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Lipopolysaccharide Enhances the Production of Nicotine-Induced Prostaglandin E_2 by an Increase in Cyclooxygenase-2 Expression in Osteoblasts

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Abstract Previous studies have indicated that lipopolysaccharide (LPS) from Gram-negative bacteria in plaque induces the release of prostaglandin E_2 (PGE_2), which promotes alveolar bone resorption in periodontitis, and that tobacco smoking might be an important risk factor for the development and severity of periodontitis. We determined the effect of nicotine and LPS on alkaline phosphatase (ALPase) activity, PGE_2 production, and the expression of cyclooxygenase (COX-1, COX-2), PGE_2 receptors Ep1–4, and macrophage colony stimulating factor (M-CSF) in human osteoblastic Saos-2 cells. The cells were cultured with 10^{-3} M nicotine in the presence of 0, 1, or 10 μg/ml LPS, or with LPS alone. ALPase activity decreased in cells cultured with nicotine or LPS alone, and decreased further in those cultured with both nicotine and LPS, whereas PGE_2 production significantly increased in the former and increased further in the latter. By itself, nicotine did not affect expression of COX-1, COX-2, any of the PGE_2 receptors, or M-CSF, but when both nicotine and LPS were present, expression of COX-2, Ep3, Ep4, and M-CSF increased significantly. Simultaneous addition of 10^{-4} M indomethacin eliminated the effects of nicotine and LPS on ALPase activity, PGE_2 production, and M-CSF expression. Phosphorylation of protein kinase A was high in cells cultured with nicotine and LPS. These results suggest that LPS enhances the production of nicotine-induced PGE_2 by an increase in COX-2 expression in osteoblasts, that nicotine-LPS-induced PGE_2 interacts with the osteoblast Ep4 receptor primarily in autocrine or paracrine mode, and that the nicotine-LPS-induced PGE_2 then decreases ALPase activity and increases M-CSF expression.

Key words nicotine; lipopolysaccharide; prostaglandin E_2; cyclooxygenase-2; Ep4 receptor

Periodontitis is an infectious disease, and bacteria are the driving force behind the observed tissue destruction [1]. The loss of alveolar bone and connective tissue attachment around teeth results from both the colonization of tooth surfaces by certain Gram-negative anaerobic bacteria, such as *Actinobacillus actinomycetemcomitans* [2,3] and *Porphyromonas gingivalis* [4], and the host response to such colonization [5]. Cigarette smoking is associated with an increased incidence of destructive periodontitis [6,7] and increased alveolar bone loss [8]. Nicotine, a major component of cigarette smoke, influences host-bacterial interactions and has been detected in the saliva and gingival crevicular fluid of smokers [9]. Nicotine has been shown to have detrimental effects on periodontal cells in a variety of ways. In an *in vitro* study, nicotine inhibited growth of gingival fibroblasts and production of fibronectin and collagen, and promoted collagen breakdown [10].

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Lipopolysaccharide (LPS), a cell wall component of the Gram-negative bacteria in dental plaque, induces the release of bone-absorbing cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α [11–13], and of prostaglandin E_2 (PGE_2) [14]. In periodontal tissue, PGE_2 is predominantly produced from arachidonic acid in gingival fibroblasts and osteoblasts when cyclooxygenase (COX)-2 is stimulated by the above-mentioned cytokines [15–17]. PGE_2 receptors are classified into four subtypes, Ep1, Ep2, Ep3, and Ep4, all of which interact with different G-proteins [18,19]. Recent studies of these receptor subtypes using knockout mice have revealed that PGE_2 fails to increase the expression of receptor activators of NF-κB (RANK) ligand in Ep4-deficient mice, resulting in the loss of bone-absorbing ability [20]. However, the involvement of nicotine and LPS in this process has not been fully elucidated, and their effects on the expression of PGE_2 receptors have not been explored in osteoblasts.

We hypothesized that alveolar bone resorption in smokers was greater than that of non-smokers under conditions of defective mouth cleaning. This hypothesis was based on reports that smoking influences the onset and progression of periodontitis [6,7], and on our previous findings that nicotine inhibits mineralized nodule formation by osteoblasts [21], that nicotine and LPS stimulate the formation of osteoclast-like cells [22], and that LPS stimulates PGE_2 production and Ep4 receptor expression in osteoblasts [16]. Consequently, we conducted the current study to determine the effects of nicotine in the presence and absence of LPS on: (1) cell proliferation; (2) alkaline phosphatase (ALPase) activity; (3) PGE_2 production; (4) expression of COX-1, COX-2, PGE_2 receptors Ep1–4, and macrophage colony stimulating factor (M-CSF); and (5) phosphorylation of protein kinase A (PKA) in osteoblasts, using the human osteosarcoma Saos-2 cell line.

