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Porphyromonas gingivalis infection and prothrombotic effects in human aortic smooth muscle cells

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ABSTRACT

Introduction: Accumulating evidence has demonstrated an association between periodontal infectious agents, such as *Porphyromonas gingivalis*, and vascular disease. Tissue factor (TF) and its specific tissue factor pathway inhibitor (TFPI) are produced by vascular cells and are important regulators of the coagulation cascade.
Materials and Methods: To assess the role of *P. gingivalis* in atherothrombosis, we infected primary human aortic smooth muscle cells (HASMC) with either *P. gingivalis 381,* its non-invasive mutant DPG3, or heat-killed *P. gingivalis 381.* Levels and activity of TF and TFPI were measured 8 and 24 hours after infection in cell extracts and cell culture supernatants.
Results: P. gingivalis 381 did not affect total TF antigen or TF activity in HASMC, but it significantly suppressed TFPI levels and activity compared to uninfected control cells, and those infected with the non-invasive mutant strain or the heat-killed bacteria. Further, *P. gingivalis*' LPS (up to a concentration of 5 μg/ml) failed to induce prothrombotic effects in HASMC, suggesting a significant role for the ability of whole viable bacteria to invade this cell type.
Conclusion: These data demonstrate for the first time that infection with a periodontal pathogen induces a prothrombotic response in HASMC.

Vascular smooth muscle cells (VSMC) are the major cellular component of the vessel wall and play a pivotal role in vascular function. They contribute to the short-term regulation of the blood pressure by altering the luminal vessel diameter, but also contribute to long-term adaptation by structural remodeling. They also constitute a significant portion of the atherosclerotic lesion and proliferation, migration and apoptosis of VSMC are essential to atherogenesis, plaque progression and rupture [1].

Accumulating evidence suggests that inflammation and coagulation are essential in the pathogenesis of vascular disease: inflammation leads to activation of coagulation, and coagulation considerably affects inflammatory activity [2]. In addition, multiple studies have shown that certain infections are implicated in atherogenesis and may serve as inflammatory stimuli that also contribute to acute events via plaque destabilization [3]. Periodontal diseases, chronic oral infections leading to destruction of tooth supporting structures and eventually tooth loss, are very common. Mild to moderate forms affect the majority of adults and severe forms have a prevalence of 10% to 15% [4]. Several studies have demonstrated a link between periodontal infections and atherosclerosis in human subjects [5–8]. It has also been postulated that since several infectious agents are likely involved in atherogenesis, the risk relates to the aggregate pathogen load [9]. As periodontal diseases are largely preventable and treatable, periodontal therapy with resultant reduction of the oral infection burden may contribute to the efforts to reduce the risk for vascular disease [10].

Porphyromonas gingivalis, a principal etiologic agent of chronic periodontitis in humans, can gain access into the bloodstream and has been identified in human atherosclerotic plaques [11,12]. Frequent low-level bacteremias, such as those resulting from chewing, brushing and flossing in periodontitis patients [13], may provide a chronic insult to the vasculature that could initiate and/or exacerbate atherogenesis. *P. gingivalis* has been shown to enhance foam cell formation in human macrophages [14] and invade vascular cells, including endothelial and smooth muscle cells [15,16]. Further, *P. gingivalis* has been shown to accelerate atherosclerosis in animal model studies [17–19]. We recently demonstrated that infection with *P. gingivalis* induces increased leukocyte adhesion to human aortic endothelial

Abbreviations: VSMC, vascular smooth muscle cells; HAEC, human aortic endothelial cells; HASMC, human aortic smooth muscle cells; TF, tissue factor; TFPI, tissue factor pathway inhibitor; LPS, lipopolysaccharide; LDH, lactate dehydrogenase.

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cells (HAEC), enhances procoagulant responses in HAEC and promotes up-regulation of adhesion molecule expression and pro-inflammatory cytokine production by this cell type [20,21].

Although *P. gingivalis* has been shown to invade VSMC, its effects on VSMC phenotype and function have not been previously explored. Therefore, we sought to investigate *P. gingivalis*' ability to modulate human aortic smooth muscle cell (HASMC) expression of tissue factor (TF), a principal initiator of the coagulation cascade. In addition, we aimed to assess *P. gingivalis*' modulation of tissue factor pathway inhibitor (TFPI), the primary physiological inhibitor that regulates TF-dependent blood coagulation.

Materials and methods

Cells, bacterial strains, and culture conditions

Clonetics® HASMC were purchased and maintained in smooth muscle cell medium (SmGM-2, Lonza Walkersville Inc, Walkersville, MD) with supplements provided by the supplier. Confluent 4th-6th passage cells were used in all experiments. *P. gingivalis* FDC381 was grown on blood agar plates (Anaerobe Systems, Morgan Hill, CA) in anaerobic chambers at 37 °C. DPG3, its fimbriae-deficient mutant constructed by insertional inactivation of the fimA gene [22], was grown on blood agar plates supplemented with erythromycin (Anaerobe Systems) in anaerobic chambers at 37 °C. Bacterial suspensions were prepared in phosphate buffered saline without Mg^{2+}/Ca^{2+} (PBS) using established growth curves and spectrophotometric analysis. In some experiments, heat-killed *P. gingivalis* 381 and ultrapure *P. gingivalis* lipopolysacharide (LPS) (Invivogen, San Diego, CA) were used as controls. The endotoxin activity of the *P. gingivalis* LPS preparation was 17.3±3.96 EU/ng, as determined by the *Limulus Amebocyte* lysate method (Lonza Walkersville Inc, Walkersville, MD).

