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Characteristics of *Porphyromonas gingivalis* lipopolysaccharide in co-culture with *Fusobacterium nucleatum*

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SUMMARY

Porphyromonas gingivalis **is associated with chronic periodontitis and forms multi-species biofilms. They can communicate within species as well as with other species found in the subgingiva, which may induce changes in the growth ratio and virulence of periodontopathogens. The lipopolysaccharide (LPS) of *P. gingivalis* shows different virulence by growth condition. The purpose of this study was to investigate the characteristics of *P. gingivalis* LPS when co-cultured with *Fusobacterium nucleatum*. After culture of *P. gingivalis* in the presence or absence of *F. nucleatum*, *P. gingivalis* LPS was extracted. THP-1 cells were treated with the LPS and induction of cytokine expression was investigated using real-time reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). For the analysis of *P. gingivalis* LPS, LPS biosynthesis-related genes such as *lpxA* and *lpxD* were evaluated with real-time RT-PCR. Finally, molecular mass of lipid A was measured by mass spectrometry after hydrolysis of the LPS. Co-cultured *P. gingivalis* LPS exhibited higher induction of expression of interleukin 1β, 6, and 8 than single-cultured *P. gingivalis* LPS. These symptoms may be caused by an increase in *m/z* 1689 lipid A through the upregulation of *lpxA* and *lpxD* expression by communication between *P. gingivalis* and *F. nucleatum*.**

INTRODUCTION

Periodontitis is associated with multi-bacterial infection with microbes such as *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* as well as with chronic inflammatory disease in subgingival tissue (Socransky & Haffajee, 2005). The periodontopathogen species co-exist in the oral cavity and there is communication between species. They form biofilms with commensal bacteria by a medium of *Fusobacterium nucleatum* (Socransky & Haffajee, 1992). Among the periodontopathogens, *P. gingivalis* is an anaerobic gram-negative species that has various virulence factors such as lipopolysaccharide (LPS), fimbriae, protease and hemagglutinin, and is strongly associated with chronic periodontitis in adults (Socransky & Haffajee, 1992).

Lipopolysaccharide, as a glycolipid, is found as a membrane component of gram-negative bacteria. It is a powerful inflammatory stimulator, and is known to be an endotoxin (Trent *et al.*, 2006). The LPS is composed of three structural domains: lipid A, core and O-antigen repeat (Raetz & Whitfield, 2002). Lipid A is an immunomodulative factor and initiates inflammatory signaling pathway by binding toll-like receptor 2 (TLR2) or TLR4 according to the structure of acyl chain (Hajjar *et al.*, 2002). Also, the virulence of LPS

has varieties depending on its structure (Hajjar *et al*., 2002). O-antigen repeat binds LPS-binding protein, is transferred to CD14 and then located on TLR2 and TLR4 (Moreno *et al*., 2004; Schroder *et al*., 2004).

The *P. gingivalis* LPS is a proinflammatory factor and is considered an inducer of chronic periodontitis (Park *et al*., 2010). The characteristics of *P. gingivalis* LPS vary according to growth conditions. The antigenicity of *P. gingivalis* LPS is different in various hemin concentrations, and the lipid A structure of *P. gingivalis* LPS is also modified by hemin concentration (Cutler *et al*., 1996; Al-Qutub *et al*., 2006). However, the characteristics of *P. gingivalis* LPS when it is co-cultured with oral bacteria such as *F. nucleatum* have not been reported. Many species of bacteria exist in the oral cavity and form biofilm. Furthermore, they communicate both within and between species using autoinducers or metabolites (Hojo *et al*., 2009). *F. nucleatum* plays a role in the formation of the *P. gingivalis* biofilm by forming a bridge between the early colonizers, like streptococci, and periodontopathogens (Kolenbrander, 2000). *F. nucleatum* adheres to early colonizers on the tooth surface and then periodontopathogens such as *P. gingivalis* and *T. forsythia* bind to the *F. nucleatum* surface and form the biofilm (Bradshaw *et al*., 1998; Marsh, 2004). Hence, *F. nucleatum* is indirectly associated with periodontitis. We have shown the effect of *P. gingivalis* growth in co-culture with *F. nucleatum* (Lee & Baek, 2010). The purpose of this study was to compare the virulence of LPS from *P. gingivalis* cultured alone with LPS from *P. gingivalis* co-cultured with *F. nucleatum* and analyzed the structural characteristics of its LPS.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Porphyromonas gingivalis ATCC 33277 and *Fusobacterium nucleatum* ATCC 25586 were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and cultured with brain–heart infusion broth (BHI; BD Bioscience, Sparks, MD) supplemented with hemin (1 μg ml^{-1}) and vitamin K (0.2 μg ml^{-1}) in anaerobic conditions at 37°C.