Materials and Methods

Cell culture

Saos-2 human osteosarcoma cells [23,24] were obtained from the RIKEN Bioresource Center (Tsukuba, Japan) and used as osteoblasts. The cells were maintained in a growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Rockville, USA) containing 10% (*V*/*V*) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, USA) and 1% (*V*/*V*) penicillin-streptomycin solution (Sigma-Aldrich, St. Louis, USA) at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2.

For treatment with nicotine and LPS, the cells were seeded onto 100-mm tissue culture plates at a density of 5×10^6 cells/cm^2. After overnight incubation, the cells were cultured for up to 14 d with DMEM containing 10% fetal bovine serum and 10^{-3} M nicotine (Wako Fine Chemicals, Osaka, Japan) in the presence of 0, 1, or 10 μg/ml LPS from *Escherichia coli* 026-B6 (L 2654; Sigma-Aldrich), or with 1 or 10 μg/ml LPS. Nicotine concentrations were chosen based on a previous report by Tipton and Dabbous [25], who examined the effect of nicotine on the proliferation of, and extracellular matrix production by, human gingival fibroblasts *in vitro,* and on our previous studies [21,22,26]. In the latter, we examined the effect of 10^{-3} and 10^{-4} M nicotine on mineralized nodule formation by Saos-2 cells *in vitro*. LPS concentrations were chosen based on a previous report by Suda *et al*. [27], who found that LPS (0.001 to 100 μg/ml) and IL-1 play multiples roles in the stimulation of osteoclastic bone resorption, and on our previous studies [16,21,22].

To determine the effects of the inhibition of endogenous PGE_2 in Saos-2 cells, we added 10^{-4} M indomethacin (Sigma-Aldrich) to the culture medium, as described in previous reports [16,28,29] and cultured the cells with 10 μg/ml LPS and 10^{-3} M nicotine for up to 14 d.

Determination of cell proliferation

Cells were placed on 96-well microplates at a density of 6×10^3 cells/cm^2 and cultured with 10^{-3} M nicotine in the presence of 0, 1, or 10 μg/ml LPS for up to 14 d. At the times indicated, the medium was replaced with fresh medium containing 10% (*V*/*V*) cell-counting reagent (Wako Fine Chemicals) [30]. After incubation for 1 h, the intensity of the reaction products was measured at 450 nm with a microtiter plate reader (SpectraMax 190 Microplate Spectrophotometer; Molecular Devices, Sunnyvale, USA). The relative cell numbers were calculated from the absorbance values on the basis of a standard curve.

Determination of ALPase activity

Cells were plated on 96-well microplates and cultured with 10^{-3} M nicotine with or without LPS and indomethacin as described under "Determination of cell proliferation". Cells were also cultured with LPS alone. At the times indicated, 200 μl of an enzyme assay solution (8 mM *p*-nitrophenyl phosphate, 12 mM $MgCl_2$, 0.1 mM $ZnCl_2$ in 0.1 M glycine-NaOH, pH 10.5) was added to the cells in each well, and the plate was incubated for 5–30 min at 37

ºC. The enzyme reaction was terminated by the addition of 50 μl of 0.5 M NaOH. The amount of *p*-nitrophenol released by the enzyme reaction was determined by measuring the absorbance at 405 nm using a microtiter plate reader. One unit of ALPase activity was defined as the amount required for the liberation of 1.0 μmol *p*-nitrophenyl per minute. The enzyme activity was recorded as mU per 10^4 cells.

Real-time polymerase chain reaction (PCR)

Cells were plated on 6-well microplates at a density of 6×10^3 cells/cm^2 and cultured with 10^{-3} M nicotine in the presence of 0, 1, or 10 μg/ml LPS for up to 14 d. Total RNA was isolated from the cultured cells on days 3, 5, 7, 10, and 14 using an RNeasy mini kit (Qiagen, Valencia, USA). The amount of RNA was equalized using a human β-actin competitive PCR kit (TaKaRa Shuzo, Shiga, Japan). The mRNA was converted into cDNA using a GeneAmp RNA PCR kit (PerkinElmer, Branchburg, USA).

