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Lipopolysaccharide from *Prevotella nigrescens* stimulates osteoclastogenesis in cocultures of bone marrow mononuclear cells and primary osteoblasts

Y.-H. Chung[1], E.-J. Chang[1], S.-J. Kim[2], H.-H. Kim[1], H.-M. Kim[1], S.-B. Lee[1], J. S. Ko[1]

[1]Department of Craniomaxillofacial Cell and Developmental Biology, BK21 Program, and Dental Research Institute, School of Dentistry, Seoul National University, Seoul, Korea and [2]Department of Periodontology, College of Dentistry, Pusan National University, Busan, Korea

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Background and Objective: Lipopolysaccharide is thought to be a major virulence factor of pathogens associated with periodontal diseases and is believed to stimulate bone resorption *in vivo*. Although *Prevotella nigrescens* has been implicated in periodontitis, its role in osteoclastogenesis has not been reported. In this study, we investigated the effects of lipopolysaccharide from *P. nigrescens* on the formation of osteoclasts and the production of cytokines related to osteoclast differentiation.

Material and Methods: Mouse bone marrow mononuclear cells were cultured in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL), with or without lipopolysaccharide. Bone marrow mononuclear cells were also cocultured with calvarial osteoblastic cells in the presence or absence of lipopolysaccharide. Osteoclast formation was determined by tartrate-resistant acid phosphatase cytochemistry. The production of osteoprotegerin (OPG), M-CSF, tumor necrosis factor alpha (TNF-α), transforming growth factor-beta (TGF-β) and prostaglandin E_2 (PGE_2) was determined by enzyme-linked immunosorbent assay (ELISA).

Results: *P. nigrescens* lipopolysaccharide inhibited osteoclast differentiation from bone marrow mononuclear cells cultured in the presence of M-CSF and RANKL. However, in the coculture system, *P. nigrescens* lipopolysaccharide stimulated osteoclastogenesis. Notably, *P. nigrescens* lipopolysaccharide decreased OPG production but increased TGF-β secretion. In addition, treatment with *P. nigrescens* lipopolysaccharide increased PGE_2 production during the late stage of the culture period. There was no difference in M-CSF and TNF-α production.

Conclusion: These results demonstrate that *P. nigrescens* lipopolysaccharide stimulates osteoclastogenesis in the coculture system by decreasing the production of OPG and increasing the production of TGF-β and PGE_2. Through the mechanisms involving these factors, *P. nigrescens* lipopolysaccharide may cause alveolar bone resorption in periodontal diseases.

Jea Seung Ko, DDS, PhD, Professor, Department of Craniomaxillofacial Cell and Developmental Biology, School of Dentistry, Seoul National University, 28 Yongon-Dong, Chongno-Gu, Seoul 110–749, Korea
Tel: +82 2 740 8668
Fax: +82 2 763 3613
e-mail: jeasko@snu.ac.kr

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It is well known that two major groups of cells – osteoblasts and osteoclasts – have equally important, but opposite, functions in skeletal homeostasis and normal bone remodeling (1). Osteoclasts are bone-resorbing multinucleated cells that originate from hematopoietic progenitors of the monocyte/macrophage lineage (2,3). Osteoblasts or bone marrow stromal cells are also involved in osteoclastogenesis through a mechanism involving cell–cell contact with osteoclast progenitors (4). Previous studies, using macrophage colony-stimulating factor (M-CSF)-deficient *op*/*op* mice, showed that M-CSF produced by osteoblasts/stromal cells is an essential factor for osteoclast differentiation (5,6). Receptor activator of NF-κB ligand (RANKL) was identified as another essential factor for osteoclastogenesis (7,8). RANKL is a member of the tumour necrosis factor (TNF) ligand family and is expressed as a membrane-associated protein by osteoblasts and stromal cells. Osteoclast precursors express RANK, the receptor for RANKL, and upon recognition of RANKL on the surface of osteoblasts/stromal cells, differentiate into osteoclasts in the presence of M-CSF (9). Osteoprotegerin (OPG), which is produced by osteoblasts/stromal cells, is a soluble decoy receptor for RANKL and blocks osteoclastogenesis by inhibiting the RANKL–RANK interaction (10).

Periodontitis is a chronic inflammatory disease characterized by gingival inflammation and alveolar bone resorption, which is often caused by infections with gram-negative bacteria. Lipopolysaccharide from gram-negative bacteria has been identified as an important factor in the pathogenesis of periodontal diseases (11). Lipopolysaccharide is a complex glycolipid composed of a hydrophilic polysaccharide portion and a hydrophobic domain, known as lipid A, which is responsible for most of the lipopolysaccharide-induced biological effects. One of the many known functions of lipopolysaccharide is the stimulation of bone resorption *in vivo* (12). However, more recent studies have demonstrated various effects of lipopolysaccharide on osteoclast differentiation *in vitro*, depending on the culture system (13–15). Lipopolysaccharide stimulates osteoblasts to secrete interleukin-1, interleukin-6, granulocyte–macrophage colony-stimulating factor (GM-CSF), prostaglandin E_2 (PGE_2) and nitric oxide, each of which seems to be involved in lipopolysaccharide-mediated periodontitis (16). Recently, mouse toll-like receptor 4 (TLR4) was identified as the receptor for lipopolysaccharide (17,18). Toll-like receptors (TLRs) are a family of mammalian proteins homologous to Drosophila Toll (19). Although one of the human Toll homologues, TLR2, has been shown to be involved in lipopolysaccharide signaling (20), further studies, including the generation of gene-disrupted mice, have shown that TLR4, but not TLR2, is essential for lipopolysaccharide responsiveness *in vivo* (21).

