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Streptococcus oralis coaggregation receptor polysaccharides induce inflammatory responses in human aortic endothelial cells

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SUMMARY

***Streptococcus oralis*, belonging to the oral viridans group streptococci, has been detected in human cardiovascular lesions including infective endocarditis and atheromatous plaques. The organism has coaggregation receptor polysaccharides (RPS) on the cell wall, which function as receptors for surface adhesins on other members of the oral biofilm community. The present study examined the capacity of *S. oralis* RPS to induce inflammatory responses in human aortic endothelial cells (HAECs). Purified RPS was used to stimulate HAECs, and the induction of cytokines, adhesion molecules and Toll-like receptors (TLRs) was examined. Involvement of RPS in HAEC invasion by *S. oralis* was also examined. RPS-stimulated HAECs produced more cytokines (interleukin-6, interleukin-8 and monocyte chemoattractant protein-1) and intercellular adhesion molecule-1 than non-stimulated HAECs. The messenger RNA (mRNA) expression of cytokines and adhesion molecules in RPS-stimulated HAECs increased markedly compared with that in non-stimulated HAECs. Upregulation of TLR-2 mRNA expression was demonstrated in RPS-stimulated HAECs. Moreover, TLR-2 mRNA expression and cytokine production were reduced by the incubation of HAECs with inhibitors against p38 mitogen-activated protein kinase and nuclear factor-κB. An RPS-defective mutant of *S. oralis* showed greater invasion into HAECs than an RPS-possessing strain. However, HAECs invaded by the RPS-defective mutant produced less cytokines than HAECs invaded by the RPS-possessing strain, indicating that RPS can stimulate HAECs intracellularly. These results suggest that *S. oralis* RPS may be an important contributor to the pathogenesis of cardiovascular diseases such as infective endocarditis and atherosclerosis.**

INTRODUCTION

Streptococcus oralis, one of commensal bacteria inhabiting the oral cavity, belongs to the oral viridans group streptococci. It has been implicated as a potential causative organism of human cardiovascular diseases including infective endocarditis and

atherosclerosis. *Streptococcus oralis* is frequently isolated from infective endocarditis (Douglas *et al.*, 1993), and ribosomal DNA of the *Streptococcus mitis* group including *S. oralis* was detected in atheromatous plaques (Lehtiniemi *et al.*, 2005).

Infective endocarditis is a life-threatening infectious disease for patients with congenital and acquired heart diseases, those with prosthetic valves, and intravenous drug users (Que & Moreillon, 2011). Despite advances in early diagnostic detection, antimicrobial therapy, and surgical techniques, the incidence of infective endocarditis appears to be increasing (Murdoch *et al.*, 2009; Castillo *et al.*, 2011). Atherosclerosis is a progressive disease involving lipid accumulation in the arterial intima resulting in the formation of vascular lesions that are characterized by inflammation, cell death, and fibrosis (Andersson *et al.*, 2010; Lundberg & Hansson, 2010). Atherosclerosis is one of the most important contributors to the development of cardiovascular disease and is the leading cause of death and illness in developed countries (Libby *et al.*, 2002).

Several virulence factors of oral streptococci have been shown to cause inflammatory responses in human cells, including surface protein antigen I/II, serotype-specific polysaccharides, and lipoteichoic acid (Vernier *et al.*, 1996; Engels-Deutsch *et al.*, 2003). Regarding polysaccharides, *Streptococcus mutans* has serotype-specific rhamnose glucose polymer (RGP) on the cell wall (Linzer *et al.*, 1987; Pritchard *et al.*, 1987), and this RGP has been shown to bind to human cells and stimulate cytokine production (Vernier *et al.*, 1996; Engels-Deutsch *et al.*, 2003). Moreover, the RGP of *S. mutans* was shown to be involved in the induction of infective endocarditis in a rat model (Nagata *et al.*, 2006). *Streptococcus oralis* has coaggregation receptor polysaccharides (RPS) on the cell wall. The RPS function as receptors for the lectin-like surface adhesins on other members of the oral biofilm community (Yoshida *et al.*, 2006). Six different types of RPS have been identified (Cisar *et al.*, 1997), and the recognition motifs within RPS have been clarified by carbohydrate engineering (Yoshida *et al.*, 2008). However, the ability of RPS to cause inflammatory responses in human cells has not been examined.

