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Porphyromonas gingivalis, a cause of preterm birth in mice, induces an inflammatory response in human amnion mesenchymal cells but not epithelial cells

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ABSTRACT

Introduction: Inflammation and infection, including dental infectious diseases, are factors that can induce preterm birth. We previously reported that mice with dental *Porphyromonas gingivalis* infection could be used as a model of preterm birth. In this model, cyclooxygenase (COX)-2 and interleukin (IL)-1β levels are increased, and *P. gingivalis* colonies are observed in the fetal membrane. However, the mechanism underlying fetal membrane inflammation remains unknown. Therefore, we investigated the immune responses of human amnion to *P. gingivalis in vitro*.

Methods: Epithelial and mesenchymal cells were isolated from human amnion using trypsin and collagenase, and primary cell cultures were obtained. Confluent cells were stimulated with *P. gingivalis* lipopolysaccharide (*P.g*-LPS) or *P. gingivalis*. mRNA expressions of IL-1β, IL-8, IL-6 and COX-2, protein expressions of nuclear factor (NF)-κB pathway components and culture medium levels of prostaglandin E_2 were evaluated.

Results: Following stimulation with 1 μg/mL *P.g*-LPS, the mRNA expression levels of IL-1β, IL-8, IL-6 and COX-2 in mesenchymal cells were increased 5.9-, 3.3-, 4.2- and 3.1-fold, respectively. Similarly, the expression levels of IL-1β, IL-8, IL-6 and COX-2 in mesenchymal cells were increased by 7.6-, 8.2-, 13.4- and 9.3-fold, respectively, after coculture with *P. gingivalis*. Additionally, stimulation with *P.g*-LPS or *P. gingivalis* resulted in the activation of NF-κB signaling and increased production of IL-1β and prostaglandin E_2. In contrast, no significant changes were observed in epithelial cells.

Discussion: Our findings suggest that mesenchymal cells might mediate the inflammatory responses to *P. gingivalis* and *P.g*-LPS, thereby producing inflammation that contributes to the induction of preterm birth.

1. Introduction

Preterm birth is the leading cause of perinatal morbidity and mortality [1]. Inflammation is recognized as an important risk factor for preterm birth, but the triggers and inflammatory pathways involved have yet to be fully characterized [2]. Therefore, elucidation of the mechanisms by which inflammation activates uterine contractions may provide important insights into new potential strategies for the

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prevention of preterm birth.

Recently, we generated an animal model of chronic inflammation-induced preterm birth using mice with a dental infection caused by *Porphyromonas gingivalis*, a common periodontal bacterial pathogen. The duration of pregnancy in mice with periodontitis was found to be 2 days shorter than that of normal pregnant mice (18.3 ± 0.9 days versus 20.5 ± 0.5 days) [3]. Notably, our model of preterm birth revealed the activation of inflammatory pathways in the fetal membrane [4,5]. Although *P. gingivalis* colonies were observed in the fetal membrane, there was little neutrophil infiltration or macrophage accumulation. These findings suggest that *P. gingivalis* or *P. gingivalis* lipopolysaccharide (*P.g*-LPS) induce local inflammation in the fetal membrane but not chorioamnionitis. However, the mechanisms underlying fetal membrane inflammation in the mouse have yet to be characterized, and it remains unclear whether *P. gingivalis* can also directly induce inflammation in human fetal membrane. Therefore, in the present study, we focused on the inflammatory responses of human fetal membrane to *P. gingivalis* and *P.g*-LPS.

The fetal membrane, especially the amnion, is the principal source of prostaglandins during human pregnancy [6]. Prostaglandins play key roles in fetal membrane rupture, uterine contraction and cervical ripening [7]. The amnion is the load-bearing structure of the fetal membrane and comprises an avascular layer of epithelial cells, underlying mesenchymal cells and extracellular matrix proteins [8]. Primary cultures of epithelial cells and mesenchymal cells isolated from the amnion have been studied to characterize their normal function and role during inflammation [9–15].

The aim of this study was to investigate the inflammatory pathways recruited by *P. gingivalis* in primary cultures of epithelial and mesenchymal cells from human amnion. Our results provide important insights into the roles of amnion cells during inflammation and establish a basis for future studies of the mechanisms of preterm birth.