Periodontitis differs from many other types of infections in that it does not appear to be caused by a single bacterium, but by a group of bacteria. Over 300 different types of bacteria have been detected in the mouth (22). Most of these bacteria are thought to be an indigenous part of the normal flora and not associated with oral disease. However, *Actinobacillus actinomycetemcomitans*, *Tannerella forsythia*, *Prevotella intermedia*, *Porphyromonas gingivalis* and *Treponema denticola* have been implicated as pathogens associated with the development and progression of periodontitis (23). The identity of *P. nigrescens* was separated from *P. intermedia*, and its association with periodontitis has been implicated (24,25). However, its role in alveolar bone resorption and osteoclastogenesis has not been reported.

In this study, we isolated lipopolysaccharide from *P. nigrescens* and investigated its effect on osteoclastogenesis by using two different culture systems. To determine the factors involved in the effect of *P. nigrescens* lipopolysaccharide, we also examined the secretion of cytokines and PGE_2, factors related to osteoclast differentiation.

Material and methods

Mice and reagents

Female mice (ICR strain) were obtained from Samtaco Korea Co. (Seoul, Korea). The α-minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). *E. coli* lipopolysaccharide (*Escherichia coli* 0111:B4) and a tartrate-resistant acid phosphatase (TRAP) staining kit were obtained from Sigma (St Louis, MO, USA). Recombinant human soluble RANKL and recombinant human M-CSF were obtained from PeproTech Inc. (Rocky Hill, NJ, USA). Cell Counting Kit-8 was obtained from Dojindo Laboratories (Kumamoto, Japan). The sources of enzyme immunoassay kits were as follows: OPG and M-CSF, R & D Systems Inc. (Minneapolis, MN, USA); TNF-α and TGF-β, Biosource Europe S.A. (Nivelles, Belgium); and PGE_2, Amersham Pharmacia Biotech. (Piscataway, NJ, USA).

Bacteria and culture conditions

P. nigrescens ATCC 33563 was grown anaerobically on the surface of enriched Trypticase soy agar containing 5% (v/v) sheep blood, or in GAM broth (Nissui, Tokyo, Japan) supplemented with 1 μg/ml menadione and 5 μg/ml hemin. Plate-grown cultures were routinely incubated for 4 d and used as the inoculums for liquid growth. Liquid-grown cells were incubated for ≈24 h, to late exponential growth phase. They were collected by centrifugation at 12,000 *g* for 20 min at 4°C, washed three times with phosphate-buffered saline (PBS, pH 7.2) and lyophilized. Culture purity was assessed by gram staining and plating on solid medium.

Lipopolysaccharide preparation

P. nigrescens lipopolysaccharide was prepared by the Tri-Reagent method followed by cold $MgCl_2$/ethanol precipitation (26). In brief, 200 μl of Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) was added to the lyophilized bacterial cells (1–10 mg)

and the cell suspension was incubated at room temperature for ≈10 min for complete cell homogenization. After incubation, 20 μl of chloroform per mg of cells was added to the bacterial suspension. The mixture was then vigorously vortexed and incubated at room temperature for an additional 10 min. The resulting mixture was centrifuged at 12,000 *g* for 10 min to separate the aqueous and organic phases. Two additional water-extraction steps were repeated and the combined aqueous phase was dried using a speed vacuum (Ilshin Engineering, Seoul, Korea). The crude Tri-Reagent extracted lipopolysaccharide was then dissolved in 500 μl of 0.375 M magnesium chloride in 95% ethanol, stored at −20°C, followed by centrifugation at 12,000 *g* for 15 min. The pellet was suspended in distilled water and lyophilized to give fluffy white solid lipopolysaccharide.

Preparation of primary calvarial osteoblasts and bone marrow mononuclear cells

The osteoblastic cells were isolated from the calvariae of 2–3-d-old ICR mice. The calvariae were digested in Hanks' balanced salt solution (HBSS) containing 0.02% type I collagenase (Gibco BRL), 0.05% trypsin and 0.53 mM EDTA, for 20 min at 37°C with vigorous shaking. The digestion procedure was repeated six times, and the cells isolated by the last three digestions were combined as an osteoblastic cell population. They were cultured in α-MEM containing 10% FBS and used for the coculture system. The bone marrow cells were collected from 5–6-wk-old female ICR mice. The ends of the tibiae and femurs were aseptically removed, and each marrow cavity was flushed by injecting HBSS containing 0.02% type II collagenase (Gibco BRL), 0.05% trypsin and 4 mM EDTA. The bone marrow cells were incubated for 12 h, and nonadherent cells were used for further culture.

