Role of JNK and NF-κB pathways in *Porphyromonas gingivalis* LPS-induced vascular cell adhesion molecule-1 expression in human aortic endothelial cells

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Abstract. An increasing number of studies have shown a correlation between *Porphyromonas gingivalis* (*P. gingivalis*) infection and atherosclerosis. A recent study demonstrated that the expression of vascular cell adhesion molecule-1 (VCAM-1) was induced by *P. gingivalis* lipopolysaccharide (LPS) in human aortic endothelial cells (HAECs). The activation of p38 mitogen-activated protein kinase (p38 MAPK) was at least partially involved in this process. Those results suggested the potential involvement of *P. gingivalis* LPS in the pathogenesis of atherosclerosis. However, the mechanism involved in *P. gingivalis* LPS-induced VCAM-1 production has not yet been elucidated. The present study examined the role of the c-Jun N-terminal kinase (JNK) and nuclear factor-κB (NF-κB) cell signaling pathways in *P. gingivalis* LPS-induced VCAM-1 expression in HAECs. Western blotting was used to investigate the activation of JNK and NF-κB pathways in HAECs exposed to *P. gingivalis* LPS. Following this, specific pharmacological inhibitors were introduced and the protein production of VCAM-1 was studied. The results showed that the JNK and NF-κB pathways in HAECs were capable of being activated by *P. gingivalis* LPS. The inhibition of NF-κB by SN50 significantly attenuated *P. gingivalis* LPS-induced VCAM-1 expression, while the inhibition of JNK by SP600125 enhanced VCAM-1 expression in *P. gingivalis* LPS-treated HAECs. Therefore, the results indicated that NF-κB was essential for the *P. gingivalis* LPS-induced VCAM-1 expression in HAECs and that JNK may be a suppressor of VCAM-1 expression in HAECs.

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Introduction

Periodontal disease, a chronic inflammatory disease of the periodontium, is initiated by bacterial infections, which may eventually result in the loss of teeth. *Porphyromonas gingivalis* (*P. gingivalis*), a Gram-negative and obligate anaerobic bacterium, has long been considered to be an important pathogen involved in human periodontal disease. Previous studies have suggested a correlation between periodontal disease and atherosclerosis and that *P. gingivalis* infection may accelerate the development of atherosclerosis (1-4).

Lipopolysaccharide (LPS) is the main component of the cell wall of Gram-negative bacteria (5) and has been increasingly suggested to be responsible for the activation of vascular endothelial cells (VECs) and the initiation of the atherosclerotic process (6,7). *P. gingivalis* LPS, which is important in *P. gingivalis* infection (8), may be released into the bloodstream following scaling and root planing or even mastication in patients with severe periodontal disease, resulting in elevated levels of circulating endotoxin, which has been shown to be correlated with an increased risk of atherosclerotic incident (9-13). It has previously been shown that *P. gingivalis* LPS has a similar ability to that of *Escherichia coli* (*E. coli*) to activate human aortic endothelial cells (HAECs) by elevating the gene expression and protein production of vascular cell adhesion molecule-1 (VCAM-1) (4,14). VCAM-1 belongs to the immunoglobulin superfamily, is important in leukocyte recruitment to sites of inflammation and accelerates the development of atherosclerosis (15-17). Therefore, *P. gingivalis* LPS-induced VCAM-1 expression may contribute to the initiation of the atherosclerotic process.

Multiple signaling mechanisms, in a number of cell lines, have been demonstrated to be involved in the induction of VCAM-1 expression in response to various stimuli. This suggests that there are divergent pathways leading to VCAM-1 expression, depending on the nature of the stimulus and the cell type (4,18-21). A major mechanism through which signals from extracellular stimuli are transmitted to the nucleus involves the activation of intracellular kinases, including those belonging to the mitogen-activated protein kinase (MAPK) superfamily. p38 MAPK and c-Jun N-terminal kinase (JNK) are two distinct and parallel MAPK pathways that have been

demonstrated to predominantly be involved in the regulation of cellular inflammation in response to stimuli, including the *E. coli* LPS-induced upregulation of VCAM-1 (22-26).