2. Materials and methods

2.1. Isolation and culture of epithelial and mesenchymal cells from human amnion

Amnion samples were obtained at elective caesarean section before the onset of labor (37–38 weeks' gestation) under protocols approved by the Institutional Review Board at Hiroshima University Hospital (E−582-1), and written informed consent was obtained from each patient. All procedures were carried out using sterile techniques.

The method for separation and isolation of amnion epithelial cells and mesenchymal cells has been described previously [9]. Epithelial cells (500,000 cells/cm^2) and mesenchymal cells (200,000 cells/cm^2) were plated in plastic dishes and cultured in a 1:1 mixture of Ham's F-12 medium and Dulbecco's Modified Eagle's Medium (F12:DMEM; Wako Pure Chemical Industries, Osaka, Japan) containing 10% heat-inactivated fetal bovine serum (Corning, NY, USA) and 2% penicillin-streptomycin-amphotericin B suspension (Wako Pure Chemical Industries). Amnion cells were characterized using antibodies specific for two different filament proteins: cytokeratin-18 (a marker of epithelial cells) and vimentin (a marker of mesenchymal cells). Immunostaining was performed using primary antibodies against cytokeratin-18 (1:15,000; Abcam, Cambridge, UK) and vimentin (1:3000; Cell Signaling Technology, Danvers, MA, USA), as described in a previous report [10]. Amnion epithelial cells displayed a cobblestone-like morphology and were positive for cytokeratin-18 and negative for vimentin. In contrast, amnion mesenchymal cells displayed a fibroblast-like morphology and were negative for cytokeratin-18 and positive for vimentin. The purity of the epithelial cells was 98%, and that of the mesenchymal cultures was 97%. The cells were also characterized using flow cytometry (FACSCalibur system and CellQuest software, BD Biosciences, San Jose, CA, USA) and antibodies against CD49f (a marker of epithelial cells; BioLegend, San Diego, CA, USA) and CD90 (a marker of mesenchymal cells; BioLegend) [16]. Amnion epithelial cells were positive for CD49f (94.3 ± 3.65%) but negative for CD90 (<1%), whereas amnion mesenchymal cells were negative for CD49f (<10%) but positive for CD90 (82.1 ± 8.66%).

2.2. Stimulation of cultured cells with P.g-LPS or P. gingivalis

Cells were grown to confluence in 2–4 days. At 80–90% confluence, the medium was replaced with fresh medium. After 12 h, the cells were incubated with vehicle, *P.g*-LPS (InvivoGen, San Diego, CA, USA) or *P. gingivalis*-W83. Before addition to the amnion cells, the *P. gingivalis*-W83 strain was incubated under anaerobic conditions in an AnaeroPack (Mitsubishi Gas Chemical Co., Tokyo, Japan) at 37 °C for 4 days. The number of *P. gingivalis* cells suspended in phosphate-buffered saline (PBS) was evaluated from the optical density (OD) at 660 nm measured using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The amnion cells were incubated in antibiotic-containing medium with vehicle or *P.g*-LPS at various concentrations (1 ng/mL, 10 ng/mL, 100 ng/mL or 1 μg/mL) or in antibiotic-free medium with vehicle or *P. gingivalis* at different multiplicities of infection (MOIs; 10, 50 or 100). After incubation for various times, mRNA and proteins were extracted from the amnion cells for evaluation of expression levels, and culture supernatants were collected and stored at −80 °C for assay of prostaglandin E_2 levels.

