Effect of *Porphyromonas gingivalis* on Human Umbilical Vein Endothelial Cells

Tomomi Hashizume, Tomoko Kurita-Ochiai, Daigo Mikuni, Ken Kawanabe, Shunichi Kanamaru, and Masafumi Yamamoto

Department of Microbiology and Immunology, Nihon University School of Dentistry at Matsudo, Matsudo, Chiba 271-8587, Japan

**Correspondence to:** Tomomi Hashizume
E-mail: hashizume.tomomi@nihon-u.ac.jp

**Abstract**

Numerous studies have suggested an association between periodontal disease and atherosclerosis. *Porphyromonas gingivalis* has been thought to be one of triggers of atherosclerosis because *P. gingivalis* DNA is detected in atheromatous plaques. In this study, we assessed the effect of *P. gingivalis* on endothelial cells to elucidate the mechanisms in *P. gingivalis*-accelerated atherosclerosis. Our results showed that *P. gingivalis* infection significantly increased the production of interleukin-8 and monocyte chemoattractant protein-1 in human umbilical vein endothelial cells (HUVEC) compared with cells without bacteria. In addition, *P. gingivalis* challenge upregulated the expression levels of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin in HUVEC at both the mRNA and protein levels. These results suggest that *P. gingivalis* stimulation induces increases in inflammatory cytokines and adhesion molecules in endothelial cells that in turn lead to the development of atheromatous plaques.

**Keywords:**
atherosclerosis, *Porphyromonas gingivalis*, human umbilical vein endothelial cells

**Introduction**

Periodontal diseases are chronic multibacterial infections and compound inflammatory diseases of the periodontium. As periodontitis progresses, the composition of subgingival bacteria is altered, allowing some species such as the major periodontopathogen *Porphyromonas gingivalis* to flourish. *P. gingivalis*, a Gram-negative anaerobe, has been considered as an important pathogen associated with human periodontal disease. In addition, *P. gingivalis* is known to promote platelet aggregation, foam cell formation, and atherogenesis in experimental models. Recently, several studies have suggested a correlation between atherogenesis and *P. gingivalis* (1). Furthermore, our previous study has shown that the intravenous injection of *P. gingivalis* accelerates atherosclerotic lesion formation in apolipoprotein E-deficient, spontaneously hyperlipidemic mice (2). Moreover, oral infection of *P. gingivalis* leads to the development of atherosclerosis in apolipoprotein E-deficient mice (3).

Several groups have investigated the effect of *P. gingivalis* infection on endothelial cells in vitro (4, 5) and have shown that this infection in endothelial cells results in increased expression of surface cell adhesion molecules and in the production of proinflammatory cytokines and chemokines. *P. gingivalis* DNA has also been detected in cardiovascular tissues and blood after oral or systemic challenge (2, 6), suggesting that the direct attachment of *P. gingivalis* to endothelial cells could be one of the reasons for accelerating atheroma in vascular disease. However, the mechanisms for linking *P. gingivalis* infection and the initial development of atherosclerosis remain to be clarified. Therefore, we assessed the effect of the *P. gingivalis* 381 strain on human umbilical vein endothelial cells (HUVEC) in vitro.

**Materials and Methods**

**Bacterial strain.**

*P. gingivalis* strain 381 was cultured on anaerobic blood agar plates (Becton Dickinson, Sunnyvale, CA)
in a model 1024 anaerobic system (Forma Scientific, Marietta, OH) with 10% H₂, 80% N₂, and 10% C₂ for 2 to 6 days. Cultures were then inoculated into brain heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 5 μg/ml of hemin and 0.4 μg/ml of menadione and grown for 2 days. The cultured bacteria were then centrifuged at 8,000 × g for 10 min at room temperature and diluted with endothelial cell basal medium-2 (EBM-2, Takara Bio, Shiga, Japan) at multiplicity of infection (MOI) levels of 100 (10⁵ cfu/ml) and 1000 (10⁶ cfu/ml).

**Cell line.**

HUVEC were purchased from Takara Bio and cultured in EBM-2 supplemented with endothelial growth medium-2 (Takara Bio) according to the manufacturer’s instructions. Cells were prepared at a concentration of 10⁶ cells/ml and cultured until confluent at 37 °C under 5% CO₂ in moist air before *P. gingivalis* treatment.

