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The possible mechanism of preterm birth associated with periodontopathic *Porphyromonas gingivalis*

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Background and Objective: Previous studies have shown that *Porphyromonas gingivalis* is found in the amniotic fluid and placentae of pregnant women with some obstetric diseases. However, the biological effects of *P. gingivalis* on intrauterine tissues remain unclear. The aim of this study was to investigate the presence of *P. gingivalis* in chorionic tissues from hospitalized high-risk pregnant women, and the effects of *P. gingivalis* lipopolysaccharide on the production of proinflammatory molecules in human chorion-derived cells.

Material and Methods: Twenty-three subjects were selected from Japanese hospitalized high-risk pregnant women. The presence of *P. gingivalis* in chorionic tissues was analyzed by PCR. Cultured chorion-derived cells or Toll-like receptor-2 (*TLR-2*) gene-silenced chorion-derived cells were stimulated with *P. gingivalis* lipopolysaccharide. Real-time PCR was performed to evaluate *TLR-2* and Toll-like receptor-4 (*TLR-4*) mRNA expression in the cells. Levels of interleukin-6 and interleukin-8 in culture supernatants of the chorion-derived cells were measured by ELISA.

Results: *P. gingivalis* DNA was detected in chorionic tissues from two women with threatened preterm labor, two with multiple pregnancy and two with placenta previa. Stimulation of chorion-derived cells with *P. gingivalis* lipopolysaccharide significantly increased *TLR-2* mRNA expression, whereas *TLR-4* mRNA expression was not changed. *P. gingivalis* lipopolysaccharide induced interleukin-6 and interleukin-8 production in chorion-derived cells, but the *P. gingivalis* lipopolysaccharide-induced interleukin-6 and interleukin-8 production was reduced in *TLR-2* gene-silenced chorion-derived cells.

Conclusion: Our results suggest that *P. gingivalis* can be detected in chorionic tissues of hospitalized high-risk pregnant women, and that *P. gingivalis* lipopolysaccharide induces interleukin-6 and interleukin-8 production via TLR-2 in chorion-derived cells.

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Periodontal disease is a chronic inflammatory disease caused by a variety of periodontopathic bacteria, resulting in the breakdown of the tooth-supporting apparatus and loss of teeth. Recent studies have indicated an association between periodontal disease and systemic diseases, including cardiovascular disease, diabetes and preterm low birth weight (preterm birth and/or low birth weight). A lot of studies on associations between periodontal disease

and preterm low birth weight have been reported since the first report by Offenbacher *et al*. in 1996 (1). We have reported that the periodontal conditions of Japanese pregnant women with a diagnosis of threatened premature labor are worse than those of normal pregnant women (2). Recently, some studies have shown that *Porphyromonas gingivalis*, a periodontopathic bacterium, is found in the amniotic fluid of pregnant women diagnosed with threatened premature labor (3) and in placentae of women with preterm delivery (4) or with pre-eclampsia (5). *P. gingivalis* is a gram-negative anaerobic bacterium, and has bioactive components including lipopolysaccharide (LPS), capsules and fimbriae on the cell surface. These components induce the production of proinflammatory cytokines and modulate the cytokine network in periodontal tissues (6). Generally, it is recognized that Toll-like receptor (TLR)-4 is the receptor for gram-negative bacterial LPS and that TLR-2 is for gram-positive peptidglycan and lipopeptides (7,8). However, it has been shown that *P. gingivalis* LPS signals through both TLR-2 and TLR-4 (9) or TLR-2 (10–13).

Delivery is started in the late stage of gestation by events including changes of various hormones and the stimulation of biomechanical molecules by fully grown fetuses. The initial signals enhance the production of the proinflammatory molecules, including interleukin (IL)-6, IL-8 and IL-1β. These factors lead to uterine contraction and cervical ripening, directly or indirectly, which result in parturition (14). There are two possible mechanisms linking periodontal disease with adverse pregnancy outcomes: (i) the proinflammatory molecules produced in periodontal tissues before the later stages of gestation cause parturition; and (ii) the bacteria in periodontal lesions migrate to the maternofetal unit via the blood and directly cause adverse pregnancy outcomes. We have previously found that pregnant women diagnosed with threatened premature labor are in worse periodontal condition and, furthermore, have high serum levels of IL-8 and IL-1β (2). As described above, some studies have shown that periodontal pathogens exist in amniotic fluid (3) and placentae (4,5); however, the biological effects of periodontal pathogens in chorionic tissues remain unclear.

The aims of the present study were to investigate the following: (i) the presence of *P. gingivalis* in the chorionic tissues of the pregnant women who were hospitalized for high-risk pregnancy; and (ii) the effects of *P. gingivalis* LPS on the production of proinflammatory molecules in human chorion-derived cells.

