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Porphyromonas Gingivalis Lipopolysaccharide Increases Monocyte Adhesion to Microvascular Endothelium by Induction of Adhesion Molecules

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***Porphyromonas gingivalis*, a major periodontal pathogen, has been implicated in the initiation and progression of periodontal disease. Endothelial dysfunction (Editor note: Aberrant and dysfunction are somewhat redundant. The authors may want to choose one or the other.) contributes to chronic periodontal inflammation. Using cDNA-representational difference analysis, we found that *P.gingivalis* lipopolysaccharide differentially induces a number of genes in human microvascular endothelial cells. Among these upregulated genes, we focused on intercellular adhesion molecule-1 (VCAM-1), which is crucial for leukocyte recruitment during vascular inflammation. *P. gingivalis* LPS significantly increased the expression of vascular cell adhesion molecule-1 (VCAM-1) as well as ICAM-1. Promoter assays revealed that the transcription of these cell adhesion molecules was mainly regulated by nuclear factor-κB (NF-κB) in endothelial cells. Furthermore, *P. gingivalis* LPS significantly increased leukocyte adhesiveness to microvascular endothelial cells and to aortic endothelium. Taken together, our results demonstrate that *P. gingivalis* LPS activates microvascular endothelial cells through NF-κB-dependent expression of cell adhesion molecules.**

Keywords : cDNA representational difference analysis; Cell adhesion molecules; human microvascular endothelial cells; *Porphyromonas gingivalis* lipopolysaccharide

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INTRODUCTION

Porphyromonas gingivalis, black-pigmented, gram-negative anaerobe, has been recognized as a major pathogen in periodontal diseases (Wang and Ohura, 2002). The *P. gingivalis* possesses a number of virulence factors such as peptidoglycan, fimbriae, gingipain, and lipopolysaccharide (LPS) which are considered as etiological agents in the initiation and progression of chronic periodontitis. *P. gingivalis* LPS has been shown to stimulate the production of proinflammatory mediators within inflamed periodontal tissue (Bainbridge and Darveau, 2001).

A key event in inflammatory processes is the recruitment and transmigration of leukocytes across the vascular endothelium of microvasculature within inflamed tissues (Rao *et al.*, 2007; Ley *et al.*, 2007). This process is predominantly mediated by endothelial cell adhesion molecules (ECAMs), such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which engage circulating leukocytes and adhere them to vascular endothelial cells lining the vessel wall (Muller, 2003).

In this study, we have used the technique of cDNA representational difference danalysis to detect genes differentially expressed in *P. gingivalis* LPS-stimulated microvascular endothelial cells. This strategy enables us to identify a number of molecules that may be involved in endothelial dysfunction in *P. gingivalis* LPS-mediated inflammatory response. Of differentially expressed genes, a transcript encoding ICAM-1 was further characterized. We found that *P. gingivalis* LPS significantly enhances the expression of ICAM-1 and VCAM-1 via NF-κB activation,

which increases leukocyte adhesion to endothelial cells. These findings indicated that *P. gingivalis* LPS exerts pro-inflammatory effects on microvascular endothelial cells by promoting leukocyte-endothelial cell interaction.

MATERIALS AND METHODS

Reagents

Mouse monoclonal anti-ICAM-1 and anti-VCAM-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human α-tubulin antibody was purchased from Biogenex (San Ramon, CA, USA). Calcein-AM was obtained from Molecular Probes (Eugene, OR, USA). *P. gingivalis* LPS was purchased from Invivogen (Carlsbad, CA, USA).

Cell culture

Human microvascular endothelial cells (HMECs) were obtained from the CDC (Atlanta, GA, USA). These cells were maintained in MCDB (Gibco, Gaithersburg, MD, USA) supplemented with 10 % FBS (Invitrogen), 1 % antibiotics (Hyclone, Logan, UT, USA) 1 μg/ml hydrocortisone (Sigma, St. Louis, MO, USA) and 10 ng/ml hEGF (Upstate Biotechnology, Lake Placid, NY, USA) at 37 °C under a humidified 5 % CO_2 incubator. U937 monocytic cells were grown in RPMI-1640 (Invitrogen) with 10 % FBS (Invitrogen) and 1 % antibiotics (Hyclone).