Bacterial co-culture

The *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 25586 were co-cultured using a Millicell cell culture insert (Millipore, Billerica, MA). BHI broth (40 ml) was mixed with hemin (1 μg ml^{-1}) and vitamin K (0.2 μg ml^{-1}) and 20 ml BHI was transferred to two new tubes. Both *P. gingivalis* and *F. nucleatum* were inoculated into each tube. A Millicell cell culture insert was hung into each well of a six-well plate, and *P. gingivalis* and *F. nucleatum* were inoculated both inside and outside the Millicell cell culture insert using the manufacturer's recommended volume, respectively. Contamination of each bacterium in the separating chamber was evaluated by observation using a microscope. Single culture of *P. gingivalis* was performed by inoculating the bacteria into the inside of the Millicell cell culture insert in a six-well plate and putting fresh medium in the outside of the insert.

Lipopolysaccharide extraction

Lipopolysaccharide was extracted from *P. gingivalis* cultured alone or with *F. nucleatum* according to the method described by Lee *et al.* (2006). The *P. gingivalis* was harvested by centrifugation at 5000 ***g*** for 10 min at 4°C and washed with phosphate-buffered saline (pH 7.2). The bacterial LPS was extracted using an LPS extraction kit (iNtRON Biotech., Kyunggi, Korea) according to the manufacturer's protocol with a slight modification. Briefly, the bacteria were mixed with lysis buffer, vortexed until the cell clump disappeared, and chloroform was added. After vortexing for 20 s, the mixture was centrifuged at 13,000 ***g*** for 15 min at 4°C and the upper phase was transferred to a new tube. The preparation was incubated with endonuclease (100 μg ml^{-1}) for 1 h at 37°C and subsequently with proteinase K (250 μg ml^{-1}) for 1 h at 55°C. The preparation was performed using lysis buffer and the protocol as described above, and was incubated with purification buffer from the kit for 10 min at − 20°C followed by centrifugation at 13,000 ***g*** for 15 min at 4°C. After removing supernatant, the pellet was washed with 1 ml 70% ethanol, air-dried and dissolved with endotoxin-free distilled water. After lyophilization, the dry weight of LPS was measured. The LPS was dissolved with endotoxin-free distilled water at the concentration of 1 mg ml^{-1}.

To compare the patterns for oligosaccharide and lipid A, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; 12% polyacrylamide gel) was performed, and the LPS was stained with silver nitrate according to the method reported by Tsai & Frasch (1982).

Cell cultivation and treatment with LPS

THP-1 cells, a monocytic cell line, were purchased from ATCC and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin sulfate). The cells (1×10^6 cells ml^{-1}) were plated in six-well plates and treated with LPS from *P. gingivalis* cultured alone or co-cultured with *F. nucleatum* at various concentrations for 8 h including 1% human serum at 37°C in a CO_2 incubator. The cells were used to analyze expression of inflammatory cytokines by real-time reverse transcription–polymerase chain reaction (RT-PCR) and the conditioned media were collected to measure interleukin-1β (IL-1β), IL-6 and IL-8 using enzyme-linked immunosorbent assay (ELISA).