In vitro osteoclast formation assay

The isolated calvarial osteoblasts were seeded at the density of 5×10^5 cells per 10-cm culture dish and grown to confluence. The cells of passage 2 were then detached from the culture dishes with trypsin-EDTA (Gibco BRL). Subsequently, the cells (1×10^4 cells/well) were cocultured with the bone marrow mononuclear cells (1×10^5 cells/well), in α-MEM containing 10% FBS, in 48-well plates. The culture volume was adjusted to 1 ml per well with α-MEM/10% FBS. Cocultures were performed by incubation with *P. nigrescens* lipopolysaccharide or *E. coli* lipopolysaccharide (0.1–1 μg/ml) for 9 d. Co-cultured cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP, a maker enzyme of osteoclasts). TRAP-positive multinucleated cells were counted.

For RANKL-induced osteoclastogenesis, bone marrow cells were plated in 96-well plates, at a density of 1×10^5 cells/well, in 0.2 ml of α-MEM containing 10% FBS in the presence of M-CSF (30 ng/ml). On day 3, the medium was changed to contain RANKL (70 ng/ml), M-CSF (30 ng/ml) and *P. nigrescens* or *E. coli* lipopolysaccharide (0.1–5 μg/ml). Osteoclast formation was evaluated on day 8. TRAP-positive cells containing more than three nuclei were counted.

Measurement of OPG, M-CSF, TNF-α, TGF-β and PGE_2 production

Primary osteoblasts (1×10^4) were cultured in 48-well culture plates, alone or together with bone marrow mononuclear cells (1×10^5) in α-MEM containing 10% FBS. Cells were treated with or without lipopolysaccharide (0.1–1 μg/ml) during the entire period of culture. After 3, 6 and 9 d, the conditioned culture media were collected and centrifuged. The supernatants were then subjected to enzyme immunoassays for determination of the concentrations of OPG, M-CSF, TNF-α, TGF-β and PGE_2.

Cell viability assay

Cell viability was evaluated with the Cell Counting Kit-8. The Cell Counting Kit-8 contains 5 mM WST-8, 0.2 mM 1-methoxy-PMS, and 150 mM NaCl. Primary bone marrow mononuclear cells were prepared as described above. Cells were plated in 96-well plates, at a density of 1×10^5 cells/well, in 0.2 ml of α-MEM containing 10% FBS in the presence of M-CSF (30 ng/ml) and an indicated dose of *P. nigrescens* or *E. coli* lipopolysaccharide. On day 3, the medium was changed and RANKL (70 ng/ml) was added. On day 8, 10 μl of the solution of Cell Counting Kit-8 was added to each well and the plate was incubated for an additional 2 h. Finally, the absorbance of each well was measured at 450 nm, with a reference at 655 nm, using the Benchmark microplate reader (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All experiments were performed at least three times and representative results are shown. In some experiments, data were expressed as means ± standard deviation (SD). The statistical significance of differences was determined by the Student's *t*-test. *p*-values of < 0.05 were considered significant.

Results

Effect of *P. nigrescens* lipopolysaccharide on RANKL-induced osteoclastogenesis in mouse bone marrow mononuclear cell cultures

To examine the role of direct interaction of *P. nigrescens* lipopolysaccharide with osteoclast precursors in their differentiation, bone marrow mononuclear cells (osteoclast precursors) were induced to differentiate to osteoclasts by treatment with soluble RANKL in the absence of osteoblasts or stromal cells. *E. coli* lipopolysaccharide was included for comparison with *P. nigrescens* lipopolysaccharide. Incubation of the osteoclast precursor cells with soluble RANKL (70 ng/ml) and M-CSF (30 ng/ml) for 5 d resulted in the formation of numerous multinucleated TRAP-positive osteoclasts (Fig. 1A). When these cells were treated with *P. nigrescens* lipopolysaccharide or *E. coli* lipopolysaccharide alone in the absence of RANKL, no