2.3. RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR)

mRNA was isolated using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands) with DNase I (Sigma-Aldrich, St. Louis, MO, USA) treatment. Reverse transcription was performed using a ReverTra Ace kit (Toyobo, Osaka, Japan), as described in the manufacturer's protocol. mRNA (1 μg) was used for cDNA synthesis. Real-time RT-PCR was performed using an ABI 7300HT system with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA). The primer sequences were as follows: human IL-8 forward, 5′-GAGAGTGATTGAGAGTGGACCAC-3′ and reverse, 5′-CACAACCCTCTGCACCCAGTTT-3′; human IL-6 forward, 5′-CAAATTCGGTACATCCTCGAC-3′ and reverse, 5′-GTCAGGGGTGGTTATTGCATC-3′; human IL-1β forward, 5′-AAACAGATGAAGTGCTCCTTCCAGG-3′ and reverse, 5′-TGGAGAACACCACTTGTTGCTCCA-3′; human COX-2 forward, 5′-GAATCATTCACCAGGCAAATTG-3′ and reverse, 5′-TCTGTACTGCGGGTGGAACA-3′; human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5′-TGATGACATCAAGAAGGTGGT-3′ and reverse, 5′-TCCTTGGAGGCCATGTGGGCC-3′. GAPDH expression was used as an internal control, and the mRNA expression of each of the other genes was normalized to that of the GAPDH gene. Each sample was run in duplicate, and output levels are reported as the average for two wells.

2.4. Protein extraction and western blotting

Cells were sonicated in 500 μL RIPA buffer (Sigma-Aldrich) containing 5 μL protease inhibitors (Sigma-Aldrich). The sonicates were centrifuged (12000 rpm for 30 min), and the supernatants were retained and stored at −80 °C. Western blot analysis was performed to determine the protein levels of nuclear factor (NF)-κB. Protein extracts (250 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 4–12% gels, and the proteins were transferred to membranes using an iBlot 2 Dry Blotting System (Life Technologies, Carlsbad, CA, USA). Membranes were then incubated overnight with the following primary antibodies: anti-phosphorylated-NF-κB p65 (1:1000; Cell Signaling Technology), *anti*-NF-κB p65 (1:3000; Cell Signaling Technology), anti-phosphorylated-IκB kinase (IKK; 1:1000; Cell Signaling Technology), anti-phosphorylated-IκBα (1:1000; Cell Signaling Technology), anti-phosphorylated-p38 mitogen-activated protein kinase

(MAPK; 1:3000; Cell Signaling Technology), *anti*-p38 MAPK (1:3000; Cell Signaling Technology) or *anti*-cyclophilin B (1:20,000; Abcam). Cyclophilin B was used as a loading control. The relative densities of the bands were assessed using ImageJ 1.49 (National Institutes of Health, Bethesda, MD, USA). The OD values of the bands were normalized to the OD values of cyclophilin B.

2.5. Measurement of IL-1β and prostaglandin E_2 levels in the culture medium

The levels of IL-1β and prostaglandin E_2 in the culture medium were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA), in accordance with the manufacturer's protocol, and a microtiter plate reader was used to measure absorbance values at 450 nm.

2.6. Statistical analysis

Statcel 3 add-in software for Microsoft Excel (Microsoft, Redmond, WA, USA) was used for statistical analyses of the data. Comparisons between groups were made using the Mann-Whitney *U* test. A *P*-value less than 0.05 was considered to indicate a significant difference.

3. Results

3.1. Stimulation with P.g-LPS or P. gingivalis enhanced the expression of pro-inflammatory cytokines in amnion mesenchymal cells but not amnion epithelial cells

To assess the effects of *P.g*-LPS on IL-1β expression, primary amnion cells were treated with *P.g*-LPS (1 μg/mL) or *P. gingivalis* (MOI: 100) for 3–48 h, and IL-1β mRNA levels were determined. The expression levels of IL-1β mRNA were very low in amnion epithelial cells under control conditions and not affected by *P.g*-LPS or *P. gingivalis* (Fig. 1A and B). On the other hand, the levels of IL-1β transcripts in amnion mesenchymal cells were significantly elevated 5.9- and 7.6-fold after stimulation for 12 h with *P.g*-LPS and *P. gingivalis*, respectively ($N = 8$ samples from different women who underwent elective cesarean section; Fig. 1A and B). We next treated cells with different concentrations of *P.g*-LPS (1 ng/mL, 10 ng/mL, 100 ng/mL or 1 μg/mL) or *P. gingivalis* (MOI: 10, 50 or 100) for 12 h. The mRNA expression levels of IL-1β in mesenchymal cells increased in a concentration-dependent manner after stimulation for 12 h with *P.g*-LPS ($N = 8$; Fig. 1C), and there was a trend toward a concentration-dependent effect of *P. gingivalis* ($N = 8$; Fig. 1D). Furthermore, the IL-1β concentration in the culture medium of amnion mesenchymal cells was increased 4.1-fold (from 13.8 ± 11.9 pg/mL to 56.7 ± 21.7 pg/mL) following stimulation with *P.g*-LPS (1 μg/mL) for

