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Cytokine production in human periodontal ligament cells stimulated with *Porphyromonas gingivalis*

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Background and Objective: Although some functions and characterizations of human periodontal ligament (hPDL) cells have been reported, the role of hPDL cells in periodontal disease is poorly understood. We have previously reported that hPDL cells produce many kinds of inflammatory cytokines by stimulation with *Prevotella intermedia*. In this study, we examined the production of cytokines in hPDL cells stimulated with *Porphyromonas gingivalis* as compared with *P. intermedia*.

Material and Methods: hPDL cells cultured in Dulbecco's modified Eagles's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics. After three to four passages, hPDL cells were stimulated with *P. intermedia* (ATCC25601) or *P. gingivalis* (ATCC33277) for 24 h. Total RNA was extracted by ISOGEN and the expression of cytokine mRNA was determined using reverse transcription–polymerase chain reaction. Cytokines in the culture supernatants were assessed by enzyme-linked immunosorbent assay.

Results: The expression of interleukin-1β, interleukin-6, interleukin-8, tumor necrosis factor-α (TNF-α), receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) mRNA was detected in hPDL cells after stimulation with *P. gingivalis* as well as *P. intermedia*. There were no significant differences in the kind of cytokines expressed in hPDL cells between *P. gingivalis* and *P. intermedia*. However, *P. gingivalis* induced a significantly higher production of cytokines in hPDL cells than *P. intermedia* ($p < 0.05$).

Conclusion: This study demonstrated that hPDL cells produce many kinds of cytokines as a result of bacterial stimulation, including stimulation with *P. gingivalis* and *P. intermedia*. These results suggest that hPDL cells may play a role in cytokine production in periodontal disease.

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Various factors are known to be involved in the mechanism of periodontal tissue destruction in periodontitis. In particular, the presence of periodontopathogenic bacteria is a direct cause of periodontal tissue destruction. Chronic bacterial stimulation of periodontal tissue causes persistent or excessive periodontal tissue breakdown, with hard tissue destruction. Thus, periodontitis occurs in the presence of persistent dental plaque infection in the special environment of the periodontal pocket. Inflammatory cells and osteoclasts are considered to be chronically activated, leading to the loss of attachment and alveolar bone destruction (1).

In recent years, several immunological studies have reported on periodontal tissue breakdown, using inflammatory exudates in the gingival crevice (2,3), gingiva (4) and periodontal ligaments (5,6). However, much of the function and structure of human periodontal ligament (hPDL) cells, consisting of fibroblasts, osteoblasts and osteoclasts, is unknown (7). Typical bacteria that form dental plaques in the periodontal pocket include *Porphyromonas gingivalis* and *Prevotella intermedia* (8,9). It has been reported that *P. gingivalis* leads to periodontal tissue destruction and alveolar bone resorption through interleukin-6 and interleukin-8 release from hPDL cells (10), and these inflammatory cytokines play a role in the destruction and disintegration of the extracelluar matrix (11). We have previously reported that hPDL cells may play an important role as cells producing inflammatory cytokines in periodontal diseases (12). In addition, hPDL cells stimulated with *Escherichia coli* lipopolysaccharide induce both receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) expression, which relate with bone metabolism, by up-regulating interleukin-1β and tumor necrosis factor-α (TNF-α) (5). Furthermore, it has been reported that *P. gingivalis* inhibits fibroblast growth more strongly than *P. intermedia* (13), suggesting that the bacteria differ in their pathogenicity. However, the effect of periodontopathogenic bacteria on the expression of cytokines in hPDL cells has not been reported.

In this study, we examined the ability of hPDL cells to produce cytokines, mainly those involved in inflammation and bone metabolism, after stimulation by the periodontopathogenic bacteria, *P. gingivalis* and *P. intermedia*.

Materials and methods

Preparation of hPDL cells

hPDL cells were obtained from a healthy erupted maxillary third molar from three donors (a 21-yr-old woman, a 23-yr-old man and a 24-yr-old woman), removed for orthodontic reasons and used with informed consent. The tissue was minced and cultured as explants in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO, USA) containing 10% fetal bovine serum (FBS) and antibiotics, as previously described (12,14). After three to four passages, the cells were used for experiments. This experimental procedure was approved by the Ethics Committee, Kyoto Prefectural University of Medicine.