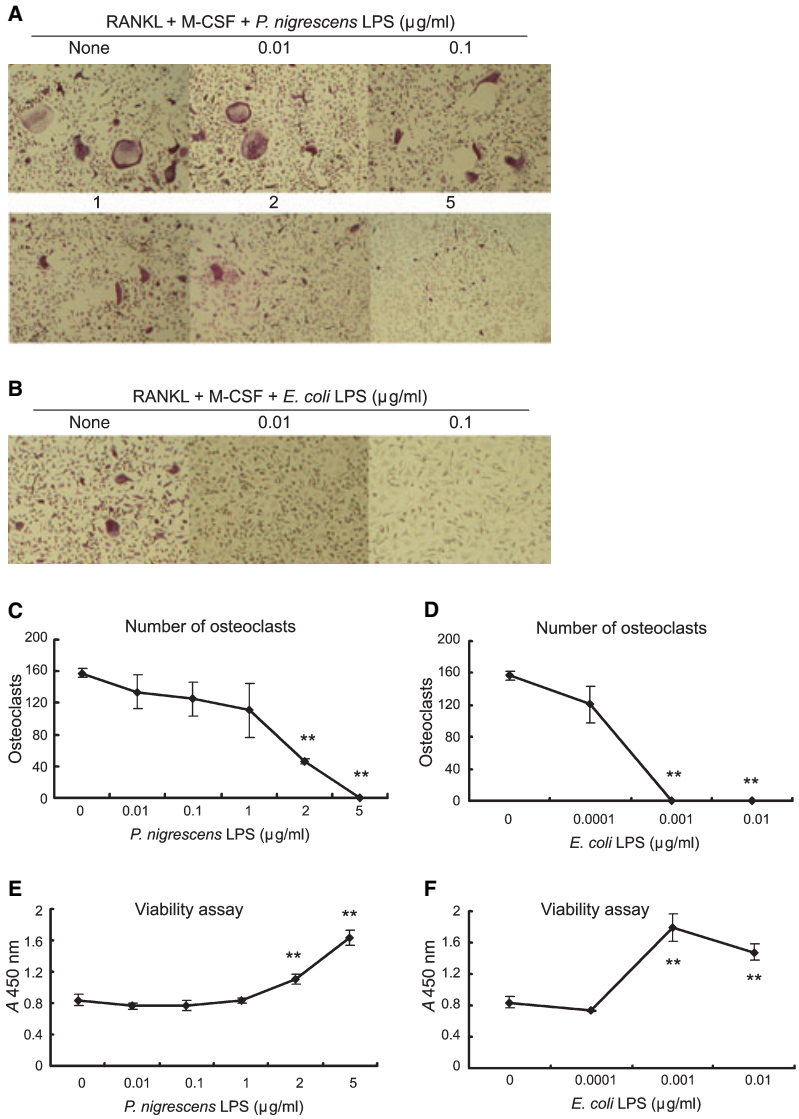

Fig. 1. Effect of *Prevotella nigrescens* lipopolysaccharide on receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclastogenesis from bone marrow precursors in the absence of supporting cells. (A and B) Mouse bone marrow mononuclear cells, grown for 3 d in medium containing macrophage colony-stimulating factor (M-CSF) (30 ng/ml), were incubated with M-CSF (30 ng/ml) and RANKL (70 ng/ml) for 5 d in the presence or absence of *P. nigrescens* lipopolysaccharide (0.01–5 μg/ml) or *Escherichia coli* lipopolysaccharide (0.001–0.01 μg/ml). Cells were stained for tartrate-resistant acid phosphatase (TRAP) and photographed. (C and D) Numbers of TRAP-positive cells containing more than three nuclei were counted. Results are expressed as means ± standard deviation (SD) of five cultures. $^{**}P < 0.01$ vs. the nontreated group. (E and F) Cells were treated, as described above, and cell viability was evaluated using the Cell Counting Kit-8. Data are expressed as means ± SD of five cultures. $^{**}P < 0.01$ vs. the nontreated group.

osteoclast formation was observed (data not shown). The addition of *P. nigrescens* lipopolysaccharide to the bone marrow cell cultures inhibited the RANKL-induced osteoclastogenesis (Fig. 1A,C). *E. coli* lipopolysaccharide displayed a more potent inhibitory effect on the osteoclast differentiation in this culture system (Fig. 1B,D). As the reduction in formation of osteoclasts by lipopolysaccharide could be caused by a decrease in cell numbers caused by potential cytotoxicity, a cell viability assay was conducted. Measurement of cell viability showed that the inhibition of osteoclastogenesis by both *P. nigrescens* lipopolysaccharide and *E. coli* lipopolysaccharide was accompanied by increased cell numbers (Fig. 1E,F). Thus, *P. nigrescens* lipopolysaccharide, as well as *E. coli* lipopolysaccharide, inhibits RANKL-induced osteoclastogenesis from bone marrow precursor cells in the absence of osteoblasts or stromal cells without reducing cell viability.

Effect of *P. nigrescens* lipopolysaccharide on osteoclast formation in a coculture system

We next examined the effects of *P. nigrescens* lipopolysaccharide on osteoclastogenesis using a coculture system in which osteoblastic cells support osteoclast differentiation. Mouse bone marrow mononuclear cells and primary osteoblastic cells were cocultured in the presence of *P. nigrescens* lipopolysaccharide or *E. coli* lipopolysaccharide for 9 d and osteoclast formation was evaluated. No TRAP-positive multinucleated cells were detected in the untreated cocultures of osteoclast precursors and primary osteoblastic cells (Fig. 2A). In the cultures treated with the *P. nigrescens* lipopolysaccharide (0.1–1 μg/ml), a number of TRAP-positive multinucleated cells were formed (Fig. 2A,C). Treatment with *E. coli* lipopolysaccharide stimulated osteoclast formation to an even greater extent (Fig. 2B,D). The osteoclasts formed in *E. coli* lipopolysaccharide-treated cultures contained a greater number of nuclei than those formed in *P. nigrescens* lipopolysaccharide-treated cultures (Fig. 2B).

