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Porphyromonas gingivalis induces IL-8 and IFN-gamma secretion and apoptosis in human extravillous trophoblast derived HTR8/SVneo cells via activation of ERK1/2 and p38 signaling pathways

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ABSTRACT

Introduction: Preterm birth is a major cause for infant mortality and morbidity. A large number of studies have suggested a link between periodontal disease and preterm birth. The purpose of this study was to investigate the interaction between a periodontopathic bacterium *Porphyromonas gingivalis* and human extravillous trophoblast derived HTR8/SVneo cells.

Methods: Production of cytokines in HTR8 cells was measured via ELISA. Annexin V/PI flow cytometry was performed to assess apoptosis. Protein expression was measured by western blot. Specific pharmacological inhibitors were used to inactivate relevant signaling pathways (p38 MAPK, SB203580; ERK1/2, U0126; JNK, SP600125; NF-κB, JSH-23) to determine their roles in inflammation and apoptosis.

Results: HTR8 cells released significant amounts of IL-8 and IFN-γ during exposure to *P. gingivalis*. Meanwhile, the percentages of both early and late apoptotic cells increased significantly in response to *P. gingivalis*. The most significant effect on inflammation was found using SB203580 and U0126, followed by SP600125 and JSH-23. Moreover, U0126 and SB203580 both partially but significantly suppressed *P. gingivalis*-induced apoptosis, with a large effect by U0126. Additionally, both heat-killed *P. gingivalis* and *P. gingivalis* lipopolysaccharide significantly induced IL-8 production.

Conclusion: P. gingivalis induces inflammation and apoptosis in HTR8 cells, and we demonstrated for the first time that activation of ERK1/2 and p38 MAPK pathways participates in *P. gingivalis*-induced inflammation and apoptosis. The abnormal regulation of inflammation and apoptosis in human trophoblasts by *P. gingivalis* infection may give new insights into how maternal periodontal disease and periodontal pathogens might be linked to preterm birth.

1. Introduction

Preterm birth (PTB, less than 37 weeks of gestation [1]) usually leads to preterm delivery of low-birth-weight. PTB has an incidence rate of about 12% and significantly raises the death and morbidity of infants [2,3]. PTB is responsible for 75%–85% of early neonatal deaths and often is related to long-term adverse health conditions including hearing and learning difficulties and cerebral palsy [4,5]. The severe consequences make it imperative to clarify the mechanisms underlying PTB to improve prevention and treatment.

There are various risk factors for PTB, and increased systemic inflammation plays a role in many of these factors [6,7]. Some types of bacterial infection can cause PTB, either originating in the urogenital tract or occurring transplacentally following bacteremia. Though the exact mechanism underlying how an infection causes pregnancy complications is still largely unknown, the key contributors in a compromised pregnancy may be over-inflammation and/or apoptosis at the maternal-fetal interface [8–12]. The placenta plays a central role in mediating exchanges between fetus and mother and feto-maternal immunological tolerance. Trophoblasts are specialized cells of the placenta that exert a crucial role in embryo implantation and subsequently differentiate to form the placenta [13]. Placental trophoblasts include extravillous trophoblasts and syncytiotrophoblasts. The extravillous trophoblasts

Abbreviations: PTB, preterm birth; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PBS, phosphate buffer solution; MOI, multiplicity of infection; PIC, proinflammatory cytokine; CM, conditioned medium; LSM, low serum medium; LPS, lipopolysaccharide; FBS, fetal bovine serum.

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invade into the maternal uterus and establish a feto-placental vasculature system. Human papillomavirus infection of extravillous trophoblast was reported to induce cell death and disturb the placental invasion into the uterine wall, leading to PTB [14]. It is believed that the extravillous trophoblasts are essential in the success of early pregnancy as well as the continual development of a healthy placenta. Dysfunction of the extravillous trophoblasts may lead to various pregnancy complications, including PTB.

Another factor that has been linked to PTB is the presence of maternal periodontal disease [15–17]. The bacteria in periodontal lesions can migrate to the maternal-fetal interface via the blood and directly cause pregnancy complications [18]. *Porphyromonas gingivalis*, the bacterium most associated with periodontal disease, is a gram-negative anaerobic bacterium that has bioactive components on the cell surface including lipopolysaccharide (LPS), capsules and fimbriae. *P. gingivalis* antigens invade placental tissues in women with preeclampsia or chorioamnionitis before 37 weeks of gestation [19,20] and have been found in the amniotic fluid [21]. *P. gingivalis* also has been identified in both fetal and maternal tissues of rodent and rabbit animals, causing placentitis and chorioamnionitis which are leading causes of PTB [17,22–24]. Dental infection of *P. gingivalis* can induce PTB in mice [17]. However, the molecular mechanisms underlying *P. gingivalis*-induced PTB are not yet fully understood. A previous study reported that *P. gingivalis* infection leads to significant upregulation of the apoptotic regulator p53, which is involved in the subsequent apoptosis of human trophoblasts in vitro [25].