The cDNA mixtures were diluted 5-fold in sterile distilled water, and 2 μl was subjected to real-time PCR using SYBR Green I dye. The reactions were carried out in 25 μl of an SYBR premixed ExTaq solution (TaKaRa Shuzo) with 20 μM sense and antisense primers (**Table 1**), which were designed using Primer3 software (version 0.2; Whitehead Institute for Biomedical Research, Cambridge, USA). The assays were carried out on a Smart Cycler (Cepheid, Sunnyvale, USA) and were analyzed using Smart Cycler software (version 1.2d). PCR consisted of 40 cycles of 95 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s, with measurements made at the end of the 72 ºC annealing step. All real-time PCR reactions were carried out in triplicate; gene expression levels were calculated and normalized by dividing the calculated values for the mRNA samples by those of glyceraldehyde 3-phosphate dehydrogenase mRNA isolated at the same time.

Enzyme-linked immunosorbent assay (ELISA)

The cells were plated on 6-well tissue culture plates and cultured with 10^{-3} M nicotine with or without LPS and indomethacin as described under "Real-time PCR". Cells were also cultured with LPS alone. At the times indicated, the medium was replaced with serum-free DMEM without LPS, nicotine, or indomethacin, and the cells were cultured for 24 h at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2. The amount of PGE_2 in the culture medium was determined using a commercially available ELISA kit (Biomedica Medizinprodukte, Vienna, Austria) according to the manufacturer's instructions. Triplicate assays were carried out on each specimen, and the data were converted to pg/ml. The protein amount of COX-2 in the cells was determined by general ELISA method using mouse monoclonal immunoglobulin G antibody against human COX-2 (Rockland Immunochemicals, Gilbertsville,

Table 1 Polymerase chain reaction primers used in this study

Target	Primer sequences (sense and antisense)	GenBank accession No.
COX-1	5′-ACCTTGAAGGAGTCAGGCATGAG-3′	U63846
	5′-TGTTCGGTGTCCAGTTCCAATA-3′	
COX-2	5′-CTGGGTTTCCGATTTTCTCA-3′	AY462100
	5′-CAAGCCCATGTGAATGACTG-3′	
Ep1	5′-TCTACCTCCCTGCAGCGGCCACTG-3′	NM_000955
	5′-GAAGTGGCTGAGGCCGCTGTGCCGGAG-3′	
Ep2	5′-TGAAGTTGCAGGCGAGCA-3′	NM_000956
	5′-GACCGCTTACCTGCAGCTGTAC-3′	
Ep3	5′-GCAGTGCTCAACTGATGTCT-3′	NM_000957
	5′-GGACTAGCTCTTCGCATAACT-3′	
Ep4	5′-TTCCGCTCGTGGTGCGAGTGTTC-3′	NM_000958
	5′-GAGGTGGTGTCTGCTTGGGTCAG-3′	
M-CSF	5′-TTCGCGCAGTGTAGATGAAC-3′	NM_172210
	5′-CATCCAGGCAGAGACTGACA-3′	
GAPDH	5′-GAGTCAACGGATTTGGTCGT-3′	NM_002046
	5′-GACAAGCTTCCCGTTCTCAG-3′	

USA), biotin-conjugated secondary antibody, and horseradish peroxidase-conjugated streptavidin. Triplicate assays were carried out on each sample, and the absorbance at 492 nm was recorded.

PKA assay

The cells were plated on 6-well tissue culture plates and cultured with 10^{-3} M nicotine with or without LPS and indomethacin as described under "Real-time PCR". The medium was replaced with serum-free DMEM without LPS, nicotine, or indomethacin on day 14, and the cells were cultured for 24 h at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2. After the culture medium was discarded, the cells were lysed with extraction buffer (0.05% Triton X-100, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulphonyl fluoride, 0.5 mM EDTA, and 25 mM Tris-HCl, pH 7.4). The cell membranes were destroyed by sonication, the supernatant fractions were collected, and their protein concentrations were determined using Bio-Rad DC Protein Assay Reagent B (Bio-Rad Laboratories, Hercules, USA).

PKA activity was determined using the PegTag Non-Radioactive Detection of PKA assay kit (Promega, Madison, USA) with a fluorescent PKA-specific peptide (L-R-R-A-S-L-G) as a substrate. When electrophoresed on a 0.8% agarose gel at neutral pH, the non-phosphorylated peptide substrate has a +1 net charge and migrates toward the negative electrode, whereas the phosphorylated peptide substrate has a −1 net charge and migrates toward the positive electrode. The intensities of the bands in the gel were quantified using an Epson GT-9500 digital scanner (Seiko Epson, Tokyo, Japan) and Digital Science 1D digital image analysis software (version 2.0) (Eastman Kodak, New Haven, USA).

Statistical analysis

All experiments were carried out in triplicate. Data shown represent the mean±SD. Significant differences were determined using ANOVA and Bonferroni's modification of Student's *t*-test. Differences with $P<0.05$ were considered significant.