Material and methods

Study population

Subjects for oral and bacterial examinations were selected from pregnant women who were hospitalized for high-risk pregnancy in the Department of Obstetrics and Gynecology, Kagoshima City Hospital. Exclusion criteria were the use of antimicrobials during pregnancy, viral infection, autoimmune disease, diabetes or gestational diabetes. Written informed consent was obtained from all women. Approval for this study was granted by the human ethical committee of Kagoshima City Hospital.

Oral examinations

Oral examinations were performed on the women during their second trimester by one periodontist. The numbers of remaining, carious or treated teeth were recorded. Assessments of periodontal parameters, including plaque index (15), gingival index (16), clinical attachment level, probing pocket depth and bleeding on probing, were carried out at four sites (mesial, distal, buccal and lingual/palatal) per tooth on the right side of the maxilla/left side of the mandible or the left side of the maxilla/right side of the mandible. The presence of two or more teeth showing probing pocket depth ≥ 4 mm and clinical attachment level ≥ 3 mm at the same site was defined as periodontitis. If the patients who were not diagnosed with periodontitis had bleeding on probing, they were defined as having gingivitis.

Bacterial examinations

Subgingival plaque, saliva and chorionic tissues were obtained to analyze the presence of *P. gingivalis*. At the time of oral examination, subgingival plaque samples were obtained by inserting a sterilized paper point for 15 s into the periodontal pockets of sites showing signs of gingival inflammation, including gingival redness and swelling. Saliva samples were collected using Salivettes (SARSTEDT, Nümbrecht, Germany). Samples were stored at −80°C until analysis. Samples of chorionic tissues were obtained from all subjects after delivery. The chorionic tissue samples were immediately fixed in 4% paraformaldehyde and then embedded in paraffin. Total DNA of subgingival plaque, saliva and chorionic tissue samples was purified by using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). Polymerase chain reaction was carried out by using the Taq PCR Core kit (Qiagen) to detect *P. gingivalis* in the DNA samples. Table 1 shows the primer sequences of *P. gingivalis* used in this study. The PCR was performed using 34 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR products were separated by electrophoresis in 2% agarose gel and visualized using ethidium bromide and an ultraviolet light transilluminator. The detection limit of *P. gingivalis* DNA in the PCR was 0.04 pg.

Primary culture of human chorion-derived cells

Human fetal membrane tissues were obtained by elective cesarean section from normal pregnant women hospitalized in Kagoshima University Medical and Dental Hospital at the month of normal parturition to isolate chorion-derived cells. The study protocol satisfied the ethical standards of the Kagoshima University Medical and Dental Hospital human ethical committee. Written informed consent was obtained from all subjects. Chorion-derived cells were isolated and cultured using a modified version of the method

Table 1. Primer sequences used in PCR and real-time PCR

Primer sequence (forward/reverse)		Size (bp)	Accession number	References
PCR				
Porphyromonas gingivalis 16s rRNA				
Forward	5′-AGGCAGCTTGCCATACTGCG-3′	404	L16492	Ashimoto *et al.* (33)
Reverse	5′-ACTGTTAGCAACTACCGATGT-3′			
Toll-like receptor-2				
Forward	5′-TCACCTACATTAGCAACAG-3′	368	NM_003264	Hatakeyama *et al.* (34)
Reverse	5′-GATCTGAAGCATCAATCTC-3′			
Toll-like receptor-4				
Forward	5-TGGATACGTTTCCTTATAAG-3′	506	NM_138554	Hatakeyama *et al.* (34)
Reverse	5′-GAAATGGAGGCACCCCTTC-3′			
Glyceraldehyde 3-phosphate dehydrogenase				
Forward	5′-CTGCACCACCAACTGCTTAGC-3′	382	M17701	Gonda *et al.* (35)
Reverse	5′-CTCAGTGTAGCCCAGGATGCC-3′			
Real-time PCR				
Toll-like receptor-2				
Forward	5′-GGCCAGCAAATTACCTGTGTG-3′	67	NM_003264	Patni *et al.* (36)
Reverse	5′-AGGCGGACATCCTGAACCT-3′			
Toll-like receptor-4				
Forward	5′-CAGAGTTTCCTGCAATGGATCA-3′	85	NM_138554	Patni *et al.* (36)
Reverse	5′-GCTTATCTGAAGGTGTTGCACAT-3′			
Glyceraldehyde 3-phosphate dehydrogenase				
Forward	5′-GAAGGTGAAGGTCGGAGTC-3′	226	BC029340	Lin *et al.* (37)
Reverse	5′-GAAGATGGTGATGGGATTTC-3′			