Effect of *P. nigrescens* lipopolysaccharide on the secretion of OPG

OPG is a decoy receptor that binds RANKL and thereby blocks RANKL interaction with RANK in osteoclast differentiation. Consequently, the relative level of OPG to RANKL is a key factor in determining the extent of osteoclastogenesis. We therefore sought to examine the effects of *P. nigrescens* lipopolysaccharide on the expression of OPG and RANKL in the coculture

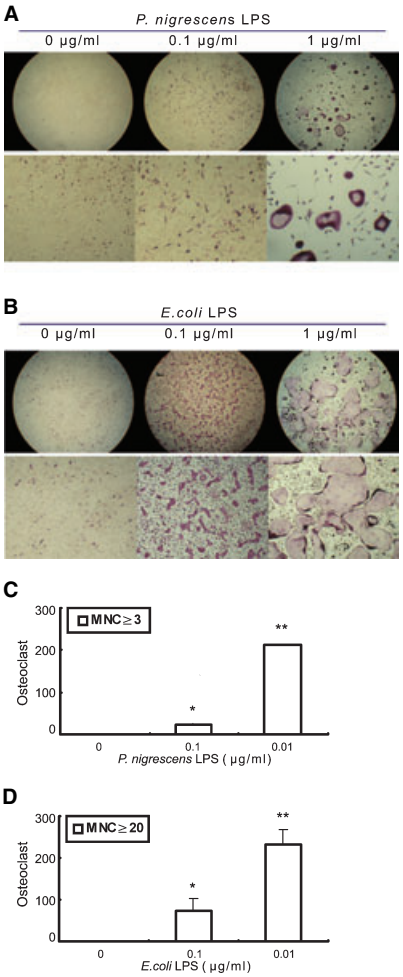

Fig. 2. Effect of *Prevotella nigrescens* lipopolysaccharide on osteoclast formation in cocultures of bone marrow osteoclast precursors and osteoblastic cells. (A and B) Mouse primary osteoblastic cells and bone marrow mononuclear cells were cocultured, for 9 d, without or with *P. nigrescens* or *Escherichia coli* lipopolysaccharide (0.1–1 μg/ml). The cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP). Magnifications: ×50 (top panels) and ×200 (bottom panels). (C and D) TRAP-positive multinucleated cells containing more than three or 20 nuclei were counted. Values are expressed as means ± SD of five cultures. $*P < 0.05$, $**P < 0.01$ vs. the nontreated group. MNC, multinucleated cells; LPS, lipopolysaccharide.

system. As RANKL is a membrane integral protein, quantification of its level by enzyme immunoassays was unsuccessful. In reverse transcription–polymerase chain reaction (RT–PCR) analyses, RANKL mRNA levels did not seem to be affected by *P. nigrescens* lipopolysaccharide (data not shown). We next measured the levels of OPG in the culture supernatant by enzyme-linked immunosorbent assay (ELISA). In the cocultures, *P. nigrescens* lipopolysaccharide caused a significant decrease in OPG secretion from day 3 (Fig. 3A). Consistent with its more potent effects on osteoclastogenesis, *E. coli* lipopolysaccharide reduced OPG to a greater extent (Fig. 3C). To determine whether this inhibitory effect on OPG production in the coculture is attributable to the action of lipopolysaccharide on the osteoblastic cells, OPG levels were determined in conditioned media of the primary osteoblastic cell single cultures treated with *P. nigrescens* lipopolysaccharide or *E. coli* lipopolysaccharide. *P. nigrescens* lipopolysaccharide at 1 μg/ml slightly decreased OPG levels in the osteoblast culture (Fig. 3B). The extent of inhibition by *P. nigrescens* lipopolysaccharide was lower in the osteoblast single culture than in the coculture (Fig. 3A,B). In contrast, *E. coli* lipopolysaccharide exerted a similar extent of inhibition on OPG secretion in the osteoblast single culture (Fig. 3D). These results indicate that reduction in OPG secretion is one mechanism by which *P. nigrescens* lipopolysaccharide stimulates osteoclast formation in the coculture system.

Effect of *P. nigrescens* lipopolysaccharide on the expression of TGF-β

Although osteoclast differentiation is essentially governed by RANKL, other cytokines also have various effects on