Results

Cell proliferation

Proliferation of Saos-2 cells cultured in the presence of 10^{-3} M nicotine and 0, 1, or 10 μg/ml LPS was determined on days 3, 5, 7, 10, and 14 of the culture (**Fig. 1**). In the presence of nicotine alone or both nicotine and LPS, cell proliferation decreased slightly compared to the control after 5 d of culture.

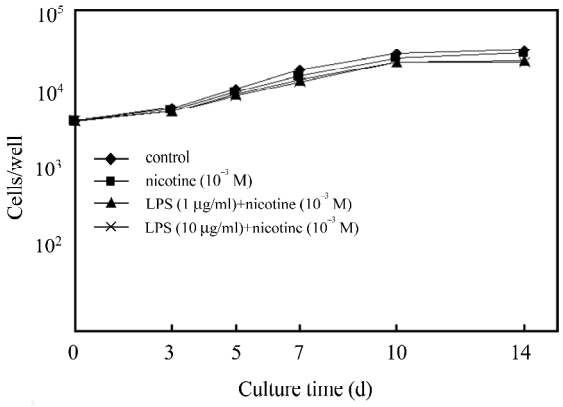

Fig. 1 Effect of nicotine and lipopolysaccharide (LPS) on cell proliferation

Saos-2 cells were cultured with 10^{-3} M nicotine and 0, 1, or 10 μg/ml LPS. Measurements were made on day 3, 5, 7, 10, and 14 of the culture.

ALPase activity

ALPase activity in Saos-2 cells cultured in the presence of 10^{-3} M nicotine and/or 1 or 10 μg/ml LPS was determined on day 5, 7, 10, and 14 of the culture (**Fig. 2**). In

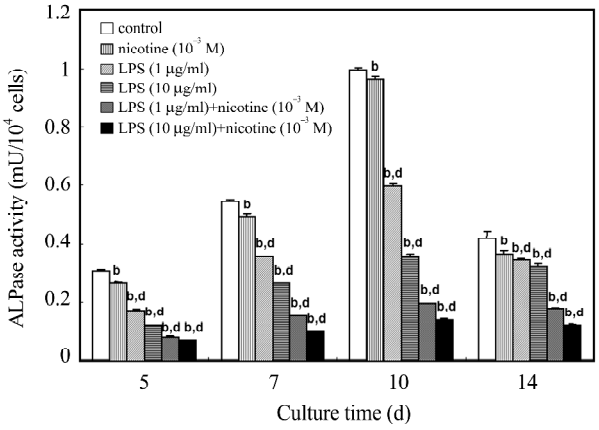

Fig. 2 Effect of nicotine and lipopolysaccharide (LPS) on alkaline phosphatase (ALPase) activity

Saos-2 cells were cultured with 10^{-3} M nicotine and/or 1 or 10 μg/ml LPS, and ALPase activity was determined on day 5, 7, 10, and 14 of the culture. The data shown are the mean±SD for three separate experiments. [b] $P<0.01$, treatment with nicotine or both nicotine and LPS compared to control; [d] $P<0.01$, treatment with both nicotine and LPS compared to nicotine treatment alone.

both the presence and absence of nicotine and LPS, ALPase activity increased gradually through day 10, then decreased by day 14. Nonetheless, on day 5, 7, 10, and 14, the overall amount of ALPase activity was less in nicotine-treated and LPS-treated cells than in the non-treated controls. Furthermore, in both the presence and absence of nicotine, ALPase activity decreased in an LPS dose-dependent manner, at each time point indicated.

There was little difference in the values between nicotine-treated and control cells. Therefore, all data were recorded by the numerical value as follows: the values of control cells on day 5, 7, 10, and 14 were 0.309±0.005, 0.547±0.005, 0.994±0.008, and 0.420±0.024, respectively; the values of nicotine-treated cells on day 5, 7, 10, and 14 were 0.266±0.003, 0.494±0.010, 0.963±0.009, and 0.365±0.013, respectively.

PGE_2 production

ELISA was used to determine PGE_2 production in Saos-2 cells cultured in the presence of 10^{-3} M nicotine and/or 1 or 10 μg/ml LPS on day 3, 5, 7, 10, and 14 of culture (**Fig. 3**). In both the presence and absence of nicotine and LPS, PGE_2 production increased gradually with time through to day 14. In the presence of nicotine and LPS alone, production of PGE_2 increased compared with the non-treated control on each day. Furthermore, in both the presence and absence of nicotine, PGE_2 production increased in an LPS dose-dependent manner, at each time point indicated.