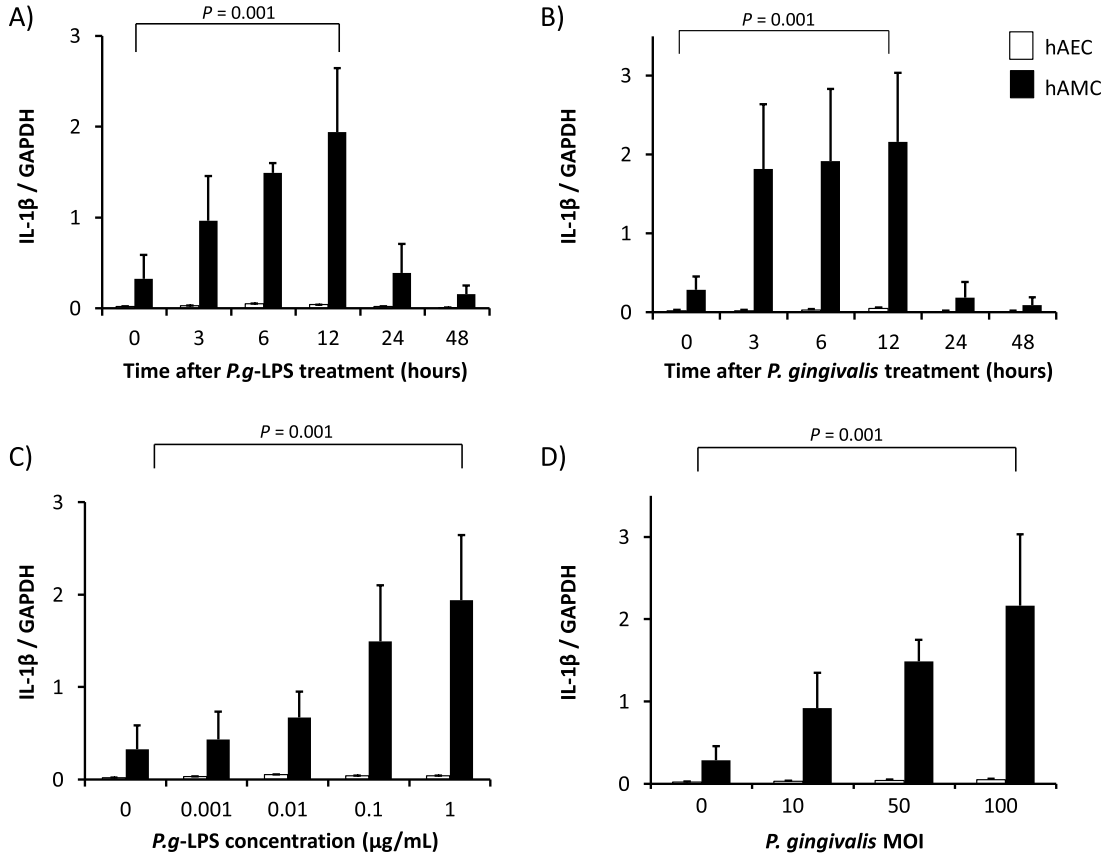

Fig. 1. Expression of interleukin (IL)-1β mRNA in human amnion epithelial cells (hAEC) and human amnion mesenchymal cells (hAMC). (A) Time-dependent changes in the expression of IL-1β mRNA following stimulation with *P. gingivalis* lipopolysaccharide (*P.g*-LPS; 1 μg/mL) were determined using real-time reverse transcription-polymerase chain reaction (RT-PCR; $N = 4$ for 3 h, 6 h, 24 h and 48 h; $N = 8$ for 0 h and 12 h). (B) Time-dependent changes in the expression of IL-1β mRNA following stimulation with *P. gingivalis* (at a multiplicity of infection [MOI] of 100) were determined using real-time RT-PCR ($N = 4$ for 3 h, 6 h, 24 h and 48 h; $N = 8$ for 0 h and 12 h). (C) Changes in the expression levels of IL-1β mRNA following stimulation with various concentrations of *P.g*-LPS for 12 h were determined using real-time RT-PCR ($N = 4$ for 1 ng/mL, 10 ng/mL and 100 ng/mL; $N = 8$ for control and 1 μg/mL). (D) Changes in the expression levels of IL-1β mRNA following stimulation for 12 h with *P. gingivalis* at various MOIs were determined using real-time RT-PCR ($N = 4$ for MOIs of 10 and 50; $N = 8$ for control and MOI of 100). All expression levels were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). *N* denotes the number of samples, each of which was obtained from a different woman who underwent elective cesarean section. Each sample was run in duplicate, and the average value was used for the analysis.

12 h ($N = 4$ for each group) and 19.3-fold (from 15.7 ± 11.7 pg/mL to 304 ± 202 pg/mL) following stimulation with *P. gingivalis* (MOI: 100) for 12 h ($N = 4$ for each group). The concentration of IL-1β in the culture medium was very low for amnion epithelial cells under control conditions and not affected by *P.g*-LPS or *P. gingivalis* (data not shown). We also examined the mRNA expression levels of IL-8 and IL-6. The mRNA expression levels of IL-8 and IL-6 in mesenchymal cells were also increased 3.3- and 4.2-fold, respectively, after stimulation with *P.g*-LPS (1 μg/mL) for 12 h and 8.2- and 13.4-fold, respectively, after stimulation with *P. gingivalis* (MOI: 100) for 12 h (Table 1). IL-8 and IL-6 mRNA were not detectable in amnion epithelial cells irrespective of whether they had been challenged with *P.g*-LPS or *P. gingivalis* (data not shown).

3.2. Stimulation with P.g-LPS or P. gingivalis activated the NF-κB pathway in amnion mesenchymal cells but not amnion epithelial cells

