INFECTION AND IMMUNITY, Sept. 1995, p. 3576–3581 Vol. 63, No. 9
0019-9567/95/$04.00+0

Inflammatory Cytokine Gene Expression in Human Periodontal Ligament Fibroblasts Stimulated with Bacterial Lipopolysaccharides

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Received 28 December 1994/Returned for modification 28 February 1995/Accepted 6 June 1995

The effects of *Porphyromonas gingivalis* lipopolysaccharide (P-LPS) and *Escherichia coli* lipopolysaccharide (E-LPS) on the gene expression and production of inflammatory cytokines of human periodontal ligament fibroblasts (HPLF) were examined by a Northern (RNA blot) assay and enzyme-linked immunosorbent assay, respectively. mRNAs for interleukin-6 (IL-6), IL-8, and transforming growth factor beta (TGF-β) were detected in HPLF cells, but IL-1α, IL-1β, tumor necrosis factor alpha, transforming growth factor alpha, and granulocyte-macrophage colony-stimulating factor were not detected by reverse transcription-PCR. The expression of TGF-β mRNA was not influenced by either LPS. P-LPS (1 to 10 μg/ml) and E-LPS (100 μg/ml) markedly stimulated the expression of IL-6 and IL-8 mRNAs compared with the control. The synthesis of IL-6 and IL-8 was also stimulated by 10 and 100 μg of both LPSs per ml, but IL-8 synthesis was not stimulated with E-LPS at 1 μg/ml. Secretion of IL-6 and IL-8 into the culture medium was detected at 6 and 3 h, respectively, after exposure to P-LPS (10 μg/ml). These findings suggested that *P. gingivalis* leads to periodontal tissue destruction and alveolar bone resorption through IL-6 and IL-8 released from HPLF cells stimulated with its LPS.

Periodontal disease is characterized by the loss of periodontal ligament and alveolar bone in periodontal tissue. Therefore, it is very important to study the role of the periodontal ligament in the pathogenesis of periodontal disease.

The periodontal ligament, which lies between two mineralized tissues, alveolar bone and cementum, is a noncalcified connective tissue consisting mainly of periodontal ligament fibroblasts and collagen, and periodontal ligament fibroblasts exhibiting characteristics of osteoblastic and fibroblastic cells, because it shows alkaline phosphatase (ALPase) activity similar to that of osteoblasts (9). On the other hand, the gram-negative anaerobic bacterium *Porphyromonas gingivalis* has been believed to be an important periodontopathic bacterium (27) because it is isolated at a high frequency from subgingival sites of advancing lesions in adult periodontitis patients (28), and serum antibody titers against this organism are significantly increased in the serum of patients with this disease (16). Furthermore, many investigators have indicated that the lipopolysaccharide (LPS) of *P. gingivalis* (P-LPS) may lead to destruction of the periodontal tissue by a direct action or indirectly by inducing inflammatory reactions (2, 4, 26). Certainly, LPS can activate osteoclasts directly and indirectly and thus lead to bone resorption (3, 7).

P-LPS differs from the LPSs of members of the family *Enterobacteriaceae* in its chemical structure and biological properties (5, 10, 12, 15, 30). Previously, it has been reported that P-LPS stimulates the induction of interleukin-1α (IL-1α), IL-1β, IL-6, and IL-8 by human gingival fibroblasts (18, 31). However, the significance of P-LPS to periodontal ligament fibroblasts remains unknown.

In this study, the effects of P-LPS on the gene expression and production of inflammatory cytokines in human periodontal ligament fibroblasts (HPLF) were examined.

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MATERIALS AND METHODS

Preparation and culture of HPLF. HPLF cells obtained by explant cultures of the periodontal ligaments of teeth extracted from healthy humans, as described by Kawase et al. (9), were cultured in Dulbecco's modified Eagle's medium (D-MEM; Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Gibco, Grand Island, N.Y.) under 5% CO_2. The cells were plated at a concentration of 2×10^3 to $3 \times 10^3/cm^2$. Upon reaching confluence, the HPLF cells were incubated with LPS at 1, 10, or 100 μg/ml or without LPS (control) for 0, 3, 6, 12, and 24 h.