Previous studies have shown the activation of p38 MAPK and JNK by *P. gingivalis* LPS in a number of cell lines; however, studies concerning human VECs are limited (27-29). In addition, the majority of the previous studies investigated the response of human umbilical VECs (HUVECs) to *P. gingivalis* LPS and were, therefore, less relevant to atherosclerosis. We recently demonstrated the involvement of the p38 MAPK pathway in *P. gingivalis* LPS-induced VCAM-1 expression in HAECs (4). However, the mechanisms responsible for *P. gingivalis* LPS-induced VCAM-1 expression in VECs have yet to be elucidated. Therefore, further study is required to demonstrate whether the activation of JNK by *P. gingivalis* LPS is associated with VCAM-1 expression in HAECs. In addition, it is of interest that many of the genes regulated by MAPKs are dependent on nuclear factor-κB (NF-κB) for transcription. NF-κB has also been shown to be involved in the expression of adhesion molecules at the transcriptional level, in various cell types (19,20,30). Since it has been shown that discrepancies in chemical structures, immunobiological activities and the activation of intracellular signaling pathways exist between *P. gingivalis* and *E. coli* LPS (31,32), *E. coli* LPS was also introduced in the present study.

The experiments in the present study were performed to investigate the roles of JNK and NF-κB in *P. gingivalis* LPS-induced VCAM-1 protein expression in HAECs. It was demonstrated that NF-κB was required for this expression, whereas JNK was a suppressor of VCAM-1 expression. These results provide a novel insight into the mechanisms of *P. gingivalis* LPS action in the initiation of atherosclerosis.

Materials and methods

Reagents. P. gingivalis LPS was purchased from Invitrogen Life Technologies (San Diego, CA, USA), while *E. coli* LPS (055:B5) and β-actin monoclonal antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). The monoclonal antibodies for JNK (56G8), phosphorylated JNK (p-JNK; G9), NF-κB, and nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor-α (IκB-α) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). VCAM-1 (E-10) monoclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The JNK (JNK Inhibitor II, SP600125) and NF-κB (SN50) inhibitors were obtained from Calbiochem (San Diego, CA, USA). SP600125 was dissolved in dimethylsulfoxide (DMSO), while SN50 was dissolved in ultrapure water at a concentration of 50 mM for stock solutions.

Cells and culture conditions. HAECs and Endothelial Cell Medium (ECM) were obtained from ScienCell (Carlsbad, CA, USA). ECM was prepared for use by mixing 5 ml Endothelial Cell Growth Supplement (ECGS), 5 ml penicillin/streptomycin solution (P/S) and 25 ml fetal bovine serum (FBS) into 500 ml basal medium, in accordance with the manufacturer's instructions. Frozen cells were thawed in a water bath at 37˚C and then seeded in 100-mm culture dishes at a density of $5x10^3$ cells/cm^2. The cells were maintained at 37˚C in humidified 5% CO_2. Twenty-four hours subsequent to seeding, the culture medium was changed to remove the residual DMSO and the unattached cells and was then changed every 2 days. When the cultures reached 90% confluence, the cells were treated with 0.25% (w/v) trypsin/0.02% (w/v) EDTA until all the cells were completely detached. The cells were subsequently resuspended and diluted with ECM, prior to the cell suspension being plated onto 100-mm culture dishes. The culture medium was changed every 2 days. HAECs at 85% confluence and in the third to sixth passages were used in all the experiments. The cells were seeded in 60-mm culture dishes at a cell density of $5x10^5$ cells/dish.

Preparation of cell extracts and western blot analysis. HAECs were pretreated with the specific inhibitor SP600125 (20 μM) or SN50 (18 mM) for 1 h prior to the addition of LPS. Cells were harvested using centrifugation at 2,500 x g for 10 min. The collected cells were subsequently lysed with cell and tissue protein extraction reagent containing protease inhibitor, phosphatase inhibitor and phenylmethanesulfonyl fluoride (PMSF; Kangchen Bio-tech, Shanghai, China). The lysates were then centrifuged at 14,000 x g for 15 min at 4˚C and the debris was removed. The protein concentrations in the lysates were assessed using bicinchoninic acid (BCA) reagents (Beyotime Institute of Biotechnology, Jiangsu, China), in accordance with the manufacturer's protocol.

