

Induction of Osteoclastogenesis and Matrix Metalloproteinase Expression by the Lipooligosaccharide of *Treponema denticola*

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Alveolar bone destruction is a characteristic feature of periodontitis. *Treponema denticola* is known to be involved in periodontitis. To elucidate the role of *T. denticola* in alveolar bone destruction in periodontitis, the effects of lipooligosaccharide (LOS) from *T. denticola* on osteoclast formation and on expression of osteoclast differentiation factor (ODF) and osteoprotegerin (OPG) mRNAs were examined in a coculture system by using mouse calvaria and bone marrow cells. In addition, the effect of *T. denticola* LOS on expression of matrix metalloproteinases (MMPs), which are involved in bone resorption, was estimated in mouse calvaria-derived osteoblastic cells. When the mouse calvaria and bone marrow cells were challenged with LOS (0.1 to 10 $\mu\text{g/ml}$) for 4 days, the number of tartrate-resistant acid phosphatase-positive multinucleated cells increased in a dose-dependent manner. The expression of ODF mRNA increased, while OPG mRNA expression decreased. Polymyxin B changed the effect of LOS (10 $\mu\text{g/ml}$) on ODF and OPG mRNA expression to the control level. LOS (10 $\mu\text{g/ml}$) stimulated prostaglandin E₂ (PGE₂) production in the cocultures. Adding indomethacin, an inhibitor of prostaglandin synthesis, resulted in a reduction in the number of osteoclasts induced by LOS and eliminated the effect of *T. denticola* LOS on ODF and OPG mRNA expression. *T. denticola* LOS increased the levels of mRNAs encoding MMP-3, -8, -9, -10, -13, and -14. Expression of one of these mRNAs, MMP-9 mRNA, was significantly induced by *T. denticola* LOS. These findings suggest that LOS from *T. denticola* stimulates osteoclastogenesis and MMP expression. Up-regulation of ODF and down-regulation of OPG by a PGE₂-dependent mechanism were involved in the osteoclastogenesis induced by *T. denticola* LOS.

Osteoclasts are multinucleated cells with bone-resorbing activity and play a crucial role in bone resorption. Osteoclast formation requires the presence of osteoblast or stromal cells (39). These cells express the osteoclast differentiation factor (ODF) (also known as a receptor activator of the nuclear factor- κB [RANK] ligand) that promotes osteoclastogenesis (21, 48). The osteoclast precursors that express RANK (the receptor for ODF) recognize ODF through a cell-to-cell interaction with osteoblasts and differentiate into osteoclasts. Osteoprotegerin (OPG), which is also secreted by osteoblast lineage cells, is a soluble decoy receptor that neutralizes the biological activity of ODF (34, 42, 47). Osteoclastogenesis is controlled by multiple factors, such as 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃], parathyroid hormone (PTH), prostaglandin E₂ (PGE₂), and interleukin-1 (IL-1) (26, 38). The regulation of ODF-OPG expression by these agents (1, 12–14, 25, 44, 47, 48) suggests that the effects of these factors on bone resorption may be mediated through control of ODF and/or OPG production and that osteoclast formation is determined principally by the ratio of ODF to OPG. Therefore, a shift to a higher ratio of ODF to OPG may be a major cause of bone loss in many metabolic disorders, including osteoporosis and periodontitis (15). Recently, it was reported that the ODF-

RANK interaction is not the sole pathway that causes osteoclast progenitors to differentiate into osteoclasts (19). Tumor necrosis factor alpha (TNF- α), which is involved in bone resorption, can be substituted for the ODF to induce osteoclast differentiation.

The matrix metalloproteinases (MMPs) are a family of structurally and functionally related enzymes that are responsible for the proteolytic degradation of the extracellular matrix components. More than 20 different MMPs have been identified. These proteins can be classified into the following subgroups based on their substrate specificities and structural homologies: collagenase (MMP-1, -8, and -13), gelatinase (MMP-2 and -9), stromelysin (MMP-3, -10, and -11), membrane-type MMPs (MMP-14, -15, -16, -17, -23, -24, and -25), and other MMPs, including matrilysin (MMP-7) and metalloelastase (MMP-12) (11, 22, 36). Previous reports suggested that MMPs are involved in degrading the bone matrix. Various MMPs, including MMP-2, -3, -9, -11, -12, -13, and -14, are expressed in the osteoblasts. Bone resorption factors, such as PTH, 1 α ,25(OH)₂D₃, IL-1, IL-6, TNF- α , and PGE₂, regulate the production of various MMPs in the osteoblasts (20, 40). Furthermore, a nonselective MMP inhibitor neutralized bone destruction stimulated by these bone resorption factors (20, 40, 41). This strongly suggests that the bone resorption factors function at least in part through MMP induction. Bone resorption first requires the osteoblasts to release collagenase to remove the nonmineralized organic matrix that covers the bone surfaces. Osteoclasts are then chemotactically attracted

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to the resorption site, where they settle onto the calcified matrix (2). Taken together, these reports suggest that the MMPs may be important for the bone resorption process.