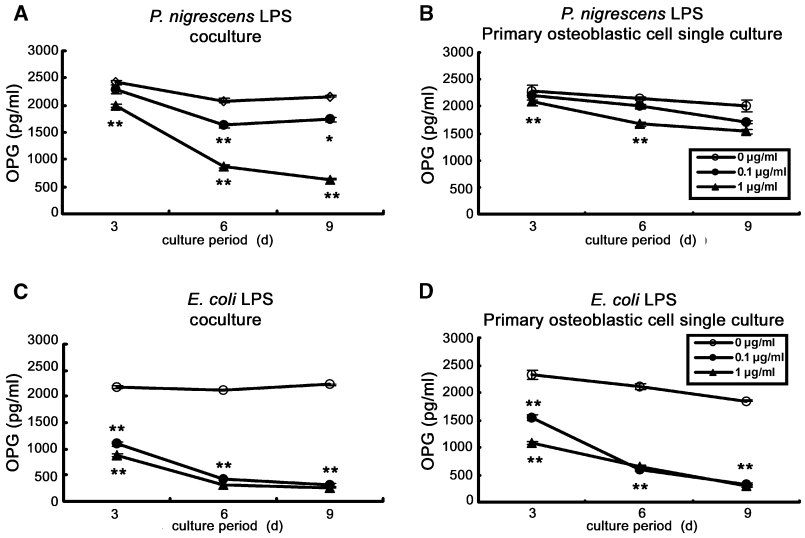

Fig. 3. Effect of *Prevotella nigrescens* lipopolysaccharide on osteoprotegerin (OPG) production. (A) Mouse primary osteoblastic cells and bone marrow mononuclear cells were cocultured without or with *P. nigrescens* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The culture media were collected and the concentrations of osteoprotegerin (OPG) were determined by enzyme-linked immunosorbent assay (ELISA). Data are expressed as means ± standard deviation (SD) of four samples. $*P < 0.05$, $**P < 0.01$ vs. the nontreated group. (B) Primary osteoblastic cells were cultured without or with *P. nigrescens* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The culture media were collected and the concentrations of OPG were determined by ELISA. Results are expressed as means ± SD of four samples. $**P < 0.01$ vs. the nontreated group. (C) The coculture was conducted without or with *Escherichia coli* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The concentrations of OPG in conditioned media were determined. Data are expressed as means ± SD of four samples. $**P < 0.01$ vs. the nontreated group. (D) Osteoblastic cells were cultured without or with *E. coli* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The concentrations of OPG in conditioned media were determined by ELISA. Results are expressed as means ± SD of four samples. $**P < 0.01$ vs. the nontreated group.

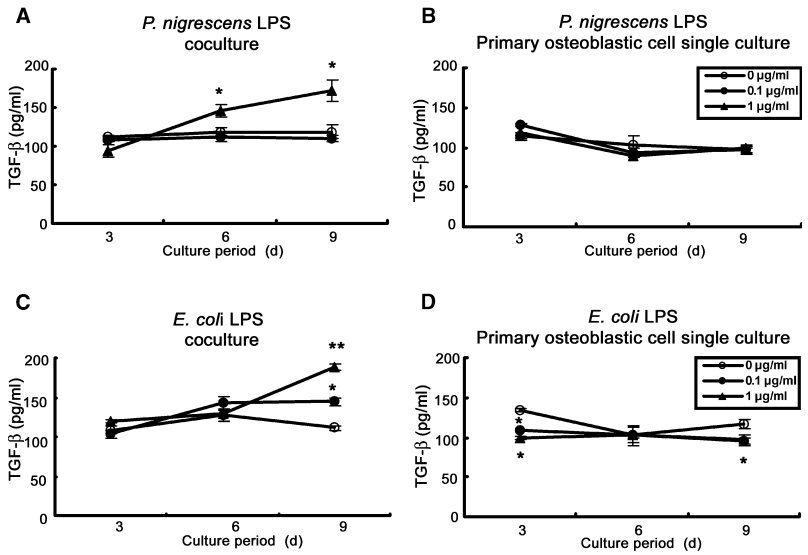

Fig. 4. Effect of *Prevotella nigrescens* lipopolysaccharide on transforming growth factor-β (TGF-β) expression. (A) Mouse bone marrow mononuclear cells and primary osteoblastic cells were cocultured without or with *P. nigrescens* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The culture media were collected and the concentrations of TGF-β were determined by enzyme-linked immunosorbent assay (ELISA). Data are expressed as means ± SD of four samples. $*P < 0.05$ vs. the nontreated group. (B) Primary osteoblastic cells were cultured without or with *P. nigrescens* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The culture media were collected and the concentrations of TGF-β were determined by ELISA. Results are expressed as the means ± SD of four samples. (C) The coculture was conducted without or with *Escherichia coli* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The concentrations of TGF-β in conditioned media were determined. Data are expressed as means ± SD of four samples. $*P < 0.05$, $**P < 0.01$ vs. the nontreated group. (D) Osteoblastic cells were cultured without or with *E. coli* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The concentrations of TGF-β in conditioned media were determined by ELISA. Results are expressed as means ± SD of four samples. $*P < 0.05$ vs. the nontreated group.

osteoclastogenesis by modulating RANK signaling. We therefore examined the influence of *P. nigrescens* lipopolysaccharide on cytokine production in the coculture and the osteoblastic cell single culture. The concentrations of M-CSF, TNF-α and TGF-β in the culture supernatants were measured by ELISA. Neither *P. nigrescens* lipopolysaccharide nor *E. coli* lipopolysaccharide had any significant effect on M-CSF production in the coculture and the osteoblast single culture (data not shown). In addition, *P. nigrescens* lipopolysaccharide did not affect TNF-α secretion in either culture systems (data not shown). *E. coli* lipopolysaccharide significantly increased TNF-α production in the coculture, but not in the osteoblast single culture (data not shown). The effect of *P. nigrescens* lipopolysaccharide on TGF-β was observed only in the coculture (Fig. 4A,B). Treatment of *P. nigrescens* with 1 μg/ml lipopolysaccharide increased TGF-β in 6- and 9-d cultures in the coculture system (Fig. 4A). In the osteoblast single culture, *P. nigrescens* lipopolysaccharide had no effect on TGF-β production (Fig. 4B). *E. coli* lipopolysaccharide increased TGF-β only at the highest concentration tested in the 9-d coculture, at the highest concentration tested (Fig. 4C,D).