Preparation of LPS from *P. gingivalis*. P-LPS was extracted from *P. gingivalis* 381 by the hot phenol-water method of Westphal and Jann (37). Briefly, *P. gingivalis* 381 was incubated anaerobically in heart infusion broth (Difco, Detroit, Mich.) supplemented with hemin (1 mol%), vitamin K_1 (0.5 mol%), yeast extract (0.5 mol%), and cysteine (80 mol%) at 37°C. The cells were heated at 121°C for 15 min to remove capsules and then harvested by centrifugation at 7,000 × *g* for 20 min at 4°C. The cells were washed once with saline and twice with distilled water and then dried with acetone. LPS was extracted from the dried cells with hot phenol-water, digested with RNase A (Sigma, St. Louis, Mo.), DNase I (Sigma), and proteinase K (Sigma), and then purified by repeated ultracentrifugation at 10,500 × *g* for 12 h at 15°C. The extract was sonicated for 5 min in a mixture of phenol-chloroform-petroleum ether (PCP, 12:5:8, by volume). After centrifugation, the sediment was washed twice with PCP mixture and three times with acetone and then lyophilized. *Escherichia coli* LPS (E-LPS) O111:B4 was purchased from Sigma.

cDNA probes. A human IL-8 cDNA clone containing a 0.5-kb insert (5′ end) was supplied by H. A. Young (Laboratory of Experimental Immunology, National Cancer Institute Frederick Cancer Research and Development Center, Frederick, Md.). The human IL-6 cDNA plasmid pBSF 2.38.1, containing a 0.43-kb *Taq*I-*Ban*II restriction fragment, was provided by T. Kishimoto (Division of Immunology, Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan). For Northern (RNA) blots, inserts were excised with the appropriate restriction enzymes and purified by agarose gel electrophoresis. A human β-actin cDNA probe was purchased from Nippon Gene (Osaka, Japan). The internal standard against which the total mRNA in each lane was quantified was β-actin. The cDNA probes for IL-6, IL-8, and β-actin were labeled with [^{32}P]CTP (specific activity, 110 TBq/mmol: ICN, Costa Mesa, Calif.) with an oligolabeling kit (Pharmacia, Uppsala, Sweden).

Assessment of cell number. Confluent HPLF cells were stimulated or not with LPS (1, 10, and 100 μg/ml) for 24 h. After incubation, cultured cells were detached by using phosphate-buffered saline (PBS) containing 0.05% trypsin and 0.025% EDTA. The cells were harvested and counted with a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

ALPase activity. ALPase activity was measured as described by Puzas and Brand (23). Briefly, the cells were rinsed twice with cold PBS. The cell layer was immersed in 0.5 ml of assay buffer, which consisted of 0.25 M 2-amino 2-methyl-1-propanol and 2 mM magnesium chloride (pH 10.1) containing 5 ng of *p*-nitrophenyl phosphate as a substrate, and incubated at 37°C for 15 min. The

reaction was terminated with 0.25 M NaOH, and the amount of *p*-nitrophenol released was measured spectrophotometrically at 405 nm.

Analysis of mRNA by Northern hybridization. Total cellular RNA was extracted with LiCl as described by Kubota et al. (11). Confluent cells in 100-mm dishes were trypsinized and pelleted by centrifugation at 1,000 × *g* for 10 min at 4°C. The pellets were dissolved in lysis buffer, consisting of 6 M urea, 3 M LiCl, 0.1% sodium dodecyl sulfate (SDS), and 70 mM β-mercaptoethanol in 10 mM sodium acetate (NaAc), pH 5.5.