NF-κB is one of the most important regulators of pro-inflammatory gene expression, and activation of this pathway depends specifically on the phosphorylation of IKKβ and IκBα [17]. Western blot analysis was performed to detect NF-κB pathway components in primary amnion cells. Amnion mesenchymal cells treated with *P.g*-LPS (1 μg/mL) or *P. gingivalis* (MOI: 100) for 12 h showed elevated levels of phosphorylated IKKβ, phosphorylated IκBα and phosphorylated NF-κB but not total NF-κB p65 (Fig. 2A and B). In contrast, there were no significant changes in amnion epithelial cells (data not shown). These results suggest that *P.g*-LPS and *P. gingivalis* activate the NF-κB pathway in amnion mesenchymal cells, thereby enhancing the levels of pro-inflammatory cytokines. We also investigated whether *P.g*-LPS or *P. gingivalis* recruited the MAPK pathway, because phosphorylated-p38 MAPK was upregulated in fetal membrane tissues from mice with dental infection that were used as a model of preterm birth [5]. However, phosphorylated-p38 MAPK and p38 MAPK were not detected in either epithelial or mesenchymal cells from human amnion, irrespective of whether they had been challenged with *P.g*-LPS or *P. gingivalis* (data not shown).

3.3. Stimulation with P.g-LPS or P. gingivalis enhanced the expression of COX-2 mRNA and the synthesis of prostaglandin E_2 in amnion mesenchymal cells but not amnion epithelial cells

Prostaglandin E_2 is believed to be involved in the premature rupture of membranes, initiation of myometrial contractions during labor and cervical ripening during parturition. Thus, we quantified the effects of *P.g*-LPS and *P. gingivalis* on prostaglandin E_2 synthesis in primary amnion cells. In amnion mesenchymal cells, the mRNA expression of COX-2 was increased 3.1- and 9.3-fold following stimulation with *P.g*-LPS (1 μg/mL) and *P. gingivalis* (MOI: 100), respectively, for 12 h (Table 2). Furthermore, for amnion mesenchymal cells, the prostaglandin E_2 concentration in the culture medium was increased 4.0- and 2.9-fold following stimulation with *P.g*-LPS and *P. gingivalis*, respectively (Table 2). In contrast, amnion epithelial cells had lower basal levels of COX-2 mRNA expression and prostaglandin E_2 concentration than mesenchymal cells, and the levels remained unchanged following stimulation with *P.g*-LPS or *P. gingivalis* (data not shown).

