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Mechanisms of tissue factor induction by *Porphyromonas gingivalis* in human endothelial cells

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Associations between periodontal infection and cardiovascular disease have been documented. *Porphyromonas gingivalis* is a well-established periodontal pathogen, and tissue factor (TF) is a key initiator of the coagulation cascade. In this context, *P. gingivalis* has been reported to enhance TF expression in human endothelial cells. The present study investigated the underlying mechanisms of TF induction by *P. gingivalis* in human umbilical vein endothelial cells. *P. gingivalis* increased TF expression in a dose- and time-dependent manner. Not only live bacteria but also glutaraldehyde-fixed bacteria increased TF expression to the same extent. However, sonicates of *P. gingivalis* did not induce TF expression. Cytochalasin D and SMIFH2, which are inhibitors of actin polymerization and actin nucleation, respectively, inhibited the TF expression induced by *P. gingivalis*. Finally, TF production was decreased or increased in the presence of various signaling inhibitors, including mitogen-activated protein kinases. These results suggest that *P. gingivalis* induces endothelial TF expression by a bacterial internalization-dependent mechanism and through diverse signal transduction mechanisms.

Keywords: *Porphyromonas gingivalis*, Tissue factor, Endothelial cells

Introduction

Periodontal diseases are among the most widespread infectious diseases in humans, and they are characterized by plaque-induced inflammation in the supporting tissues of the teeth [1,2]. A relationship between periodontal diseases and systemic diseases has been increasingly recognized over the past decades. The systemic diseases involved include cardiovascular disease, gastrointestinal and colorectal cancer, diabetes and insulin resistance, and Alzheimer's disease, as well as respiratory tract infection and adverse pregnancy outcomes [3]. The presence of periodontal pathogens and their metabolic by-products in the mouth may modulate the immune response beyond the oral cavity, thus promoting the development of systemic conditions [4].

Porphyromonas gingivalis is a well-established pathogen in severe forms of adult periodontal diseases. *P. gingivalis* is a gram-negative black pigmented anaerobe that colonizes in periodontal pockets and spreads into deeper tissues [5,6]. It has been detected within atheromatous plaques and been shown to induce inflammatory, immune, and procoagulant responses [7,8]. *P. gingivalis* invades endothelial cells, and it has been proposed that endothelial cell dysfunction is an early manifestation of atherosclerotic vascular disease [9]. There is evidence that *P. gingivalis* is able to induce and maintain a chronic state of inflammation at distant sites, including atheromatous plaques, and this species has been shown to accelerate atherosclerosis in animal models [10,11].

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Tissue factor (TF) is a key initiator of the coagulation cascade. It is a 47-kD membrane-bound glycoprotein expressed in both vascular and nonvascular cells. TF is constitutively expressed in subendothelial cells such as vascular smooth muscle cells leading to rapid initiation of coagulation when the vessel is damaged. Endothelial cells do not express TF under physiological conditions. In response to various stimuli, however, TF expression and activity can be induced in endothelial cells. TF binds factor VIIa resulting in activation of factor IX and factor X, leading to fibrin formation [12-14].

Previous studies demonstrated that *P. gingivalis* induces procoagulant effects in human aortic endothelial cells and smooth muscle cells [15,16]. They revealed that *P. gingivalis* enhanced TF expression in endothelial cells, but not in vascular smooth muscle cells. However, they did not report regarding the mechanisms of TF induction by *P. gingivalis*. The purpose of the present study was to investigate the mechanisms responsible for TF induction in endothelial cells.

Materials and Methods

1. Reagents

PD98059, SB203580, SP600125, GF109203X, and U73122 were purchased from Calbiochem (San Diego, CA, USA). Wortmannin, genistein, SC-514, cytochalasin D (CD), SMIFH2 were purchased from Sigma (St. Louis, MO, USA).

2. Bacterial culture

P. gingivalis 381 was grown in Trypticase soy broth supplemented with yeast extract (1 mg/mL), hemin (5 μg/mL), and menadione (1 μg/mL). The bacteria were incubated anaerobically (85% N_2, 10% H_2, and 5% CO_2) at 37℃. For experiments, bacterial concentrations were determined by measuring optical density at 600 nm using a spectrophotometer.

3. Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Cascade Biologics (Portland, OR, USA) and cultured in Medium 200 supplemented with 10% fetal bovine serum and 50 μg/mL gentamicin at 37℃ in 5% CO_2. For glutaraldehyde (GA) fixation, *P. gingivalis* was washed twice in phosphate-buffered saline (PBS) and then incubated with 2.5% GA in PBS for 2 hours at room temperature. After fixation, the bacteria were washed twice in PBS and then resuspended in a small volume of PBS. For sonication, *P. gingivalis* was washed twice in PBS and the bacterial cells were disrupted in iced bath using a Vibra-Cell VCX-600 ultrasonic processor (Sonics & Materials, Newtown, CT, USA) with 13 mm diameter horn. Amplitude of the ultrasonic device is maintained at 20% for 20 minutes.

4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA) as specified by the manufacturer and was quantified spectrophotometrically. First-strand cDNA was synthesized from 1 μg of RNA using random primers (Promega, Madison, WI, USA) and Molony murine leukemia virus reverse transcriptase (Promega). Two μL of cDNA products were amplified in 25 μL volumes under a layer of mineral oil using a GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Each PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.2 μM each dNTP, 1 U Taq DNA polymerase, and 0.5 μM of each primer. Each cycle consisted of denaturation at 94℃ (30 s), annealing at 57℃ (30 s), and extension at 72℃ (60 s). The sequences of primers were 5′-TGAAGGATGTGAAGCAGACG-3′, 5′-GCCAGGATGATGACAAGGAT-3′ for TF (525 bp); 5′-CAGCCAGATGCAATCAATGC-3′, 5′-GTGGTCCATGGAATCCTGAA-3′ for monocyte chemoattractant protein (MCP-1) (198 bp); and 5′-AGCGGGAAATCGTGCGTG-3′, 5′-CAGGGTACATGGTGGTGCC-3′ for β-actin (300 bp). The PCR products of 10 μL were fractionated on 1.2% (w/v) agarose gels containing RedSafe (iNtRON Biotechnology, Seongnam, Korea), visualized by UV transillumination, and photographed.

5. Enzyme-linked immunosorbent assay (ELISA)

After the cell culture supernatant was removed, cells were lysed by Cell Lysis Butter 1 (R&D Systems, Minneapolis, MN, USA). After cell debris was removed by centrifugation, the levels of TF were quantified using commercial ELISA kits (R&D Systems) according to the manufacturer's directions.

6. Statistical analysis

Our experiments were conducted in three independent experiments to confirm the reproducibility of the results. The data

are presented as means with standard deviations. Statistical analysis of one-way analysis of variance with Tukey-Kramer multiple comparisons test was performed using GraphPad In-Stat (GraphPad Software, La Jolla, CA, USA). A *p*-value < 0.05 was considered statistically significant.

Results

1. Dose- and time-dependent TF induction by *P. gingivalis*

We first determined the dose-dependency of *P. gingivalis*-stimulated TF expression. Various doses of *P. gingivalis* were added to HUVEC cultures and the TF expression was determined by RT-PCR. TF expression was increased dose-dependently by *P. gingivalis*. The induction of TF expression was evident from the multiplicity of infection (MOI) of 50 (Fig. 1A).

