1 μM) or indomethacin (1 μM). TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts. *Significant difference ($p < 0.05$) compared with the untreated cells. †Significant difference ($p < 0.05$) compared with Td92- or LPS-treated cells.

bacteria interactions in periodontitis. In the present study, Td92, one of the outer membrane proteins of *T. denticola*, was identified as a stimulator of osteoclastogenesis. Lipopolysaccharide, a major cell wall component of gram-negative bacteria, stimulates osteoclast formation via the regulation of RANKL/OPG in osteoblasts (27). Therefore, LPS is generally used as a positive control in studies of osteoclast formation in periodontitis. When Td92 was compared with LPS, the osteoclastogenic activity of Td92 was similar to that of LPS. It is uncertain how Td92 is able to penetrate into periodontal tissues to interact with cells of alveolar bone surface. To invade periodontal tissues, *T. denticola* must first overcome the barrier composed of the epithelium and the basement membrane. *T. denticola* has been shown to exhibit a cytopathic effect, motility and chemotaxis to penetrate the epithelium (28,29). In addition, *T. denticola* possesses a chymotrypsin-like protease, which facilitates migration through the basement membrane by destruction of type IV collagen, laminin and fibronectin (30). The treponemal outer membrane is fragile and forms vesicles (17,31); therefore, Td92 might be released from outer membrane to periodontal tissues. Thus, after *T. denticola* penetrates into periodontal tissues, the released Td92 might affect cells of the alveolar bone surface, including osteoclasts and osteoblasts.

With regard to periodontitis, RANKL and OPG are the essential cytokines in the regulation of osteoclastogenesis during the process of bone destruction. Osteoblasts infected with *P. gingivalis* exhibited elevated RANKL mRNA expression (32), and osteoblasts treated with LPS from *P. nigrescens* showed reduced OPG expression (5). Elevated RANKL expression, as well as decreased OPG expression, detected in the gingival crevicular fluid of diseased sites indicate a close correlation between RANKL/OPG and the incidence of alveolar bone destruction in periodontitis (33,34). It is known that RANKL is expressed in two active forms: soluble and membrane bound (1,2). Therefore, the level of RANKL in both supernatants and cell lysates of osteoblast cultures was assessed. The cell lysates showed increased levels of RANKL with Td92 treatment. However, the culture supernatants did not show detectable levels of RANKL in the Td92-treated group (data not shown). These results indicate that Td92 primarily induces membrane-bound RANKL in osteoblasts. This study also demonstrated that Td92 markedly reduced the level of OPG expression. In order to confirm the involvement of RANKL/OPG in Td92-induced osteoclastogenesis, OPG, a decoy receptor of RANKL, was administered to the Td92-treated

co-cultures. The osteoclast formation induced by Td92 was significantly inhibited by OPG. These results demonstrate that Td92 functions as a potent virulence factor in bone-resorptive periodontitis, by inducing osteoclastogenesis via RANKL up-regulation and OPG down-regulation.

Td92 is a recombinant protein; therefore, this study confirmed the endotoxin decontamination by using the NF-κB reporter cell line, CHO/CD14/TLR4, and heat treatment. Td92 did not induce the NF-κB reporter in CHO cells to express membrane CD25 via TLR4-dependent NF-κB activation. However, LPS, a ligand of TLR4, induced NF-κB-regulated CD25 expression in flow cytometry (data not shown). When Td92 was heat treated, osteoclast formation was not observed, and the application of heat treatment to Td92 reversed the RANKL/OPG regulatory activity of Td92. However, heat-treated LPS continued to exhibit osteoclast formation activity and RANKL/OPG regulatory activity identical to that of unheated LPS. Therefore, the effect of Td92 on osteoclast formation or RANKL/OPG expression could be concluded to be the effect of the protein, not the contaminated endotoxin.

Prostaglandin E_2 is a potent proinflammatory molecule that is involved in the pathogenesis of periodontal disease. Previous studies showing elevated PGE_2 levels at the site of periodontitis implicate PGE_2 as one of the primary pathogenic molecules in periodontitis (35–37). In bone, PGE_2 has been shown to perform a critical function as a mediator of RANKL expression (2,38,39). The elevation of PGE_2 in LPS-treated osteoblasts suppressed the expression of OPG (27). Therefore, the correlation between PGE_2 and RANKL/OPG is one of the principal foci of studies about the bone resorption of periodontitis. In the present study, Td92 induced significant PGE_2 production in osteoblasts, and it was inhibited by NS398 or indomethacin, inhibitors of PGE_2 synthesis. Additionally, the up-regulation of RANKL expression by Td92 was inhibited by NS398 or indomethacin. In contrast, the down-regulation of OPG expression by Td92 was recovered by treatment with NS398 or indomethacin. NS398 or indomethacin also inhibited Td92-induced osteoclast formation. These data suggest that PGE_2 plays a significant role in Td92-induced osteoclastogenesis, via the regulation of RANKL/OPG expression in osteoblasts.

In summary, the results of this study demonstrated that Td92 stimulates osteoclastogenesis via the up-regulation of RANKL expression and the down-regulation of OPG expression, as well as via an increase in PGE_2 production. The regulation of RANKL/OPG is mediated by PGE_2. Therefore, the osteoclastogenic effect of Td92 may potentially contribute to alveolar bone resorption in periodontitis. In order to better understand coincidences or differences among the other Tp92 homologs in terms of the alveolar bone resorption of periodontitis, the Tp92 homologs of other *Treponema* species will be further investigated.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (R13-2003-013-04002-0). H.-K. Jun is the recipient of a scholarship from the BK21 Program (Craniomaxillofacial Life Science 21).

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