Periodontitis is an inflammatory disease, and loss of alveolar bone is a hallmark of this disease (32). *Treponema denticola* is one of the bacteria which have been implicated in the etiology of periodontitis (6, 9, 35). This bacterium has multiple virulence factors that include adhesins, proteolytic and hydrolytic enzymes, cytopathic activity, and immunomodulation (3, 9). For the virulence factor associated with bone resorption, it was reported that the outer membrane of the bacterium increased Ca^{2+} release in an organ culture of fetal radii and ulnae (10). Although this suggested that the heat-stable material of *T. denticola* in the outer membrane might be involved in bone resorption, the components of this bacterium which stimulate bone resorption and the underlying mechanism have not been studied. Recently, we reported that whole-cell sonicates of *Treponema lecithinolyticum* associated with aggressive periodontitis induced osteoclast formation (5). In this case, heat-stable components were involved.

In this study, lipooligosaccharide (LOS) from *T. denticola* was isolated, and its effects on osteoclastogenesis and on expression of both ODF and OPG mRNAs were investigated in a coculture system consisting of mouse calvaria and bone marrow cells in order to determine the role of *T. denticola* in bone resorption. In addition, regulation of the expression of several MMPs by *T. denticola* LOS was estimated in mouse calvaria-derived osteoblastic cells. This is the first report showing that LOS from *T. denticola* induces osteoclast formation and that this process is dependent on up-regulation of ODF expression and down-regulation of OPG expression through PGE_2 synthesis. We also found that *T. denticola* LOS increased the expression of the mRNAs of several MMPs in osteoblastic cells.

MATERIALS AND METHODS

Materials. Mice (ICR strain) were obtained from Bio Korea Co. (Seoul, Korea). The α minimum essential medium (α -MEM), bovine serum albumin, and heat-inactivated fetal bovine serum (FBS) were purchased from GIBCO BRL (Grand Island, N.Y.). Indomethacin, polymyxin B, and a tartrate-resistant acid phosphatase (TRAP) (a marker of osteoclasts) staining kit were obtained from Sigma (St. Louis, Mo.).

Preparation of *T. denticola* sonicates. *T. denticola* ATCC 33521 was cultured anaerobically in OMIZ-PAT broth for 3 to 5 days, as described previously (46). The bacterial cells were harvested by centrifugation at $5,000 \times g$ for 10 min at 4°C . The cells were then washed three times with phosphate-buffered saline. The bacterial cells were disrupted for 5 min with an ultrasonic processor (Sonic Dismembrator; Fisher Scientific, Pittsburgh, Pa.) by using an output power of 8 W with 20-s intervals. The cell debris was removed after centrifugation at $15,000 \times g$ for 5 min at 4°C , and the supernatant was collected. The protein concentrations were determined by using a Coomassie brilliant blue protein assay reagent (Pierce, Rockford, Ill.).

Isolation of LOS. *T. denticola* LOS was isolated by the method described by Walker et al. (45). The bacteria were cultured and harvested as described above. The cell pellets were repeatedly frozen and thawed for 40 cycles and then centrifuged at $6,000 \times g$ for 10 min to remove the cellular debris. The supernatant was centrifuged at $36,000 \times g$ for 30 min at 4°C , and the resulting pellet containing the membrane fraction was washed twice in 60 ml of 0.05 M Tris-HCl (pH 7.2) and suspended in Tris-HCl. The detergent-soluble outer membrane fraction was obtained by extracting the membrane fraction with 1% Zwittergent 3.14 (Calbiochem Co., La Jolla, Calif.). To obtain the LOS, the detergent-soluble fraction was digested with proteinase K (50 $\mu\text{g}/\text{ml}$) at 37°C overnight. Two volumes of 0.375 M MgCl_2 in 95% ethanol (-20°C) was added, and the preparation was kept at -20°C for 40 min and then centrifuged at $15,000 \times g$ for 20

min. The pellet was suspended in a solution containing 2% sodium dodecyl sulfate, 0.1 M EDTA, and 10 mM Tris-HCl (pH 8.0). The proteinase K digestion-ethanol precipitation procedure was repeated twice. The LOS was purified by centrifugation at $48,000 \times g$ for 2 h at 4°C and suspended in a small volume of distilled water. After the LOS was heated at 90°C for 30 min, it was quantified by lyophilization, and the weight was measured. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15% polyacrylamide gel) of the LOS was performed, and the gels were silver stained.

Preparation of primary calvaria and bone marrow cells. The osteoblastic cells were isolated from the calvariae of 1- to 2-day-old ICR mice as previously described (5). The calvariae were digested in 10 ml of α -MEM containing 0.2% collagenase (Wako Pure Chemicals, Osaka, Japan) and 0.1% dispase (GIBCO BRL) for 20 min at 37°C with vigorous shaking and then centrifuged at $1,500 \times g$ for 5 min. The first supernatant was discarded, another 10 ml of the collagenase-dispase enzyme solution was added, and the preparation was incubated for 20 min. The digestion procedure was repeated four 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 an antibiotic solution (100 U of penicillin per ml, 100 μg of streptomycin per ml, 25 μg of amphotericin B per ml) and used for the coculture system. The bone marrow cells were collected from 5- to 8-week-old mice. The ends of the tibiae and femurs were removed, and each marrow cavity was flushed by slowly injecting medium at one end with 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 per 10-cm culture dish and grown to confluence. The cells 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 cells (1×10^5 cells/well) in α -MEM containing 10% FBS in 48-well plates (Corning Inc., Corning, N.Y.). The culture volume was adjusted to 400 μl per well with α -MEM containing 10% FBS. Either a bacterial sonicate or LOS was added to each coculture with or without polymyxin B or indomethacin after the medium was exchanged on day 3. The coculture was then maintained for an additional 4 days. Osteoclast differentiation was monitored by using a TRAP staining kit according to the manufacturer's instructions. TRAP-positive multinucleated cells having more than three nuclei per well were counted as osteoclasts. ODF and OPG mRNA expression in the cocultures was determined after the mRNA was isolated with TRIzol reagent (Life Technologies, Inc., Grand Island, N.Y.).