Real-time RT-PCR

THP-1 cells were harvested by centrifugation at 1200 ***g*** for 5 min at 4°C and washed twice with cold phosphate-buffered saline. Total RNA from THP-1 cells was isolated with a TRIzol® RNA isolation kit (Invitrogen Life Tech., Carlsbad, CA) according to the manufacturer's instructions. For investigation of bacterial genes, total RNA from *P. gingivalis* was isolated with a TRIzol® Max bacterial RNA isolation kit (Invitrogen Life Tech) according to the manufacturer's protocol. The cDNA was synthesized by mixing total RNA (1 μg) and Maxime™ RT Premix (iNtRON, Kyunggi, Korea) in a 20-μl reaction volume and incubating the mixture at 45°C for 1 h. To inactivate polymerase, the samples were heated at 95°C for 5 min. Complementary DNAs were mixed with 10 μl SYBR Premix Ex Taq (Takara Co., Kyoto, Japan), 0.4 μM of each primer pair and ROX dye in a 20-μl final volume and subjected to 40 PCR cycles (95°C for 15 s, 60°C for 15 s and 72°C for 33 s) with an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The PCR products were investigated for each specific amplification product using a dissociation curve of amplification. A negative control was carried out without RT. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for THP-1 cells and 16s rRNA for *P. gingivalis*, a housekeeping gene, were used as a reference to normalize expression levels and to quantify changes of inflammatory cytokine for THP-1 cells and expression of LPS related genes between control and LPS-treated cells or single-cultured *P. gingivalis* and co-cultured *P. gingivalis*, respectively. The critical threshold cycle (Ct) was defined as the cycle at which fluorescence became detectable above background and was inversely proportional to the logarithm of the initial number of template molecules. The sequences of primers for real-time RT-PCR were as follows: 5′-AGC TGT ACC CAG AGA GTC C-3′ and 5′-ACC AAA TGT GGC CGT GGT TT-3′ the IL-1β gene; 5′ AAC CTG TCC ACT GGG CAC A-3′ and 5′-TCT GGC TCT GAA ACA AAG GAT-3′ the IL-6 gene; 5′-GTG AAG GTG CAG TTT TGC CA-3′ and 5′-TCT CCA CAA CCC TCT GCA C-3′ for the IL-8 gene; 5′-GTG GTG GAC CTG ACC TGC-3′ and 5′-TGA GCT TGA CAA AGT GGT CG-3′ for GAPDH gene; 5′-GCG GAA AAC CAT TCT ATT ATC AA-3′ and 5′-ACT AAG ACT TTA AGG AAA TGC ATT-3′ for the hemin-binding protein gene; 5′-TCC ATG GTT CCG CGG ACA ATG-3′ and 5′-CTC CCG CCT CTC GAA GGA CAT-3′ for the LpxApg gene; 5′-TTT GAC GTT GCT CAA CAA GCC-3′ and 5′-GAC AGG GCT GTT ATG GAT TCA -3′ for the LpxDpg gene; 5′-TGC AAC TTG CCT TAC AGA GGG-3′ and 5 '-ACT CGT ATC GCC CGT TAT TC-3′ for the pg 16s RNA gene.

ELISA

The culture supernatants of the cells treated with singley cultured or co-cultured *P. gingivalis* LPS as described above were centrifuged at 7000 ***g*** for 10 min at 4°C to remove cell debris and were analyzed for IL-1β, IL-6 and IL-8 levels using an ELISA kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol.

Mass spectrometry

To analyze the lipid A part of *P. gingivalis* LPS, the LPS was hydrolyzed with pH 3.4 solution (adjusting pH with acetic acid) supplemented with 1% SDS at 100°C for 20 min according to the method described

by Caroff *et al.* (1988). The hydrolysis product was loaded onto a stainless steel sampler of a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer and dried at room temperature. After applying a matrix solution (2,5-dihydroxybenzoic acid in methanol) and drying, the mass spectra were measured with a Voyager DE-STR MALDI-TOF spectrometer (Applied Biosystems, Foster City, CA) set with a pulsed nitrogen laser (−20 kV, 337 nm), and acquired in negative on modes. A total of 300 laser shots were summed for each spectrum. The data were processed for baseline correction and noise was filtered by DataExplorer™ software (Applied Biosystems).

Statistical analysis

Statistically significant differences between untreated cells and LPS of *P. gingivalis* cultured alone or co-cultured with *F. nucleatum* were analyzed by Mann–Whitney *U*-test using SPSS 10 (SPSS Inc., Chicago, IL). *P*-values < 0.05 were considered statistically significant.