Preparation of bacteria

P. intermedia (ATCC25611) and *P. gingivalis* (ATCC33277) are gram-negative spiral rods and *Streptococcus mutans* (*FA1*) is a gram-positive coccus. All bacteria were generously supplied by the Department of Microbiology, Kyoto Prefectural University of Medicine Graduate School of Medical Science. *P. intermedia* and *P. gingivalis* were grown anaerobically on 5% sheep blood agar plates (Nissui Pharmaceutical, Tokyo, Japan) at 37°C for 24 h.

Stimulation with bacteria in hPDL cells

The hPDL cells were seeded onto Petri dishes at a concentration of 1×10^6 cells/dish. The cells reached confluence after approximately 1 wk of culture, and then were stimulated with *P. intermedia*, *P. gingivalis* or *S. mutans* [1×10^7 colony-forming units (CFU)/ml] for 24 h.

Expression of cytokine mRNA

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan), and the expression of cytokine mRNA was determined by using reverse transcription–polymerase chain reaction (RT-PCR). We analyzed the mRNA expression of interleukin-1β, interleukin-6, interleukin-8, TNF-α, receptor activator of nuclear factor-κB (RANK), RANKL and OPG. β-actin was used as an internal control. The primer sequences are shown in Table 1. For RT–PCR, we used our previously reported procedure (15). Briefly, total RNA was extracted with ISOGEN, and cDNA was produced using Superscript RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), oligo dT primer and 2.5 mmol/l dNTP Mixture (Takara Shuzo, Otsu, Japan). PCR was performed for 35 cycles, each consisting of 1 min at 95°C for denaturation, 1 min at 50°C for annealing and 1 min at 72°C for extension. Ten microliters of each PCR product was analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide (Bio-Rad Laboratories, Hercules, CA, USA), and the bands were visualized under ultraviolet (UV) light.

Production of cytokine in hPDL cells

Assays for cytokines in the culture supernatants employed commercially available enzyme-linked immnosorbent assay (ELISA) kits obtained from the following sources: interleukin-1β, interleukin-6, interleukin-8, TNF-α (Biosource International, Camarillo, CA, USA), RANKL and OPG (Cosmo Bio, Tokyo, Japan). All assays were conducted in accordance with the manufacturer's instructions.

Morphologial changes of the cells

Before and after the stimulation with bacteria, the morphological changes of hPDL cells were observed using an inverted optical microscope (Olympus, Tokyo, Japan). The size of the cells was assessed viaually. The viability of hPDL cells was examined using the Trypan Blue exclusion test.

Statistical analysis

Data obtained from separate experiments were pooled and expressed as means ± standard error of the mean (SEM). Comparisons between groups were made using the Student's *t*-test. Differences were considered significant at $p < 0.05$.

Results

Expression of cytokine mRNA in hPDL cells

To examine the ability of *P. gingivalis* to induce the production of cytokines,

Table 1. Polymerase chain reaction primer, sequences and predicted size

Cytokine		Sequences	Predicted size
Interleukin-1β	Sense	ATAAGCCCACTCTACAGCT	443
	Antisense	ATTGGCCCTGAAAGGAGAGA	
Interleukin-6	Sense	GTACCCCCAGGAGAAGATTC	819
	Antisense	CAAACTGCATAGCCACTTTC	
Interleukin-8	Sense	GCTTTCTGATGGAAGAGAGC	585
	Antisense	GGCACAGTGGAACAAGGACT	
TNF-α	Sense	TCGGGCCAATGCCCTCCTGGCCAA	468
	Antisense	GTAGACCTGCCCAGACTCGGCAAA	
RANK	Sense	TTAAGCCAGTGCTTCACGGG	497
	Antisense	ACGTAGACCACGATGATGTCGC	
RANKL	Sense	CAGCACATCAGAGCAGAGAAAGC	517
	Antisense	CCCCAAAGTATGTTGCATCCTG	
OPG	Sense	GTACGTCAAGCAGGAGTGCAATC	472
	Antisense	TTCTTGTGAGCTGTGTTGCCG	
β-actin	Sense	GTGGGGCGCCCCAGGCACCA	541
	Antisense	CTCCTTAATGTCACGCACGATTTC	

OPG, osteoprotegerin; RANK, receptor activator of nuclear factor κB; RANKL, receptor activator of nuclear factor κB ligand; TNF-α, tumor necrosis factor-α.

cytokine-specific expression of mRNA in hPDL cells was analyzed using RT–PCR.