Osteoblastic cell cultures. To analyze expression of mRNAs of MMPs and tissue inhibitors of metalloproteinases (TIMPs), osteoblastic cells isolated from mouse calvariae were seeded into 24-well dishes at a density of 8×10^4 cells/well in 800 μl of α -MEM containing 10% FBS. When the cells had grown to 80% confluence, the medium was changed to α -MEM containing 1 mg of bovine serum albumin per ml. After incubation for 12 h, the cells were exposed to *T. denticola* LOS alone or in combination with polymyxin B for 48 h. The mRNA was isolated from the cultured osteoblastic cells by using TRIzol reagent according to the manufacturer's protocol (Life Technologies, Inc.).

RT-PCR. Expression of ODF, OPG, MMP, and TIMP mRNAs was determined by reverse transcription (RT)-PCR. Total RNA (1 μg) from nontreated or treated cells was used as a template for cDNA synthesis in a 20- μl reaction mixture performed with an RT kit (CLONTECH, Palo Alto, Calif.) used according to the manufacturer's instructions. The RNA (1 μg) and oligo(dT)₁₈ primers (1 mM) were denatured at 70°C for 5 min and incubated for 1 to 2 min on ice. The denatured RNA and oligo(dT)₁₈ primers were added to the reaction mixture (1 U of Moloney murine leukemia virus reverse transcriptase per μl , $1 \times$ reaction buffer, 500 μM dATP, 500 μM dCTP, 500 μM dGTP, 500 μM dTTP, 20 U of recombinant RNase inhibitor) and incubated at 42°C for 60 min, followed by 94°C for 5 min.

The cDNA (4 μl) was amplified by PCR in a 50- μl reaction mixture containing $1 \times$ PCR buffer, each deoxynucleoside triphosphate at a concentration of 200 μM , 200 pM forward primer, 200 pM reverse primer, and 0.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom) in a DNA thermal cycler (Biometra, Göttingen, Germany). The amplification reaction was performed for 35 cycles, and the primer sequences and annealing temperatures used are shown in Table 1. The PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining. The relative intensities of the gel bands were measured by using an image-analyzing program (TINA 2.0e; Neuro-Image Analysis Centre, Oxford, United Kingdom). In order to exclude contaminating DNA from 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 ODF, OPG, MMPs, TIMPs, and β -actin

Molecule	Direction	Primer sequence	Annealing temp ($^{\circ}$ C)	Product size (bp)
ODF	Forward	5'-ATCAGAAGACAGCACTCACT-3'	45.3	750
	Reverse	5'-ATCTAGGACATCCATGCTAATGTTTC-3'		
OPG	Forward	5'-TGAGTGTGAGGAAGGGCGTTAC-3'	45.5	636
	Reverse	5'-TTCCTCGTTCTCTCAATCTC-3'		
MMP-3	Forward	5'-GTACAGAGCTGTGGGAAGTCAATG-3'	60	287
	Reverse	5'-ATCAGCTCCATAGTGTGGAGTCC-3'		
MMP-7	Forward	5'-TGTTGATGGCAGCTATGCAGCTCA-3'	60	387
	Reverse	5'-CTAAGTTCCTGGGATCTGCATAC-3'		
MMP-8	Forward	5'-TGACTCTGGTGATTTCTTGCTAA-3'	60	164
	Reverse	5'-GTGAAGGTCAGGGGCGATGC-3'		
MMP-9	Forward	5'-CTGTCCAGACCAAGGTACAGCCT-3'	60	263
	Reverse	5'-GTGGTATAGTGGACACATAGTGG-3'		
MMP-10	Forward	5'-GATGTACCCAGTCTACAGTTCTC-3'	60	409
	Reverse	5'-GTAGCCTGCTTGACTTCATTTC-3'		
MMP-11	Forward	5'-GGAGAAGACAGACCTCACCTATAG-3'	60	376
	Reverse	5'-CTTAGCTGCTGTGGTGTGTTGTAG-3'		
MMP-12	Forward	5'-CCAGGAAATGCAGCAGTTCTTGG-3'	60	252
	Reverse	5'-CTTAGAGGAGTCACATCACTCCAG-3'		
MMP-13	Forward	5'-CATTACAGCTATCCTGGCCACCTTC-3'	60	250
	Reverse	5'-CAAGTTGCCAGTCACCTCTAAGC-3'		
MMP-14	Forward	5'-GAGATCAAGGCCAATGTCGAGG-3'	60	382
	Reverse	5'-TTAGATCCTCATTTTGGACAGTCC-3'		
TIMP-1	Forward	5'-CCTTATACCAGCCGTTATAAGATCAAGAT-3'	60	346
	Reverse	5'-GTCCACAAACAGTGAGTGTCACTC-3'		
TIMP-2	Forward	5'-GCAATGCAGACGTAGTGATCAGAG-3'	60	371
	Reverse	5'-GATCATGGGACAGCGAGTGATCTT-3'		
β -Actin	Forward	5'-GGACTCCTATGGTGGGTGACGAGG-3'	58	366
	Reverse	5'-GGGAGAGCATAGCCCTCGTAGAT-3'		

PGE₂ production assay. Osteoblastic cells (1×10^4 cells/well) were cocultured with bone marrow cells (1×10^5 cells/well) in 200 μ l of α -MEM containing 10% FBS in 48-well plates (Corning Inc.). After the cells had grown to confluence, the cocultures were treated with LOS (10 μ g/ml) in either the presence or the absence of polymyxin B and incubated for an additional 24 h. PGE₂ production in the cocultures was determined by using a PGE₂ enzyme immunoassay kit according to the manufacturer's instructions (Amersham Pharmacia Biotech).