described previously by Uchide *et al.* (17). After removal of decidua, amnion and blood clots from the fetal membranes, chorion tissues were washed with phosphate-buffered saline containing antibiotics (120 units/mL penicillin G sodium, 120 μg/mL streptomycin sulfate, 16 μg/mL gentamicin sulfate and 0.3 μg/mL amphotericin B), and were minced into fragments (approximately 3 mm^2). Up to 10 g of the fragments was transferred to a solution composed of 0.25% trypsin (Gibco BRL, MD, USA) and 0.04% collagenase type I (Gibco BRL), followed by agitation for 10 min at 37°C. After the suspensions were filtered through a sterilized 100 μm mesh, the filtrates were discarded. The residues were resuspended in 50 mL of fresh trypsin/collagenase solution, followed by agitation for 20 min at 37°C. After the released cells were collected by filtration, the cell-collecting procedures were repeated once more. The released cells were cultured in culture medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (D-MEM/F-12; Gibco BRL) supplemented with 0.244% $NaHCO_3$ and antibiotics (120 units/mL penicillin G sodium and 120 μg/mL streptomycin sulfate) with 20% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 in air at 37°C.

Stimulation of chorion-derived cells with *P. gingivalis* LPS or *Escherichia coli* LPS

Chorion-derived cells were seeded at a density of 3×10^4 cells/cm^2 in collagen type I-coated dishes for 24 h. The medium was then replaced with culture medium with 0.1 or 10% FBS. After 24 h, the cells were stimulated with vehicle, *P. gingivalis* LPS (InvivoGen, San Diego, CA, USA), or *E. coli* LPS (InvivoGen). After 12 h of incubation, total RNA was extracted for assay of *TLR-2* and *TLR-4* mRNA expression. After 24 h of incubation, culture supernatants were collected for assay of IL-6 and IL-8 production.

Gene silencing of *TLR-2* and *TLR-4* in chorion-derived cells

Gene silencing of *TLR-2* and *TLR-4* in chorion-derived cells was performed by using small interfering (si)RNA transfection following the reverse transfection protocol provided by Invitrogen (Invitrogen, Carlsbad, CA, USA). Lipofectamine RNAiMAX (Invitrogen) was mixed with siRNA (*TLR-2* siRNA, sc-40256; *TLR-4* siRNA, sc-40260; control siRNA, sc-37007; Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was diluted to a final concentration of 1 nM with Opti-MEM® I Reduced Serum Medium (Invitrogen). The siRNA–Lipofectamine™ RNAiMAX complexes were incubated at room temperature for 20 min in 12-well plates. Chorion-derived cells (density 1.5×10^4 cells/cm^2) suspended in antibiotic-free culture medium with 20% FBS were added to the wells and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 48 h. The medium was then replaced with the antibiotic-free culture medium with 0.1%FBS. After 12 h, the cells were stimulated with vehicle or *P. gingivalis* LPS (InvivoGen). After 24 h of incubation, culture supernatants were collected for assay of IL-6 and IL-8 production, and total RNA was extracted for assay of *TLR-2* mRNA expression.

Analysis of proinflammatory molecule levels

The levels of IL-6, IL-8, MMP-3, MMP-9 and prostaglandin E_2 (PGE_2) in the culture supernatants were

determined by using commercially available ELISAs (IL-6, IL-8, MMP3, and MMP9 from Invitrogen; and PGE_2 from R&D SystemS, Minneapolis, MN, USA).

Analysis of *TLR-2* and *TLR-4* mRNA expression

Total RNA was extracted by using ISOGEN (Wako, Osaka, Japan) according to the manufacturer's instructions. The total RNA samples were digested with DNase I (Invitrogen) at 37°C for 30 min and at 75°C for 5 min to remove contaminating genomic DNA. Reverse transcription of the DNase-treated samples was carried out by SuperScript III First-strand synthesis Super Mix (Invitrogen) according to the manufacturer's instructions. Both PCR and quantitative real-time PCR were carried out to investigate the expression of *TLR-2* and *TLR-4* mRNA on the chorion-derived cells. Table 1 shows the primer sequences used in the PCR and real-time PCR methods. The PCR was performed using 34 or 37 cycles for *TLR-2*, 33 or 37 cycles for *TLR-4* and 22 cycles for glyceraldehydes 3-phosphate dehydrogenase (*GAPDH*) of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The PCR products were separated by electrophoresis in 2% agarose gel and visualized using ethidium bromide and an ultraviolet light transilluminator. Quantitative real-time PCR was performed in the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using the QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. Real-time PCR was carried out using 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s. The comparative threshold cycle number (*Ct*) method ($2^{-\Delta\Delta Ct}$) was used to quantify the results obtained by real-time PCR. All quantifications were normalized to *GAPDH* mRNA levels.