cDNA-representational difference analysis (RDA)

cDNA-RDA was performed according to GeneFisher PCR subtraction system (TAKARA, Shuzo, Tokyo, Japan) on total RNA isolated from HMECs after 8 h of *P. gingivalis* LPS treatment. Briefly, cDNAs were prepared by reverse transcription of mRNA, isolated by Micro-Fast Track kit, using an oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen). cDNAs were digested with *Dpn*II and ligated to the adaptors. Amplicons were made by PCR amplification of the adaptor-ligated *Dpn*II cDNA fragments. Driver DNA was prepared by digesting amplicons with *Dpn*II, and tester DNA was prepared by gel purification of digested amplicons followed by ligation to other adaptors. The first subtractive hybridization was performed using a tester-driver ratio of 1:100 to obtain difference product 1 (DP1). Subtractive hybridization was repeated using tester-driver ratios of 1:800 for the second round to obtain difference product 2 (DP2). The DP2 products were ligated into pGEM-T vector, and then all constructs were sequenced by automatic DNA sequencing analysis.

RT-PCR

Total RNA was isolated from HMECs with a TRIzol reagent kit (Invitogen). cDNA synthesis was performed on 3 μg of total RNA with a reverse transcription kit (Promega, Madison, WI, USA). The oligonucleotide primers for PCR were designed as follows: β-actin, 5'-GACTACCTCAT-GAAGATC-3' and 5'-GATCCACATCTGCTGGAA-3'; ICAM-1, 5'-CGATGACCATCTACAGCTTTCCG-G-3' and 5'-GCTGCTACCACAGTGATGATGACAA-3'; VCAM-1, 5'-GATACAACCGTCTTGGTCAGCCC-3' and 5'-CAGT-TGAAGGATGCGGGAGTA-TATG-3'.

Transient transfection and reporter gene analysis

Cells were plated on 24-well plates and transfected with the luciferase construct and pCMV-*β*-gal using Lipofectamine plus reagents (Invitrogen). Cell extracts were prepared 48 h after transfection, and then analyzed with the *β*-galactosidase enzyme assay for luciferase activity using an assay kit (Promega) and a luminometer (Turner Biosystems, Sunnyvale, CA, USA). Each extract was assayed at least three times and relative luciferase activity was calculated as RLU/*β*-galactosidase. ICAM-1 and VCAM-1 luciferase reporter constructs with full-length (ICAM-1: -1350 to +45 bp, VCAM-1: -1716 to +119 bp) and truncated forms (ICAM-1: -485 to +45 bp, VCAM-1: -213 to +119 bp) were used as previously reported (Kim *et al.*, 2008). These luciferase vectors were kindly provided by Dr. Young-Geun Kwon (Yonsei University, Korea)

In vitro monocyte adhesion assay

HMECs were plated on 24 well plates at 5×10^4 cells/well and incubated with or without *P. gingivalis* LPS for 16 h. U937 cells were then added (5×10^5 cells/ml) to the confluent monolayers of HMECs and incubated for 30 min. Non-adherent monocytes were removed by washing twice with PBS. Adherent cells were fixed and washed twice with PBS. Binding of monocytes to the endothelial cells were stained with Diff-Quick (Sysmex, Kobe, Japan), and the adherent cells were counted in 3 separate fields in each well by microscopy (Nikon, Tokyo, Japan). The average number of adherent monocytes was calculated in the three fields for each set of four wells.

Ex vivo monocyte adhesion assay

Male Sprague-Dawley rats (SD, 6 weeks of age) were obtained from Samtako (Seoul, Korea). The aortas were opened longitudinally, and incubated with *P. gingivalis* LPS for 16 h. The aortas were then incubated for 30 min with 1×10^6 fluorescence-labeled monocytes. After incubation, unbound monocytes were rinsed away; the adherent monocytes were counted in 3 consistent fields using fluorescence microscopy (Nikon).