Statistical analyses. The statistical significance of differences was determined by the Mann-Whitney U test. A *P* value of <0.05 was considered significant.

RESULTS

Effect of *T. denticola* LOS on osteoclast formation in a coculture system. The purified lipopolysaccharide (LPS) preparation from *T. denticola* did not show a typical ladder-like band pattern in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels after staining with silver nitrate, as previously described (28, 33). It migrated to a position between 7 and 14 kDa (based on the protein marker) and was not stained with Coomassie brilliant blue (data not shown). These data mean that the LPS preparation from *T. denticola* is LOS.

In the coculture treated with either the *T. denticola* sonicate (10 μ g/ml) or LOS (0.1 to 10 μ g/ml) for 4 days, a number of TRAP-positive multinucleated cells were formed, while the nontreated cultures did not contain TRAP-positive multinucleated cells. LOS increased the number of osteoclasts in a dose-dependent manner (Fig. 1).

Effect of *T. denticola* LOS on expression of ODF and OPG mRNAs. Expression of ODF and OPG mRNAs was investigated with the cocultures treated with LOS for 4 days by RT-PCR (Fig. 2). The nontreated cells exhibited steady-state levels of ODF and OPG mRNA expression, as described in

other reports (16, 25). ODF mRNA expression increased and OPG mRNA expression decreased after stimulation with *T. denticola* LOS (0.1 to 10 μ g/ml). To confirm the effect of LOS on ODF and OPG mRNA expression, an inhibition assay was

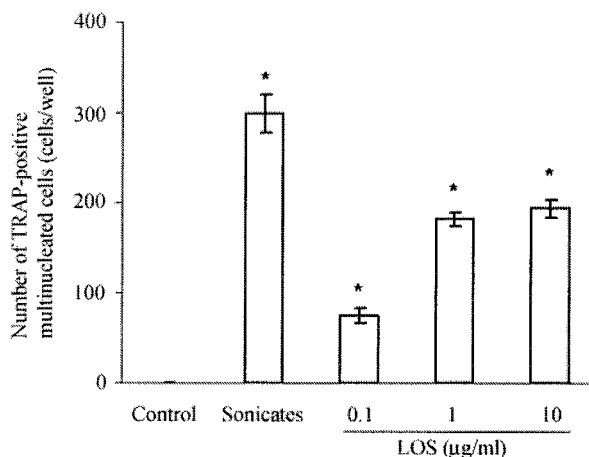


FIG. 1. Formation of TRAP-positive multinucleated cells in the coculture system treated with whole-cell sonicates and LOS from *T. denticola*. Mouse bone marrow and calvaria cells were cocultured to confluence and treated with *T. denticola* sonicates (10 μ g/ml) or LOS (0.1 to 10 μ g/ml) for an additional 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 \pm standard errors for four cultures. An asterisk indicates that the *P* value is <0.05 for a comparison with the results for the nontreated cultures.

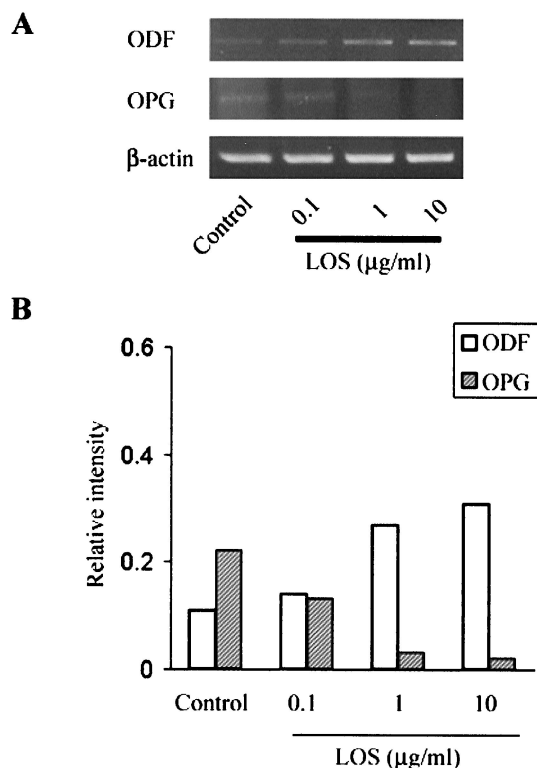


FIG. 2. ODF and OPG mRNA expression in cocultures treated with *T. denticola* LOS. After the cocultures were treated with the LOS from *T. denticola* (0.1 to 10 µg/ml), as described in the text, the ODF, OPG, and β-actin mRNA levels were examined by RT-PCR (A). Signals in the RT-PCR were quantified and normalized to β-actin mRNA expression by using an image analyzer (B). The experiments were repeated three times, and similar results were obtained in all experiments.

performed with polymyxin B, which is known to form a stable complex with the lipid A of LPS and to neutralize LPS activity (24). Polymyxin B (50 µg/ml) eliminated the effect of LOS (10 µg/ml) on ODF and OPG mRNA expression (Fig. 3).