There was little difference in values between nicotine-treated and control cells. Therefore, all data were recorded by the numerical value as follows: the values of control cells on day 3, 5, 7, 10, and 14 were 7.7±2.8, 89.2±8.2, 75.5±18.1, 120.5±17.4 and 127.4±8.2, respectively; the values of nicotine-treated cells on day 3, 5, 7, 10, and 14 were 25.1±10.8, 130.9±6.6, 130.8±3.7, 142.7±7.8, and 153.5±15.6, respectively.

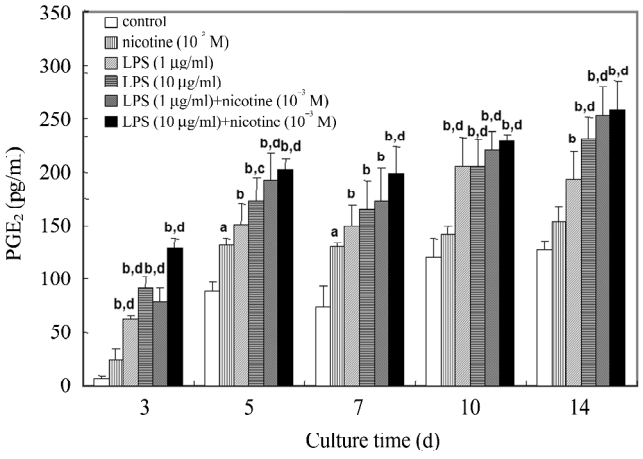

Fig. 3 Effect of nicotine and lipopolysaccharide (LPS) on prostaglandin E_2 (PGE_2) production

Saos-2 cells were cultured with 10^{-3} M nicotine and/or 1 or 10 μg/ml LPS, and PGE_2 production was determined using enzyme-linked immunosorbent assay on day 3, 5, 7, 10, and 14 of the culture. The data shown are the mean±SD for three separate experiments; [a] $P<0.05$, [b] $P<0.01$, treatment with nicotine or both nicotine and LPS compared to control. [c] $P<0.05$, [d] $P<0.01$, treatment with both nicotine and LPS compared to nicotine treatment alone.

Expression of COX-1 and COX-2

Real-time PCR was used to determine COX-1 and COX-2 gene expression in Saos-2 cells cultured in the presence of 0 or 10^{-3} M nicotine and 0, 1, or 10 μg/ml LPS on day 14 of culture [**Fig. 4(A,B)**]. In the presence of nicotine alone, expression of COX-1 and COX-2 did not change compared to the non-treated control levels. In the presence of both nicotine and LPS, COX-1 expression either decreased significantly or did not change [**Fig. 4(A)**] and COX-2 expression increased markedly [**Fig. 4(B)**], compared with the nicotine-only control.

COX-2 protein expression in Saos-2 was also determined using ELISA on day 14 of culture. The protein expression showed a tendency similar to the gene expression [**Fig. 4(C)**].

Expression of PGE_2 receptors

Real-time PCR was used to determine expression of the Ep1, Ep2, Ep3, and Ep4 receptor genes in Saos-2 cells cultured in the presence of 0 or 10^{-3} M nicotine and 0, 1, or 10 μg/ml LPS on day 14 of culture (**Fig. 5**). In the absence of LPS, nicotine had no effect on expression of Ep1, Ep3, or Ep4 [**Fig. 5(A,C,D)**], but it did significantly decrease expression of Ep2 [**Fig. 5(B)**]. When both nicotine and LPS were present, expression of Ep1 and Ep2 decreased significantly compared with the nicotine-only control [**Fig. 5(A,B)**], whereas expression of Ep3 and Ep4 increased markedly in an LPS dose-dependent manner [**Fig. 5(C,D)**].

Effect of indomethacin on ALPase activity, PGE_2 production, and M-CSF expression

ALPase activity, PGE_2 production, and M-CSF expression were determined for Saos-2 cells cultured in the presence of 10^{-3} M nicotine and 10 μg/ml LPS with or without

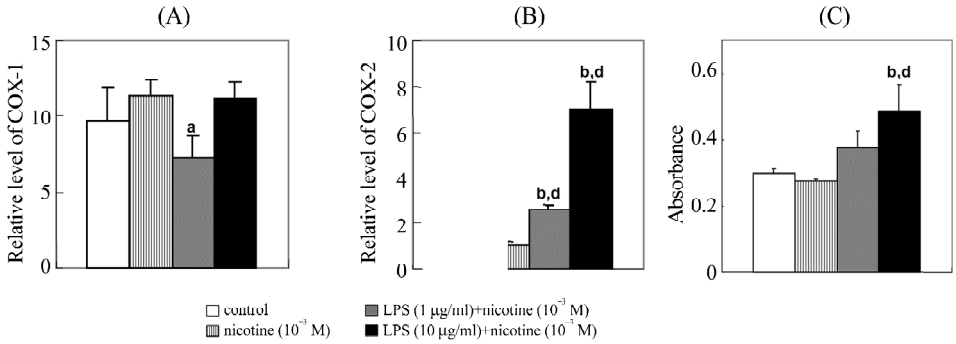

Fig. 4 Effect of nicotine and lipopolysaccharide (LPS) on cyclooxygenase (COX)-1 and COX-2 expression