RESULTS

Identification of ICAM-1 gene in *P. gingivalis* LPS-stimulated microvascular endothelial cells

To identify genes whose expression was altered in response to *P. gingivalis* LPS in endothelial cells, we

performed cDNA representational difference analysis (RDA) using cDNA isolated from untreated and microvascular endothelial cells treated with *P. gingivalis* LPS (10 μg/ml). This concentration showed no cytotoxic effect on microvascular endothelial cells (data not shown). After two rounds of subtractive hybridization/PCR, the DP2 products (Fig. 1A) were subcloned into pGEM-T vectors and sequenced. Among clones, some clones were identified as ICAM-1 (Fig. 1B).

P. gingivalis LPS regulates the expression of ICAM-1 and VCAM-1 in microvascular endothelial cells

Cell adhesion molecules, such as ICAM-1 and VCAM-1, on endothelial cells mediate leukocyte adhesion to the endothelium during inflammation (Rao *et al*., 2007). Therefore, we investigated whether *P. gingivalis* LPS could induce the expression of ICAM-1 and VCAM-1 on human microvascular endothelial cells. Treatment of HMECs with *P. gingivalis* LPS (10 μg/ml) or *P. gingivalis* LPS even at low concentration (1 μg/ml) significantly increased the mRNA expression of ICAM-1 and VCAM-1 in various time points (Fig. 2A and 2B).

P. gingivalis LPS induces the promoter activity of ICAM-1 and VCAM-1 genes through NF-κB

To examine whether the induction of ICAM-1 and VCAM-1 mRNA levels by *P. gingivalis* LPS is mediated by transcriptional activation of these genes, we carried out promoter analysis with luciferase reporter constructs. The full-length promoter regions of both cell adhesion molecules have transcription factor binding sites, such as NF-κB, TRE, and GATA for the 1.3kb ICAM-1 promoter and NF-κB, TRE and GATA for the 1.8kb VCAM-1 promoter. These luciferase reporter constructs were transfected into HMECs and then incubated with *P. gingivalis* LPS, respectively. *P. gingivalis* LPS significantly increased luciferase reporter activities in full-length constructs-transfected HMECs (Fig. 3A).

Both ICAM-1 and VCAM-1 promoters contain NF-κB binding sites which are located in ~ 200 bp (ICAM-1), and 65 and 75 bp (VCAM-1) upstream of the transcription start site, respectively. These sites are critical for the induction of ICAM-1 and VCAM-1 mRNA transcription (Kim *et al*., 2008). The treatment of *P. gingivalis* LPS markedly stimulated promoter activities in these truncated ICAM-1

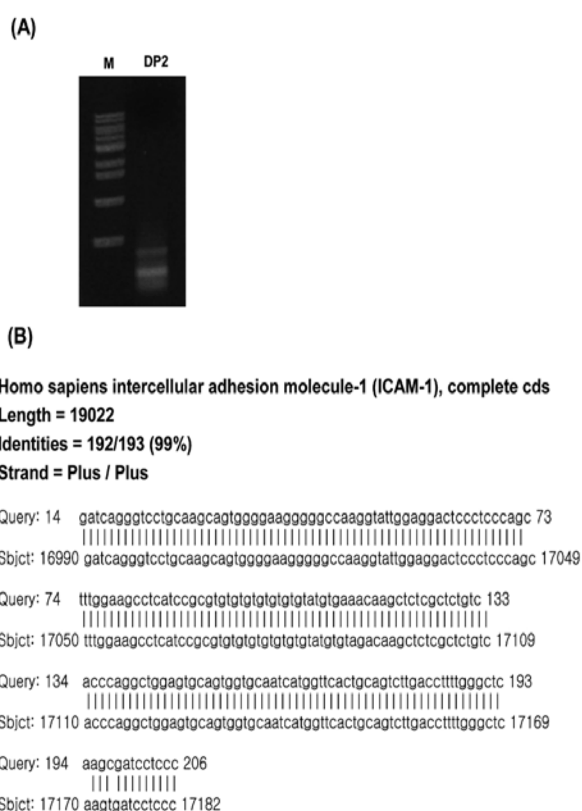

Fig. 1. Representational difference analysis of gene expression between untreated and *P. gingivalis* LPS-stimulated HMECs. A, DP2 was run on a 1.5 % agarose gel and stained with ethidium bromide. Visible smearing bands were excised from the gel and subcloned into the pGEM-T vectors. M, DNA marker; DP2, difference product 2. B, Comparison of nucleotide sequence of identified clone with human ICAM-1 gene.

