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Possibility of Involvement of *Porphyromonas gingivalis* in Coronary Heart Disease

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Porphyromonas gingivalis has been implicated in periodontal diseases. Accumulating evidence suggests that cardiovascular disease is the most prevalent medical problem in patients with periodontal diseases. In order to check the possibility that *P. gingivalis* is involved in coronary heart disease, the present study was performed to observe *P. gingivalis* adherence and invasion of human coronary artery endothelial cells (HCAEC) and production of cytokines and growth factors by HCAEC upon *P. gingivalis* infection. ^{3}H-labeled *P. gingivalis* 381 was incubated with HCAEC for 90 min. The radioactivity of the washed HCAEC was a measure of the absorbed (adhering and invading) *P. gingivalis*. The absorption radioactivity of the HCAEC infected by *P. gingivalis* was determined to be 59.58% of the input bacterial cells. In contrast, the absorption radioactivity of the cells infected by *S. gordonii* Challis which was employed as a control was negligible (0.59%). DPG3, a *P. gingivalis* mutant defective of fimbriae, appeared to be impaired to some extent in capability of adherence/invasion as compared to that of the parental strain 381, showing 43.04% of the absorption radioactivity. The absorption radioactivity of the HCAEC infected by *P. gingivalis* 381 in the presence of excessive fimbriae at the concentrations of 50 μg and 100 μg/ml was 57.27 and 45.44%, respectively. Invasion of HCAEC by *P. gingivalis* 381 was observed by an antibiotic (metronidazole) protection assay and transmission electron microscopy (TEM). In the antibiotic protection assay, invasion by the bacterium was measured to be 0.73, 1.09, and 1.51% of the input bacterial cells after incubation for 30, 60, and 90 min, respectively. Invasion by DPG3 was shown to be 0.16% after 90-min incubation. In comparison of invasion efficiency at 90 min of the incubation, the invasion efficiency of DPG3 was 0.37% while that of its parental strain 381 was 2.54%. The immunoblot analysis revealed fimbriae of *P. gingivalis* did not interact with the surface of HCAEC. These results suggest that fimbriae are not the major contribution to the adherence of *P. gingivalis* to HCAEC but may be important in the invasion of HCAEC by the bacterium. The presence of cytochalasin D (1 μg/ml) and staurosporine (1 μM) reduced the invasion of HCAEC by *P. gingivalis* 381 by 78.86 and 53.76%, respectively, indicating that cytoskeletal rearrangement and protein kinase of HCAEC are essential for the invasion. Infection of *P. gingivalis* induced HCAEC to increase the production of TNF-α by 60.6%. At 90 min of the incubation, the HCAEC infected with *P. gingivalis* cells was apparently atypical in the shape, showing loss of the nuclear membrane and subcellular organelles. The overall results

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suggest that *P. gingivalis* may cause coronary heart disease by adhering to and invading endothelial cells, and subsequently damaging the cells.

Key Words: *P. gingivalis*, Coronary heart disease, Adherence, Invasion, Endothelial cells

INTRODUCTION

Coronary heart disease (CHD) is the leading cause of death in most developed countries. Sclerotic and thromboembolic events of coronary artery lead to myocardial infarction. The lesions result from an excessive, inflammatory fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the artery wall. A large number of cytokines, growth factors, and vasoregulatory molecules participate in this process (31). Although smoking, hypertension, and high cholesterol, especially low density lipoprotein oxidized on endothelial cells are the major risk factors for CHD (4), some infectious agents have been shown to be implicated in CHD, including *Chlamydia pneumoniae* (40), viruses (42), and *Helicobacter pylori* (27).

In recent years, several studies have found evidence linking periodontal disease with an increased risk for atherosclerosis and thromboembolisms which may result in CHD (4,12). It has been reported that men with periodontitis are 25% more likely to develop CHD and CHD is, therefore, found to be most common condition shared by periodontitis patients (7,12,41). A study of carotid atheromas by polymerase chain reaction provided evidence for periodontopathic bacteria like *P. gingivalis* were found (14).