No expression of interleukin-1β, interleukin-6, interleukin-8, RANK, RANKL or OPG mRNA was observed in the control cells before stimulation with bacteria, whereas in stimulated hPDL cells, interleukin-1β, interleukin-6, interleukin-8, TNF-α, RANKL and OPG mRNA were detected (Fig. 1). However, there were no significant differences in the types of cytokines expressed in hPDL cells upon stimulation with either *P. intermedia* or *P. gingivalis*. Each experiment was performed eight or nine times. We carried out the same experiment using other hPDL cells, and obtained the same results.

Production of cytokine in hPDL cells

To examine the production of cytokines after stimulation with *P. intermedia* or *P. gingivalis*, hPDL cells were incubated with either *P. intermedia* or *P. gingivalis* for 24 h, and the culture supernatants were analyzed by ELISA. The production of interleukin-1β, interleukin-6, interleukin-8 and RANKL increased significantly after stimulation with *P. intermedia*. On the other hand, *P. gingivalis* induced significantly higher production of interleukin-1β, interleukin-8, TNF-α and RANKL in PDL cells than *P. intermedia and S. mutans* ($p < 0.05$, Fig. 2), but the production of OPG was significantly lower ($p < 0.05$) (data not shown).

Morphological changes of the hPDL cells

Bacterial stimulation caused no changes in the morphology of hPDL cells, and did not affect cellular viability (data not shown). hPDL cells did not exhibit morphological changes (Fig. 3).

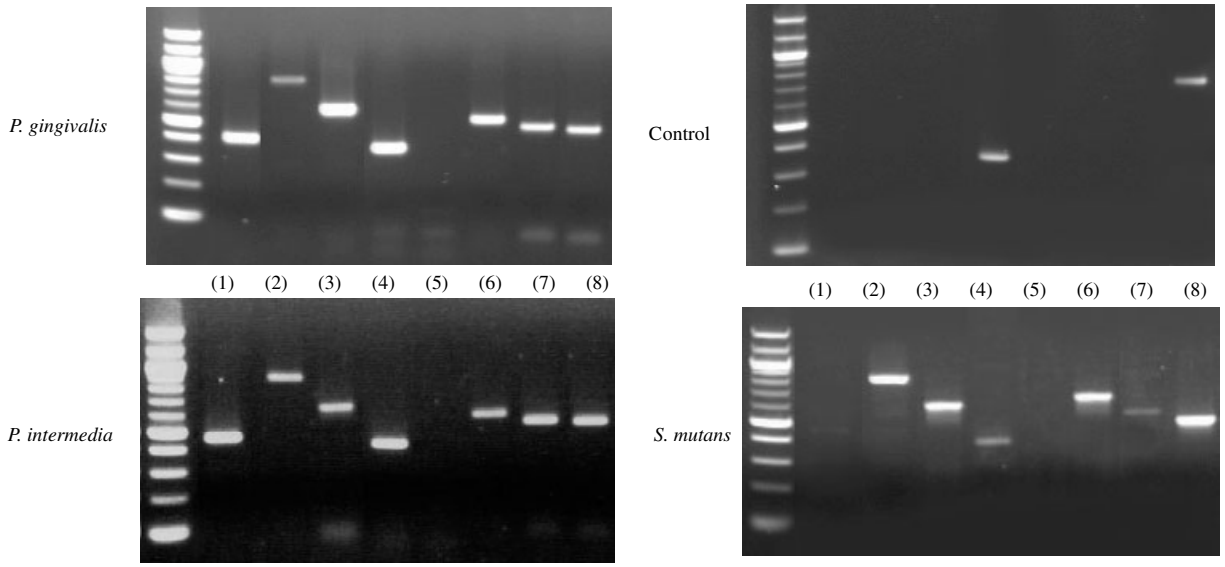

Fig. 1. Expression of cytokine mRNA in in human periodontal ligament (hPDL) cells stimulated with *Porphyromonas gingivalis*, *Prevotella intermedia* or *Streptococcus mutans*. The expression of interleukin-1β, interleukin-6, interleukin-8, receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) in hPDL cells stimulated with *P. gingivalis*, *P. intermedia* or *S. mutans* is shown. Lane 1, interleukin-1β; lane 2, interleukin-6; lane 3, interleukin-8; lane 4, tumor necrosis factor-α (TNF-α); lane 5, receptor activator of nuclear factor κB (RANK); lane 6, receptor activator of nuclear factor κB ligand (RANKL); lane 7, osteoprotegerin (OPG); and lane 8, β-actin. Unmarked lane, molecular weight markers.