Saos-2 cells were cultured with 10^{-3} M nicotine and 0, 1, or 10 μg/ml LPS, and expression levels of COX-1 (A) and COX-2 (B) genes and COX-2 (C) protein were determined using real-time polymerase chain reaction and enzyme-linked immunosorbent assay on day 14 of the culture. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. The data shown are the mean±SD for three separate experiments. [a] $P<0.05$, [b] $P<0.01$, treatment with nicotine or both nicotine and LPS compared to control; [d] $P<0.01$, treatment with both nicotine and LPS compared to nicotine treatment alone.

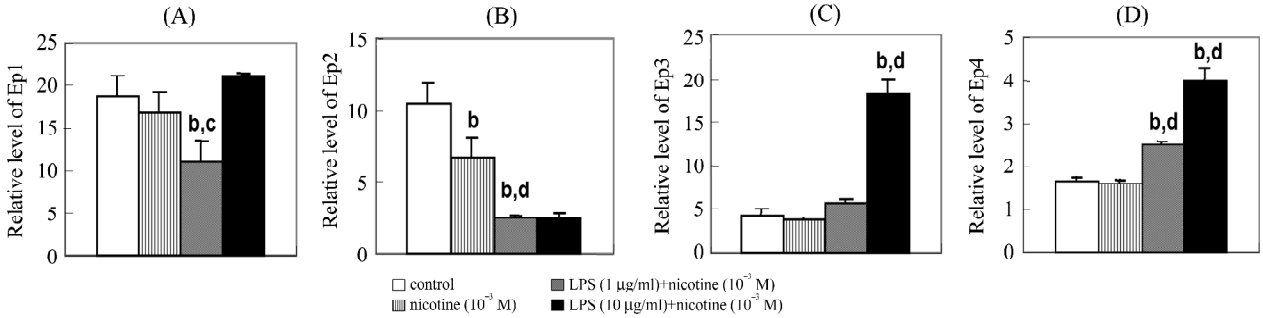

Fig. 5 Effect of nicotine and lipopolysaccharide (LPS) on expression of prostaglandin E_2 (PGE_2) receptors

Saos-2 cells were cultured with 10^{-3} M nicotine and 0, 1, or 10 μg/ml LPS, and expression of the Ep1 (A), Ep2 (B), Ep3 (C), and Ep4 (D) receptor genes was determined using real-time polymerase chain reaction on day 14 of the culture. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. The data shown are the mean±SD for three separate experiments. [b] $P<0.01$, treatment with nicotine or both nicotine and LPS compared to control; [c] $P<0.05$, [d] $P<0.01$, treatment with both nicotine and LPS compared to nicotine treatment alone.

10^{-4} M indomethacin for up to 14 d (**Fig. 6**). In the absence of indomethacin, ALPase activity was reduced by treatment with both nicotine and LPS, but simultaneous treatment with indomethacin returned ALPase activity to the control level by day 7 [**Fig. 6(A)**]. Furthermore, in the absence of indomethacin, PGE_2 production and *M-CSF* expression were increased by treatment with both nicotine and LPS, but simultaneous treatment with indomethacin returned them to the control levels by day 14 [**Fig. 6 (B,C)**].

Phosphorylation of PKA

PKA phosphorylation in Saos-2 cells cultured in the presence of 10^{-3} M nicotine with or without 10 μg/ml LPS was examined on day 14 of the culture (**Fig. 7**). The gel bands indicated a high level of phosphorylated PKA in cells cultured with both nicotine and LPS, compared with those cultured in the presence of nicotine alone or the non-treated control.