The mixture was then transferred to a siliconized microcentrifuge tube, sonicated briefly, and incubated at 0°C for 16 h. The RNA was collected by centrifugation at 12,000 × *g* for 30 min and suspended in 200 μl of an RNase-free solution of 0.1 M NaAc, pH 5.5, containing SDS (5 mg/ml). The RNA was isolated by successive extraction with equal volumes of phenol followed by phenol-chloroform (1:1). The aqueous phase was transferred and precipitated overnight at −20°C with 0.3 M NaAc and 2.5 volumes of ethanol. The RNA was dissolved in 20 μl of RNase-free water. Thereafter, 1 μl of RNA was diluted to 50 μl with distilled water, and then the absorbance was measured at 280 and 260 nm. Samples of total RNA (20 μg) were fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde, and then the RNA bands were transferred to a Bio Trans membrane (0.22-μm pores; ICN). The RNA bound to the membrane was prehybridized at 42°C for 2 h with 50% formamide–5× Denhardt's solution (0.01% Ficoll, 0.01% polyvinylpyrrolidone, 0.01% bovine serum albumin [BSA])–5× SSC (0.75 M sodium chloride, 0.075 M trisodium citrate)–50 mM sodium phosphate (pH 5.5)–0.1% SDS–250 μg of non-homologous DNA per ml. Hybridization proceeded at 42°C for 16 to 24 h in 50% formamide–5× Denhardt's solution–5× SSC–50 mM sodium phosphate (pH 5.5)–0.1% SDS–250 μg of nonhomologous DNA per ml, with 10^7 cpm of ^{32}P-labeled cDNA probe per ml.

After hybridization, the membrane was washed twice for 10 min each at room temperature in 2× SSC (30 mM sodium citrate, 0.3 M NaCl [pH 7.0])–0.1% SDS and then twice for 10 min each at 50°C in 0.1× SSC–0.1% SDS. The membranes were exposed to X-OMAT AR X-ray film with an intensifying screen at −70°C.

Reverse transcription. First-strand cDNA was synthesized by using Moloney murine leukemia virus RNase H$^-$ reverse transcriptase (Gibco) (24) in a 20-μl reaction mixture containing 20 M Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM each dATP, dCTP, dGTP, and dTTP, 100 μg of BSA per ml, 2 μg of RNA, 200 U of enzyme, and 0.2 μg of each 3′ primer. The reaction mixtures were incubated at 37°C for 1 h and chilled on ice. The first-strand cDNA reaction mixture was added to 80 μl of 20 mM Tris-HCl (pH 8.3) containing 50 mM KCl, 2.5 M $MgCl_2$, 100 μg of BSA per ml 0.1 μg of each primer per ml, and 1.25 U of Amplitaq DNA polymerase (Takara, Kyoto, Japan). The reaction mixture was overlaid with mineral oil, and PCR amplification was done in a Perkin-Elmer Cetus DNA thermal cycler. After an initial denaturation step at 94°C for 5 min, the following amplification program was followed: 60 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 2 min, and extension at 72°C for 3 min. IL-1α, β, IL-6, IL-8, transforming growth factor alpha (TGF-α), TGF-β, tumor necrosis factor alpha (TNF-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) primers were purchased from Clontech (Palo Alto, Calif.).

Aliquots (20 μl) of the PCR mixtures were resolved by electrophoresis on a 2% Nusieve (3:1) agarose (FMC, Rockland, Maine) gel and stained with ethidium bromide (0.5 μg/ml). The DNA products were visualized by UV fluorescence.

Measurement of IL-6 and IL-8. The amounts of IL-6 and IL-8 secreted into the culture medium were determined by an enzyme-linked immunosorbent assay (ELISA) with human IL-6 ELISA (Genzyme, Cambridge, Mass.) and human IL-8 ELISA (Amersham) kits, respectively.

Statistical analysis. All statistical analyses were made with Student's *t* test.

RESULTS

Cell proliferation. We examined the effects of P-LPS and E-LPS on the proliferation of HPLF cells. The cells were incubated with various doses of P-LPS and E-LPS. P-LPS did not inhibit the proliferation of the HPLF cells. E-LPS enhanced the growth of the HPLF cells at a concentration of 1 μg/ml; however, there were no marked effects at other concentrations (Fig. 1). No morphological changes were found after incubation with P-LPS and E-LPS by phase-contrast microscopy (data not shown).

ALPase activity. Since HPLF cells possess high ALPase activity, which is a marker of HPLF differentiation, we examined the effects on it of P-LPS and E-LPS. Both LPSs diminished the ALPase activity of HPLF cells at all concentrations examined (Fig. 2).