Adherence of bacteria to cell/tissue surface is the crucial step in infection process (13). Subsequent invasion of cells by bacteria is an important strategy for the sequestration of bacteria from the immune system (10). Therefore, invasion is an important feature of pathogenicity of bacteria (36). *Porphyromonas gingivalis* is one of the important bacteria involved in periodontal diseases. *P. gingivalis* has been observed within gingival tissues *in vivo*, suggesting that the bacterium may pass through the epithelial barrier (32). Recent *in vitro* studies have shown that *P. gingivalis* can adhere to and subsequently invade various cell types including gingival epithelial cells (1,21,44), KB oral epithelial cell (9,28,33,34), and junctional (30) and pocket epithelial cells (35). *P. gingivalis* is able to multiply and persist within human oral epithelial (25) and gingival epithelial cells (20).

The ability of *P. gingivalis* to advance into deeper epithelial layer may play an important role in the systemic spread of the bacterium (30, 32). Since periodontal tissue is extremely well vascularized, trauma to gingiva, even associated with toothbrushing, frequently causes bacteremia with plaque bacteria. *P. gingivalis* is found in very high proportion of patients with periodontitis, and in very high number in their plaque (45), therefore, there are more chances for *P. gingivalis* to enter the blood stream.

Lipopolysaccharide of *P. gingivalis* interacts with endothelial cells inducing an inflammatory response (5). *P. gingivalis* is known to have platelet-aggregation-associated protein (PAAP) on its surface which triggers platelet aggregation (17). It is well known that platelet aggregation by bacteria influence experimental endocarditis (15,16). Therefore, it is conceivable that if *P. gingivalis* can adhere to and invade coronary artery endothelial cells, subsequently it may result in atherosclerotic and thromboembolic events which lead to CHD.

The present study was performed to ascertain the involvement of *P. gingivalis* in CHD by observing *P. gingivalis* adherence to and invasion of human coronary artery endothelial cells, and changes in morphology of the endothelial cells and production of cytokines and growth factors

by the cells upon adherence and invasion of *P. gingivalis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. gingivalis* 381 and DPG3, afimbrial *P. gingivalis* mutant were used. *Streptococcus gordonii* Challis was also selected as a control. *P. gingivalis* 381 and DPG3 were grown in half-strength (18.5 mg/ml) brain heart infusion broth (BHI; Difco) supplemented with 5 mg of yeast extract per ml, 5 μg of hemin per ml, and 0.2 μg of vitamin K1 per ml and buffered at pH 7.4. The cells were grown with or without ^{3}H-thymidine (5 μCi/ml) at 37℃ for 2 days in an anaerobic chamber (80% N_2, 10% H_2, 10% CO_2) as described previously (24). *S. gordonii* was grown in a normal BHI anaerobically for 2 days.

Endothelial cells. Primary cultured normal human coronary artery endothelial cells (HCAEC) were purchased from Clonetics (San Diego, CA, U. S. A.; CC-2685). HCAEC were cultured in EGM®-MV BulletKit® (Clonetics) according to the manufacturer's instruction and used for the experiment before 10 population doublings. For the experiment, HCAEC were plated and incubated in a 24-well plate.

Purification of fimbriae. Fimbriae used for this study were purifed from *P. gingivalis* 381 by the method using guanidine HCl described in the previous study (23). Briefly, *P. gingivalis* cells were grown and harvested by centrifugation to collect the cell pellet. The cell pellet was subjected to mild ultrasonication for 10 min. The crude fimbriae of the sonic extract were collected and brought to 40% saturation by the stepwise addition of solid ammonium sulfate. The precipitated proteins were dialyzed against 10 mM Tris-HCl (pH 8.0) for 3 days.