Table 1
Levels of interleukin (IL)-8 and IL-6 mRNA.

	Control	*P.g*-LPS	Control$^{(-)}$	*P. gingivalis*
IL-8 mRNA	0.18 ± 0.11	0.60 ± 0.30*	0.12 ± 0.04	1.00 ± 0.57**
IL-6 mRNA	0.24 ± 0.16	1.00 ± 0.46**	0.22 ± 0.15	2.95 ± 2.45**

Expression levels of IL-8 and IL-6 mRNA in amnion mesenchymal cells in the presence/absence of stimulation with *P. gingivalis* lipopolysaccharide (*P.g-LPS*; 1 μg/mL for 12 h) or *P. gingivalis* (MOI: 100 for 12 h) were determined using real-time reverse transcription-polymerase chain reaction ($N = 8$ for each group). The expression of IL-8 and IL-6 was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). *N* denotes the number of samples, each of which was obtained from a different woman who underwent elective cesarean section. Each sample was run in duplicate, and the average value was used for the analysis. Control$^{(-)}$: medium without antibiotics. *$P < 0.05$, **$P < 0.01$.

4. Discussion

Important findings of the present study were that stimulation of amnion mesenchymal cells with *P.g*-LPS or *P. gingivalis* resulted in the up-regulation of inflammatory cytokines, activation of NF-κB signaling, increased expression of COX-2 and enhanced production of prostaglandin E_2. By contrast, no such changes were observed in amnion epithelial cells after stimulation with *P.g*-LPS or *P. gingivalis*. Our novel data suggest that amnion mesenchymal cells may mediate the inflammatory responses to *P. gingivalis* and *P.g*-LPS, and it is possible that the resulting inflammation is a factor that induces preterm birth.

It has been reported that preterm birth is associated with the occurrence of inflammatory events, such as leukocyte activation and increased levels of pro-inflammatory cytokines, in the myometrium, fetal membranes and cervix [18]. Both sterile intra-amniotic inflammation and intra-amniotic infection due to microbial invasion of gestational tissues have been established as important factors associated with preterm birth. NF-κB is involved in the regulation of many genes, including those encoding COX-2 and a number of pro-inflammatory cytokines [12]. The NF-κB pathway is activated following stimulation by pro-inflammatory cytokines, LPS or bacteria. Phosphorylated IKK promotes the phosphorylation of IκB, leading to further phosphorylation and translocation of the p65 protein from the cytoplasm to the nucleus. This protein then binds to the promoter regions of various genes. In the current study, we provide evidence that stimulation of human amnion mesenchymal cells by *P.g*-LPS or *P. gingivalis* results in activation of the NF-κB pathway as well as upregulation of pro-inflammatory cytokines and increased biosynthesis of prostaglandin E_2. However, p38 MAPK pathways were not activated by *P.g*-LPS or *P. gingivalis*, consistent with a previous suggestion that sterile inflammation initiates p38 MAPK signaling whereas infectious inflammation stimulates the NF-κB pathway [19]. Thus, it is likely that the NF-κB signaling pathway plays an important role in the development and/or progression of inflammation in human amnion cells in response to a pathogen. Notably, both preclinical and clinical studies have implicated NF-κB signaling in preterm birth in the presence of inflammation [5,20,21].

Amnion is the major source of prostaglandin E_2 throughout gestation and parturition [11,12]. Amnion is composed of epithelial cells, mesenchymal cells, fibroblasts and extracellular matrix. The present study demonstrated that amnion epithelial cells and mesenchymal cells had differing responses to *P.g*-LPS and *P. gingivalis*, with only the latter cell type exhibiting up-regulation of inflammatory cytokine expression, enhanced expression of COX-2 mRNA and increased synthesis of prostaglandin E_2. These findings indicate that amnion mesenchymal cells can mediate immune responses. Immunohistochemical analyses have shown that infected placentas contain mesenchymal cells with high levels of IL-6 expression [10]. Moreover, stimuli such as IL-1β, IL-6, thrombin, fibronectin and LPS from *Escherichia coli* have been reported to activate NF-κB, enhance COX-2 mRNA expression and increase prostaglandin E_2 production in mesenchymal cells but not epithelial cells [9–15]. Therefore, the findings of the current investigation are consistent with previous research.