RESULTS

SDS–PAGE of single- or co-cultured *P. gingivalis* LPS

To investigate the pattern of single- or co-cultured *P. gingivalis* LPS, 12% SDS–PAGE was performed after LPS extraction from single- and co-cultured *P. gingivalis*. The migration patterns of both forms of LPS were similar to the ladder form (Fig. 1). Furthermore, the bands of single- and co-cultured *P. gingivalis* LPS were located at the same size compared with the molecular marker. The similarity of migration patterns of both LPS indicated that the length of the O-antigen repeat of both LPS was the same.

Induction of inflammatory cytokine expression by single- or co-cultured *P. gingivalis* LPS

Next, induction of cytokine expression on THP-1 cells by single- or co-cultured *P. gingivalis* LPS was investigated. THP-1 cells were treated with single- or co-cultured *P. gingivalis* LPS at various concentrations, and then the expressions and production of cytokines were evaluated by real-time RT-PCR and

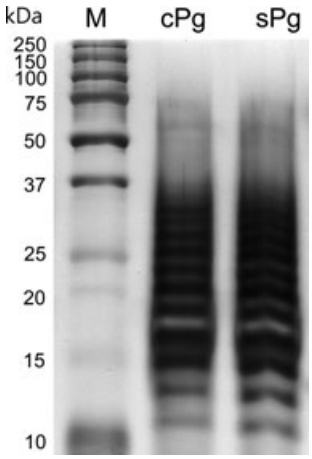

Figure 1 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of single- and co-cultured *Porphyromonas gingivalis* lipopolysaccharide (LPS). After cultivating *P. gingivalis* in the presence or absence of *Fusobacterium nucleatum*, the LPS was purified from *P. gingivalis* of each condition. The LPS underwent SDS–PAGE (12% acrylamide), and the gel was stained with silver nitrate. M; marker, cPg; co-cultured *P. gingivalis* LPS and sPg; single-cultured *P. gingivalis* LPS.

ELISA, respectively. Single- and co-cultured *P. gingivalis* LPS significantly induced cytokine expression such as IL-1β, IL-6 and IL-8 (Fig 2A–C). Also, the production of cytokines was induced by single- and co-cultured *P. gingivalis* LPS (Fig. 2D–F). Although both LPS induced cytokine expression in a dose-dependent manner, the LPS from co-cultured *P. gingivalis* induced more expression and production of cytokines than the single-cultured *P. gingivalis* LPS.

Expression of LPS synthesis-related genes

As single- and co-cultured *P. gingivalis* LPS induced different levels of cytokine expression, human cells responded differently to structurally different acyl-chains of lipid A (Hajjar *et al.*, 2002), and the O-repeat unit did not show differences for either LPS in the data of SDS–PAGE. Therefore, the lipid A biosynthesis-related genes were investigated. UDP-*N*-acetylglucosamine acyltransferase as the *LpxA* gene and UDP-3-*O*-(3-hydroxymyristoyl) *N*-acetylglucosamine deacetylase as the *LpxD* gene were expressed more strongly in co-cultured *P. gingivalis* than in single-cultured *P. gingivalis* (Fig. 3A,B). Furthermore, hemin-binding protein of *P. gingivalis*, related with the biosynthesis of LPS (Al-Qutub *et al.*, 2006), was expressed at higher levels in co-cultured *P. gingivalis* LPS compared with single-cultured *P. gingivalis* (Fig. 3C).