Statistical analysis

Data are expressed as the means ± SD. Statistical significance of differences between groups was analyzed by Mann–Whitney *U*-test or one-way analyses of variance (ANOVA) with Bonferroni correction by using SSPS13.0 (APPA Inc., Chicago, IL, USA).

Results

Twenty-three hospitalized pregnant women were enrolled in this study. We examined the existence of *P. gingivalis* in the chorionic tissues of all subjects. Eleven of the 23 women were diagnosed with threatened preterm labor, three with premature rupture of membranes, one with polyhydramnios and seven with no obstetric inflammation signs. Eight women had multiple pregnancy and four were diagnosed with placenta previa. The clinical profiles and microbiological findings of the women are presented in Table 2. Figure 1 shows detection of

Table 2. Clinical profiles and microbiological findings of the pregnant women in this study

Subject number	Age	Diagnosis	*P. gingivalis* in chorionic tissue	PB or TB	LBW or NBW	Periodontal condition	*P. gingivalis* in saliva	*P. gingivalis* in subgingival plaque
S1	33	TPL (no obstetric inflammation sign)	+	PB	LBW	Gingivitis	+	+
S2	37	TPL (PROM)	+	PB	NBW	Periodontitis	−	−
	34	TPL (no obstetric inflammation sign)	−	TB	NBW	Gingivitis	−	−
	35	TPL (no obstetric inflammation sign)	−	PB	NBW	Gingivitis	+	−
	25	TPL (no obstetric inflammation sign)	−	PB	NBW	Gingivitis	+	−
	38	TPL (no obstetric inflammation sign)	−	PB	NBW	Periodontitis	+	+
	33	TPL (no obstetric inflammation sign)	−	PB	LBW	Periodontitis	+	+
	32	TPL (no obstetric inflammation sign)	−	PB	LBW	Periodontitis	+	−
	25	TPL (PROM)	−	PB	NBW	Periodontitis	+	+
	36	TPL (PROM)	−	PB	LBW	Periodontitis	−	−
	22	TPL (polyhydramnios)	−	TB	LBW	Gingivitis	−	−
S3	25	Multiple pregnancy	+	PB	LBW/LBW	Gingivitis	+	+
S4	30	Multiple pregnancy	+	PB	LBW/LBW	Gingivitis	−	+
	37	Multiple pregnancy	−	PB	LBW/LBW	Gingivitis	+	−
	39	Multiple pregnancy	−	PB	LBW/LBW	Gingivitis	−	+
	27	Multiple pregnancy	−	PB	LBW/LBW	Gingivitis	+	+
	25	Multiple pregnancy	−	PB	LBW/LBW	Gingivitis	+	+
	40	Multiple pregnancy	−	PB	LBW/LBW	Periodontitis	−	−
	29	Multiple pregnancy	−	PB	LBW/LBW	Periodontitis	−	−
S5	29	Placenta previa	+	PB	LBW	Gingivitis	−	−
S6	32	Placenta previa	+	PB	LBW	Gingivitis	−	−
	29	Placenta previa	−	PB	NBW	Gingivitis	−	+
	36	Placenta previa	−	PB	NBW	Periodontitis	−	+

LBW, low birth weight; NBW, normal birth weight; PB, preterm birth; PROM, premature rupture of membranes; TB, term birth; TPL, threatened preterm labor; and periodontitis, the presence of two or more teeth showing probing pocket depth ≥ 4 mm and clinical attachment level ≥ 3 mm at the same site.

P. gingivalis by PCR in six chorionic tissue samples. *P. gingivalis* was detected in two samples in the threatened premature labor group, two samples in the multiple pregnancy group and two samples in the placenta previa group. *P. gingivalis* was found in either subgingival plaque or saliva in three of these women. The means (SD) of gestational age at delivery and neonatal body weight were 34 wk + 3 d (0 wk + 3 d) and 2059.0 g (285.4 g) in the *P. gingivalis*-positive in chorionic tissues group and 34 wk + 5 d (0 wk + 5 d) and 2141.3 g (738.5 g) in the *P. gingivalis*-negative in chorionic tissues group. There were no significant differences in the means of gestational age at delivery and neonatal body weight between the *P. gingivalis*-positive in chorionic tissues group and the *P. gingivalis*-negative in chorionic tissues group.