Expression of inflammatory cytokine genes in HPLF cells. Reverse transcription-PCR studies indicated that inflammatory cytokine genes were expressed in HPLF, as shown in Fig. 3. mRNAs for IL-6, IL-8, and TGF-β were expressed in HPLF cells, whereas mRNAs for IL-1α,β, TNF-α, GM-CSF, and TGF-α were undetectable. Thus, we examined the effects of P-LPS and E-LPS on expression of the IL-6, IL-8, and TGF-β genes by Northern blotting. The TGF-β gene was not influ-

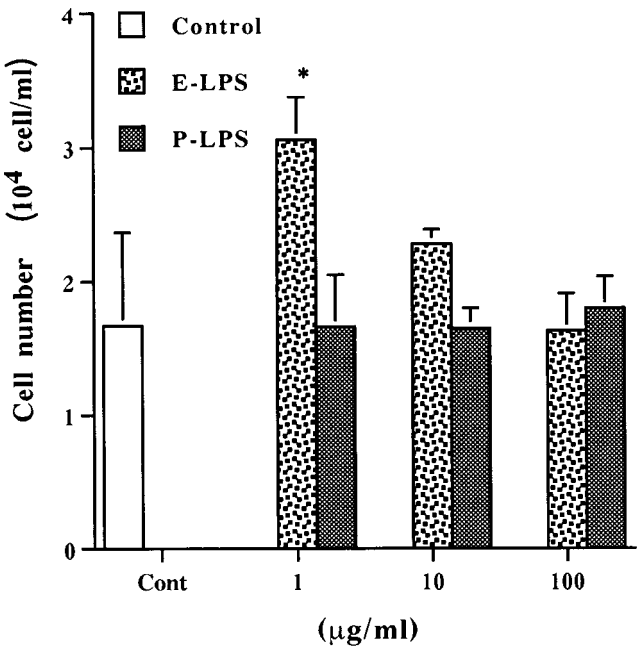

FIG. 1. Dose-response effects of P-LPS and E-LPS on growth of HPLF cells. The cells were incubated for 24 h with various concentration of P-LPS or E-LPS. $^*P < 0.05$, significantly different from the control.

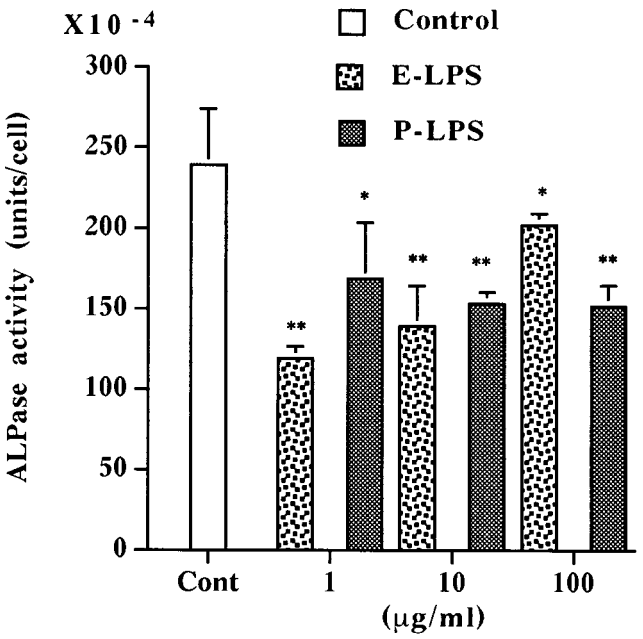

FIG. 2. ALPase activity of HPLF cells incubated with the indicated concentrations of P-LPS or E-LPS for 24 h. Both LPSs suppressed the ALPase activity of HPLF cells. $^*P < 0.05$, significantly different from control; $^{**}P < 0.025$, significantly different from control.