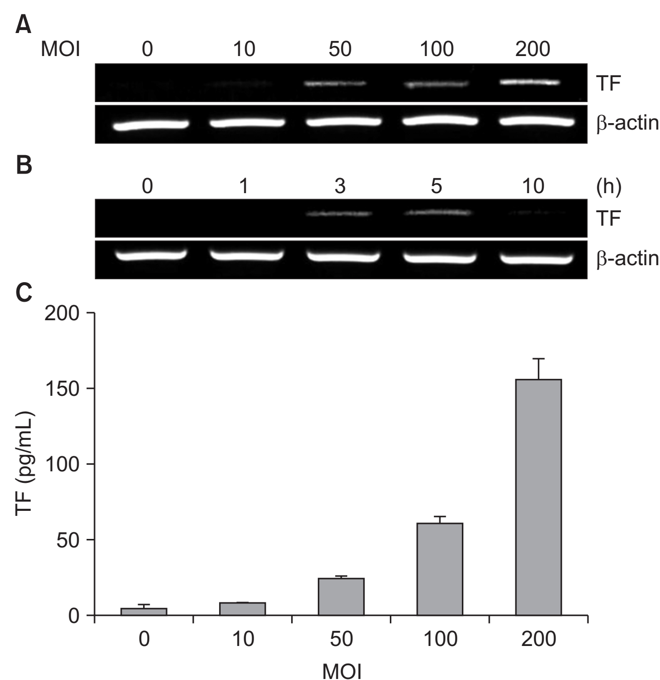

Fig. 1. Dose- and time-dependent tissue factor (TF) expression by *Porphyromonas gingivalis*. (A) Human umbilical vein endothelial cells (HUVEC, 2 × 10^4 in 12-well plates) were infected with increasing multiplicity of infections (MOIs) of *P. gingivalis* for 5 hours. (B) HUVEC were infected with *P. gingivalis* (MOI = 1:200) for various times. Total RNA was isolated and levels of TF mRNA were determined by reverse transcription-polymerase chain reaction. Shown are photographs of agarose gels from one experiment, representative of three. (C) HUVEC were infected with increasing MOIs of *P. gingivalis* for 18 hours. Levels of TF protein in cell lysates were measured by ELISA. Data are the mean ± standard deviation of a representative experiment performed in triplicate. Similar results were obtained in two other experiments.

Time course experiments showed that the mRNA expression was induced around 3 hours and maintained around 5 hours after *P. gingivalis* stimulation. The TF mRNA level declined nearly to the resting level around 10 hours (Fig. 1B). Levels of TF protein in lysed cell samples was determined by ELISA. The ELISA results showed that *P. gingivalis* dose-dependently increased the production of TF protein by HUVEC (Fig. 1C). These results demonstrated that *P. gingivalis* stimulates HUVEC to express TF in dose- and time-dependent manners.

2. Comparison between live, killed, and sonicated *P. gingivalis*

Next, we determined if there is difference between live and killed *P. gingivalis* in stimulating HUVEC to induce TF expression. In parallel, *P. gingivalis* sonicates were also compared. HUVEC cultures were incubated with the same number of live, GA-fixed, or sonicated *P. gingivalis* (MOI = 1:200) and the TF expression was measured by RT-PCR and ELISA. Not only live, but also killed *P. gingivalis* strongly stimulated HUVEC to express TF. However, *P. gingivalis* sonicates did not increase the TF expression at all (Fig. 2).

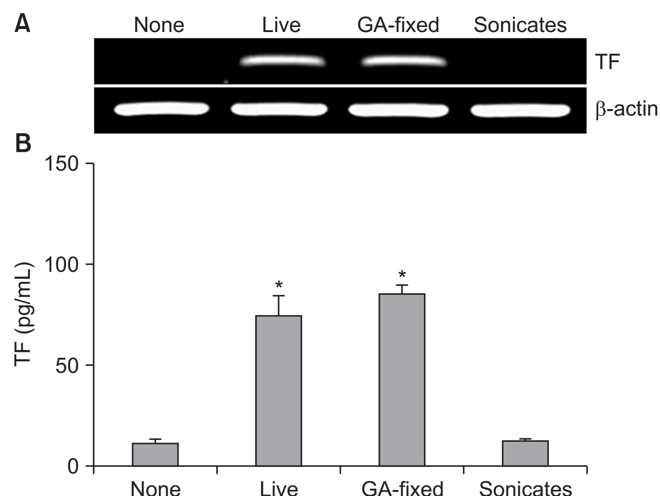

Fig. 2. Comparison between live, killed, and sonicated *Porphyromonas gingivalis* in their ability to induce tissue factor (TF) expression. (A) Human umbilical vein endothelial cells (HUVEC) cultures were incubated with the same amount (multiplicity of infection [MOI] = 1:200) of live, glutaraldehyde (GA)-fixed, or sonicated *P. gingivalis* for 5 hours and the TF expression was measured by reverse transcription-polymerase chain reaction. Shown are a photograph of agarose gels from one experiment, representative of three. (B) HUVEC were incubated with live, killed, sonicated *P. gingivalis* for 18 hours. Levels of TF protein in cell lysates were measured by ELISA. Data are the mean ± standard deviation of a representative experiment performed in triplicate. The asterisks indicate significant differences ($p < 0.05$). Similar results were obtained in two other experiments.