Co-incubation of HASMC with P. gingivalis and its LPS

HASMC were seeded (10^5/well) in 24-well plates (Corning, Acton, MA) for 24 hours and were then infected with viable, or heat-killed, *P. gingivalis* 381 or DPG3 at a multiplicity of infection (MOI) of 1:100 (i.e. 100 bacteria per cell) for 90 minutes in a 37 °C, 5% CO_2 environment. In order to approximate *in vivo* conditions, the bacteria were not centrifuged onto the HASMC to promote intimate contact. For all experiments MOI was calculated based on the number of cells per well when seeded. After the incubation period, cells were washed with PBS and maintained in SmGM-2 medium without antibiotics for 8 or 24 hours. The cell culture supernatant was collected and stored at -70 °C until analyzed. In additional experiments, HASMC were co-incubated with various concentrations of *P. gingivalis* LPS for either 8 or 24 hours. After the incubation period, cell culture supernatants were harvested and kept frozen until further analyses.

Antibiotic protection assay and assessment of HASMC necrosis

In order to confirm the potential of *P. gingivalis* 381 to invade HASMC in our experiments, recovery of viable microorganisms from antibiotic-treated cells was assessed. Confluent HASMC on a 96-well plate (Corning) were infected with *P. gingivalis* 381 or DPG3 (MOI 1:100) for 90 min at 37 °C, 5% CO_2. After washing, metronidazole (200 mg/ml, Sigma) was added to each well for 1 hour. Control experiments confirmed that extracellular bacteria were killed at this antibiotic concentration. Following metronidazole treatment, cells were washed, lysed with sterile deionized water, and lysates were plated on blood agar plates. Colonies were counted after a three-day incubation under standard anaerobic conditions. To assess non-specific necrosis, smooth muscle cell membrane integrity was measured using a lactate dehydrogenase (LDH) assay (Roche, Mannheim, Germany). Data are given as percent specific LDH release.

Determination of TF levels, TF activity, TFPI levels, and TFPI activity

After the cell culture supernatants were removed, cells were lysed by 3 freeze-thaw cycles. TF was extracted for 30 minutes in TRIS buffered saline containing 0.1% Triton X-100 at 37 °C, samples were collected and stored at -70 °C until analyzed. Expression of total TF in cell extracts was assessed by ELISA (American Diagnostica, Stamford, CT). TF procoagulant activity in cell extracts was determined using a two-stage chromogenic activity assay (American Diagnostica). In the first stage of this assay, the TF in the sample is allowed to complex with factor (F) VIIa to generate TF/FVIIa complexes and convert FX into FXa. In the second stage, FXa cleaves Spectrozyme® FXa, a highly specific FXa chromogenic substrate and a chromophore is released. Absorbance is read and compared to values from a standard curve generated using known amounts of active TF. Levels of total TFPI and TFPI activity were quantified in culture supernatants, using a commercially available ELISA kit and a three-stage chromogenic activity assay, respectively (American Diagnostica). The latter measures the ability of TFPI to inhibit the catalytic activity of the TF/FVIIa complex to activate Fx to FXa. After incubation of samples with TF/FVIIa and FX, the residual activity of the TF/FVIIa complex is measured using Spectrozyme® FXa. The TFPI activity present in the sample is interpolated from a standard curve constructed using known TFPI activity levels.

Data and statistical analysis

All experiments were performed in duplicate wells for each condition and repeated at least three times. Data are presented as mean±SD and n represents the number of experiments. Statistical comparisons were performed using Student's t-tests and the resultant

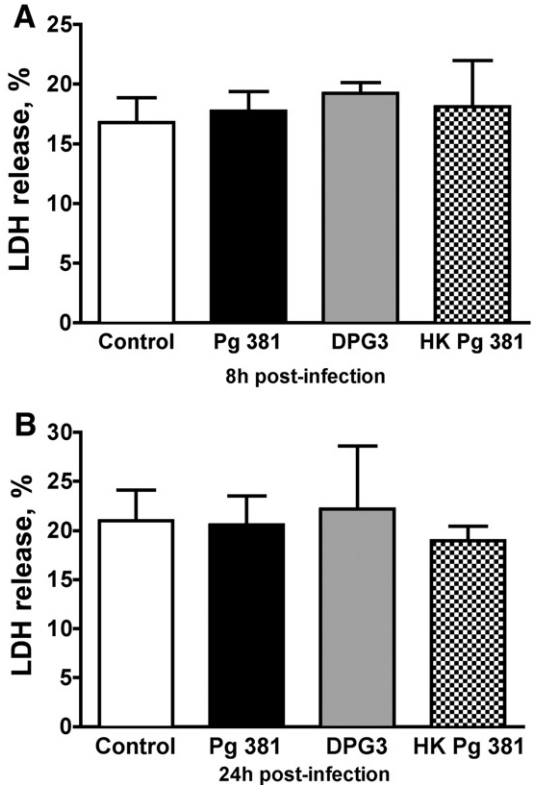

Fig. 1. *HASMC necrosis assessed by lactate dehydrogenase (LDH) release*. Infection of HASMC with *P. gingivalis 381* (Pg 381) at MOI 1:100 did not increase LDH release after 8 h (A) compared to non-infected control, non-invasive mutant DPG3, or heat-killed *P. gingivalis 381* (HK Pg 381). This was also the case at the 24 h time point (B); n=4 for all. Mean values are shown and error bars denote standard deviations.