The effects of *P. gingivalis* LPS and *E. coli* LPS on gene expression of *TLR-2* and *TLR-4* in chorion-derived cells were investigated in the culture medium with 0.1 or 10% FBS. In the culture medium with 0.1% FBS, stimulation of the cells with *P. gingivalis* LPS significantly increased *TLR-2* mRNA expression levels (approximately fivefold) compared with control cultures, whereas *E. coli* did not affect *TLR-2* expression levels (Fig. 2A,B). In the culture medium with 10% FBS, *TLR-2* expression in chorion-derived cells was not upregulated by stimulation with *P. gingivalis* LPS, whereas *E. coli* LPS significantly increased *TLR-2* mRNA expression levels (approximately fourfold) compared with control cultures (Fig. 2D,E). Neither *P. gingivalis* LPS nor *E. coli* LPS influenced *TLR-4* expression in both culture media (Fig. 2A,C,D,F).

The levels of IL-6, IL-8, MMP3, MMP9 and PGE_2 in culture supernatants of chorion-derived cells stimulated with *P. gingivalis* LPS for 24 h were examined in the culture medium with 0.1% FBS. As shown in Fig. 3A,B, *P gingivalis* LPS increased IL-6 and IL-8 secretion in a dose-dependent manner. Levels of MMP3, MMP9 and PGE_2 were not significantly changed by stimulation with *P. gingivalis* LPS (data not shown).

We examined to what extent TLR-2 and/or TLR-4 were involved in *P. gingivalis* LPS- and *E. coli* LPS-induced IL-6 and IL-8 production in chorion-derived cells. Figure 4A shows the gene expression of *TLR-2* in control siRNA-, *TLR-2* siRNA- and *TLR-4* siRNA-treated chorion-derived cells

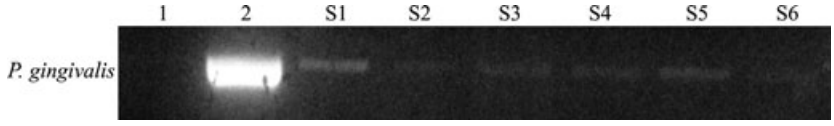

Fig. 1. Detection of *Porphyromonas gingivalis* in the chorionic tissues of six women by RT-PCR. Lanes S1–S6 show the *P. gingivalis* in each sample. Lane 1 is nontemplate control. Lane 2 is the positive control (DNA of *P. gingivalis* ATCC53477).