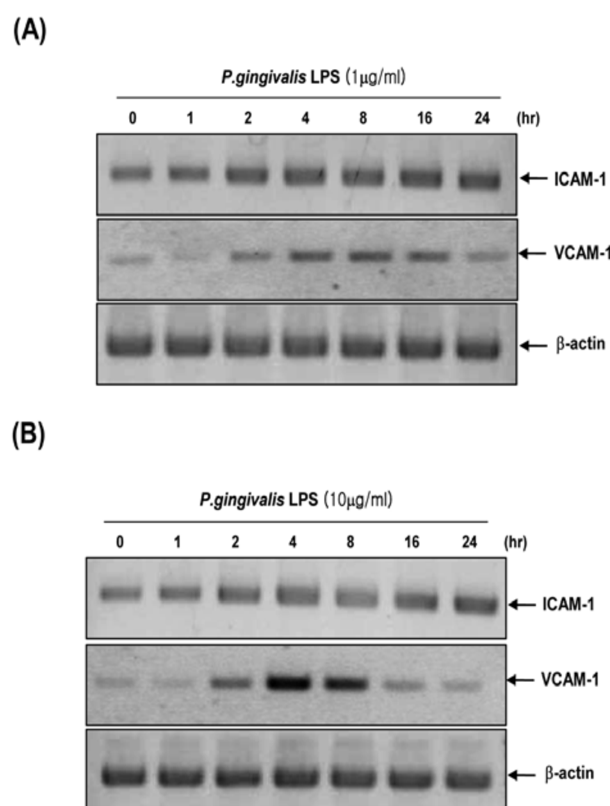

Fig. 2. *P. gingivalis* LPS increases the expression of ICAM-1 and VCAM-1 mRNAs in HMECs. HMECs were incubated with or without *P. gingivalis* LPS (1 μg/ml) (A) or (10 μg/ml) (B) for indicated times. Total RNAs were isolated and then analyzed by RT-PCR using specific primers for human ICAM-1 and VCAM-1. β-actin served as an internal control.