Effect of *P. nigrescens* lipopolysaccharide on the production of PGE_2

PGE_2 has a profound effect on osteoclast differentiation, and *E. coli* lipopolysaccharide was shown to decrease OPG expression potentially via stimulation of PGE_2 production in osteoblasts (15). To determine whether PGE_2 is involved in the stimulatory effect of *P. nigrescens* lipopolysaccharide on osteoclastogenesis in the coculture, we analyzed PGE_2 levels in the coculture and the osteoblastic cell culture. In the coculture system, 1 μg/ml *P. nigrescens* lipopolysaccharide increased the production of PGE_2 in 6- and 9-d cultures (Fig. 5A). No stimulatory effect on PGE_2 production was observed in the osteoblastic cell culture treated with *P. nigrescens* lipopolysaccharide (Fig. 5B). As reported previously, *E. coli* lipopolysaccharide increased PGE_2 secretion in both cultures, but with a more prominent effect in the coculture (Fig. 5C,D).

Discussion

In the present study, we determined whether lipopolysaccharide from *P. nigrescens*, a potential pathogenic bacterial strain in periodontitis, has activities for regulating osteoclastogenesis. When tested in a single culture of mouse bone marrow osteoclast precursor cells, in which osteoclast differentiation is dependent on exogenous RANKL addition, *P. nigrescens* lipopolysaccharide suppressed osteoclastogenesis (Fig. 1). In contrast, in cocultures of the bone marrow precursor cells and primary osteoblastic cells, *P. nigrescens* lipopolysaccharide induced osteoclastogenesis in the absence of any other osteoclastogenic factors, such as $1,25(OH)_2D_3$, dexamethasone and parathyroid hormone (Fig. 2). These effects of *P. nigrescens* lipopolysaccharide were consistent with the effects of *E. coli* lipopolysaccharide, although the latter was more potent. These results indicate that *P. nigrescens* lipopolysaccharide has the potential to cause alveolar bone resorption in periodontitis.

Inflammatory cytokines, such as interleukin-1 and TNF-α, have been reported to induce osteoclast formation indirectly by stimulating osteoblasts (27–29). Likewise, lipopolysaccharide, an inflammatory component of gram-negative bacteria, has been reported to induce osteoclastogenesis indirectly (16). The

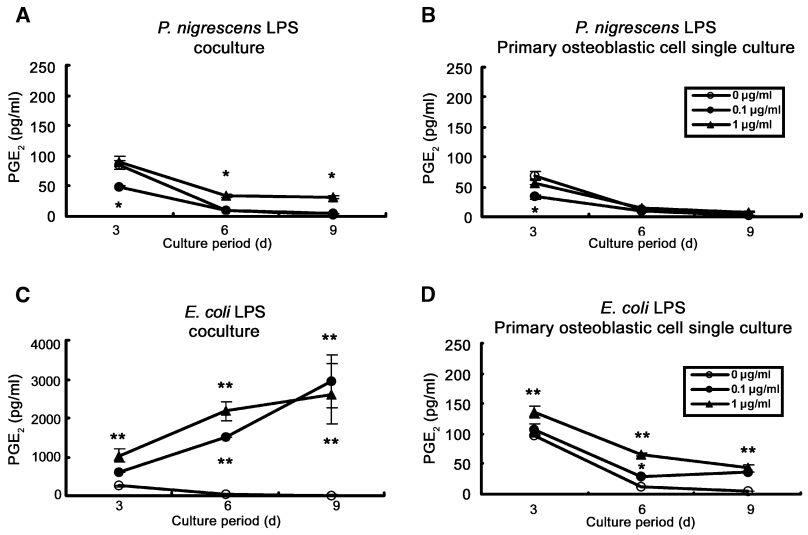

Fig. 5. Effect of *Prevotella nigrescens* lipopolysaccharide on prostaglandin E_2 (PGE_2) secretion. (A) Bone marrow mononuclear cells and osteoblastic cells were cocultured without or with *P. nigrescens* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The culture media were collected and the concentrations of PGE_2 were determined by enzyme-linked immunosorbent assay (ELISA). Data are expressed as means ± SD of four samples. $^*P < 0.05$ vs. the nontreated group. (B) Primary osteoblastic cells were cultured without or with *P. nigrescens* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The culture media were collected and the concentrations of PGE_2 were determined by ELISA. Results are expressed as means ± SD of four samples. $^*P < 0.05$ vs. the nontreated group. (C) The coculture was conducted without or with *E. coli* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The concentrations of PGE_2 in conditioned media were determined. Data are expressed as means ± SD of four samples. $^{**}P < 0.01$ vs. the nontreated group. (D) Osteoblastic cells were cultured without or with *E. coli* lipopolysaccharide (0.1–1 μg/ml) for the indicated number of days. The concentrations of PGE_2 in conditioned media were determined by ELISA. Results are expressed as means ± SD of four samples. $^*P < 0.05$, $^{**}P < 0.01$ vs. the nontreated group.

mechanism by which osteoblasts send a second signal to osteoclast precursors in response to primary osteolytic signals has been the subject of intense investigation. It has been demonstrated that osteoblastic cells regulate osteoclastogenesis by expressing RANKL, M-CSF and OPG. In this context, lipopolysaccharide stimulates host cells, including gingival fibroblasts, and recruits leukocytes, including monocytes and macrophages, or osteoblasts producing cytokines and local mediators (30–32). The main function of lipopolysaccharide is known to be supported by mediators such as lipopolysaccharide-binding protein, cytokines, prostaglandins, prostacyclins and nitric oxide. Lipopolysaccharide induces RANKL expression in osteoblasts (33) and stimulates these cells to secrete interleukin-1, PGE_2 and TNF-α, each of which seems to be involved in lipopolysaccharide-mediated bone resorption (16). TGF-β has also been shown to enhance osteoclast differentiation in hematopoietic cells stimulated with RANKL and M-CSF (34,35).