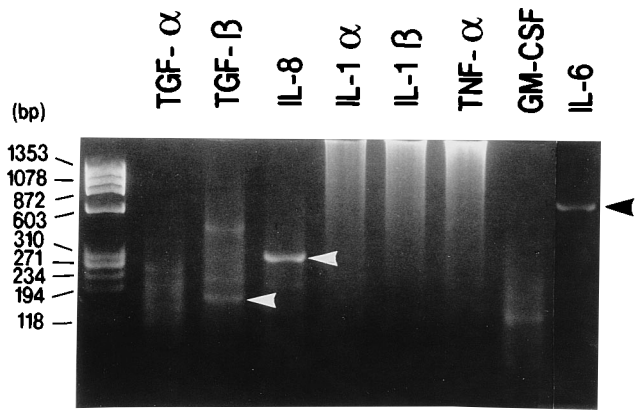

FIG. 3. Expression of various inflammatory cytokines in HPLF cells. These cytokine genes were analyzed by reverse transcription-PCR. mRNAs for IL-6 (628 bp), IL-8 (289 bp), and TGF-β (161 bp) were expressed in HPLF cells (arrowheads); however, mRNAs for IL-1α, IL-1β, TNF-α, and GM-CSF were not detected.

enced by either P-LPS or E-LPS (data not shown), whereas IL-6 mRNA (1.3 kb) expression in HPLF was stimulated by LPS. As shown in Fig. 4, the IL-6 mRNA level was markedly enhanced by P-LPS at concentrations above 1 μg/ml. The control gene for β-actin was not affected by P-LPS or E-LPS. Furthermore, IL-8 mRNA expression in HPLF was also stimulated by LPS. The level of IL-8 mRNA was markedly enhanced by P-LPS at a concentration of 1 μg/ml or more. However, E-LPS did not enhance the level of IL-8 mRNA at concentrations below 10 μg/ml. As shown in Fig. 5, the expression of IL-8 mRNA (1.8 kb) was also induced by P-LPS.

Effects of LPS on IL-6 and IL-8 production by HPLF. The effects of LPS on IL-6 production by HPLF are shown in Fig. 6 and 7. IL-6 production in cultured HPLF was significantly increased by stimulation with P-LPS and E-LPS at concentrations above 1 μg/ml (Fig. 6). The secretion of IL-6 into the culture medium began after 6 h of exposure to P-LPS and increased until 24 h in a time-dependent manner (Fig. 7). The effects of LPS on IL-8 production by HPLF are shown in Fig. 8 and 9. IL-8 production was greatly increased by stimulation with P-LPS or E-LPS at concentrations above 1 μg/ml. The effects of E-LPS on IL-8 production were dose dependent (Fig. 8). The secretion of IL-8 into the culture medium started after 3 h of exposure and increased until 24 h in a time-dependent manner (Fig. 9).

DISCUSSION

P. gingivalis is implicated in the development of periodontal disease (16, 27, 28). The components of this bacterium may modify host responses that eventually destroy periodontal tissue. LPS is a major cell wall component of gram-negative bacteria, and it possesses marked immunobiological and pharmacological activities, such as the stimulation of bone resorption (3, 7). The structure and biological potency of LPSs from members of the family *Bacteroidaceae* are quite different from those of enterobacterial LPSs (5, 10, 12, 15, 30).

The ALPase activity of HPLF was low when cells were in the proliferative phase, but it was increased by stimulation with $1\alpha25(OH)_2D_3$ or dexamethasone. These responses were similar to those of human alveolar bone cells. Therefore, HPLF may act as "osteoblastic" fibroblasts (9). Thus, ALPase is a marker of differentiation of HPLF cells and mineralization of tissues. In this study, both LPSs decreased the ALPase activity of the HPLF cells, and therefore they suppressed the differentiation of HPLF.

We examined the expression of inflammatory cytokine genes stimulated by LPS in HPLF by reverse transcription-PCR. mRNAs for TGF-β, IL-6, and IL-8 were expressed in HPLF, but not those for IL-1α, IL-1β, TGF-α, TNF-α, or GM-CSF (Fig. 3).