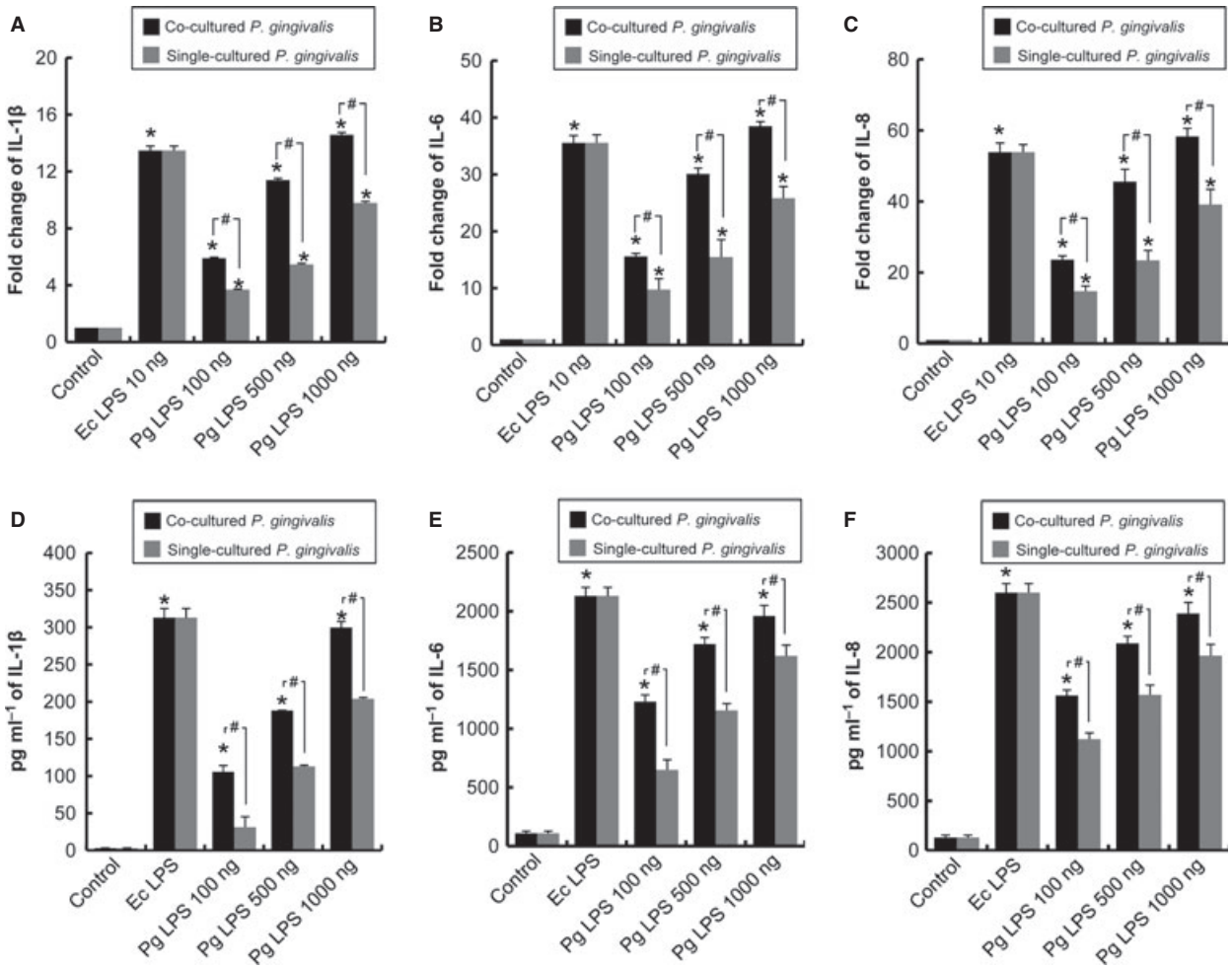

Figure 2 Comparison of bioactivity of single- and co-cultured *Porphyromonas gingivalis* lipopolysaccharide (LPS). THP-1 cells (1×10^6 cells ml^{-1}) were treated with single- or co-cultured *P. gingivalis* LPS for 8 h. The induction of cytokine expression was analyzed from LPS-treated cells using real-time reverse transcription polymerase chain reaction (A–C). The culture supernatants were measured level of interleukin-1β (IL-1β), IL-6 and IL-8 by enzyme-linked immunosorbent assay (D–F). The experiments were performed three times in duplicate and representative data are shown. An asterisk represents significant difference compared with untreated control cells, and # represents a significant difference compared with single-cultured *P. gingivalis* LPS and co-cultured *P. gingivalis* LPS ($P < 0.05$). *Escherichia coli* LPS was used for positive control.