In our study, although *P. nigrescens* lipopolysaccharide did not have osteoclastogenic effects as potent as *E. coli* lipopolysaccharide, it still exerted significant effects on osteoclastogenesis and the production of OPG, TGF-β and PGE_2 in the coculture system. It has been reported that suppression of OPG mRNA expression by PGE_2 is crucially involved in *E. coli* lipopolysaccharide-induced osteoclast formation (15). The results of our OPG ELISA are consistent with the reported effects of *E. coli* lipopolysaccharide on OPG mRNA levels. In our study, *P. nigrescens* lipopolysaccharide had a prominent suppressive effect on OPG secretion in the coculture (Fig. 3A), and *P. nigrescens* lipopolysaccharide also displayed weak stimulatory effects on TGF-β and PGE_2 production in the coculture (Figs 4A,5A). The effects of *P. nigrescens* lipopolysaccharide on OPG and PGE_2 production were much weaker than those of *E. coli* lipopolysaccharide. Therefore, the weaker stimulatory effect of *P. nigrescens* lipopolysaccharide on osteoclastogenesis in the coculture may be associated with the smaller changes in OPG and PGE_2 levels caused by *P. nigrescens* lipopolysaccharide than by *E. coli* lipopolysaccharide. As the biological activity of lipopolysaccharide depends on the chemical structure of its lipid A portion, the differential potency in OPG and PGE_2 production may be attributed to structural differences of lipid A between *P. nigrescens* and *E. coli* lipopolysaccharide molecules.

All the changes in OPG, TGF-β and PGE_2 levels induced by *P. nigrescens* lipopolysaccharide were minimal or absent in the osteoblastic cell single culture (Figs 3B, 4B and 5B). The more prominent effect in the coculture than in the osteoblast single culture may be hypothesized to occur because *P. nigrescens* lipopolysaccharide influences both osteoblasts and bone marrow mononuclear cells. Alternatively, the effect of *P. nigrescens* lipopolysaccharide may be synergized by a second signal provided by the bone marrow mononuclear cells to osteoblasts in the coculture. Currently, we are not able to draw a conclusion in preference of either hypothesis. *E. coli* lipopolysaccharide was different from *P. nigrescens* lipopolysaccharide, not only in its potency in osteoclastogenic activity in the coculture system but also in its mode of OPG suppression. The suppression of OPG production by *E. coli* lipopolysaccharide was comparable between the coculture and the osteoblast single culture (Fig. 3C,D). In contrast, the effect of *P. nigrescens* lipopolysaccharide on OPG was much greater in the coculture than in the osteoblast culture (Fig. 3A,B). Taking together our results and reported data (15), OPG reduction via PGE_2 synthesis in osteoblasts appears to be the major determinant in the osteoclastogenic effect of *E. coli*

lipopolysaccharide in the coculture system. *P. nigrescens* lipopolysaccharide may require additional contributing factors, other than OPG suppression, to stimulate osteoclast differentiation in the coculture.

Zou & Bar-Shavit (14) showed that lipopolysaccharide from *E. coli* O55:B5 inhibited RANKL-induced osteoclast differentiation from bone marrow cells in the absence of stromal cells or osteoblasts. However, *E. coli* lipopolysaccharide promoted osteoclastogenesis in RANKL-primed primary bone marrow monocytes. In our study, *P. nigrescens* lipopolysaccharide did not induce osteoclast differentiation from bone marrow mononuclear cells, but stimulated osteoclast generation in cocultures of bone marrow mononuclear cells and osteoblastic cells. This suggests that *P. nigrescens* lipopolysaccharide is likely to induce osteoclast differentiation indirectly through osteoblast modulation in the coculture. In bone marrow cell cultures in the absence of other supporting cells, *P. nigrescens* lipopolysaccharide also had an inhibitory effect on osteoclastogenesis when simultaneously treated with RANKL, while it exerted a stimulatory effect when the cells were primed with RANKL before lipopolysaccharide treatment. Thus, depending on the experimental design, lipopolysaccharide inhibits, induces, or does not affect, osteoclast differentiation.

In conclusion, this study provides evidence that *P. nigrescens* lipopolysaccharide can induce osteoclastogenesis from bone marrow precursor cells when supporting osteoblastic or stromal cells are present. *P. nigrescens* lipopolysaccharide may stimulate osteoclast formation in the coculture system by decreasing the concentration of OPG, as well as by increasing the concentration of TGF-β and PGE_2. Further studies on *in vivo* bone-resorptive activity of *P. nigrescens* lipopolysaccharide will help to evaluate the role of *P. nigrescens* in periodontitis.

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