This is the first report examining the role of *S. oralis* RPS in inducing inflammatory responses in human aortic endothelial cells (HAECs). We examined the ability of purified *S. oralis* RPS to induce the production of proinflammatory cytokines including interleukin-6 (IL-6), IL-8, and monocyte chemoattractant protein-1 (MCP-1); adhesion molecules including E-selectin, P-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1); and toll-like receptors (TLRs) from HAECs. These cytokines, adhesion molecules, and TLRs are involved in the initiation and progression of cardiovascular diseases (Edfeldt *et al.*, 2002; Libby *et al.*, 2002; Hansson, 2005; Yeh *et al.*, 2006; Shun *et al.*, 2009). In addition, we examined the invasion of HAECs by the RPS-possessing wild-type strain and an RPS-deficient mutant, and measured the subsequent production of proinflammatory cytokines. We found that the *S. oralis* RPS has the capacity to induce inflammatory responses in HAECs. In addition, RPS is involved in the invasion of HAECs by the bacteria and subsequent cytokine induction.

MATERIALS AND METHODS

Bacterial strains

Streptococcus oralis ATCC 10557 from the stock culture collection in the Department of Preventive Dentistry, Kagoshima University, Graduate School of Medical and Dental Sciences, Japan was grown anaerobically in Todd–Hewitt broth (Becton, Dickinson and Company, Sparks, MD). *Streptococcus oralis* TC2, an RPS-defective mutant (*S. oralis* ATCC 10557 containing *ermAM* in place of the truncated *wzy* and *wzy*) (Yoshida *et al.*, 2008) was grown anaerobically in the same medium containing 10 μg ml^{-1} erythromycin.

Isolation of *S. oralis* RPS

The *S. oralis* RPS was purified as previously described (Cisar *et al.*, 1997). Briefly, after harvesting *S. oralis* ATCC 10557 cells from 128-l stationary-phase cultures, the cells were treated with 0.1% Triton-X-100 to disrupt membranes and then digested with ribonuclease A (Sigma-Aldrich, St Louis, MO) followed by *Streptomyces griseus* protease (Sigma-Aldrich) to facilitate the removal of cytoplasmic material. Surface polysaccharides were solubilized by mutanolysin (Sigma-Aldrich) digestion of the resulting crude cell walls. Protein was precipitated from

mutanolysin digests by adding cold trichloroacetic acid to a final concentration of 5%. The soluble fraction was neutralized and dialysed against water before purification of RPS by gradient elution from a diethylaminoethyl Sepharose FF anion-exchange column (GE Healthcare Sciences, Uppsala, Sweden), in which RPS was eluted as a single peak. Purified polysaccharides were detected by a dot blot assay using an antibody against *S. oralis* ATCC 10557 RPS and an assay for total carbohydrate content by the phenol–sulfuric acid reaction (Dubois *et al.*, 1956) with glucose as a standard. The RPS-containing fractions were pooled and dialysed extensively against distilled water, and then lyophilized. The purity of lyophilized preparation was verified by nuclear magnetic resonance spectroscopy (Yoshida *et al.*, 2005), and no components other than RPS constituents were detected. The molecular weight of RPS was more than 1×10^5 and 1 μg RPS is equivalent to approximately 1×10^8 bacterial cells.

Cell culture

Human aortic endothelial cells (Invitrogen Corporation, Carlsbad, CA) were maintained in Humedia-EG2 medium supplemented with low-serum growth supplement (Kurabo Industries Ltd, Osaka, Japan) at 37°C in 5% CO_2. Sixth-passage cells were used in all experiments.

Invasion of HAECs by *S. oralis*

Antibiotic protection assays were performed to examine the invasion of HAECs by *S. oralis* based on the method of Stinson *et al.* (2003), with some modifications (Nagata *et al.*, 2011). The HAECs were plated in 48-well plates at a density of 2×10^4 cells per well 24 h before adding the bacterial suspensions. The multiplicity of infection (MOI) was calculated from the number of HAECs per well when seeded. Bacterial cells were collected by centrifugation at 1700 ***g*** for 10 min, washed with phosphate-buffered saline (PBS), and suspended in medium. Bacterial suspensions (0.2 ml) were added to HAECs at an MOI of 1000 and incubated at 37°C in 5% CO_2 for 2 h. The HAECs were washed and externally adherent bacteria were killed by incubating the infected HAECs for 1 h with 0.4 ml medium containing gentamicin (200 μg ml^{-1}) and penicillin G (20 μg ml^{-1}). After exposure to antibiotics, the resulting monolayers were detached by adding 0.1 ml trypsin–ethylenediaminetetraacetic acid (Kurabo Industries). Then, 0.4 ml 0.1% Tween-20 was added, the plates were incubated at 37°C for 20 min, and finally the lysates were pipetted vigorously and plated on Todd–Hewitt broth agar plates. The colony-forming units of invasive bacteria were counted after the plates were incubated anaerobically for 2 days at 37°C.