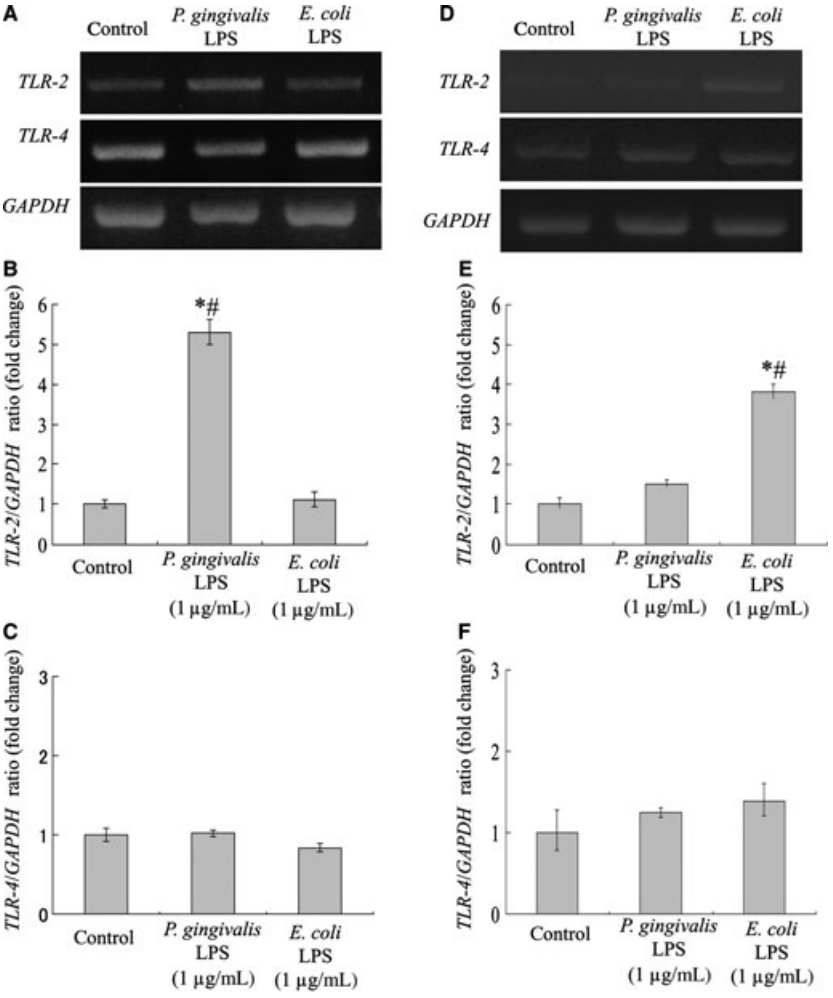

Fig. 2. Gene expression of *TLR-2* and *TLR-4* in the chorion-derived cells stimulated with *P. gingivalis* lipopolysaccharide (LPS) or *E. coli* LPS by RT-PCR (A,D) and real-time PCR (B,C,E,F) in the culture medium with 0.1% fetal bovine serum (FBS; A–C) or 10% FBS (D–F). The cells were stimulated with vehicle, *P. gingivalis* LPS (1.0 μg/mL) or *E. coli* LPS (1.0 μg/mL) in the culture medium with 0.1 or 10% FBS. After 12 h, total RNA was extracted and the gene expression of *TLR-2* and *TLR-4* analyzed by RT-PCR (A,D). In addition, quantitative analyses of gene expression of *TLR-2* (B,E) and *TLR-4* (C,F) were performed by real-time PCR. Values are means ± SD ($n = 3$). Data are representative of three separate experiments. *Significantly different from control at $p < 0.05$. Significantly different from *E. coli* LPS (1.0 μg/mL) or *P. gingivalis* LPS (1.0 μg/mL) at $p < 0.05$.

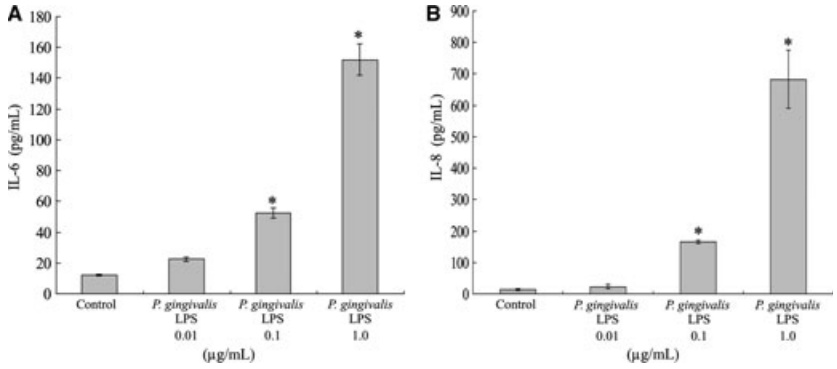

Fig. 3. Levels of interleukin (IL)-6 (A) and IL-8 (B) in culture supernatants of chorion-derived cells stimulated with *P. gingivalis* LPS. The cells were stimulated with vehicle or *P. gingivalis* LPS (0.01, 0.1 or 1.0 μg/mL). After 24 h, IL-6 and IL-8 levels in the culture supernatants were measured by ELISA. Values are means ± SD ($n = 4$). Data are representative of three separate experiments. *Significantly different from control at $p < 0.05$.