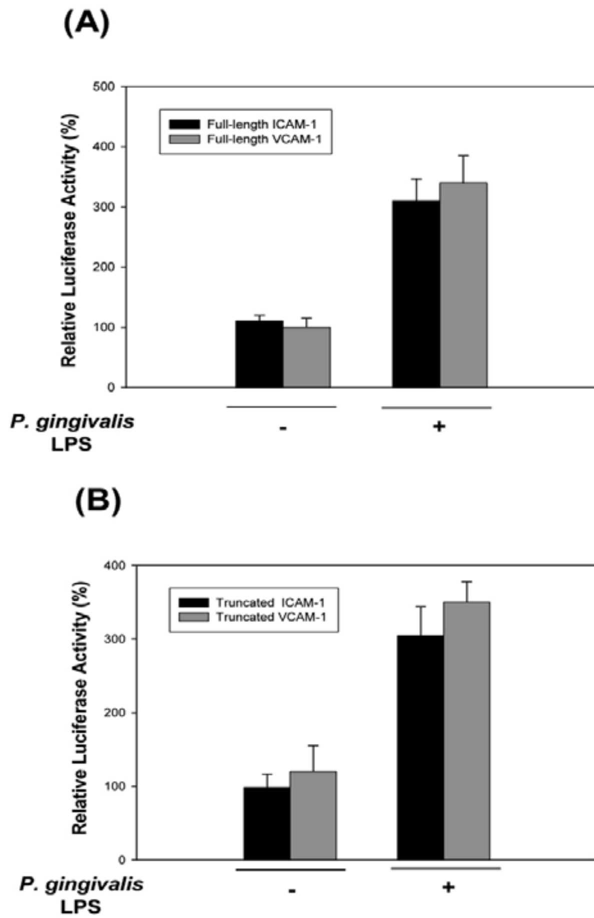

Fig. 3. *P. gingivalis* LPS induces the transcriptional activities of ICAM-1 and VCAM-1 genes through NF-κB. A, HMECs were transiently transfected with full-length ICAM-1 and VCAM-1 luciferase vectors that contain ICAM-1 (1.2kb) or VCAM-1 (1.8kb) promoter regions, respectively and then treated with or without *P. gingivalis* LPS (10 μg/ml) for 12 h. B, HMECs were transfected with truncated ICAM-1 and VCAM-1 promoters that contain NF-κB binding site located ~200 bp (ICAM-1) and 65 and 75 bp (VCAM-1) upstream of the transcription start site, respectively and incubated with or without *P. gingivalis* LPS (10 μg/ml). Data is the mean ± SE of triplicate experiments relative to the luciferase light units in untreated cells (set at 100 %).

and VCAM-1 promoters (Fig. 3B). Reporter activities of truncated ICAM-1 and VCAM-1 promoters are similar to those of full-length ICAM-1 and VCAM-1 promoters. These results indicated that NF-κB binding sites are important for *P. gingivalis* LPS-induced transcriptional activation of ICAM-1 and VCAM-1 genes.

P. gingivalis LPS induces adhesion of leukocytes to endothelial cells *in vitro* and *ex vivo*

Based on uperegulation of ICAM-1 and VCAM-1 by *P. gingivalis* LPS, we next investigated whether *P. gingivalis* LPS regulates the adhesion of leukocytes to endothelium. As shown in Fig. 4A, incubation of *P. gingivalis* LPS with HMECs significantly increased the adhesion of U937 monocytic cells to HMECs. To test whether *P. gingivalis* LPS enhances monocyte binding to the *ex vivo* aortic endothelium, we performed an *ex vivo* adhesion assay using fluorescence-labeled monocytes and an aorta isolated from