Discussion

Periodontitis is a complex and persistent inflammatory disorder with a pathophysiology that is related to both microbial plaque and the host response to its accumulation. LPS from the cell wall of Gram-negative bacteria in plaque is involved in the alveolar bone resorption associated with periodontal disease [31]. Smoking is also an important risk factor for periodontal disease [7]. The present study was designed to clarify the relationship between smoking and mouth cleanliness by examining PGE_2 production in relation to LPS and nicotine, as indices of mouth cleaning and

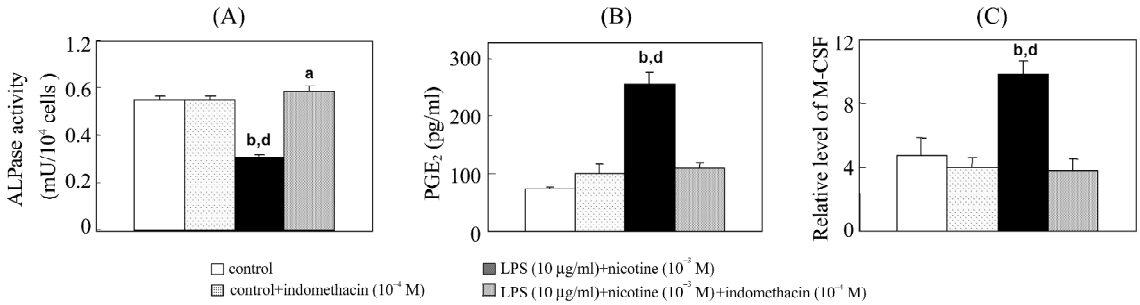

Fig. 6 Effect of indomethacin on alkaline phosphatase (ALPase) activity, prostaglandin E_2 (PGE_2) production, and macrophage colony stimulating factor (M-CSF) expression

Saos-2 cells were cultured in the presence of 10^{-3} M nicotine and 10 μg/ml lipopolysaccharide (LPS) with or without 10^{-4} M indomethacin, and ALPase activity (A) was determined on day 7 of the culture. PGE_2 production (B) was determined using enzyme-linked immunosorbent assay, and expression of the M-CSF gene (C) was determined using real-time polymerase chain reaction on day 14 of the culture. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. The data shown are the mean±SD for three separate experiments. [a] $P<0.05$, [b] $P<0.01$, treatment with nicotine or both nicotine and LPS compared to control; [d] $P<0.01$, treatment with both nicotine and LPS compared to nicotine treatment alone.

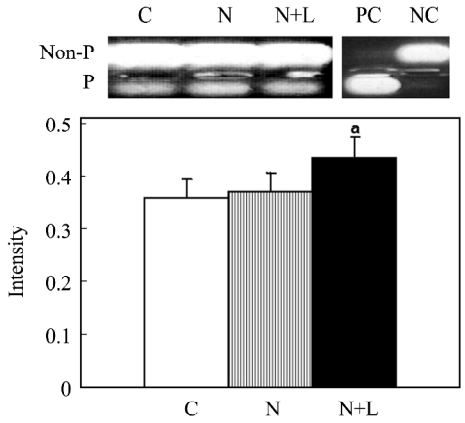

Fig. 7 Effect of nicotine and lipopolysaccharide (LPS) on phosphorylation of protein kinase A (PKA)

Saos-2 cells were cultured with 10^{-3} M nicotine with or without 10 μg/ml LPS, and phosphorylation of PKA was examined using the PegTag Non-Radioactive Detection of PKA assay kit on day 14 of the culture. The intensities of the bands were quantified using a scanner and digital image analysis software. C, control; N, treatment with 10^{-3} M nicotine; N+L, treatment with both 10^{-3} M nicotine and 10 μg/ml LPS; NC, negative control; Non-P, non-phosphorylation; P, phosphorylation; PC, positive control. The data shown are the mean±SD for three separate experiments. [a] $P<0.05$, treatment with both nicotine and LPS compared to the control.

smoking. In determining the experimental concentration of nicotine, we assumed that nicotine directly stimulates the gingival epithelium; thus, we chose to use the quantity 10^{-3} M. Our previous studies had already shown that 10^{-3} M nicotine does not kill Saos-2 cells [21,22,26]. Nicotine stimulates nicotinic acetylcholine receptors (nAChR) by binding at the acetylcholine binding site of the nAChR α-subunits [32]. Walker *et al.* [33] reported that nicotine has a direct effect on human bone cells in modulating proliferation, up-regulating the c-*fos* transcription factor by way of the nAChR α4-subunit, and the synthesis of the bone matrix protein, osteopontin. Our previous study reported that nicotine treatment caused a significant increase in expression of the genes encoding the nAChR α7-subunit and the c-*fos* transcription factor. In addition, the nicotine antagonist D-tubocurarine blocked the enhancement of matrix metalloproteinase-1 gene expression by nicotine [26]. Taken together, these reports suggest that the types of nAChR α-subunits were different in the tumor cells and normal cells, even in human osteoblasts, and that nicotine at least binds to the nAChR α7-subunit of Saos-2 cells.