Protein samples (40 μg) were denatured and loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, prior to being electrotransferred onto a 0.2-μm polyvinylidene fluoride (PVDF) transfer membrane (Pall Corp., Pensacola, FL, USA). The membranes were subsequently blocked at room temperature with 5% (w/v) skimmed milk or 5% (w/v) bovine serum albumin (BSA) in Tris-buffered saline and Tween 20 [TBST; Tris-HCl 50 mM, NaCl 150 mM, 0.05% (w/v) Tween-20, pH 7.4] for ≥1 h. The blocked membranes were incubated overnight at 4˚C with the specific antibodies against VCAM-1 (1:100), β-actin (1:5,000), p-JNK (1:2,000), JNK (1:1,000), NF-κB (1:1,000) or IκB-α (1:1,000) in TBST. Subsequent to being washed with TBST, the membranes were incubated with a fluorescent-labeled secondary antibody (1:1,000) for 1 h, and the immunoreactive products were analyzed using the Odyssey® Infrared Imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis. Statistical analyses were performed using an independent samples t-test with the SPSS 20.0 statistical software package (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

P. gingivalis LPS activated JNK signaling pathway transduction in HAECs. To investigate whether *P. gingivalis* and *E. coli* LPS mediated JNK phosphorylation, the activation of this kinase was examined using an antibody specific for tyrosine-phosphorylated JNK and western blotting. As shown in Fig. 1A and C, the *P. gingivalis* LPS-induced phosphorylation of JNK was observed within 15 min subsequent to stimulation and reached a peak at 30 min. In *E. coli* LPS-treated HAECs, JNK phosphorylation was observed as early as 5 min and

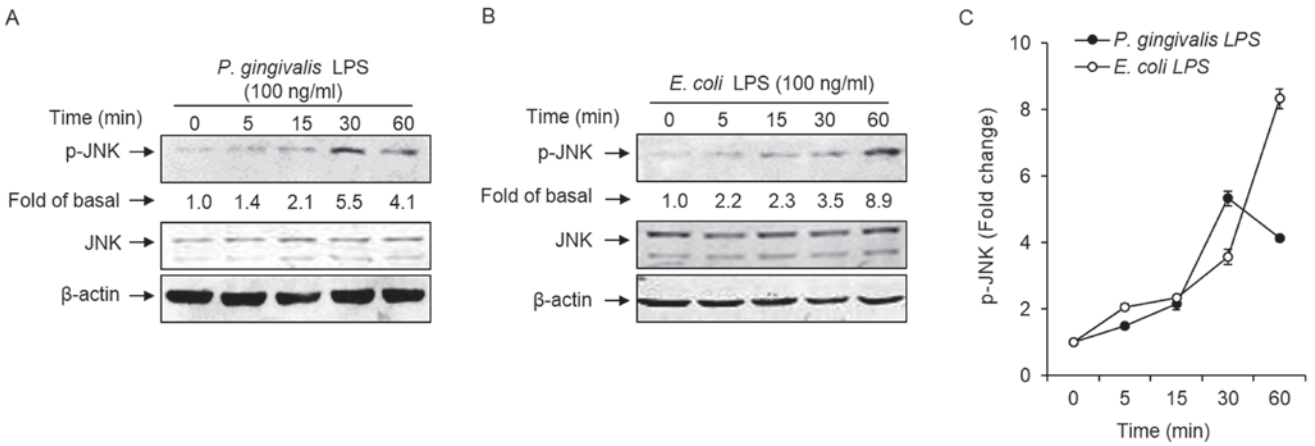

Figure 1. Lipopolysaccharide (LPS) induces the tyrosine phosphorylation of c-Jun N-terminal kinase (JNK) in human aortic endothelial cells (HAECs). Levels of JNK and phosphorylated JNK (p-JNK) were assessed using western blot analysis. HAECs were stimulated with (A) *Porphyromonas gingivalis* (*P. gingivalis*) LPS and (B) *Escherichia coli (E. coli)* LPS for the indicated periods of time. The image shows one of three similar experiments. The intensity of the western blotting bands shown was quantified using scanning densitometry and standardized to the equivalent β-actin protein levels. (C) Fold induction of p-JNK expression normalized to the equivalent β-actin protein levels vs. cells cultured in medium alone (basal level). Data are expressed as the mean ± standard deviation of three independent experiments.

reached the highest level at 60 min, which was the end of the observation period (Fig. 1B and C).