Mass analysis of lipid A of single- and co-cultured *P. gingivalis* LPS

To confirm the difference of expression of lipid A-biosynthesis genes, lipid A of both LPS was analyzed using MALDI-TOF mass spectra. The LPS was hydrolyzed according to the method described by Caroff *et al.* (1988), and the hydrolyzed products were analyzed using MALDI-TOF mass spectrometry. As shown in Fig. 4, the mass of the hydrolyzed products as lipid A showed peaks characteristic of common *m/z* such as 1249, 1449 and 1689 *m/z*. However, the density of each peak was different. The peaks of lipid A from single-cultured *P. gingivalis* LPS were similar. However, in mass of lipid A from co-cultured *P. gingivalis* LPS, the density of 1689 *m/z* was higher than 1249 and 1449 *m/z*.

DISCUSSION

Porphyromonas gingivalis is a gram-negative bacterium and is considered a causative pathogen of periodontitis. It induces not only aggressive periodontitis but also chronic periodontitis (Socransky & Haffajee, 2005). Furthermore, this bacterium is associated with

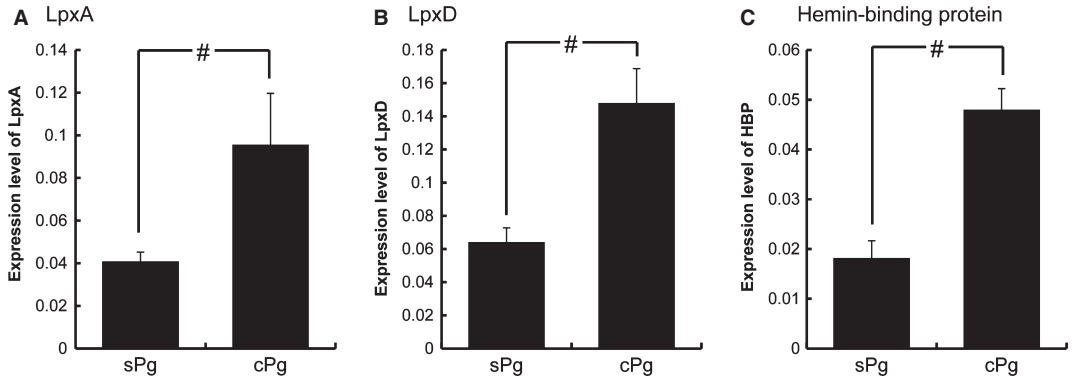

Figure 3 Comparison of lipopolysaccharide (LPS) biosynthesis-related genes. *Porphyromonas gingivalis* was cultivated in the presence or absence of *Fusobacterium nucleatum* until exponential phase, and then total RNA was eluted using the bacterial RNA isolation kit. After synthesizing cDNA, real-time polymerase chain reaction was performed with each specific primer for LpxA, LpxD and hemin-binding protein, and analyzed expression level. The experiments were performed three times in duplicate and representative data are shown. # represents a significant difference compared with single-cultured *P. gingivalis* and co-cultured *P. gingivalis* ($P < 0.05$).

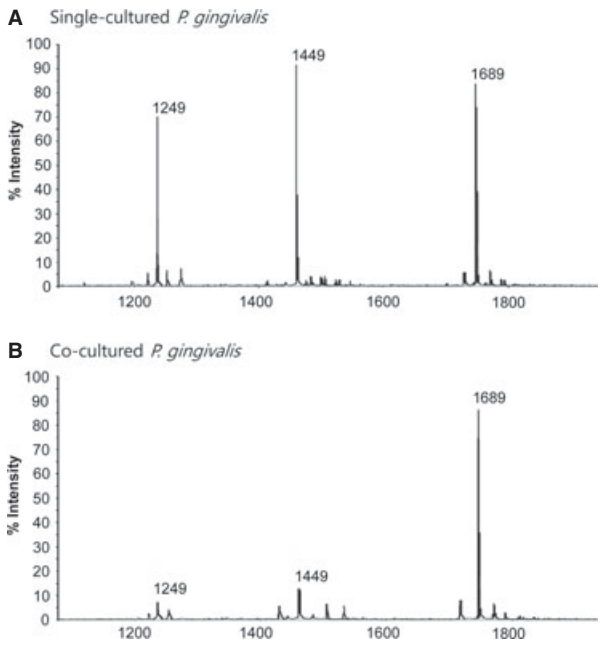

Figure 4 Analysis of single- or co-cultured *Porphyromonas gingivalis* lipid A by mass spectrometry. The *P. gingivalis* lipid A was obtained using the methods described by Caroff *et al.* (1988). Mass of lipid A was analyzed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The data were baseline corrected and noise filtered using DataExplorer™ software. All values given are average masses.

systemic diseases like atherosclerosis (Wada & Kamisaki, 2010).