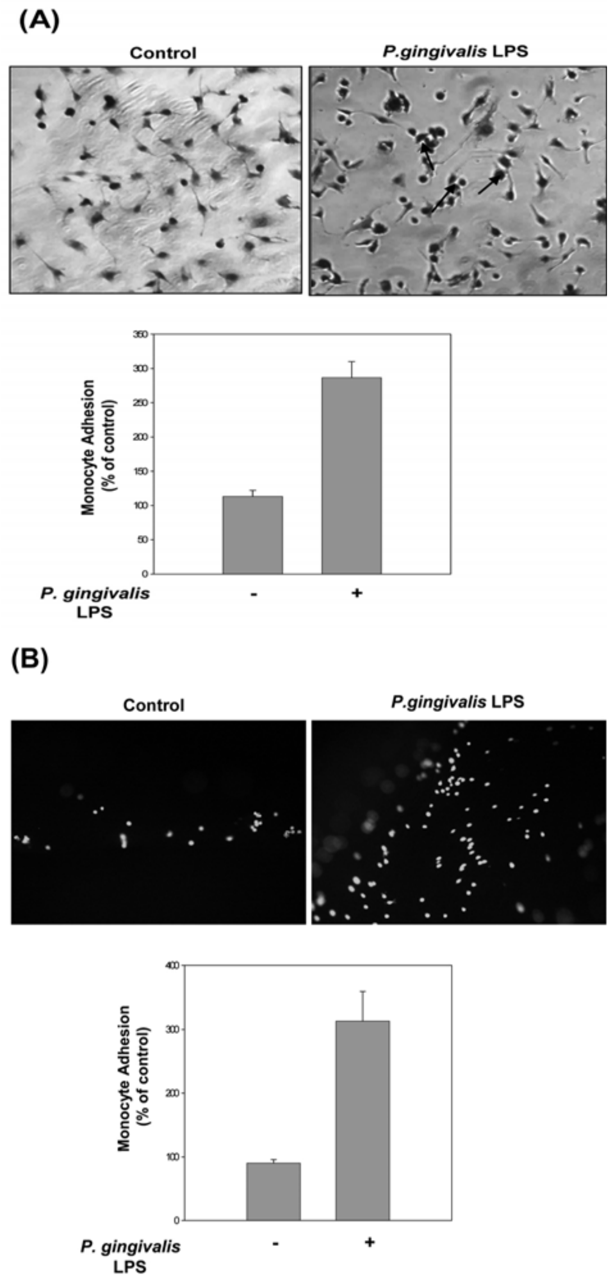

Fig. 4. *P. gingivalis* LPS increases the adhesion of leukocytes to endothelial cells *in vitro* and *ex vivo*. A, HMECs were exposed with or without *P. gingivalis* LPS (10 μg/ml) for 16 h. U937 monocytes were added to HMECs, and numbers of adherent cells were counted. Arrow indicates the adhesion of U937 monocytes to HMECs. B, Rat aorta was isolated, and the incubated with or without *P. gingivalis* LPS (10 μg/ml) for 16 h. Fluorescence-stained monocytes were identified and counted by fluorescence microscopy at 40 × magnification. Data is the mean ± SE relative to adhesion of untreated cells (set at 100 %) in triplicate experiments.

an SD rat. The numbers of fluorescence-labeled adherent monocytes on the surface of the aortic endothelium were markedly increased by treatment of *P. gingvialis* LPS (Fig. 4B).

DISCUSSION

In this study, we performed cDNA-RDA to identify genes differentially expressed under the treatment of *P. gingivalis* LPS in HMECs. We identified ICAM-1 gene, one of the endothelial cell adhesion molecules, which is up-regulated in *P. gingivali*s LPS-stimulated HMECs. We also showed that *P. gingivalis* LPS induces ICAM-1 and VCAM-1 expression via NF-κB activation in HMECs, leading to adhesion of leukocytes to endothelial cells *in vitro* and *ex vivo*.

Periodontal disease is a typical chronic inflammatory disorder which results from infection with periodontal pathogens (Socransky and Haffajee, 1992). The LPS secreted by *P. gingivalis*, a major periodontal pathogen, may be able to activate various nonimmune cell types such as gingival epithelial cells, periodontal ligament cells, and microvascular endothelial cells in periodontal tissues (Bainbridge and Darveau, 2001). Especially, microvascular endothelial cells lining the microvasculature may play a key role in inflammatory reactions through the recruitment of immune cells into periodontal tissues (Walter *et al*., 2004; Mao *et al*., 2004). In the progress of periodontal diseases, endothelial dysregulation in microvasculature has been associated with the pathogenesis of chronic periodontitis and gingivitis (DeCarlo *et al*., 2008; Zoellner *et al*., 2002). Our work demonstrated the effect of *P. gingivalis* LPS on microvascular endothelial cells, particularly the ability to regulate leukocyte adhesion. Blockage of endotheial dysregulation may be able to contribute to protecting against periodontogen-mediated vascular inflammation. Thus, development of anti-vascular inflammatory agents will be beneficial for treatment of periodontal diseases.

P. gingivalis LPS engages with toll-like receptor 4 (TLR4) or TLR2 on cell surface of different cell types, and activates intracellular second messenger such as a number of kinases and transcriptional factors (Wang *et al*., 2000; Harokopakis and Hajishengallis, 2005). It has been reported that *P. gingivalis* LPS-dependent NF-κB activation is mediated by TLR4 in HMECs (Coats *et al*., 2003). Taken these facts into consideration, *P. gingivalis* LPS may stimulate leukocyte adhesion to microvascular endothelial cells through TLR4-dependent activation of microvascular endothelial cells. Of course, further investigations are required to elucidate whether TLR4 participates in the *P. gingivalis* LPS-induced microvascular endothelial dysfunction.

In summary, our results demonstrated the use of cDNA-RDA as an efficient molecular screening method for differentially regulated genes in P. *gingivalis* LPS-treated microvascular endothelial cells; We focused that *P. gingivalis* LPS exerts pro-inflammatory effects on microvascular endothelial cells by induction of ICAM-1 and VCAM-1 expression via the NF-κB activation. Our findings suggest a pathological role of *P. gingivalis* LPS in microvascular endothelial dysfunction. We also identified a number of up-regulated genes having known or unknown functions. Further studies on these genes will be useful to provide a global gene expression profile and their novel functions during *P. gingivalis* LPS-mediated microvascular inflammation.

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