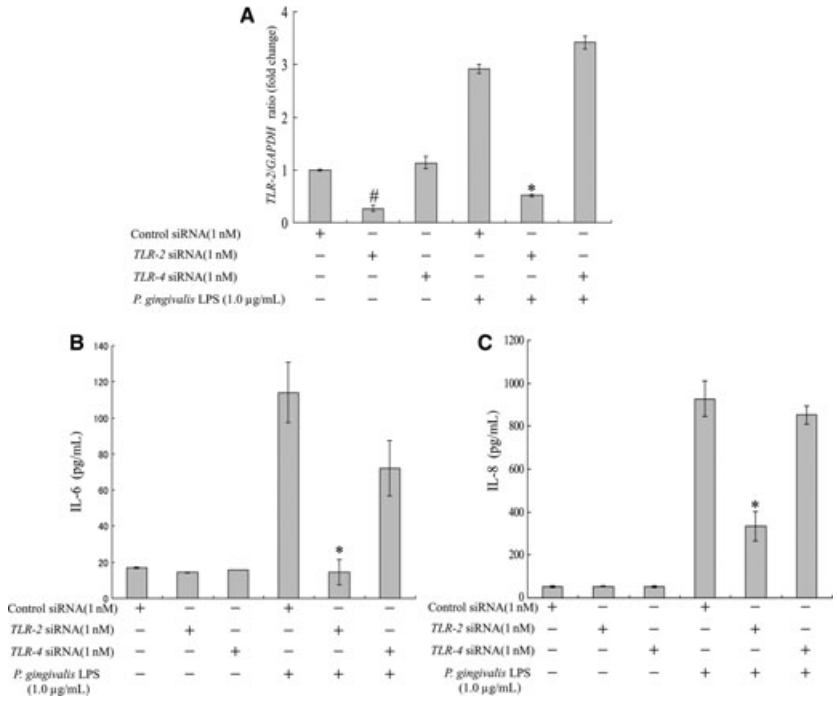

Fig. 4. Gene expression of *TLR-2* (A) and the levels of IL-6 (B) and IL-8 (C) of culture supernatants in control siRNA-, *TLR-2* siRNA- or *TLR-4* siRNA-treated chorion-derived cells stimulated with or without *P. gingivalis* LPS. The control siRNA-, *TLR-2* siRNA- or *TLR-4* siRNA-treated chorion-derived cells were stimulated with or without *P. gingivalis* LPS (1.0 μg/mL). After 24 h of incubation, gene expression levels of *TLR-2* in the cells were analyzed by real-time PCR (A). In addition, IL-6 and IL-8 levels in the culture supernatants were measured by ELISA (B,C). Values of gene expression levels are means ± SD ($n = 3$), and data are representative of two separate experiments. Values of IL-6 and IL-8 levels are means ± SD ($n = 4$), and data are representative of three separate experiments. Significantly different from control siRNA- or *TLR-4* siRNA-treated cells stimulated without *P. gingivalis* LPS at $p < 0.05$. *Significantly different from control siRNA- or *TLR-4* siRNA-treated cells stimulated with *P. gingivalis* LPS at $p < 0.05$.

stimulated with vehicle or *P. gingivalis* LPS. The gene expression of *TLR-2* in *TLR-2* gene-silenced cells was significantly decreased compared with the chorion-derived cells treated with control siRNA. *P. gingivalis* LPS-induced *TLR-2* mRNA expression was significantly reduced in *TLR-2* gene-silenced chorion-derived cells. Transfection of chorion-derived cells with *TLR-4* siRNA did not affect *TLR-2* gene expression. The gene expression of *TLR-4* in *TLR-4* siRNA-treated chorion-derived cells was significantly decreased compared with control siRNA-treated chorion-derived cells (data not shown). *P. gingivalis* LPS-induced IL-6 was reduced to control levels in *TLR-2* gene-silenced chorion-derived cells (Fig. 4B). *P. gingivalis* LPS-induced IL-8 production was significantly reduced by approximately 70%, but not to control levels, in *TLR-2* gene-silenced chorion-derived cells (Fig. 4C). There were no significant differences in the levels of IL-6 and IL-8 induced by *P. gingivalis* LPS between *TLR-4* siRNA-treated chorion-derived and control siRNA-treated chorion-derived cells (Fig. 4B,C).

Discussion

In the present study, we found that *P. gingivalis* existed in the chorionic tissues of hospitalized high-risk pregnant women. It has been indicated that infectious periodontal disease may be a risk factor for preterm low birth weight (18). Vergnes *et al.* (19) have shown that the odds ratio of the risk of preterm low birth weight in mothers with periodontal disease is 2.83 by meta-analysis. Previous studies have detected *P. gingivalis* in the amniotic fluid of women with threatened premature labor (3), in the placenta of women with pre-eclampsia (5) and in the placentae of women with chorioamnionitis (4). These findings imply the possibility that the presence of *P. gingivalis* in the chorionic tissues may affect the abnormal pregnancy outcomes. In the present clinical study, however, despite showing the presence of *P. gingivalis* in chorionic tissues from six high-risk pregnant women, we did not reveal the pathological roles of *P. gingivalis* existing in the chorionic tissue *in vivo*. In addition, there was no significant association between the detection of *P. gingivalis* in the chorionic tissues and gestational age of delivery or neonatal body weight. This result might be attributed to the fact

that there were only six women in whom *P. gingivalis* was detected in chorionic tissue samples and that most of the subjects in this study were high-risk pregnant women who had some obstetric causes and risk factors for abnormal pregnancy outcomes. To investigate the correlation between detection of *P. gingivalis* in chorionic tissues and abnormal pregnancy outcomes, large-scale studies focusing on the pathological roles of *P. gingivalis* in chorionic tissues are needed.

There are two possible pathways through which *P. gingivalis* can translocate to the chorionic tissues, as follows: (i) bacteria in the oral cavity migrate to the chorionic tissues as a result of hematogenous spread; and (ii) bacteria in the vagina and cervix spread to the uterus. Leon *et al.* (3) have suggested that *P. gingivalis* in the oral cavity is disseminated to the amniotic fluid. Han *et al.* (20) reported a case of stillbirth caused by *Fusobacterium nucleatum* that originated in the mother's mouth. Furthermore, *Fusobactium nucleatum* intravenously injected into pregnant mice is transmitted to the placenta and causes adverse pregnancy outcomes (21). In addition, it has been reported that bacteremia occurs after chewing and tooth brushing in individuals with gingival inflammation (22). It has been suggested that *P. gingivalis* detected in atherosclerotic plaques was derived from the oral cavity through bacteremia (23). These findings imply the possibility that the source of *P. gingivalis* detected in chorionic tissues in the present study was the oral cavity. However, in the present study, *P. gingivalis* was detected only in the subgingival plaque and/or saliva of three of the six women in whom *P. gingivalis* was detected in chorionic tissue samples. Therefore, we could not exclude the possibility that the bacterium in chorionic tissues came from the vagina and cervix. We could not investigate whether *P. gingivalis* was present in the vagina and cervix because we could not obtain vaginal and cervical samples owing to humanitarian considerations. Further studies focusing on the dissemination of periodontal pathogens in the chorionic tissues are needed.