Involvement of JNK in P. gingivalis LPS-induced VCAM-1 expression. To demonstrate the involvement of the JNK cell signaling pathway in *P. gingivalis* and *E. coli* LPS-induced VCAM-1 expression in HAECs, SP600125, a selective pharmacological inhibitor (33), was used to block the JNK signaling pathway. As shown in Fig. 2, pretreatment of the HAECs with SP600125 at a concentration of 20 μM for 1 h significantly inhibited the *P. gingivalis* and *E. coli* LPS-induced JNK phosphorylation ($P<0.01$). However, SP600125 failed to suppress the upregulation of VCAM-1 induced by the *P. gingivalis* and *E. coli* LPS. Notably, blocking the intracellular JNK signaling pathway with SP600125 significantly elevated the expression of VCAM-1 in LPS-treated HAECs ($P<0.01$; Fig. 3).

P. gingivalis LPS-activated NF-κB signaling pathway transduction in HAECs. To determine the activation of the transcription factor NF-κB in HAECs exposed to *P. gingivalis* and *E. coli* LPS, western blotting was used to assess the degradation of IκB-α, an indicator of NF-κB activation, as well as the activation pattern of p65, a component of NF-κB, using antibodies specific for IκB-α and NF-κB p65, respectively. As shown in Fig. 4, IκB-α was significantly degraded, corresponding with the upregulation of NF-κB p65, following 30 min of exposure of the HAECs to *P. gingivalis* and *E. coli* LPS. SN50, a synthetic cell permeable peptide and inhibitor of NF-κB (34), has been shown to significantly attenuate the degradation of IκB-α and the elevated expression of NF-κB p65.

NF-κB inhibitor suppresses P. gingivalis LPS-induced VCAM-1 expression. To demonstrate the involvement of the NF-κB signaling pathway in *P. gingivalis* and *E. coli* LPS-induced VCAM-1 expression in HAECs, SN50 was used to block the NF-κB signaling pathway. As shown in Fig. 5, pretreatment of the HAECs with SN50 at a concentration of 18 μM for 1 h

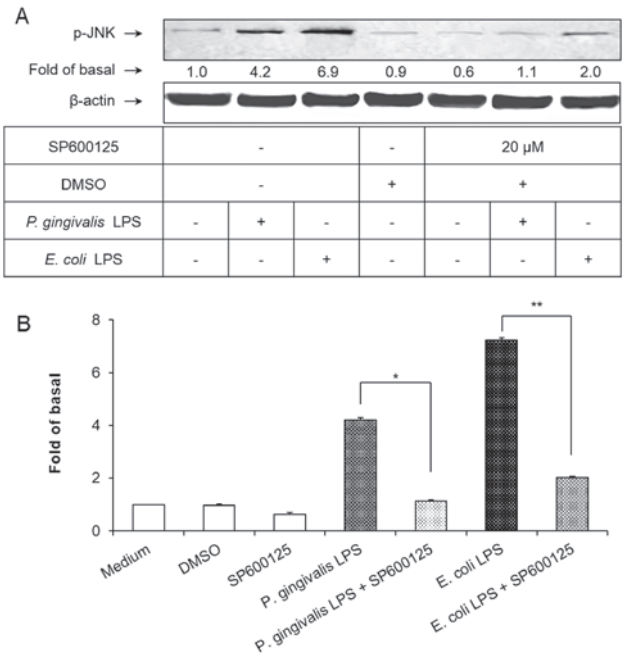

Figure 2. The c-Jun N-terminal kinase (JNK) inhibitor, SP600125, inhibits lipopolysaccharide (LPS)-induced JNK phosphorylation. (A) Cells were preincubated with SP600125 for 1 h prior to stimulation with *Porphyromonas gingivalis* (*P. gingivalis*) LPS (30 min) or *Escherichia coli* (*E. coli*) LPS (60 min) at a concentration of 100 ng/ml. The image shows one of three similar experiments. The intensity of the western blotting bands shown was quantified using scanning densitometry and standardized to the equivalent β-actin protein levels. (B) Fold induction of phosphorylated-JNK (p-JNK) expression compared with the basal level. Data are expressed as the mean ± standard deviation of three independent experiments. Statistically significant differences were determined using the independent samples t-test. $^{*}P<0.01$, $^{**}P<0.01$. DMSO, dimethylsulfoxide.

significantly inhibited the induction of VCAM-1 expression in HAECs exposed to *P. gingivalis* and *E. coli* LPS. These results suggested that the activation of NF-κB was essential for the *P. gingivalis* and *E. coli* LPS-induced VCAM-1 expression in HAECs.