In this study, we performed, for the first time, co-culture of human primary amnion cells with *P. gingivalis*. Periodontitis has been reported to be associated with preterm birth and fetal growth restriction [22–25]. Three plausible mechanisms link periodontitis with adverse pregnancy outcomes: (i) the production of pro-inflammatory molecules by periodontal tissues, leading to chronic inflammation and early onset of labor; (ii) the induction of inflammation by bacteria that have migrated from periodontal lesions to the maternal/fetal unit via the blood; and (iii) the production of LPS by bacteria in intrauterine tissues. Nevertheless, the actual mechanisms remain uncharacterized. *P. gingivalis* has

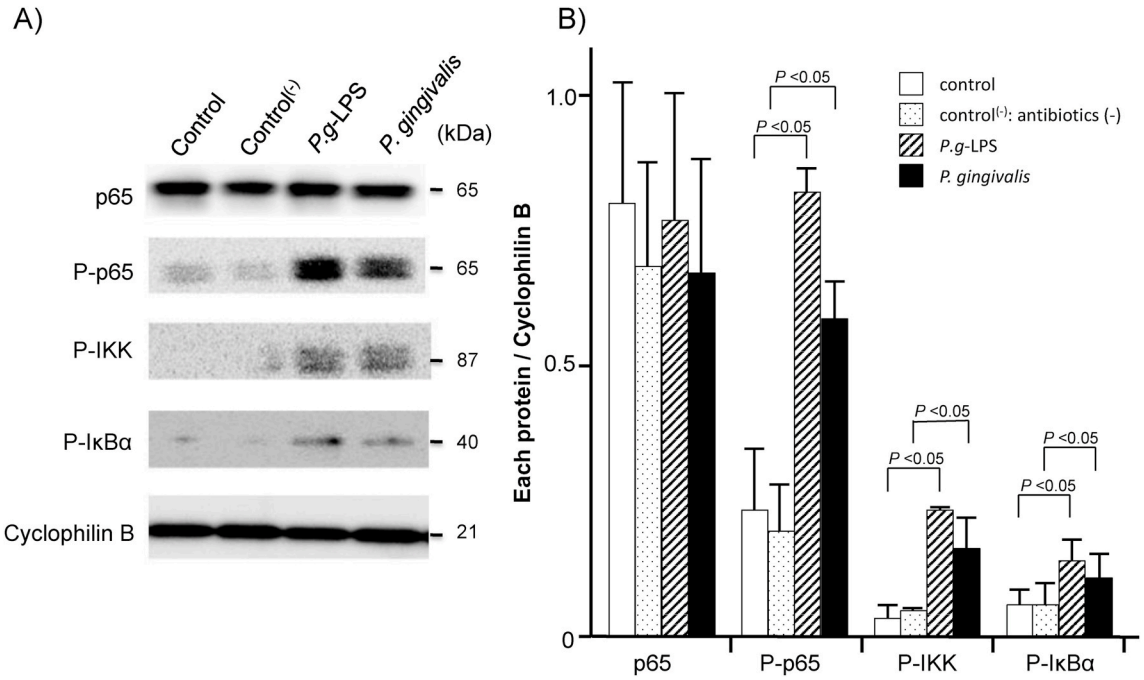

Fig. 2. Expression of components of the nuclear factor (NF)-κB pathway in human amnion mesenchymal cells. (A) Representative western blots showing the expression of p65, phosphorylated-p65 (P-p65), phosphorylated-IκB kinase (P-IKK), phosphorylated-IκBα (P-IκBα) and cyclophilin B. Cells were treated with *P. gingivalis* lipopolysaccharide (*P.g*-LPS; 1 μg/mL, 12 h) or *P. gingivalis* (multiplicity of infection of 100, 12 h), and cell lysates were subjected to western blotting. Control$^{(-)}$: medium without antibiotics. (B) Optical density values for each band normalized to that of cyclophilin B ($N = 3$ samples, each of which was obtained from a different woman who underwent elective cesarean section).