Here we investigated the interaction between *P. gingivalis* and human extravillous trophoblast derived HTR8/SVneo cells and analyzed whether mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways were involved in *P. gingivalis*-induced inflammation and apoptosis, and we demonstrated that activation of ERK1/2 and p38 MAPK pathways participated in *P. gingivalis*-induced inflammation and apoptosis in HTR8 cells.

2. Material and methods

2.1. Chemicals and reagents

Roswell Park Memorial Institute (RPMI)-1640, Fetal bovine serum (FBS), and trypsin containing ethylene diamine tetraacetic acid (EDTA; Gibco, BRL, MD, USA); Annexin V-FITC/PI Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA); ELISA kit (NeoBioscience, Shenzhen, China); *P. gingivalis* LPS (InvivoGen, San Diego, CA, USA); p38 inhibitor SB203580, ERK1/2 inhibitor U0126, JNK inhibitor SP600125 and NF-κB inhibitor JSH-23 (Selleck, Houston, Texas, US); antibodies against p38, ERK1/2, caspase-3, phospho-p38, phospho-ERK1/2, cleaved-caspase-3 and β-actin (Sigma, St. Louis, MO, USA) were used here.

2.2. Bacterial culture

P. gingivalis ATCC 33277 (American Type Culture Collection, Manassas, VA, USA) was used in this study. Bacterial cells were cultivated in a trypticase soy broth added with 1 mg/ml yeast extract, 5 μg/ml hemin and 1 μg/ml menadione under anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2) as described previously [25]. After cultivation, the bacterial cells were centrifuged at 6000 rcf for 10 min, washed with phosphate buffer solution (PBS) three times and resuspended in PBS or RPMI-1640. Heat-killed *P. gingivalis* was obtained by heat-inactivating live *P. gingivalis* at 60 °C for 1 h. To obtain conditioned medium, RPMI-1640 medium containing live *P. gingivalis* was kept in a wetted 5% CO_2 atmosphere at 37 °C for 24 h and then centrifuged to remove the bacterial cells.

2.3. Cell culture

Charles Graham (Kingston, Ontario, Canada) provided the HTR8/SVneo trophoblast cell line. HTR8 cells were cultivated in RPMI-1640 added with 10% FBS, 100 μg/ml streptomycin and 100 U/ml penicillin. Cells were kept in a wetted 5% CO_2 atmosphere at 37 °C and the medium was changed every two or three days. When confluency was 80%–90%, the cells were subcultured in 0.25% trypsin at 37 °C. *P. gingivalis* was used to infect the cells at a multiplicity of infection (MOI) of 50, 100 or 200 according to previous reports [20,25].

2.4. Cytokine assay

The expression of interferon (IFN)-γ, tumor necrosis factor (TNF)-α and interleukin (IL)-1β, -6, -8, and -10 in the supernatants were all detected in triplicate using a human ELISA kit as per the protocol. The optical absorbance at 450 nm was measured with a microplate reader (Victor, Vienna, US). The final levels were determined by comparison to a standard curve.

2.5. Western blot analysis

Both adherent and free cells were harvested. After washing with ice-cold PBS, the cells were lysed at 4 °C for 30 min. After centrifugation of the cell lysate at 12,000 rpm for 15 min, the supernatant was harvested. The protein level in the supernatant was measured by a bicinchoninic acid (BCA) assay kit. The protein lysate (20 μg/lane) was separated via 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane. The membrane was blocked in 10% nonfat dry milk in Tris-buffer for 1 h, and then treated first with the primary antibody for 1 h and then with the peroxidase-conjugated secondary antibody. After that, an enhanced chemiluminescence (ECL) kit was used to measure the immunoreactive proteins. The difference in protein loading was corrected by stripping and reprobing each membrane with anti-actin antibody. Densitometry of bands was analyzed using ImageJ.

2.6. Flow cytometric analysis of apoptosis

Apoptosis in HTR8 cells was investigated via Annexin V-FITC Apoptosis Detection Kit as required by the manufacturer. After washing with PBS, the cells were suspended in a binding buffer and stained with fluorescein isothiocyanate (FITC) and propidium iodide (PI) solutions. Samples, each containing 10,000 stained cells, were investigated using a flow cytometer (BD Bioscience).

2.7. Statistical analysis

Data were presented as mean ± standard deviation (SD) of at least 3 independent experiments. Between-group differences were investigated through one-way analysis of variance (ANOVA) or *t*-test, with a significance level at $P < 0.05$.

3. Results

3.1. P. gingivalis stimulates the release of proinflammatory cytokines (PICs) from HTR8 cells

To comprehensively assess the release of cytokines from HTR8 cells following infection with *P. gingivalis*, we cultured the cells without or with *P. gingivalis* (MOI 50:1, 100:1 or 200:1) for 6, 12, 24 or 48 h. Cytokine levels in the supernatants, including IFN-γ, IL-1β, -6, -8, -10 and TNF-α, were determined via ELISA. Results show the