Effect of indomethacin on osteoclast formation and expression of ODF and OPG mRNAs regulated by *T. denticola* LOS. In order to determine whether PGE₂ was involved in osteoclastogenesis induced by *T. denticola* LOS, PGE₂ production in the coculture treated with LOS (10 µg/ml) in the presence or absence of polymyxin B was determined. A low level of PGE₂ was detected in the untreated cultures, and the PGE₂ concentration was higher in the cultures treated with LOS. Polymyxin B (50 µg/ml) decreased the PGE₂ production stimulated by LOS (Fig. 4). To confirm the involvement of PGE₂ in osteoclast formation and the expression of ODF and OPG mRNAs, cocultures were treated with LOS in the presence or absence of indomethacin, which is a prostaglandin inhibitor. In the coculture treated simultaneously with indomethacin (1 µM) and LOS (10 µg/ml) for 4 days, the number of TRAP-positive multinucleated cells was significantly less than the numbers of such cells in the cultures treated with LOS alone (Fig. 5A). The ODF mRNA was down-regulated and the OPG mRNA was up-regulated by indomethacin (Fig. 5B and C).

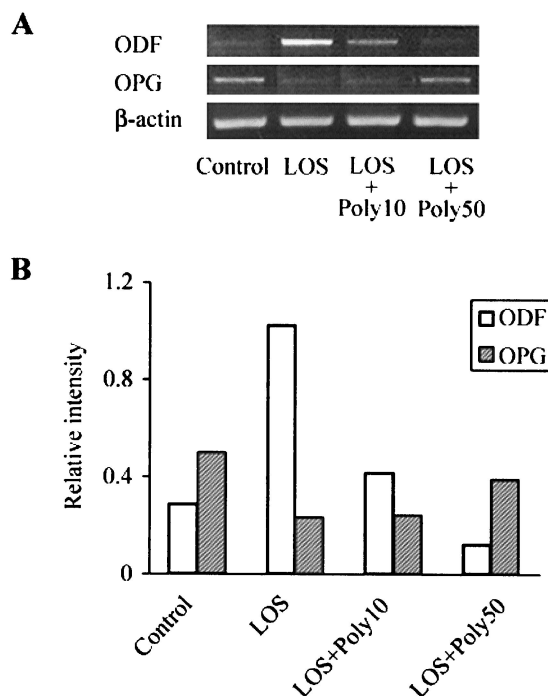


FIG. 3. Effect of polymyxin B on the ODF and OPG mRNAs induced by *T. denticola* LOS. After cocultures were treated with LOS (10 µg/ml) in the presence or absence of 10 µg of polymyxin B per ml (Poly10) or 50 µg of polymyxin B per ml (Poly50) for 4 days, the ODF, OPG, and β-actin mRNA levels were examined by RT-PCR (A). The signals in the RT-PCR were quantified and normalized to β-actin mRNA expression by using an image analyzer (B). The experiments were repeated three times, and similar results were obtained in all experiments.

Indomethacin alone did not affect osteoclast formation and the expression of ODF and OPG mRNAs (data not shown).

Expression of mRNAs of MMPs and TIMPs of osteoblastic cells treated with *T. denticola* LOS. MMPs, including MMP-3, -7, -8, -9, -10, -12, -13, and -14, and TIMPs, including TIMP-1 and -2, were examined by RT-PCR to determine expression of their mRNAs in the osteoblastic cells treated with *T. denticola* LOS (10 µg/ml) for 48 h (Fig. 6A and B). The MMP-3, -8, -9, -10, -13, and -14 and TIMP-1 and -2 mRNAs were expressed in the untreated osteoblastic cells, whereas MMP-7 and -12 mRNA expression could not be detected in treated or untreated cells. *T. denticola* LOS (10 µg/ml) increased the MMP-3, -8, -9, -10, -13, and -14 mRNA levels. Of these, MMP-9 mRNA expression was markedly enhanced by *T. denticola* LOS. *T. denticola* LOS slightly decreased TIMP-2 mRNA expression, whereas it did not influence TIMP-1 mRNA expression. To confirm the effect of *T. denticola* LOS on MMP expression, the levels of expression of MMP-9, whose mRNA exhibited a marked change in expression, were compared for cells treated with LOS (10 µg/ml), with polymyxin B (50 µg/ml), and with LOS plus polymyxin B (Fig. 6C and D). Polymyxin B (50 µg/ml) increased MMP-9 expression to some extent compared to the expression in the untreated cells. However, polymyxin B (50 µg/ml) decreased the MMP-9 expression stimulated by *T. denticola* LOS to the same level observed for the cells treated with polymyxin B alone.