Porphyromonas gingivalis exists in subgingival tissue and forms biofilms with some of the bacterial species in the particular sequence. The formation of oral biofilm is briefly explained as following steps: (i) gram-positive cocci as streptococci species adhere to tooth surface, (ii) *F. nucleatum* binds gram-positive biofilm, (iii) periodontopathogens form biofilm on *F. nucleatum* surface (Kolenbrander, 2000; Marsh, 2004). The communication among the oral bacteria is more active in biofilm than in planktonic conditions because the bacterial density is higher. According to previous studies, *F. nucleatum* communicates with periodontopathogens and provides the condition for biofilm formation of *P. gingivalis*. For these reasons, we examined and reported the effect of growth ratio on co-culturing *P. gingivalis* with oral bacteria. The growth of *P. gingivalis* increased in the presence of *F. nucleatum*, but decreased in the presence of *Streptococcus gordonii* (Lee & Baek, 2010). Their character is known to be changed by growth conditions like hemin concentration (Mukherjee, 1985). The *P. gingivalis* LPS also induced modification of biosynthesis by hemin concentration as a growth condition (Cutler *et al.*, 1996; Al-Qutub *et al.*, 2006). Responses when *P. gingivalis* is cultured with *F. nucleatum* to the characteristics of *P. gingivalis* LPS have not been investigated. Therefore, we compared virulence of LPS between single- and co-cultured *P. gingivalis* with *F. nucleatum* and analyzed structural characteristics of its LPS.

Lipopolysaccharide is a powerful immunostimulator, and induces inflammation and shock (Raetz & Whitfield, 2002). LPS reacts strongly to induce local or systemic disease (Cooke *et al.*, 2002; Andreasen

et al., 2008), so the study of LPS is ongoing. *Porphyromonas gingivalis* LPS is also an inflammatory factor (Park *et al*., 2010), and has been studied in relation to oral disease (Kumada *et al*., 1995; Lamont & Jenkinson, 1998; Coats *et al*., 2009). In this study, when the LPS of *P. gingivalis* was treated on THP-1 cells, the LPS induced expression of inflammatory cytokines. We first showed change of virulence of *P. gingivalis* LPS by co-cultivation of oral bacteria. When *P. gingivalis* was cultured with or without *F. nucleatum*, co-cultured *P. gingivalis* LPS with *F. nucleatum* induced higher levels of cytokine expression than single-cultured *P. gingivalis*.