**Detection of cytokines and chemokines.**

HUVEC were incubated with *P. gingivalis* at an MOI of 100 or 1000 for 21 h. Culture supernatants were then collected and analyzed using enzyme-linked immunosorbent assay kits (Thermo Scientific, Yokohama, Japan) for interleukin (IL)-6, IL-8, and monocyte chemoattractant protein (MCP)-1 (CCL2) according to the manufacturer’s instructions.

**Flow cytometry.**

HUVEC were treated with *P. gingivalis* at an MOI of 100 or 1000 for 21 h. Cells dissociated with trypsin EDTA were stained with fluorescein isothiocyanate-conjugated anti-human CD54 [intercellular adhesion molecule (ICAM)-1] (R&D System, Tokyo, Japan), CD106 [vascular cell adhesion molecule (VCAM)-1] (R&D System), phycoerythrin-conjugated anti-human CD62E (E-selectin) (BD Biosciences, Tokyo, Japan), fluorescein isothiocyanate-conjugated anti-mouse IgG1, IgG2a, or phycoerythrin-conjugated anti-mouse IgG1 antibodies (BD Bioscience), or isotype control. 7-Amino-actinomycin D (BD Bioscience) was used for the exclusion of nonviable cells. Flow cytometric analysis was performed using a FACS Calibur (BD Bioscience).

**Quantitative reverse transcription–polymerase chain reaction.**

Total RNA was isolated from HUVEC treated with *P. gingivalis* for 16 h using an RNeasy kit (Qiagen, Germany) according to the manufacturer’s procedures. cDNA was synthesized using a PrimeScript RT reagent kit (Perfect Real Time) (Takara Bio). Quantitative reverse transcription–polymerase chain reaction (RT–PCR) was performed using the Thermal Cycler Dice Real Time System TP800 (Takara Bio) and SYBR Premix Ex Taq II (Perfect Real Time) (Takara Bio) according to the manufacturer’s instructions. The primers specific for ICAM-1 (forward primer, TCACGAGCTCCC–AGTCCTAA; reverse primer, AAAGGCAGGTTGGCAATG), VCAM-1 (forward primer, CGAAAGGCCCAGTTG-AAGGA; reverse primer, GAGCAGGAAGCTCT– CAGGAGAAA), E–selectin (forward primer, ATGC- CGTGTGCAAGCAAGATTTA; reverse primer, AGGCTAGACGAGCTTTGGCAATTA), and GAPDH (forward primer, GCACGTCAGGCTGAGA- C; reverse primer, TGGTGAAGCGACCTGGA) were designed and produced by Takara Bio.

**Statistical analysis.**

The data are presented as the mean± the standard deviation (SD) and compared using unpaired Student’s *t*-test. A *P* value of <0.05 was considered statistically significant.

**Results**

*P. gingivalis promotes the production of pro-inflammatory cytokines and chemokines in HUVEC*

Previous reports have demonstrated that IL-6, IL-8, and MCP-1 production increased in endothelial cells following infection with *P. gingivalis* (1, 4, 5). Thus, to confirm the effect of *P. gingivalis* on the production of pro-inflammatory cytokines, we analyzed the levels of IL-6, IL-8, and MCP-1 in culture supernatants of HUVEC challenged with *P. gingivalis* for 21 h. Our results showed that the culture
supernatant of HUVEC challenged with *P. gingivalis* at an MOI of 100 possessed significantly higher levels of IL-8 and MCP-1 compared with those of untreated cells, while at an MOI of 1000, the level of MCP-1, but not those of IL-6 and IL-8, was reduced compared to that of MOI of 100 (Fig. 1). On the other hand, the level of IL-6 production was slightly increased (Fig. 1). These results indicated that stimulation of endothelial cells by *P. gingivalis* promoted the upregulation of proinflammatory cytokine and chemokine synthesis.