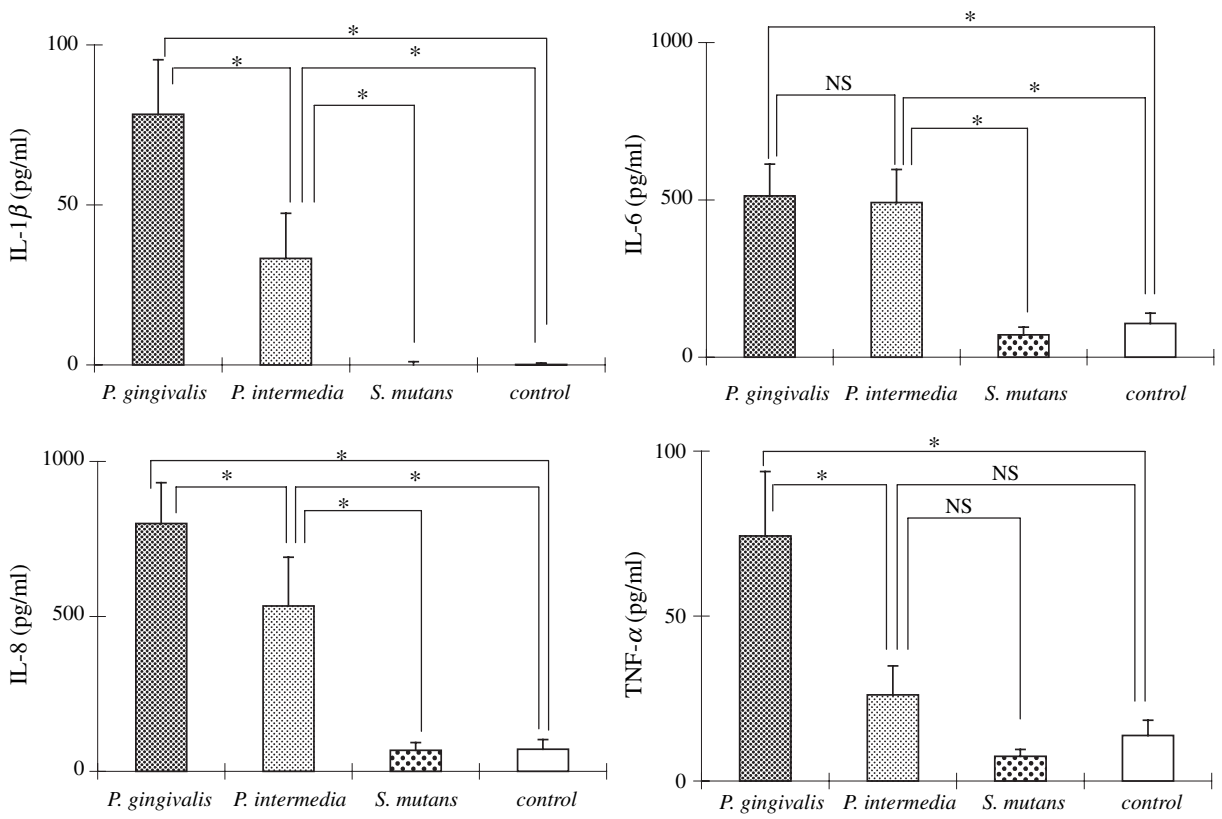

Fig. 2. Effect of *Porphyromonas gingivalis*, *Prevotella intermedia* or *Streptococcus mutans* on the production of inflammatory cytokines. The amount of inflammatory cytokines on human periodontal ligament (hPDL) cells stimulated with *P. gingivalis* was significantly higher than the amount of inflammatory cytokines stimulated by *P. intermedia*. Results are expressed as mean ± standard error (SE). $*p < 0.05$, $n = 11$. IL, interleukin; TNF-α, tumor necrosis factor-α.

Fig. 3. Photomicrographs of human periodontal ligament (hPDL) cells stimulated with *Porphyromonas gingivalis* (A), *Prevotella intermedia* (B) and control (C). hPDL cells showed no morphological changes following stimulation with *P. gingivalis* or *P. intermedia*. (Original magnification ×100).