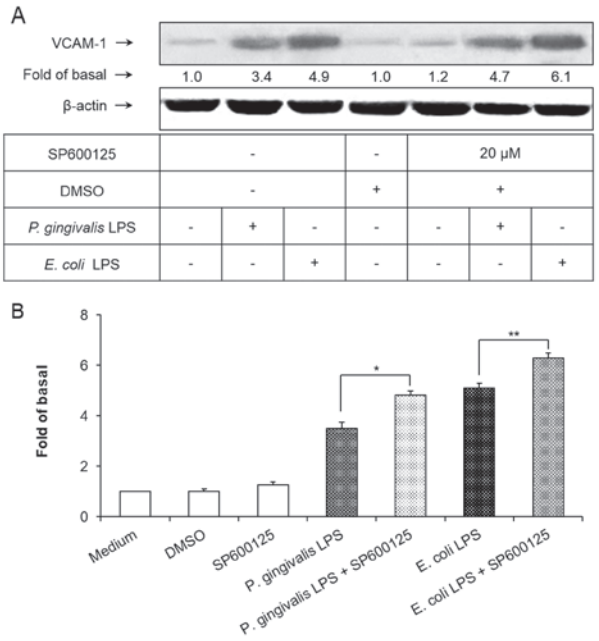

Figure 3. c-Jun N-terminal kinase (JNK) inhibitor, SP600125, inhibits lipopolysaccharide (LPS)-induced vascular cell adhesion molecule-1 (VCAM-1) protein expression. (A) Cells were preincubated with SP600125 for 1 h prior to stimulation with *Porphyromonas gingivalis* (*P. gingivalis*) LPS or *Escherichia coli* (*E. coli*) LPS for 16 h. The image shows one of three similar experiments. The intensity of the western blotting bands shown was quantified using scanning densitometry and standardized to the equivalent β-actin protein levels. (B) Fold induction of VCAM-1 expression compared with the basal level. Data are expressed as the mean ± standard deviation of three independent experiments. Statistically significant differences were determined using the independent samples t-test. *P<0.01, **P<0.01. DMSO, dimethylsulfoxide.

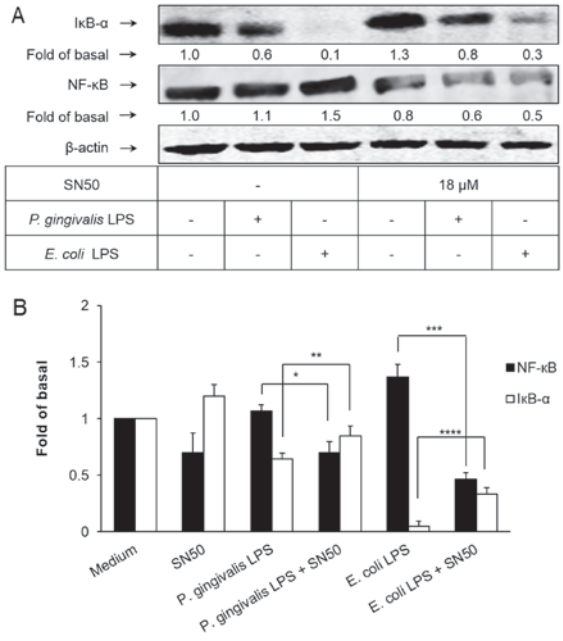

Figure 4. Nuclear factor-κB (NF-κB) inhibitor, SN50, inhibits lipopolysaccharide (LPS)-induced degradation of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor-α (IκB-α) and expression of NF-κB p65. (A) Cells were preincubated with SN50 for 1 h prior to stimulation with *Porphyromonas gingivalis* (*P. gingivalis*) LPS or *Escherichia coli* (*E. coli*) LPS for 30 min at a concentration of 100 ng/ml. The images shows one of three similar experiments. The intensity of the western blotting bands shown was quantified using scanning densitometry and standardized to the equivalent β-actin protein levels. (B) Fold induction of NF-κB expression and fold degradation of IκB-α compared with the basal level. Data are expressed as the mean ± standard deviation of three independent experiments. Statistically significant differences were determined using the independent samples t-test. *P<0.01, **P<0.01, ***P<0.05, ****P<0.01.