Determination of bacterial invasion by confocal scanning laser microscopy

To confirm invasion of bacteria, a double-fluorescence technique was used according to the previous report (Nagata *et al.*, 2011). Briefly, HAECs were grown in 35-mm collagen type I-coated glass-bottomed culture dishes (Asahi Glass Co. Ltd., Tokyo, Japan) and infected with *S. oralis* ATCC 10557 or *S. oralis* TC2 under the conditions described above. Cells were fixed with 3% paraformaldehyde (Sigma-Aldrich) for 10 min, washed with PBS, and then incubated with a rabbit anti-*S. oralis* serum diluted 1 : 500 with PBS–0.5% bovine serum albumin (BSA) for 60 min at room temperature. Following incubation, culture dishes were washed with PBS and incubated with Alexa Fluor 633-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR) diluted 1 : 500 with PBS–0.5% BSA for 30 min at room temperature to visualize attached bacteria. Invasive bacteria were then stained by first permeabilizing HAECs using 0.4% Triton-X-100 solution for 5 min, and then staining with the rabbit anti-*S. oralis* serum followed by AlexaFluor 555-conjugated goat anti-rabbit IgG (Molecular Probes) diluted 1 : 500 as described above. Actin filaments were stained with AlexaFluor 488 conjugated to phalloidin (Molecular Probes) for 30 min at room temperature to visualize the cellular cytoskeleton. Culture dishes were examined by confocal scanning laser microscopy using a TCS-SP5 microscope (Leica Microsystems GmbH, Wetzlar, Germany) with a DMI6000 B fluorescence microscope (Leica) and a 63× oil immersion objective.

Cytokine production by HAECs

Cytokine production was analysed for HAECs stimulated with purified RPS, invaded HAECs, and HAECs incubated with antibiotics-killed bacteria. To examine HAECs stimulated with purified RPS, HAECs

(2×10^4 cells per well) were mixed with RPS at concentrations of 25, 50, 100, and 200 μg ml^{-1} and incubated for 24 h. The supernatants were collected and filtered through 0.2-μm pore-size filters. To examine the invaded HAECs, cells were co-cultured with each *S. oralis* strain at an MOI of 1000 for 2 h. After exposure to antibiotics for 1 h, invaded HAECs were incubated in 0.2 ml fresh medium containing gentamicin (50 μg ml^{-1}) for 2 or 24 h. To examine the HAECs incubated with antibiotics-killed bacteria, bacterial cells were treated for 1 h in medium containing gentamicin (200 μg ml^{-1}) and penicillin G (20 μg ml^{-1}). Killed bacterial suspensions (0.4 ml) were added to HAECs at an MOI of 1000 and incubated for 1 h. Subsequently, HAECs were washed and further incubated in 0.2 ml fresh medium containing gentamicin (50 μg ml^{-1}) for 24 h. The supernatants were collected and processed similarly. Concentrations of IL-6, IL-8, and MCP-1 were analysed using enzyme-linked immunosorbent assay (ELISA) kits (Peprotec, London, UK for IL-8 and MCP-1; Mabtech AB, Stockholm, Sweden for IL-6) following the manufacturer's instructions. Unstimulated HAECs were used as controls.