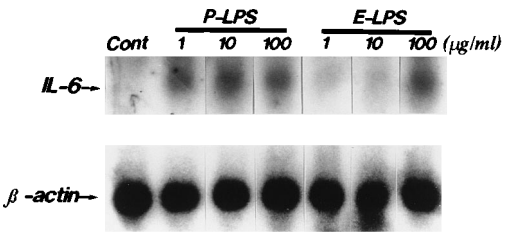

FIG. 4. Effects of P-LPS and E-LPS on induction of IL-6 mRNA expression in HPLF cells. HPLF cells were incubated without (control) or with various concentrations of P-LPS or E-LPS. Total RNA was prepared 24 h later. IL-6 and β-actin cDNAs were used as probes for Northern blotting.

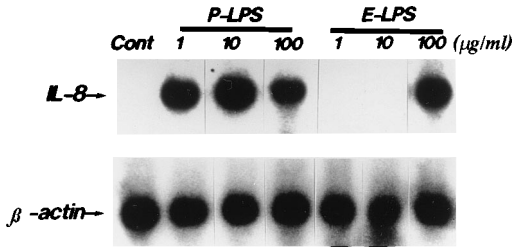

FIG. 5. Effects of P-LPS and E-LPS on induction of IL-8 mRNA expression in HPLF cells. HPLF cells were incubated without (control) or with various concentrations of P-LPS or E-LPS. Total RNAs were prepared 24 h later. IL-8 and β-actin cDNAs were used as probes for Northern blotting.

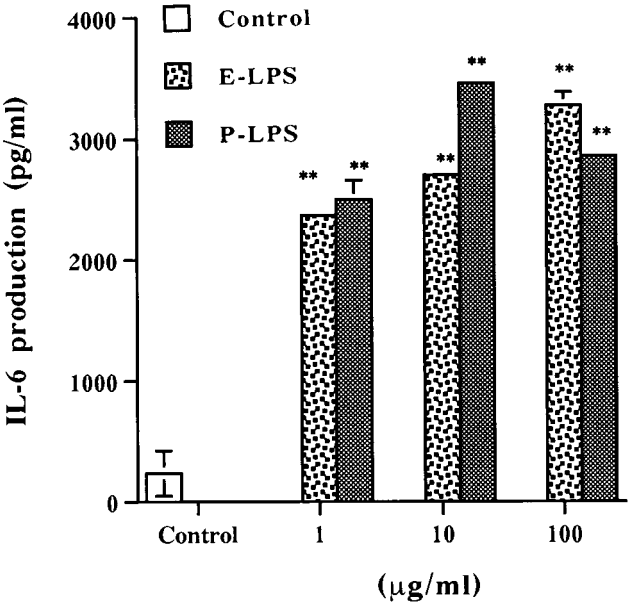

FIG. 6. Dose effects of P-LPS and E-LPS on IL-6 production in HPLF cells. HPLF cells were incubated without (control) or with various concentrations of P-LPS or E-LPS, and the culture supernatants were collected 24 h later. The amount of IL-6 produced was measured with an ELISA. $^{**}P < 0.025$, significantly different from control.

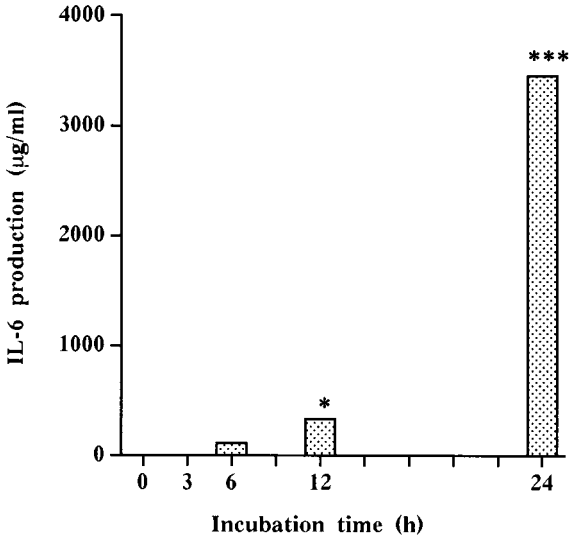

FIG. 7. Time course of IL-6 production in HPLF cells incubated with P-LPS (10 μg/ml). The amounts of IL-6 produced were measured by means of an ELISA with antiserum against human IL-6. $^{*}P < 0.05$, significantly different from 0-h value; $^{***}P < 0.01$, significantly different from 0-h value.