It is generally recognized that TLR-4 is the receptor for gram-negative bacterial LPS, including *E. coli* LPS (24), and that TLR-2 is the receptor for gram-positive bacterial peptidoglycan and lipopeptides (7,8). However, it has been shown that *P. gingivalis* LPS signals through TLR-2/TLR-4 (9) or TLR-2 (10–13). It has been considered that CD14, including a membrane-bound form and a soluble form, is involved in recognition of LPS (25). We examined the gene expression of *TLR-2* and *TLR-4* in chorion-derived cells in the culture medium with 0.1 and 10% FBS. *E. coli* LPS did not increase *TLR-2* mRNA expression in the presence of 0.1% FBS, but increased *TLR-2* mRNA expression in the presence of 10% FBS. These results might indicate that the effect of *E. coli* LPS on chorion-derived cells was enhanced by soluble CD14 contained in the FBS. However, in the presence of 10% FBS, *P. gingivalis* LPS did not induce the gene expression of *TLR-2*. We thought that this result might be caused by influences of some factors contained in FBS, because the gene expression of *TLR-2* on chorion-derived cells stimulated with *P. gingivalis* LPS in the presence of 0.1% FBS was significantly increased. Therefore, we used the medium with 0.1% FBS to investigate the effects of *P. gingivalis* LPS on human chorion-derived cells in this study.

It has been indicated that the start of parturition is modulated by interaction between proinflammatory molecules, including cytokines, PGE_2 and MMPs, in various obstetric tissues. Bacteria and bacterial products cause preterm birth by stimulating production and release of these molecules, which induce preterm delivery (26). Katz *et al.* (4) have suggested that *P. gingivalis* colonization of placental tissues may contribute to preterm delivery. In the present study, we investigated the effects of *P. gingivalis* LPS on production of proinflammatory molecules using cultured chorion-derived cells. We showed that *P. gingivalis* LPS induced IL-6 and IL-8 generation by chorion-derived cells at significantly higher levels than control cultures, whereas could not induce production of PGE_2, MMP-3 and MMP-9. It has been reported that IL-6 and IL-8 increase the production of MMPs and PGE_2 in cytotrophoblastic, amniotic and decidual cells (27–29). In our clinical study, we did not examine the levels of *P. gingivalis* in chorionic tissues and pathological roles of *P. gingivalis* in inflammation in chorionic tissues; however, the present findings suggest that *P. gingivalis* detected in chorionic tissues might affect preterm labor by stimulating production of proinflammatory molecules, including IL-6 and IL-8, in the microenvironment. Further studies are needed to investigate the pathological roles of *P. gingivalis* existing in intrauterine tissues.

In this study, we showed that the application of *TLR-2* siRNA into chorion-derived cells significantly decreased the *P. gingivalis* LPS-induced IL-6 and IL-8 production. These results suggest that the production of IL-6 and IL-8 stimulated by *P. gingivalis* LPS is induced primarily through TLR-2, which may be autocyclically upregulated. Previous studies have shown that nuclear factor-κB activation is involved in the upregulation of TLR-2 expression in human endothelial cells (30), and that *P. gingivalis* induces nuclear factor-κB in human gingival fibroblasts (31). Further studies are required to investigate the signaling pathways of *P. gingivalis* LPS in the chorion-derived cells.

In conclusion, we suggest that *P. gingivalis* can be detected in chorionic tissues of hospitalized high-risk pregnant women, and that *P. gingivalis* LPS induces IL-6 and IL-8 production via TLR-2 in chorion-derived cells. Previous studies have suggested the possibility that the presence of *P. gingivalis* in amniotic cavity and placenta is involved in preterm birth or threatened preterm labor (3,4). Madianos *et al.* (32) have found that the absence of a protective maternal antibody response to some periodontal pathogens is associated with the translocation of periodontal bacteria to the fetus. There may be a need for further study focusing both on pathogenic factors and on host factors in pregnant women to elucidate the link between

abnormal pregnancy outcomes and periodontal disease.

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