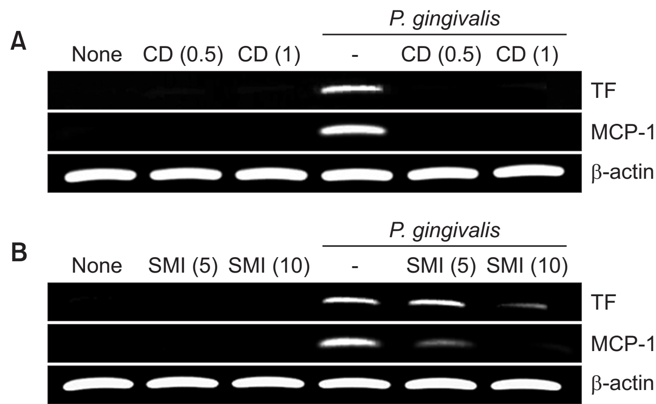

Fig. 3. Effect of cytochalasin D (CD) (A) and SMIFH2 (SMI) (B) on the induction of tissue factor (TF) and monocyte chemoattractant protein (MCP-1) by *Porphyromonas gingivalis*. Human umbilical vein endothelial cells were pretreated with CD (0.5–1 μg/mL) or SMIFH2 (5–10 μM) and then infected with *P. gingivalis* (multiplicity of infection [MOI] = 1:200) for 3 hours. Total RNA was isolated and levels of TF mRNA were determined by reverse transcription-polymerase chain reaction. Shown are photographs of agarose gels from one experiment, representative of three.

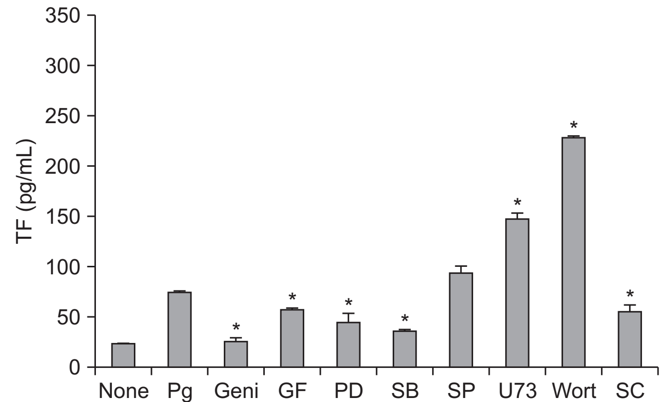

Fig. 4. Effects of various signaling inhibitors on the production of tissue factor (TF) by *Porphyromonas gingivalis*. Human umbilical vein endothelial cells were pretreated for 1 hour with GF109203X (GF, 1 μM), wortmannin (Wort, 100 nM), U73122 (U73, 10 μM), genistein (Geni, 50 μM), PD98059 (PD, 50 μM), SB203580 (SB, 10 μM), SP600125 (SP, 10 μM), or SC-514 (SC, 30 μM) and then infected with *P. gingivalis* (Pg, multiplicity of infection [MOI] = 1:200) for 18 hours. Levels of TF protein in the cell lysates were measured by ELISA. Data are the mean ± standard deviation of a representative experiment performed in triplicate. The asterisks indicate significant differences compared to *P. gingivalis* stimulation without inhibitors (p < 0.05).