The crude fimbriae were clarified by centrifugation and then mixed with solid guanidine HCl (UltraPURE, enzyme grade; Gibco BRL) in a total volume of 2~3 ml, adjusting the final molarity of guanidine HCl to 8 M. After stirring for 1 h at room temperature, the mixture was loaded onto a Sepharose CL-6B (Pharmacia LKB) column (1.5x110 cm) equilibrated with 6 M guanidine HCl (practical grade, Sigma) which was dissolved in deionized water and filtered. The column was eluted with 6 M guanidine HCl at flow rate of 10 ml/h and 2.2 ml fractions were collected. The fractions were monitored at 280 nm for the presence of proteins and dialyzed against stepwise decreasing concentrations of guanidine HCl and finally against 5 mM Tris-HCl, pH 8.0. The dialyzed fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis using anti-fimbriae (22). The fimbrial protein-rich fractions were pooled and concentrated by ammonium sulfate precipitation. Gel filtration of the fimbrial fractions using guanidine HCl was repeated on the same column to obtain pure fimbriae.

Radioactivity absorption assay. For the assay, HCAEC were plated (2,500 cells/cm^2) in a 24-well plate and incubated at 37℃ under the conditions of 5% CO_2. Confluent monolayers of HCAEC were washed with EBM® culture medium (Clonetics) without fetal calf serum and then infected with ^{3}H-labeled bacteria (5x10^7 cells) in a total of 1 ml EBM® in each well of the plate for 90 min with or without the purified fimbriae from *P. gingivalis* (50 and 100 μg/ml). After incubation, the HCAEC were washed with EBM®, treated with 0.5 N NaOH, and then measured in a scintillation counter the absorption radioactivity which was a measure of the adherence and invasion efficiencies of the bacteria. All assays in the study were performed in triplicate.

Immunoblot analysis. To identify the protein (s) that binds to HCAEC, first, HCAEC grown in the 24-well plate were lysed in distilled water, washed with PBS, pH 7.2, and incubated with the sonic extract of *P. gingivalis* 381 for 1 h.

After washing 3 times, the mixture was collected by centrifugation and treated with 1 X sample buffer and subjected to SDS-PAGE. The separated proteins on the gel were transferred to a nitrocellulose membrane by using a Semi-Dry Blotting Unit (FB-SDB-2020; Fischer Scientific) for 50 min at 2.5 mA/cm^2 of gel. The membrane was blocked with 1% BSA in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl and incubated with rabbit anti-*P. gingivalis* whole cell for 3 h at room temperature. After washing, the membrane was incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad) for 1 h. The membrane was then extensivley washed and the bound antibodies were visualized by the addition of 4-chloro-1-naphthol (22).

Invasion assay. To measure the invasion efficiency of HCAEC by *P. gingivalis*, an antibiotic protection assay was carried out (37). HCAEC were incubated in 1 ml of EBM® in each well of the 24-well plate with or without cytochalasin D (1 μg/ml) and staurosporine (1 μM) for 30 min. After washing with EBM®, the HCAEC were infected with 5x10^7 cells of *P. gingivalis* 381 and DPG3 for 30, 60, and 90 min. The HCAEC were washed with EBM® and further incubated with 100 μg of metronidazole for 60 min to kill external adherent cells. After washing with PBS (pH 7.2), the HCAEC were lysed in 1 ml of sterile distilled water per well for 30 min and vigorously agitated. The lysates were serially diluted, plated on sheep blood agar plates, and incubated anaerobically for 3～7 days. Colony forming units of the invading bacteria were enumerated.