Porphyromonas gingivalis LPS stimulates TLR2 or TLR4 (Darveau *et al*., 2004). The *P. gingivalis* LPS has diverse structures of lipid A that include various phosphate groups and amount and position of acyl-chain (Al-Qutub *et al*., 2006). Especially, the structure of lipid A in *P. gingivalis* LPS shows more diversity than other gram-negative bacteria (Kumada *et al*., 1995). Therefore, the structure of the lipid A is changed according to growth condition or hemin concentration (Cutler *et al*., 1996; Al-Qutub *et al*., 2006), and the LPS shows different virulence for human cells or mouse cells according to variations of lipid A structure (Hajjar *et al*., 2002; Darveau *et al*., 2004). Mouse TLR4 recognizes acyl chains with fewer than 12 carbon atoms in pentamers, but human TLR4 recognizes acyl chains including more than 14 carbon atoms (Hajjar *et al*., 2002). Lipid A of *P. gingivalis* has an acyl-chain of various numbers (propamer, butamer and pentamer), and its acyl-chain shows various carbon numbers (Al-Qutub *et al*., 2006). To check the relation between the virulence change and the lipid A structure of *P. gingivalis* by *F. nucleatum*, the change of *P. gingivalis* LPS biosynthesis-related genes by communication with *F. nucleatum* was examined. The genes *lpxA* and *lpxD* were found in *P. gingivalis* genome and the functions of these genes were analyzed using complement *Escherichia coli*. *Porphyromonas gingivalis lpxA* and *lpxD* directly contribute to *E. coli* lipid A heterogeneity (Bainbridge *et al*., 2008). According to this paper, a major cluster of *E. coli* lipid A showed *m/z* 1798. However, when the *lpxA* and *lpxD* genes of *P. gingivalis* were transfected in *E. coli* using plasmids, the acyl-chains of lipid A became longer, and the mass of lipid A become greater, and mass peak of lipid A showed *m/z* 1826 and 1854. So, *lpxA* and *lpxD* of *P. gingivalis* were related to lipid A biosynthesis and heterogeneity. Also, *P. gingivalis* lipid A heterogeneity with varying hemin concentrations was shown to be due to hemin uptake through hemin binding protein (Al-Qutub *et al*., 2006). In this study, when *P. gingivalis* was cultured with *F. nucleatum*, *lpxA* and *lpxD* genes were expressed at higher levels compared with single-cultured *P. gingivalis*. Furthermore, hemin-binding protein was expressed more in co-cultured *P. gingivalis*. These results indicate that the biosynthesis of *P. gingivalis* LPS may be affected by communication with *F. nucleatum*. For confirmation of lipid A heterogeneity by communication with *F. nucleatum*, the masses of single- and co-cultured *P. gingivalis* lipid A were analyzed by MALDI-TOF mass spectrometry after hydrolysis of the LPS. While lipid A of single-cultured *P. gingivalis* LPS showed mass ion peaks at *m*/*z* 1249, 1449 and 1689, and the density of each peak was the same; lipid A of co-cultured *P. gingivalis* LPS displayed peaks at *m*/*z* 1249, 1449 and 1689, and the peak at 1689 *m*/*z* was higher than the other peaks. Nichols *et al.* (2012) suggested that TLR2 stimulation by *P. gingivalis* may be mediated by phosphorylated dihydroceramide lipids rather than *P. gingivalis* LPS and lipid A. Molecular mass of phosphorylated dihydroceramide exhibits < 1000 *m/z*, and when the extract was analyzed with mass spectra after *P. gingivalis* LPS isolation, the LPS extract contained approximately 25-fold less contaminating phosphorylated dihydroceramide. However, in this study, single- or co-cultured *P. gingivalis* LPS extracts did not show differences in the induction levels of CD25 expression using a CHO/CD14/TLR2 reporter cell, which is a nuclear factor-κB reporter cell line and expresses a membrane CD25 (data not shown). That indicates that the level of phosphorylated dihydroceramide may be no different. Finally, we focused on the heterogeneity of *P. gingivalis* lipid A and analyzed its structure with mass spectra. Coats *et al.* (2009) showed activities of *P. gingivalis* LPS for TLR4 after modifying *P. gingivalis* LPS using mutated LPS-related genes (Coats *et al*., 2009). *Porphyromonas gingivalis* express lipid A of various structures with molecular masses 1248, 1448 and 1688 *m/z* in low hemin concentrations. They performed mutation of each LPS-related gene, and generated 1773- and 1587-deficient mutants. The 1773 knockout (KO) mutant or 1587 KO mutants of *P. gingivalis* have lipid A with molecular mass 1248 and 1688 *m/z* or 1368 and 1448 *m*/*z*,

respectively. 1773KO *P. gingivalis* have slight bioactivity and showed antagonistic effects on *E. coli* LPS for a TLR4 response. However, 1587KO *P. gingivalis* have strong bioactivity and an agonistic effect on the TLR4 response. These results support our data that co-cultured *P. gingivalis* LPS is more virulent than single-cultured *P. gingivalis* LPS.

In conclusion, the virulence of *P. gingivalis* LPS is enhanced by co-cultivation with *F. nucleatum*, which may be a result of lipid A modification of the LPS by increasing expression of *LpxA* and *LpxD*. In further study, we investigate the communication molecules between *P. gingivalis* and *F. nucleatum* related to virulence or lipid A heterogeneity.

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