PGE_2 is formed through the action of PGE synthase on prostaglandin H_2, which in turn arises from the action of COX enzymes on arachidonic acid that is released from cell membrane phospholipids. Two types of COX enzymes regulate this process. COX-1 appears constitutively, whereas COX-2 is induced by inflammation. We showed previously that LPS causes a significant dose-dependent increase in PGE_2 production and COX-2 expression, whereas it causes a significant decrease in COX-1 expression [16]. In the present study, nicotine or LPS alone caused increases in PGE_2 production. These effects increased further when nicotine and LPS were present at the same time, in a dose-dependent manner, whereas COX-1 expression decreased significantly or did not change. However, PGE_2 production induced by treatment with both nicotine and LPS was blocked by simultaneous treatment with indomethacin. These results suggest that LPS enhances nicotine-induced PGE_2 production through an in-

crease in COX-2 expression in osteoblasts.

Osteoclast precursors express RANK, recognize RANK ligand through cell-to-cell interactions with osteoblasts [34], and differentiate into osteoclasts in the presence of M-CSF [35–37]. PGE_2 is just one of the factors known to increase RANK ligand expression in osteoblasts; other known factors are 1α, 25-dihydroxyvitamin D_3, parathormone, and IL-11 [38–40]. In the present study, the induction of M-CSF expression caused by treatment with both nicotine and LPS was blocked by simultaneous treatment with indomethacin. These results suggest that nicotine and LPS might stimulate osteoclast formation through increases in PGE_2 and M-CSF production in osteoblasts.

PGE_2 receptors have been classified into four subtypes: Ep1, which increases the intracellular Ca^{2+} concentration; Ep2 and Ep4, which increase intracellular cAMP/PKA levels; and Ep3, which acts predominantly to decrease intracellular cAMP/PKA levels [41]. Recent evidence points to Ep4 as the major Ep receptor mediating the anabolic effects of PGE_2 [18,19,42,43]. Sakuma *et al*. [44,45] reported that LPS-induced osteoclast formation was barely observable in cell cultures prepared from Ep4 knockout mice, and that the urinary excretion of deoxypyridinoline, a sensitive marker for bone resorption, was not increased in Ep4 knockout mice injected with LPS. Suda *et al*. [27] reported that LPS stimulates osteoclastogenesis through two parallel events: the direct enhancement of RANK ligand; and the suppression of osteoprotegerin (OPG) expression, which is mediated by PGE_2 production. These results suggest that PGE_2 and Ep4 are the key factors in the enhancement of osteoclastogenesis by LPS *in vivo* and *in vitro*. In this study, expression of the PGE_2 receptors did not change in response to nicotine alone, whereas Ep3 and Ep4 expression markedly increased when both nicotine and LPS were present. In addition, levels of phosphorylated PKA were higher in cells cultured with both LPS and nicotine than in those cultured in the presence of nicotine alone or in the non-treated control. These results suggest that nicotine-and-LPS-induced PGE_2 interacts with the osteoblast Ep4 receptor predominantly through autocrine or paracrine action, and it might also enhance osteoclastogenesis by an increase in RANK ligand expression and a decrease in OPG production in osteoblasts. Ep1 expression in the presence of both nicotine and LPS differed according to LPS concentration; the expression in 1 μg/ml LPS-treatment decreased significantly compared with control, whereas the expression in 10 μg/ml LPS-treatment did not decrease. In contrast, our previous study [16] showed that the expression decreased significantly in both LPS concentrations without nicotine compared with control. We will examine these results in detail to clarify this difference.

We showed previously that ALPase activity in osteoblasts and chondrocytes decreases after treatment with inflammatory cytokines or LPS [16,46–48]. ALPase plays an important role in bone calcification [21,46]. In the present study, ALPase activity decreased in cells cultured with nicotine or LPS alone, and this effect was enhanced when nicotine and LPS were present at the same time. In addition, the nicotine-and-LPS-induced reduction in ALPase activity returned to the control level when indomethacin was also present. These results suggest that nicotine-and-LPS-induced PGE_2 suppresses calcification by decreasing ALPase activity.

In this research, we used nicotine at a high concentration because we assumed that it directly stimulates the gingival epithelium. We will use different lower concentrations of nicotine in future research.

In conclusion, our results indicate that LPS enhances the production of nicotine-induced PGE_2 by an increase in COX-2 expression. Furthermore, nicotine-and-LPS-induced PGE_2 is likely to interact primarily with the osteoblast Ep4 receptor in autocrine or paracrine modes, and it might promote osteoclast formation by increasing M-CSF expression. In addition, our results indicate that alveolar bone resorption in smokers is greater than it is in non-smokers, and this tendency is exacerbated by insufficient mouth cleaning.

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