Enzyme-linked immunoabsorbent assay (ELISA). To measure the production of interleukin-1β (IL-1β), IL-6, IL-8, tumor necrosis factor-α (TNF-α), transforming growth factor (TGF-β1), and platelet-derived growth factor-BB (PDGF-BB) by HCAEC infected with *P. gingivalis* 381, an ELISA was performed as described elsewhere. Briefly, HCAEC grown in a 24-well plate were washed EBM® and infected with *P. gingivalis* suspended in a 1 ml of EBM® (5x10^7 cells) for 90 min. The culture medium was collected from each well and centrifuged to obtain the supernatant. The supernatant was transferred to a 96-well plate (flat bottom) and stored at 4℃ overnight. After washing, the proteins bound to the bottom were incubated with antibodies to IL-1β (goat anti-human; Santa Cruz Biotechnology, CA, U. S. A.), IL-6 (rabbit anti-human; HyCult Biotechnology, Uden, The Netherlands), IL-8 (rabbit anti-human; HyCult Biotechnology), TNF-α (mouse anit-human; HyCult Biotechnology), TGF-β1 (rabbit anti-human; Santa Cruz Biotechnology), and PDGF-BB (rabbit anti-human; Santa Cruz Biotechnology). Antibodies other than the ones of rabbit origin were treated with rabbit IgG antibody to the respective species used for raising the antibodies. Unbound antibodies were removed by washing and the bound ones were detected by biotinylated goat anti-rabbit IgG conjugated with avidin peroxidase (Vectastain; Vector Laboratories, Burlingame, CA, U. S. A.) and visualized by using 3,3'-diaminobenzidine tetrahydrochloride substrate kit (Zymed Laboratories, San Francisco, CA, U. S. A.). The extent of color was determined at 450 nm as based on the reference at 655 nm.

Transmission electron microscopy (TEM). HCAEC incubated in a culture dish (35x10 mm) were washed with EBM® and then incubated with 5x10^7 cells of *P. gingivalis* 381 for 90 min. After washing with PBS, the HCAEC were fixed with 2.5% glutaraldehyde in PBS, washed with PBS, and postfixed with 1% osmium tetroxide. The HCAEC were dehydrated in a series of increasing concentrations of ethanol. The monolayer of the HCAEC was directly embedded in Epon 812 by pouring the resin onto the monolayer. The monolayer was ultrathin sectioned parallel to the horizontal axis of the cells, contrasted with lead citrate and uranyl acetate, and then examined by TEM with a JEM-1010

Table 1. Absorption radioactivity of HACEC infected by radiolabeled bacteria[a]

Bacteria and additive		Absorption (%)
P. gingivalis 381	No	59.58±1.01[b]
	fimbriae (50 μg/ml)	57.27±4.10
	fimbriae (100 μg/ml)	45.44±1.72
DPG3		43.04±2.12
S. gordonii Challis		0.59±0.10

[a]; $5x10^7$ cells were added in a total of 1 ml of EBM®
[b]; The aborption values are the means of triplicate independent determinations from a typical experiment± standard deviation

(JEOL, Tokyo, Japan).

RESULTS

Absorption of bacteria to HCAEC. Bacterial cells adhering to and invading HCAEC were quantitated by measuring the absorption radioactivity remaining in the HCAEC after washing. While 59.58% of the input radioactivity of *P. gingivalis* 381 cells was found to adhere to and invade the HCAEC (Table 1), an apparent absorption radioactivity was not detected when the HCAEC was infected with *S. gordonii* Challis (0.59%). The absorption radioactivity of the HCAEC infected by afimbriated DPG3 was reduced to some extent (43.04%) as compared to the parental strain, *P. gingivalis* 381. The addition of 50 μg of *P. gingivalis* fimbriae did not change the absorption radioactivity of the HCAEC infected by *P. gingivalis* 381, but excessive amount of fimbriae (100 μg/ml) decreased the radioactivity down to 45.44%.