Discussion

The gram-negative, black-pigmented bacteria *P. gingivalis* and *P. intermedia* used in this study are typical adult periodontal disease-causing bacteria that form dental plaques in the periodontal pocket. Van Winkelhoff *et al.* reported that *P. gingivalis* and *P. intermedia* were significantly more prevalent in patients with periodontal destruction, and *P. gingivalis* is the strongest bacterial marker for destructive periodontal disease (8). Tanaka *et al.* showed that the number of both bacteria was greater in the area of chronic inflammation than in the area of acute exacerbation of chronic inflammation in adult periodontal disease (16). However, it is well known that the presence alone of periodontopathogenic bacteria, such as *P. gingivalis*, *P. intermedia* and *Actinobacillus actinomycetemcomitans*, is insufficient for the development of periodontal disease and is only a risk factor for periodontal disease (17).

Therefore, it is necessary to examine the effect of both species of bacteria on hPDL cells to elucidate the process of periodontal tissue breakdown. On the other hand, it is well known that dental caries is caused by *S. mutans*. Van der Reijden *et al.* reported a negative correlation between the percentage of *S. mutans* and the percentage of *P. gingivalis* in subgingival plaque (18). Thus, *S. mutans* is thought to be non-periodontopathogenic against PDL cells in the periodontal pocket. In the present study, we examined the ability of *P. gingivalis* to induce the production of cytokines in hPDL cells. In hPDL cells, TNF-α mRNA was constitutively detected before and after the stimulation of these bacteria. However, the expression of interleukin-1β, interleukin-6, interleukin-8, RANKL and OPG was induced in response to exposure to these bacteria in hPDL cells. These results indicate that hPDL cells are involved in periodontal tissue inflammation.

It has been reported that hPDL cells stimulated with *P. gingivalis* lipopolysaccharide induced the expression of inflammatory cytokine mRNA (10), and that the expression of inflammatory cytokine mRNA was enhanced by costimulation with *P. gingivalis* lipopolysaccharide and excessive mechanical stress (19). Our results are in agreement with their results, and we demonstrated that *P. gingivalis* and *P. intermedia* induce cytokine production not only at the mRNA level, but also at the protein level.

RANKL and OPG in hPDL cells are known to play important roles in the differentiation of osteoclats (20). It has been reported that more severe periodontal disease was associated with a greater amount of RANKL and a smaller amount of OPG from gingival crevicular exudates in periodontal disease patients (21). In the present study, *P. gingivalis* induced a higher production of RANKL than *P. intermedia*, suggesting that *P. gingivalis* has a higher pathogenicity for hPDL cells than *P. intermedia*. Using lipopolysaccharide from different strains of *P. gingivalis*, Shipira *et al.* reported that different strains of *P. gingivalis* had different levels of pathogenicity (22). Thus, more careful study is needed to elucidate the relationship between the inducibility of RANKL or OPG and the pathogenicity of *P. gingivalis*.

Furthermore, Kon *et al.* reported that in a mouse model of iliac bone fracture healing, interleukin-1β and TNF-α mRNA, in addition to RANKL, OPG and macrophage colony-stimulating factor (M-CSF) mRNA, were expressed in the early and late stages of healing (23). Nukaga *et al.* found that interleukin-1β stimulation of hPDL cells increased RANKL mRNA expression (24). To date, we have shown that TNF-α mRNA is involved in the expression of RANKL and OPG mRNA in the wall of peri-apical cysts (13). These observations suggest that inflammatory cytokines are closely related to the expression of RANKL and OPG mRNA.

hPDL cells did not show morphological changes when cocultured with *P. gingivalis* or *P. intermedia*, which is in agreement with the report that oral infections, such as periodontal disease and caries, are caused by low-virulence bacteria (25). As shown in Fig. 1, the hPDL cells used in this study appeared morphologically to be fibroblasts (7); however, we consider that hPDL cells should be separated into fibroblasts, osteoblasts and osteoclasts in future studies, because osteoblasts and osteoclasts are also expected to be involved in the pathogenesis of periodontal disease.

In conclusion, we demonstrated that hPDL cells could produce many types of cytokines by stimulation with *P. gingivalis and P. intermedia*. These results suggest that hPDL cells may play a role in cytokine production in periodontal disease.

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