Table 2
Levels of cyclooxygenase-2 (COX-2) mRNA and prostaglandin E_2.

	Control	*P.g*-LPS	Control$^{(-)}$	*P. gingivalis*
COX-2 mRNA	0.67 ± 0.45	2.34 ± 1.34**	0.49 ± 0.36	4.61 ± 2.66**
PGE_2 (ng/mL)	6.17 ± 2.63	24.8 ± 3.61*	8.32 ± 0.89	24.6 ± 10.3*

Expression levels of COX-2 mRNA in amnion mesenchymal cells in the presence/absence of stimulation with *P. gingivalis* lipopolysaccharide (*P.g-LPS*; 1 μg/mL for 12 h) or *P. gingivalis* (MOI: 100 for 12 h) were determined using real-time reverse transcription-polymerase chain reaction ($N = 8$ for each group). *N* denotes the number of samples, each of which was obtained from a different woman who underwent elective cesarean section; each sample was run in duplicate, and the average value was used for the analysis. The expression of COX-2 was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Prostaglandin E_2 (PGE_2) concentrations in the culture medium in the presence/absence of stimulation with *P.g-LPS* (1 μg/mL for 12 h) or *P. gingivalis* (MOI: 100 for 12 h) were determined using enzyme-linked immunosorbent assay ($N = 4$ for each group). *N* denotes the number of samples, each of which was obtained from a different woman who underwent elective cesarean section. Control$^{(-)}$: medium without antibiotics. *$P < 0.05$, **$P < 0.01$.

been detected in the amniotic fluid of women with threatened premature labor, the placenta of women with preeclampsia and preterm birth [26–28], and the fetal membrane of mice with chronic gingivitis [3–5]. These findings imply that the presence of *P. gingivalis* or *P.g*-LPS in the intrauterine tissues may be harmful for pregnancy. *P.g*-LPS was reported to induce IL-6 and IL-8 production in chorion-derived cells *in vitro* [24]. Moreover, IL-1β production was increased in tissue cultures of fetal membranes treated with *P. gingivalis* [29]. Consistent with this prior research, we showed that both *P.g*-LPS and *P. gingivalis* were able to induce inflammation in primary mesenchymal cells from human amnion. Toll-like receptors (TLRs) are important for the detection of pathogens [30]. TLRs participate in the first line of defense against invading pathogens and play a notable role in the regulation of inflammation and immune cells through activation of NF-κB signaling and up-regulation of inflammatory cytokines. Fetal membrane inflammation might be induced via TLR-2 in mice with chronic gingivitis [5], and the presence of *P. gingivalis* and *P.g*-LPS promotes TLR-2 expression and the induction of inflammatory cytokines in chorion-derived cells [24].

In conclusion, primary mesenchymal cells, but not epithelial cells, from human amnion exhibited activation of NF-κB signaling, enhanced expression of IL-1β, IL-6, IL-8 and COX-2 and increased production of prostaglandin E_2 in response to stimulation with *P.g*-LPS or *P. gingivalis*. This suggests that human amnion mesenchymal cells play a key role in the immune response to *P.g*-LPS and *P. gingivalis*. However, there are many interactions between different amnion cell types and between fetal membranes *in vivo*. Further studies using this primary culture system are ongoing to characterize the mechanisms through which inflammatory pathways are regulated following exposure to *P. gingivalis*.

Author's contributions

1. Conception and design (H.K., S.U., H.M.)
2. Financial support (none).
3. Administrative support (S.U., H.M., Y.K.)
4. Provision of study materials or patients (I.K., J.S., S.S., M.M., T.T.)
5. Collection and/or assembly of data (H.K., Y.T., Y.M., S.S.)
6. Data analysis and interpretation (H.K., J.S.)
7. Manuscript writing (S.K., Y.K.)
8. Final approval of manuscript (all).
9. Other (please be specific) (none).

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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