HCAEC-binding proteins of *P. gingivalis*. To identify the proteins that can interact with the surface of HCAEC, the sonic extract of *P. gingivalis* 381 incubated with HCAEC was subjected to SDS-PAGE, followed by immunoblot using rabbit antiserum to *P. gingivalis* whole cell. The antiserum detected 13 faint but distinct bands of proteins with apparent molecular masses ranging from 14 to 50 kDa (Fig. 1). No distinct band of the 43-kDa fimbrial protein

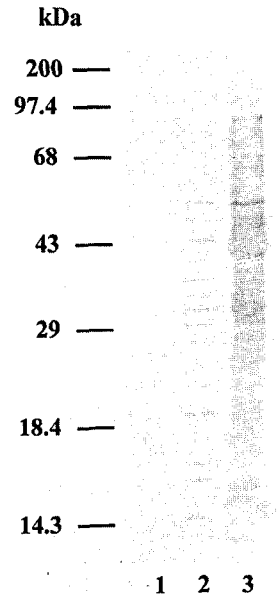


Figure 1. Immunoblot analysis of sonic extract of *P. gingivalis* 381 incubated with HCAEC. The sonic extract of *P. gingivalis* 381 was mixed with HCAEC for 1 h. After washing 3 times, the mixture was treated with 1 X sample buffer and subjected to SDS-PAGE. The separated proteins on the gel were transferred to a nitrocellulose membrane, blocked and then incubated with rabbit anti-*P. gingivalis* whole cell for 3 h at room temperature. After washing, the membrane was incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate (Bio-Rad) for 1 h. The membrane was then extensively washed and the bound antibodies were visualized by the addition of 4-chloro-1-naphthol. Lanes: 1, HCAEC only; 2, HCAEC and sonic extract of *P. gingivalis*; 3. sonic extract of *P. gingivalis* only.

Table 2. Invasion of HCAEC by *P. gingivalis*

Bacteria	Invasion (%)	Invasion efficiency (%)
P. gingivalis 381		
30-min incubation	0.73±0.05	
60-min incubation	1.09±0.04	
90-min incubation	1.51±0.07	2.54±0.11[a]
DPG3		
90-min incubation	0.16±0.01	0.37±0.02

[a]; Percentage of the inoculum of *P. gingivalis* ($5x10^7$ cells/ml) protected from metronidazole killing after the infection period relative to the number of bacteria absorbed to HCAEC. Values are the means of triplicate independent determinations from a typical experiment±standard deviation

Table 3. Invasion of HCAEC by *P. gingivalis* in the presence of metabolic inhibitors[a]

Inhibitors	Invasion inhibition (%)
No	0
Cytochalasin D (1 μg/ml)	78.86±4.70
Staurosporine (1 μM)	53.76±4.32

[a]; Inhibition of *P. gingivalis* 381 invasion of HCAEC by metabolic inhibitors was measured under the condition as described in Table 2

Table 4. Production of cytokines and growth factors by HCAEC infected with *P. gingivalis*

Cytokines and growth factor	Relative production (%)
IL-1β	98.1[a]
IL-6	105.2
IL-8	87.0
TNF-α	160.6
TGF-β1	109.9
PDGF-BB	111.4

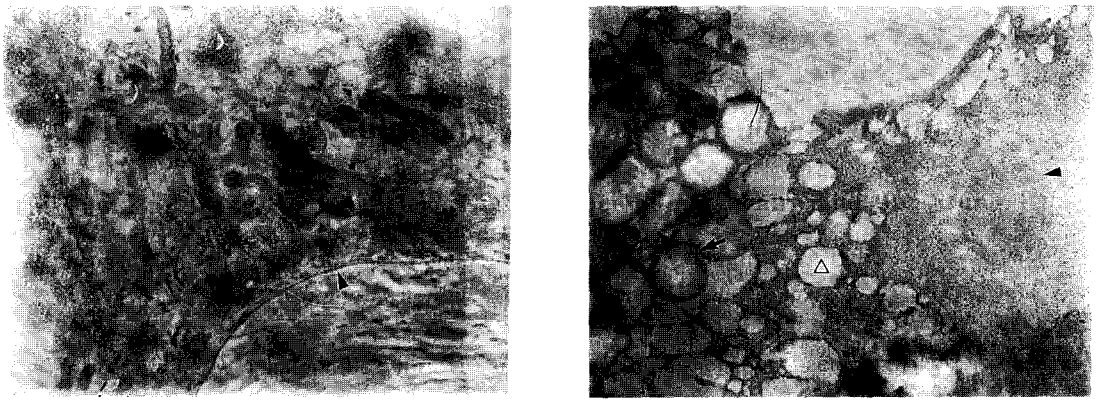
[a]; Percentage of the production by *P. gingivalis* 381-infected HCAEC relative to the production by non-infected HCAEC