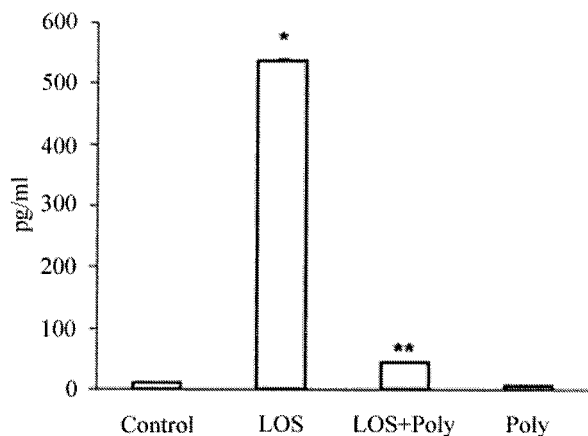


FIG. 4. Effect of LOS on PGE₂ production in cocultures. The cocultures were treated with LOS (10 μ g/ml) with or without polymyxin B (Poly) (50 μ g/ml) for 24 h. The PGE₂ concentration was determined by using a PGE₂ enzyme immunoassay kit. The data are the means \pm standard errors for three cultures. One asterisk indicates that the *P* value is <0.05 for a comparison with the results for the nontreated cultures. Two asterisks indicate that the *P* value is <0.05 for a comparison with the results for the LOS-treated cultures.

DISCUSSION

To determine the role of *T. denticola* in bone resorption, the effects of *T. denticola* sonicates and LOS on osteoclast formation and MMP expression were examined. Osteoblasts or stromal cells play an essential role in osteoclastogenesis through ODF expression (48). Therefore, to investigate the effect of *T. denticola* on osteoclastogenesis, a coculture system consisting of mouse calvaria cells which contained primary osteoblasts and bone marrow cells which included osteoclast precursors was used. In the preliminary study, *T. denticola* sonicates stimulated osteoclastogenesis in the coculture. This result prompted us to search for a component of *T. denticola* involved in osteoclastogenesis.

Yotis et al. (49) reported that *T. denticola* possesses an LPS-like molecule (8 to 14 kDa) that exhibits *Limulus* amoebocyte lysate clotting activity. Other research groups (28, 33) purified an approximately 14- to 21-kDa LOS from *T. denticola*. This LOS stimulated nitric oxide and TNF- α production in mouse macrophages, and the induction was inhibited by polymyxin B (28). In addition, Schultz et al. (31) reported that *T. denticola* LOS is quite different from the LPS of other gram-negative bacteria. Therefore, it was of interest to investigate the ability of *T. denticola* LOS to stimulate osteoclastogenesis. Gopalsami et al. (10) reported that the outer membrane of *T. denticola* increased Ca²⁺ release in an organ culture of radii and ulnae, and heat treating the outer membrane did not alter the effect on Ca²⁺ release. These authors concluded that a heat-stable LPS-like material is present in the outer membrane of *T. denticola*, which might be responsible for bone resorption. Taken together, these findings imply that the LOS from *T. denticola* may be involved in osteoclastogenesis. In the present study, *T. denticola* LOS was shown to stimulate osteoclast differentiation in a dose-dependent manner by using a coculture of osteoblast and osteoclast precursors. This means that *T. denticola* LOS induces osteoclastogen-

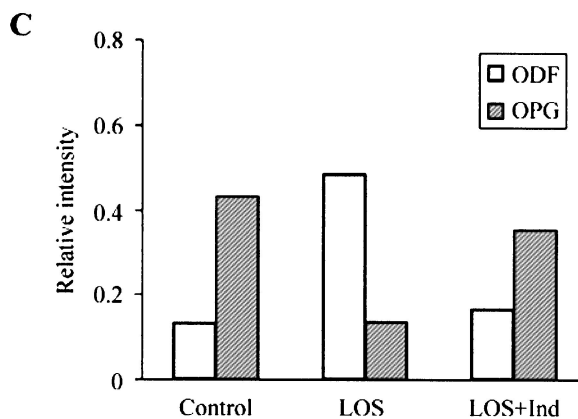
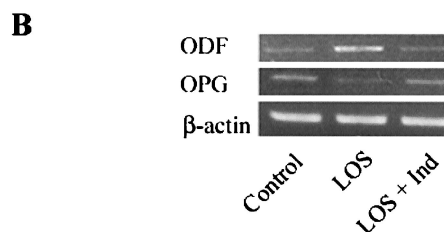
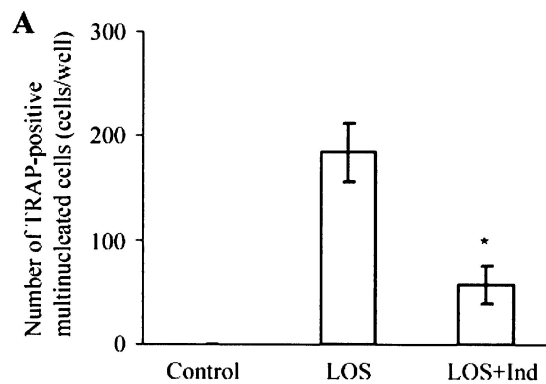


FIG. 5. Effect of indomethacin on osteoclastogenesis and the expression of ODF and OPG mRNAs modulated by *T. denticola* LOS. Cocultures were simultaneously treated with LOS (10 μ g/ml) with or without indomethacin (Ind) (1 μ M) for 4 days. The cells were then stained for TRAP to count the number of osteoclasts. The data are means \pm standard errors for three cultures (A). The RNA was isolated from the cultured cells, and the ODF, OPG, and β -actin mRNA levels were analyzed by RT-PCR (B). The RT-PCR signals shown were quantified and normalized to the β -actin mRNA expression by using an image analyzer (C). Representative results of three experiments that yielded similar results are shown. An asterisk indicates that the *P* value is <0.05 for a comparison with the results for the LOS-treated cultures.

esis. However, *T. denticola* sonicates induced more TRAP-positive cells than LOS induced. In addition, when *T. denticola* sonicates were heat treated, the osteoclast formation activity of sonicates was not completely inhibited (data not shown). Therefore, the possibility that some heat-labile components of *T. denticola* are involved in osteoclastogenesis cannot be ruled out.