3. Effects of CD and SMIFH2 on the induction of TF and MCP-1 by *P. gingivalis*

As bacterial internalization has been reported to play important roles in various infections [17-19], we examined the effects of inhibitors of actin polymerization on TF induction by *P. gingivalis*. CD prevents actin polymerization and SMIFH2 inhibits actin nucleation. HUVEC cultures were incubated with *P. gingivalis* in the presence or absence of the inhibitors and the mRNA levels of TF were examined by RT-PCR. We observed the mRNA expression of MCP-1 together because MCP-1 induction by *P. gingivalis* is dependent on bacterial internalization [18]. As shown in Fig. 3, both inhibitors inhibited *P. gingivalis*-stimulated TF expression as well as MCP-1 expression.

4. Effects of various signaling inhibitors on the production of TF by *P. gingivalis*

In order to evaluate the relative importance of various signaling pathways in *P. gingivalis*-induced TF production in HUVEC, specific pharmacological inhibitors were used. HUVEC cultures were pretreated with GF109203X (protein kinase C), wortmannin (phosphatidylinositol 3-kinase), U73122 (phospholipase C), genistein (protein tyrosine kinase), PD98059 (extracellular signal-regulated kinase, ERK), SB203580 (p38 mitogen-activated protein kinase), SP600125 (c-Jun N-terminal kinase), or SC-514 (nuclear factor kappa B, NF-κB) for 1 hour, and then the cells were incubated with *P. gingivalis* for 18 hours. TF concentrations of the cell lysates were measured by ELISA. Among the inhibitors, SB203580, PD98059, genistein, GF109203X, and SC-514 inhibited TF production stimulated by *P. gingivalis*. In contrast, TF production was significantly elevated in the presence of U73122 and wortmannin (Fig. 4).

Discussion

In the present study, we investigated the mechanisms of TF induction by *P. gingivalis* in endothelial cells. Increased TF expression was observed at the levels of both mRNA and protein. MOI as low as 1:50 evidently stimulated HUVEC to express TF and higher MOIs resulted in higher levels of TF expression. Time-course monitoring of TF mRNA expression showed that the mRNA level peaked at 3–5 hours after *P. gingivalis* stimulation and declined thereafter.

We prepared killed *P. gingivalis* by GA fixation. GA has been widely used to kill and fix bacteria [20]. There was no difference between live and GA-killed *P. gingivalis* in the ability to stimulate HUVEC to express TF. This means that activities of viable *P. gingivalis* like enzyme secretion are not required for the TF induction. However, sonicated *P. gingivalis* could not induce the TF expression. Previous studies also reported that sonicated bacteria were less stimulatory for cytokine induction

than intact bacteria [21,22].

Bacterial internalization has been reported to play important roles in stimulating host cells. Also, *P. gingivalis* internalization into endothelial cells has been proposed as an important mechanism of pathogenesis in periodontal and cardiovascular diseases [23,24]. As actin polymerization is absolute requirement for bacterial internalization, we used CD, an inhibitor of actin polymerization, to block bacterial internalization. We also examined the effect of SMIFH2, a formin inhibitor on the *P. gingivalis*-stimulated TF expression. Formin proteins are actin nucleators that bind monomeric actin and polymerizes it into filamentous actin. SMIFH2 is a cell-permeable compound that inhibits formin-dependent actin polymerization [25]. Our results showed that CD treatment prevented the TF induction. SMIFH2 treatment resulted in decreased expression of TF. These results suggest that *P. gingivalis* induce TF expression through a bacterial internalization-dependent mechanism.

Finally, we evaluated the relative importance of various signaling pathways in *P. gingivalis*-induced TF expression in HUVEC. Pharmacological inhibition of protein kinase C, p38, ERK, protein tyrosine kinases, NF-κB inhibited the TF production. In contrast, the TF production was elevated with inhibition of phospholipase C and phosphatidylinositol 3-kinase. It is yet to be determined if the signaling pathways modulate the TF expression at the transcriptional level or posttranscriptional level. The signaling pathways may also indirectly involve the TF induction by affecting the *P. gingivalis* internalization. Further studies are needed to obtain more detailed information about involvement of each signaling pathway in the *P. gingivalis*-induced TF expression in endothelial cells.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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