was detected, if any very weak.

Invasion of HCAEC by *P. gingivalis*. In the invasion assay, it was observed that 0.73, 1.09, and 1.51% of the absorbed *P. gingivalis* 381 to HCAEC actually invaded the cells (Table 2). The mutant DPG3 showed the reduced capability of invasion (0.16%). In contrast to the parental strain 381 whose invasion efficiency was 2.54% (percentage of the input *P. gingivalis* protected from metronidazole killing after the incubation period relative to the number of bacteria adhering/invading), DPG3 revealed a much lower invasion efficiency of 0.37%.

Metabolic requirement for *P. gingivalis* invasion. Inhibition of invasion of HCAEC by *P. gingivalis* with compounds that impede various metabolic functions of eucaryotic cells was examined as shown in Table 3. HCAEC pretreated with cytochalasin D, an inhibitor of actin polymerization drastically reduced the *P. gingivalis* invasion by 78.86%. Likewise, staurosporine, a broad-spectrum inhibitor of protein kinase inhibited the *P. gingivalis* invasion of HCAEC by 53.76%.

Changes in production of cytokines and growth factors by *P. gingivalis*-infected HCAEC. In order to examine the possible implication of *P. gingivalis* in the pathogenesis of CHD, changes in the production of proinflammatory cytokines and growth factors by HCAEC infected with *P. gingivalis* 381 were determined by ELISA. As compared to non-infected HCAEC, the production of IL-1β, IL-6, IL-8, TGF-β1, and PDGF-BB by the infected HCAEC was not much affected, showing that percentages of the



A B

Figure 2. Electron microscopic finding of HCAEC without (**A**) and with invading *P. gingivalis* 381 (**B**). Note that the nuclear membrane (arrow head) and subcellular organelles are visible in the case of HCAEC which is not invaded by *P. gingivalis* after 60-min challenge of the bacterium (**A**). In contrast, HCAEC with invading *P. gingivalis* cells at 90-min of challenge apparently does not have the nuclear membrane and subcellular organelles although the nucleolus (arrow head) is clearly discernible. Formation of vaculoles (open triangle) is prominent in *P. gingivalis*-invading HCAEC but *P. gingivalis* (arrow) is not enclosed within the vacuole (**B**). 25,000 X magnification.

relative production ranged from 87.0 to 111.4%. However, the relative production of TNF-α by the infected HCAEC greatly increased as much as 160.6% (Table 4).

Changes in morphology of HCAEC upon *P. gingivalis* invasion. TEM observation confirmed the invasion of HCAEC by *P. gingivalis*. After 60-min infection with *P. gingivalis* 381, most HCAEC were invaded by the bacterial cells (results were not shown). HCAEC without invading *P. gingivalis* appeared to be normal in the shape, showing distinct nuclear membrane and subcellular organelles (Fig. 2A).

After 90-min incubation, most HCAEC had *P. gingivalis* in a higher number and appeared atypical in their shape. Although the nucleolus was apparently normal, the boundary of the nucleus was not distinct, which resulted from loss of the nuclear membrane. The presence of the subcellular organelles was not evident but the appearance of the vacuoles was prominent in the HCAEC with invading *P. gingivalis*. A number of *P. gingivalis* cells were visible in the cytoplasm but no *P. gingivalis* cell appeared to be enclosed within the vacuoles (Fig. 2B).