IL-1 levels in the gingival fluid of patients with periodontal disease are higher than those in healthy individuals, and IL-1β has been detected in tissues from periodontal patients but not from healthy volunteers (17, 30, 31). Oral *Bacteroides* LPS induced cell-associated IL-1α and cell-free IL-6 in human gingival fibroblast cultures, whereas LPSs from other bacterial species had no or only weak activity in this respect. IL-1α, IL-1β, and IL-8 are induced in human gingival fibroblasts obtained from healthy individuals stimulated with oral *Bacteroides* LPS, but not in Gin.1 cells (18, 32, 34). In this study, IL-1α and IL-1β were not detected in HPLF cultures (Fig. 3).

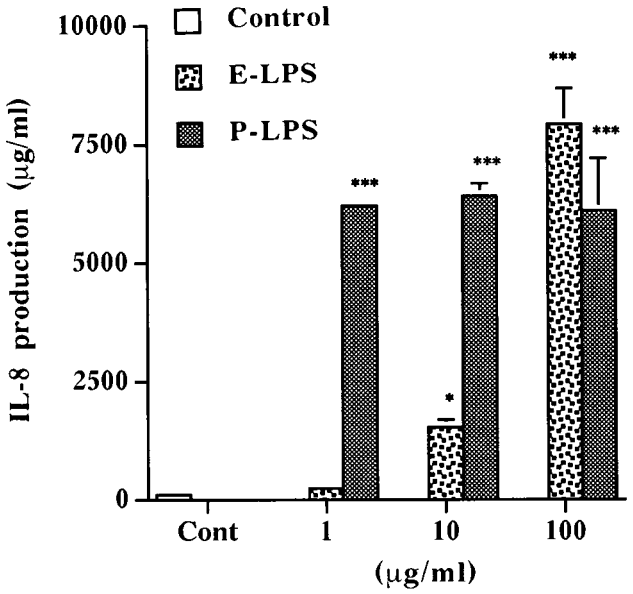

FIG. 8. Dose effects of P-LPS and E-LPS on induction of IL-8 in HPLF cells. HPLF cells were incubated without (control) or with various concentrations of P-LPS or E-LPS, and the culture supernatants were collected 24 h later. The amounts of IL-8 produced were measured with an ELISA. $^{*}P < 0.05$, significantly different from control; $^{***}P < 0.01$, significantly different from control.

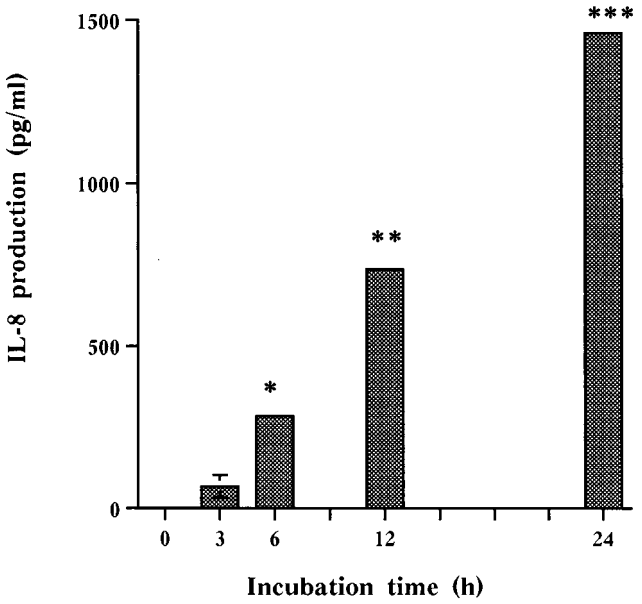

FIG. 9. Time course of IL-8 production in HPLF cells incubated with P-LPS (10 μg/ml). The amounts of IL-8 produced were measured by ELISA with antiserum against human IL-8. $^{*}P < 0.05$, significantly different from 0-h value; $^{**}P < 0.025$, significantly different from 0-h value; $^{***}P < 0.01$, significantly different from 0-h value.