It was reported previously that the osteoblast or stromal cell

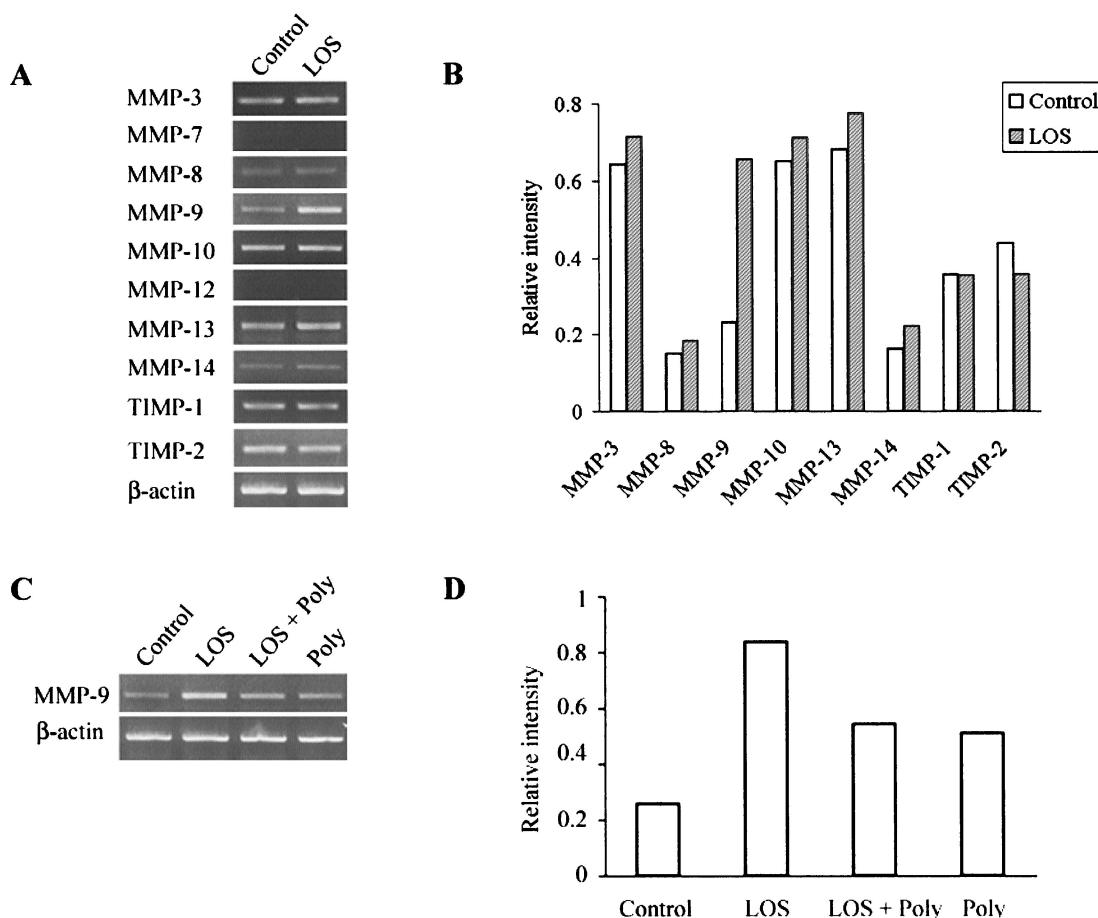


FIG. 6. Expression of MMP and TIMP mRNAs in mouse calvaria-derived osteoblastic cells treated with *T. denticola* LOS. After the osteoblastic cells were treated with *T. denticola* LOS (10 μ g/ml) in the absence (A) or in the presence (C) of polymyxin B (Poly) (50 μ g/ml) for 48 h, the RNA was isolated from the cells, and the MMP and TIMP mRNA levels were analyzed by RT-PCR. Representative results of three experiments that yielded similar results are shown. The RT-PCR signals shown in panels A and C were quantified and normalized to β -actin mRNA expression by using an image analyzer (B and D).

lines that support osteoclastogenesis showed a much higher ODF mRNA level, whereas the level of OPG mRNA was drastically reduced in the presence of either PGE₂ or 1 α ,25(OH)₂D₃ (25). Another group of workers also reported that 1 α ,25(OH)₂D₃, PTH, or IL-11 prompted an increase in the ratio of ODF to OPG (16). These findings indicate that the effects of these bone resorptive factors are mediated through regulation of the production of ODF and its endogenous receptor antagonist, OPG, and that the ODF/OPG ratio appears to be an essential factor that determines the ability of osteoblastic cells to induce osteoclast formation. Since the discovery of ODF, it has been believed that ODF is the sole factor responsible for inducing osteoclast differentiation. However, it has been shown that TNF- α stimulates osteoclast formation via an ODF-independent mechanism (19). Our results suggest that the stimulatory effect of *T. denticola* LOS on osteoclast formation is mediated through ODF up-regulation and OPG down-regulation.