DISCUSSION

Adult periodontitis is a bacterially induced chronic inflammatory disease that is the major cause of tooth loss in the adult population (37). Increasing evidence indicates that periodontal disease like adult periodontitis may itself be a risk factor for other infections and systemic diseases and abnormalities in the body such as CHD (4,12) and preterm delivery with low-birth-weight infants (29). A periodontopathic bacterium *P. gingivalis* has been reported to be involved in CHD and pregnancy-related problems (12,29).

The possibility of *P. gingivalis* involvement in CHD was further supported by the study of Despane and co-workers (16) who found that *P. gingvialis* can adhere to and actively invade transformed endothelial cells of fetal bovine heart, bovine aorta, and human umblical vein. The absorption efficiency of the bacterium was 0.1～0.5% as determined by counting colony forming units of *P. gingvialis* on blood agar plates. Of the absorbed *P. gingivalis* to the en-

dothelial cells, only 0.1~0.3% actually invaded the cells. Since the surface molecules of transformed cells may vary from those of normal cells, in the present study, we used normal endothelial cells from human coronary artery that would be more relevant and might provide a more clear picture of bacteria-endothelial cell interaction and direct evidence of *P. gingivalis* involvement in CHD. We observed that 60% of the added ^{3}H-*P. gingivalis* 381 absorbed to (adhered to and invaded) HCAEC and 2.5% of the absorbed *P. gingivalis* cells actually invaded the HCAEC (Table 1, 2). The absorption ability of *P. gingivalis* 381 to the HCAEC in the present study was much greater than that reported in the study by Despane and co-workers (16). It is unlikely that non-specific binding contributed to the higher absorption ability of the strain, since *S. godonii* Challis used as a negative control revealed a relatively much lower absorption ability (0.59%). At present, such a substantial discrepancy in absorption and invasion efficiencies between our group and Despane's group cannot be explained. Difference in types of endothelial cells and bacterial cells may be in part responsible for this discrepancy although contribution of differences in number of endothelial and bacterial cells added and assay system cannot be ruled out. It was reported that the invasion efficiency in transformed KB oral epithelial cells is lower than in primary cultured gingival epithelial cells (28). Lower invasion efficiency of *P. gingivalis* in KB oral epithelial cells may be related to alterations in surface receptors and signal transduction pathways in transformed cells (2,19). Indeed, KB oral epithelial cells present fewer receptors for *P. gingivalis* than primary gingival epithelial cells (18).

It has been shown that fimbriae have an important role in adherence and invasion of various cell types by *P. gingivalis* (6,9,38,43,44). Sparsely fimbriated strain W50 and afimbriated mutant DPG3 demonstrated to have 10 to thousands times lower invasion capability than highly fimbriated and the parent strains. The present study revealed that ability of DPG3 to adhere to HCAEC was relatively comparable to that of the parent strain 381. Besides, absorption radioactivity of the mutant DPG3 was reduced only by 27% in the presence of fimbriae. However, invasion capability of the mutant was impaired. The invasion efficiency of DPG3 was 7 times lower than that of the parent strain 381, suggesting that fimbriae may not play a major role in the adherence of *P. gingivalis* to HCAEC but may be important in the invasion. The immunoblot analysis (Fig. 1) also suggests that fimbriae may not be involved in the adherence of *P. gingivalis* to HCAEC unlike transformed endothelial and epithelial cells (6,9,38,43,44). Duncan et al. (8) suggested that *P. gingivalis* does not depend solely on fimbria-mediated binding to epithelial cells and other surface molecules may participate in this function. It may be noteworthy that as will be discussed later, arrays of surface molecules and invasion pathway of normal HCAEC may different from those of transformed endothelial cells and other cell types. *P. gingivalis* cells invading the transformed cells have been shown to be enclosed within vacuoles (6,28). *P. gingivalis* in normal HCAEC did not appear to reside within vacuoles in the present study (Fig. 2B). It is likely that characteristics of HCAEC might have influenced the invasion of HCAEC by *P. gingivalis*.