However, IL-1α and IL-1β may be detected in HPLF cultures after stimulation with inflammatory cytokines such as IL-1 and TNF-α. The effects of inflammatory cytokines on gene expression in HPLF cells remains to be examined.

TGF-β is a 25-kDa homodimeric protein that is expressed in various tissues abundant in demineralized matrix with autocrine and paracrine activities (26). TGF-β influences cellular proliferation and differentiation and stimulates the synthesis of extracellular matrix components such as collagen, fibronectin, and SPARC/osteonectin (11). TGF-β is a bifunctional regulator of various cellular and immunological processes, and therefore it can activate and inhibit immune cell function (17, 22, 35). It is likely that the coupling of bone formation and bone resorption is mediated by local factors in the bone microenvironment. TGF-β acts as a regulatory growth factor for osteoblasts, and it has been suggested that it affects their functions (19, 21, 36). We found that TGF-β was expressed in HPLF but was not influenced by bacterial LPS. These results suggested that TGF-β accumulates in inflammatory lesions and suppresses immune cell function, but does not lead to tissue destruction by stimulation with LPS (data not shown).

The cytokine IL-6 is a major mediator of the host response to injury and infections. IL-6 is a 26-kDa protein which was initially thought to be a lymphokine of T-cell origin, where its major role was in the induction of differentiation of activated B lymphocytes into antibody-secreting cells (13, 14, 33). Various immune cells such as B cells, plasma cells, and macrophages infiltrate active sites of periodontal disease (25). IL-6 is produced by patients with adult periodontitis at levels much higher than those of healthy individuals. IL-6 is released from healthy human gingival fibroblasts upon stimulation with periodontopathic bacteria (31). This study showed that P-LPS stimulated IL-6 expression at the gene level and also its synthesis (Fig. 4, 6, and 7). These results suggested that P-LPS plays an important role in the initiation and development of periodontitis via the production of IL-6 from HPLF. IL-1 and IL-6 are released

from gingival fibroblasts by stimulation with IL-1, TNF-α, and IL-6 (18). These results suggested that tissue destruction is caused by the autocrine action of various cytokines.

Many investigators have demonstrated that monocytes or macrophages and neutrophils play a functional role in the inflammatory response (1). Neutrophils are present in high proportions in the gingivae of patients with periodontitis, and the cells may contribute to inflammation and alveolar bone loss in periodontal disease. Therefore, it is very important to understand the chemotactic mechanisms by which the cells infiltrate the periodontal tissue. IL-8 is a chemoattractant cytokine produced by a variety of tissue and blood cells. It has a distinct target specificity for neutrophils (39). The importance of neutrophil functions in periodontal disease is further demonstrated in hereditary Mac1/LFA-1 deficiency, where periodontal breakdown is very rapid. Therefore, IL-8 is another important inflammatory cytokine which is induced in human monocytes, endothelial cells, neutrophils, and fibroblasts by IL-1 and TNF-α. The monocyte chemoattractant protein-1 (MCP-1) gene, which belongs to the IL-8 supergene family, has been detected in human fibroblasts, suggesting that MCP-1 plays a functional role in the marked infiltration of monocytes into the periodontal tissues of patients with periodontal disease (20). In this study, the IL-8 gene was expressed earlier than that of IL-6 in HPLF. These results suggested that the IL-8 from gingival tissue induces infiltration of neutrophils into the periodontal tissue and the release of inflammatory cytokines such as IL-6, IL-1, and TNF-α, which cause tissue destruction. The IL-8 gene is expressed in gingival fibroblasts upon stimulation with LPS from *Prevotella intermedia* and *P. gingivalis* but not from *E. coli* (30). However, the IL-8 gene was expressed in HPLF upon E-LPS stimulation. Whether these results depended upon different characteristics of HPLF and gingival fibroblasts remains unknown. Probably, these results depend on different chemical structures, especially that of lipid A in P-LPS and E-LPS.

ACKNOWLEDGMENT

We express our gratitude to T. Kawase, Department of Oral Biochemistry, Kanagawa Dental College, for his help in HPLF cell isolation.

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