PGE₂ has been shown to play a role in osteoclastogenesis (17). Two studies on the involvement of PGE₂ in ODF and OPG mRNA expression in osteoblastic cells by LPS from *Escherichia coli* had different results. Kikuchi et al. (18) re-

ported that LPS increased the ODF mRNA level and that an inhibitor of PGE₂ synthesis failed to block the effect of LPS after 2 h of exposure, while OPG gene expression remained constant after LPS stimulation. These authors suggested that LPS induced ODF mRNA in osteoblasts directly, not via PGE₂, and did not affect OPG expression. In contrast, Sakuma et al. (30) observed the effect of LPS for a longer period (24 h). They showed that LPS exposure induced ODF mRNA expression in a time-dependent manner. Induction for 4 h was not inhibited by indomethacin. However, induction for 24 h was partially inhibited by indomethacin. Furthermore, LPS exposure increased OPG mRNA expression, but the OPG levels that were induced by LPS exposure were not affected by indomethacin. This report suggested that ODF is induced by LPS in a PGE₂-dependent manner and in a PGE₂-independent manner and that OPG expression by LPS is increased independent of PGE₂. To determine if PGE₂ is involved in the effect that *T. denticola* LOS has on ODF and OPG mRNA expression, we estimated the numbers of osteoclasts and the levels of ODF and OPG mRNA expression in cocultures treated with LOS in the presence and in the absence of indomethacin for 4 days. *T. denticola* LOS stimulated PGE₂ production and osteoclast for-

mation by LOS was reduced by indomethacin in coculture, which suggests the possibility that PGE₂ is involved in LOS-regulated ODF and OPG expression. Like LPS, *T. denticola* LOS increased ODF mRNA expression, and this stimulating activity was inhibited by indomethacin. However, unlike regulation by LPS, OPG mRNA was down-regulated by *T. denticola* LOS, and the OPG mRNA level recovered to the control level after treatment with indomethacin. Taken together, these findings indicate that PGE₂ is involved in the regulation of ODF and OPG gene expression by *T. denticola* LOS. The down-regulation of OPG gene expression by *T. denticola* LOS is somewhat different from the results for LPS reported previously (18, 30). The discrepancy may result from the different culture conditions used (i.e., the cell type and the stimulation time) or from the structural differences between LPS and LOS.

It has been reported that PGE₂ and IL-1 are involved in osteoclast formation by *Actinobacillus actinomycetemcomitans* LPS and that *Porphyromonas gingivalis* LPS promotes bone resorption, which is mediated by IL-1, IL-6, TNF- α , and PGE₂ (4, 23, 43, 50). These previous studies suggest that cytokines, such as IL-1, TNF- α , and IL-6, are also involved in LPS-induced osteoclastogenesis. We are currently studying the involvement of proinflammatory cytokines in osteoclastogenesis by *T. denticola* LOS.

The bone matrix consists of various proteins, including collagen, proteoglycans, and glycoproteins (27, 29). Recent observations show that MMPs may play a role not only in dissolving the bone matrix but also in initiating bone resorption, which determines where and when bone resorption occurs. Removing nonmineralized matrix from the bone surface is essential for initiating osteoclastic bone resorption, because osteoclasts cannot attach to nonmineralized osteoid (2). In order to determine the involvement of MMPs in bone resorption by *T. denticola* LOS, we analyzed the expression of mRNAs of various MMPs. The most apparent increase was observed for MMP-9. MMP-9 has broad substrate specificity, including proteoglycans, glycoproteins, and gelatin, a denatured form of collagen (22). In addition to gelatinase activity, MMP-9 exerts chemotactic activity on osteoclasts. MMP-9 releases extracellular matrix-bound vascular endothelial growth factor, which acts as a chemoattractant for osteoclasts (8). TNF- α is a bone resorption-inducing factor, and it has been reported that the proteolytic processing of this factor from its precursor to an active form is carried out by MMP-9 (22). These reports imply that MMP-9 may play an important role in the bone resorption process by dissolving the bone matrix, releasing the chemotactic factor on osteoclasts, and activating the cytokines involved in bone resorption. Although the expression of mRNAs of MMP-3, -8, -10, -13, and -14 is increased less than that of mRNA of MMP-9 by *T. denticola* LOS in osteoblastic cells, increased expression of the mRNAs of these MMPs was observed in all three experiments. Therefore, in the future, the significance of these MMPs in the bone resorption induced by *T. denticola* LOS should be confirmed.

TIMPs are the major endogenous inhibitors that down-regulate MMP activity (7). Four TIMPs have been identified. Although each TIMP inhibits most of the MMPs, a certain degree of specificity has been observed. TIMP-1 and -2 are inhibitors of MMP-9. TIMP-1 preferentially binds MMP-9 and inhibits its activity (7, 37). The balance between the activities of

the MMPs and the TIMPs is believed to determine the rate of matrix degradation. *T. denticola* LOS did not affect TIMP-1 mRNA expression and slightly decreased TIMP-2 mRNA expression in osteoblastic cells. These observations imply that TIMPs have a minor effect on MMP-9 activity.

In conclusion, here we provide evidence that the LOS from *T. denticola* stimulates osteoclastogenesis and the expression of several MMPs, including MMP-9, in osteoblastic cells. ODF up-regulation and OPG down-regulation via PGE₂ are involved in osteoclastogenesis. *T. denticola* is known to be one of the major putative pathogens of periodontitis, a polymicrobial infection. Our results show that the pathogenesis induced by *T. denticola* may be one of the mechanisms of bone destruction in periodontitis.

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