Invasion of mammalian cells by *P. gingivalis* is a rapid and efficient process. Belton and colleagues (1) showed that *P. gingivalis* invasion of gingival epithelial cellls reached completion after 12 min of challenge. In the present study using a higher number of endothelial cells, we observed that the number of invading *P. gingivalis* cells was still increasing even after 60 min of incubation. They also found that only vital bacteria were capable of invasion and the invasion induced rearrangement of the actin cy-

toskeleton in the epithelial cells. Our study revealed that cytochalasin D, an inhibitor of actin polymerization reduced *P. gingivalis* invasion of HCAEC by 79% and staurosporine, a broad-spectrum inhibitor of protein kinase, by 54% (Table 3). It appears that endocytic pathway of engulfment is commonly involved in *P. gingivalis* invasion of various cells types such as endothelial cells (6) and KB oral epithelial cells (33). The pretreatment of the cells with cytochalasin D, staurosporine, and other inhibitors of eucaryotic metabolism resulted in reduction in number of invading *P. gingivalis*.

Using fluorescence imaging technique, Belton and colleagues (1) observed that internalized bacteria congregated in the perinuclear region of human gingival epithelial cells. In the present study, perinuclear localization of the invading *P. gingivalis* was not evidenced, but the bacterial cells scattered within the cytoplasm (Fig. 2B). It should be noted that the nuclear membrane was not longer present in the HCAEC invaded by *P. gingivalis* at 90 min of challenge. In contrast, HCAEC without invading *P. gingivalis* showed a distinct nuclear membrane and subcellular organelles (Fig. 2A).

A variety of cytokines and growth factors produced by endothelial cells and monocytes attracted to the endothelial cells have been shown to participate in the process of atherosclerosis (31) leading to CHD. Interestingly, the *P. gingivalis*-infected HCAEC produced 61% more TNF-α than non-infected HCAEC (Table 4). Increase in TNF-α production by endothelial cells is often observed when the cells are infected with some types of bacteria. Cell walls of *Streptococcus pneumoniae* stimulate cerebral endothelial cells to release TNF-α resulting in autocrinically induction of nitric oxide synthase and ICAM-1 expression, resulting in inflammatory blood-brain-barrier disruption (11). Adhesion of *Neisseria meningitidis* directly causes endothelial cells to produce TNF-α which is involved in many aspects of meningococcal pathogenesis such as coagulopathy (39). TNF-α induced by *P. gingivalis* infection seems to have suppressive effect on fetal weight (29). It is also noteworthy that TNF-α promotes adherence of *Staphylococcus aureus* to endothelial cells (3).

Overall, it is likely that *P. gingivalis* may cause CHD by adherence to and invasion of coronary artery endothelial cells, inducing or forming sclerotic and thromboembolic plaque on the cells, producing proinflammaory cytokines which may eventually cause damage the endothelial cells. *P. gingivalis* can invade HCAEC possibly through a highly efficient uptake by the endothelial cells and fimbriae seem to be responsible to a large extent for the invasion. The mechanisms by which fimbriae are involved in invasion of HCAEC by *P. gingivalis* and relationship between invasion, cytokine production, and damage of HCAEC have yet to be elucidated.

Cardiovascular disease is the leading cause of death in most developed countries, and periodontal disease is one of the most common infections in humans: at age of 60, approximately 90% of the individuals experienced or are experiencing periodontal disease. Even if periodontal disease has only a modest effect on increasing the risk of heart attack, its prevalence may make it a significant contributor to the risk for heart disease in the population as whole. Preventing periodontal disease from occurring, or treating it early if it has occurred may be very important in decrease of the risk for heart disease.

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