Discussion

Mononuclear cell (MNC) adhesion to the atherosclerosis-prone vascular endothelium has been suggested to be the initial step of atherosclerosis (15-17). The upregulation of VCAM-1 on the surface of the VECs may be important in the recruitment, rolling, firm adhesion and infiltration of MNCs at sites of inflammation in the vascular endothelium and may accelerate the development of atherosclerosis (35). *P. gingivalis* LPS has been shown to be an inducer of VCAM-1 (4,14); however, little is known about the intracellular signaling pathways leading to VCAM-1 expression in VECs exposed to *P. gingivalis* LPS. In this study, we investigated the involvement of the JNK and NF-κB signaling pathways in *P. gingivalis* LPS-induced VCAM-1 expression in HAECs. Using western blotting, we demonstrated that pretreatment of the HAECs with a JNK inhibitor, SP600125, significantly attenuated JNK phosphorylation; however, an upregulation, instead of a downregulation, of *P. gingivalis* LPS-induced VCAM-1 expression was observed in SP600125 pretreated HAECs. Furthermore, the activation of NF-κB by *P. gingivalis* LPS in the HAECs resulted in VCAM-1 expression, since the activation of NF-κB and subsequent expression of VCAM-1 was inhibited by an inhibitory peptide of NF-κB, SN50. These results suggest that NF-κB was essential for the induction of VCAM-1 protein production in the HAECs exposed to *P. gingivalis* LPS, whereas JNK may be a suppressor of VCAM-1 expression in HAECs.

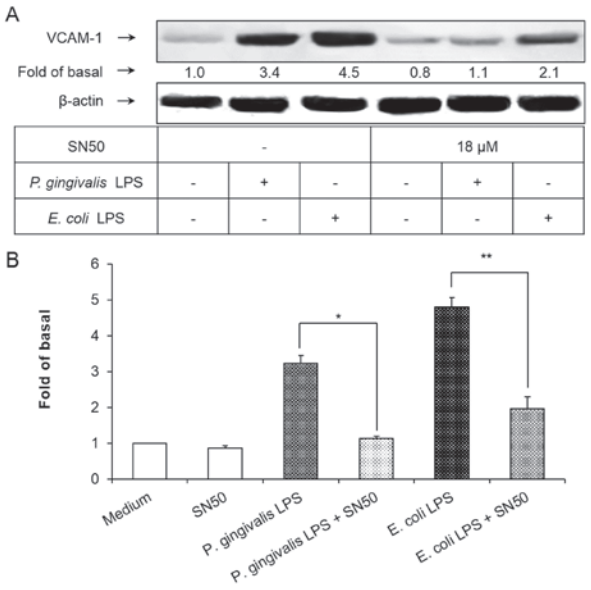

Figure 5. Nuclear factor-κB (NF-κB) inhibitor, SN50, inhibits lipopolysaccharide (LPS)-induced vascular cell adhesion molecule-1 (VCAM-1) protein expression. (A) Cells were preincubated with SN50 for 1 h prior to stimulation with *Porphyromonas gingivalis* (*P. gingivalis*) LPS or *Escherichia coli* (*E. coli*) LPS for 16 h. The intensity of the western blotting bands shown was quantified using scanning densitometry and standardized to the equivalent β-actin protein levels. (B) Fold induction of VCAM-1 expression compared with the basal level. Data are expressed as the mean ± standard deviation of three independent experiments. Statistically significant differences were determined using the independent samples t-test. *P<0.01, **P<0.01.

There are at least three distinct and parallel MAPK pathways, including p38 MAPK, JNK and the extracellular signal-regulated kinase (ERK) pathway (25). It has been demonstrated that the p38 MAPK and JNK pathways mediate signaling stimulated by bacterial components (LPS, lipoteichoic acid), inflammatory cytokines [interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α)] and stress factors (osmotic shock, heat shock, H_2O_2, UV radiation and DNA-damaging agents), while activation of ERK is important in mediating cell proliferation in response to growth factors and mitogens (36-38). We recently demonstrated that p38 MAPK mediated *P. gingivalis* LPS-induced VCAM-1 expression in HAECs (4). Therefore, the involvement of JNK in this effect was investigated in the present study.

In this study, we demonstrated the activation of the JNK pathway in *P. gingivalis* and *E. coli* LPS-stimulated HAECs. As shown in Fig. 1, *P. gingivalis* LPS-induced JNK phosphorylation was weaker and reached a peak much earlier than that induced by *E. coli* LPS. Similar results were observed in LPS-induced VCAM-1 expression and NF-κB activation (Figs. 3-5). Previous studies have revealed discrepancies in the chemical structure of lipid A, the biologically active center of LPS, between *P. gingivalis* and *E. coli* LPS (32). In addition, it has previously been demonstrated that the endotoxic activities of *P. gingivalis* LPS and its lipid A are relatively weak when compared with those of *E. coli*-derived LPS and lipid A (4,13,32). The observations in the present study were consistent with the results from those studies, suggesting the potential involvement of *P. gingivalis* LPS in chronic inflammatory processes, such as atherosclerosis and periodontal disease, rather than acute infection, such as sepsis.