**P. gingivalis enhances expression of adhesion molecules on HUVEC**

Previous studies have reported that the expression of cell adhesion molecules, including the ICAM-1, VCAM-1, and E-selectin, on endothelial cells was increased in human atherosclerosis (7, 8). Moreover, *P. gingivalis* infection of endothelial cells has been shown to increase the expression levels of cell adhesion molecules (4, 5). To confirm the effect of *P. gingivalis* on cell adhesion molecules on endothelial cells, monolayers of HUVEC were cultured with *P. gingivalis* 381. Flow cytometric analysis showed significantly upregulated expression of ICAM-1, VCAM-1, and E-selectin on HUVEC treated with *P. gingivalis* compared with cells without *P. gingivalis* (Fig. 2A). Quantitative RT-PCR analysis supported these results and showed that increased levels of ICAM-1, VCAM-1, and E-selectin were detected in HUVEC treated with *P. gingivalis* (Fig. 2B). These results supported previous studies and clearly indicated that the expression of cell adhesion molecules on endothelial cells was upregulated by *P. gingivalis*.

**Discussion**

Previous studies have demonstrated that periodontal pathogens such as *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* are detected in atherosclerotic plaque in cardiovascular diseases (9). *P. gingivalis* might be metabolically active within the atherosclerotic lesions (10). Thus, *P. gingivalis* in the oral cavity may gain access to the vasculature and either bacteremia or bacteremia followed by invasion of the vascular endothelium may be responsible for localizing *P. gingivalis*. We thus investigated the direct effect of *P. gingivalis* on endothelial cells, although it has been proposed that local or distant infections contribute to the inflammatory process that promotes the initiation and evolution of athero-
oma as well as decisively precipitating acute thrombotic complications of atheroma in atherosclerosis (11, 12). Several groups have reported that *P. gingivalis* challenge to endothelial cells upregulates cell adhesion molecules, both chemokines and cytokines, and contributes to the initial development of atherosclerosis (4, 5). Our results also showed that *P. gingivalis* at an MOI of 100 upregulated the production of IL-8, a key mediator of monocyte trafficking, and MCP-1, a chemotactic factor for neutrophils causing the development of early atheroma, on HUVEC (Fig. 1) (13).

MCP-1 production by HUVEC treated with *P. gingivalis* at an MOI of 1000 was decreased compared with that at an MOI of 100 (Fig. 1). In the case of IL-6, production increased slightly, but not significantly, at an MOI of 100, which is inconsistent with results of Ho et al. which found that HUVEC infected with *P. gingivalis* secrete significantly more IL-6 than the untreated control (14). The basis for the discrepancy is not known, but the outcome could be explained by the influence of proteases (e.g., gingipain) produced by *P. gingivalis*. Previous studies have indicated that the degradation of chemokines and cytokines is mediated by their own production of gingipain (1, 15). Although further investigation is needed to elucidate the mechanisms in the upregulation of cytokines and chemokines by *P. gingivalis* using our experimental model system, these findings suggest that *P. gingivalis* infection enhances pro-inflammatory cytokines and chemokines that in turn leads to the induction of inflammatory responses in arteries.

Our results showed that the expression of surface proteins such as ICAM-1, VCAM-1, and E-selectin on HUVEC challenged with *P. gingivalis* was significantly increased (Fig. 2). Previous reports have shown that *P. gingivalis* infection upregulated the expression of cell adhesion molecules at the protein levels (4, 5), which is supported by our results that show an increase in cell adhesion molecule expression by *P. gingivalis* at the mRNA level. Upregulation of ICAM-1, VCAM-1, and E-selectin leads to enhancement of the interaction between endothelial cells and monocytes that accelerates atherosclerosis (5, 16, 17). These results suggest that the upregulation of cell adhesion molecule expression on endothelial cells could lead to the development of atherosclerosis. However, the dose of *P. gingivalis* used in this study is much higher than those shown by other groups (1, 4, 5). It may be that a high dose of bacteria induces necrosis of HUVEC and as a result, dead cells may bind to fluorescence-conjugated antibodies non-specifically in flow cytometry analysis. However, because we used 7-amino-actinomycin D to remove nonviable cells, only living cells were analyzed. Further examination is necessary to clarify this difference.

In summary, the present study demonstrated that periodontal pathogens such as *Porphyromonas gingivalis* surely promote the secretion of pro-inflammatory cytokines and chemokines and also enhance the expression levels of cell adhesion molecules on endothelial cells at the mRNA level, in addition to the protein level previously reported. These inflammatory responses may lead to the development and acceleration of the early stage of atherosclerosis.

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