Although the present and previous studies have demonstrated the activation of the JNK pathway by *P. gingivalis* and *E. coli* LPS (26,39), the correlation between the activation of JNK and the expression of VCAM-1 has been contradictory in different cell lines. It has been shown that the JNK pathway mediated VCAM-1 expression in human tracheal smooth muscle cells (HTSMCs) exposed to TNF-α (19) and in endothelial cells (EVC304) exposed to *E. coli* LPS (21). However, Binion *et al* (20) demonstrated that in human intestinal microvascular endothelial cells (HIMECs) activation of JNK was not a requisite for the induction of VCAM-1 production by *E. coli* LPS. These results suggest that VCAM-1 is selectively expressed in a cell type and stimulus-specific manner and is mediated through the activation of diverse intracellular signaling pathways. Furthermore, the cell lines used in the majority of the previous studies concerning the activation of JNK in endothelial cells exposed to LPS were venous VECs (20,21). It has been demonstrated that there are genetic variances between artery and vein-derived endothelial cells, which contribute to the different biological and immunological responses to atherosclerotic factors (13,40,41). Of note was the fact that the HUVEC cell line used in a number of studies was the EVC304 cell line (21,42), which has been revealed to be genetically identical to the human bladder cancer-derived epithelial cell line T24/83 and has been suggested to be inappropriate for the study of endothelial cell biology (43). The involvement of the JNK pathway in LPS-induced VCAM-1 expression in HAECs remains to be determined. As shown in Fig. 2, we elucidated that SP600125, an anthrapyrazole and a reversible ATP-competitive inhibitor of JNK, significantly attenuated the phosphorylation of JNK. However, this was demonstrated to enhance the *P. gingivalis* and *E. coli* LPS-induced protein production of VCAM-1 in HAECs (Fig. 3). These observations suggested that JNK may be an intracellular suppressor for the expression of VCAM-1 in HAECs.

The observation that SP600125 enhanced VCAM-1 expression indicated that the administration of SP600125 with *P. gingivalis* and *E. coli* LPS caused an additive effect on VCAM-1 expression in the HAECs (Fig. 3). This result was consistent with those of previous studies, in which SP600125 was shown to elicit additive effects with TNF-α on VCAM-1 expression in human chondrosarcoma cells and in gingival fibroblasts (44,45), although a study using HK-2 cells revealed contradictory observations (46). There have been few studies concerning the additive effect of SP600125; therefore, further investigation is required to fully elucidate the underlying molecular mechanism of JNK in VCAM-1 expression.

As extensively studied previously, inflammatory responses following exposure to extracellular stimuli highly depend on the activation of the transcription factor NF-κB (47-50). The sequestration of NF-κB by IκB-α in the cytoplasm and the phosphorylation of IκB-α, leading to the proteasomal degradation of IκB-α, results in the activation and translocation of NF-κB into the nucleus, a process that is essential in the expression of a number of genes, such as adhesion molecules, in various cell types (14,48,50). In the present study, as shown in Fig. 4, the degradation of IκB-α and the activation of NF-κB p65 were observed following *P. gingivalis* LPS exposure and were also observed to be inhibited by SN50. Furthermore, *P. gingivalis* LPS-induced VCAM-1 expression was almost completely suppressed by SN50 (Fig. 5), indicating that the activation of NF-κB was essential for the induction of VCAM-1 expression in *P. gingivalis*-stimulated HAECs.

Within the limitations of this study, we have demonstrated that *P. gingivalis* LPS has the ability to accelerate the development of atherosclerosis by upregulating the expression of VCAM-1 in HAECs, a process that is critical in the initiation of atherosclerosis. Furthermore, we demonstrated that *P. gingivalis* LPS was able to activate the JNK and NF-κB signaling pathways in HAECs. The activation of the NF-κB pathway was essential for the induction of VCAM-1 expression in HAECs exposed to *P. gingivalis* LPS, whereas JNK may be a suppressor of VCAM-1 expression. To the best of our knowledge, this study is the first to investigate the role of JNK in *P. gingivalis* LPS-induced VCAM-1 expression in HAECs.

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