Adhesion molecule production by HAECs

Adhesion molecule production was analysed for HAECs stimulated with purified RPS. Cell ELISA was performed to examine the expression of adhesion molecules such as E-selectin, P-selectin, ICAM-1, and VCAM-1 on the HAEC surface (Loizou *et al.*, 2010). The HAECs were plated in 96-well plates at a density of 8×10^3 cells per well 24 h before stimulation, and were incubated with RPS for 2 h (E-selectin and P-selectin) or 24 h (ICAM-1 and VCAM-1) at 37°C. Subsequently, the cells were fixed with 0.1% glutaraldehyde in ice-cold PBS for 30 min at 4°C. Plates were blocked for 1 h at 37°C with PBS containing 1% BSA (PBS-BSA), followed by incubation with mouse monoclonal antibody against human E-selectin, P-selectin, ICAM-1, or VCAM-1 (AbD Serotec, Kidlington, UK) in PBS-BSA (2 μg ml^{-1}) overnight at 4°C. Next, the plates were washed three times with 0.1% Tween-20 in PBS-BSA and incubated at room temperature for 1 h with a horseradish peroxidase-conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD) at a dilution of 1 : 1000. Then the plates were washed with 0.1% Tween-20 in PBS-BSA and the expression of adhesion molecules was quantified by the addition of 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid liquid substrate solution (Sigma-Aldrich). The absorbance at 405 nm was measured using a microplate reader. Unstimulated HAECs were used as controls.

Real-time reverse transcription-polymerase chain reaction analysis

For quantitative analysis of messenger RNA (mRNA) expression by the RPS-stimulated HAECs, total RNA was isolated from HAECs using the RNeasy mini kit (Qiagen K.K., Tokyo, Japan). Total RNA (0.5 μg) from each sample was used to generate complementary DNA (cDNA; High-Capacity cDNA Reverse Transcription kits; Applied Biosystems, Foster City, CA) in a 20-μl volume. Then, 2 μl of the cDNA obtained was used for each subsequent reaction. Polymerase chain reaction (PCR) amplification was performed using the StepOne™ Real-time PCR System (Applied Biosystems) with 2× TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) for IL-6, IL-8, MCP-1, selectin, ICAM-1, VCAM-1, TLR-1, TLR-2, and TLR-4. The amplification consisted of an initial denaturation at 95°C for 20 s, followed by 50 cycles at 95°C for 1 s, and 60°C for 20 s. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was amplified in parallel with the gene of interest. Relative changes in gene expression were analysed using the comparative threshold cycle method (Livak & Schmittgen, 2001) and reported as the difference (*n*-fold) relative to the value for a calibrator cDNA (control, non-stimulated HAECs) prepared in parallel with the experimental cDNAs.

Effects of signal transduction intermediate inhibitors

To identify the pathways involved in the upregulation of TLR-2 mRNA in RPS-stimulated HAECs, an inhibition assay was performed as described in a previous report (Satta *et al.*, 2008). HAECs were incubated in medium containing an inhibitor of p38 mitogen-activated protein (MAP) kinase (10 μM SB202190, Sigma-Aldrich), c-*Jun* N-terminal kinase (JNK) (5 μM SP600125, Tocris Bioscience, Ellisville, MO), or nuclear factor-κB (NF-κB) (2 μM Bay11-7082, Sigma-Aldrich) at 37°C for 1 h. Subsequently, RPS was

added to the medium at a concentration of 200 μg ml^{-1} and the cells were incubated for 24 h. Total RNA was extracted and real-time reverse transcription-PCR analyses were performed as described above. In addition, the culture supernatants collected after incubation were subjected to cytokine determination as described above.

Statistical analysis

To analyse the statistical differences between groups, the Student's *t*-test and one-way analysis of variance, followed by Bonferroni's or Dunnett's tests, were used when appropriate.

RESULTS

Cytokine, adhesion molecule, and TLR induction by HAECs stimulated with RPS

Cytokine production was examined in HAECs stimulated by the purified RPS. Cells were incubated in medium with several concentrations of RPS, and a dose-dependent increase in the production of IL-6, IL-8, and MCP-1 was observed in RPS-stimulated HAECs (Fig. 1). Using cell ELISA, the changes in adhesion molecule production were also examined in HAECs stimulated by purified RPS. Cells were stimulated with several concentrations of RPS, and a dose-dependent increase in ICAM-1 production was observed (Fig. 2). In contrast, RPS did not induce production of VCAM-1, E-selectin, or P-selectin in RPS-stimulated HAECs.

The expression changes of mRNA for cytokines and adhesion molecules in RPS-stimulated HAECs were compared with the mRNA expression of unstimulated HAECs. The RPS-stimulated HAECs expressed increased levels of IL-6, IL-8, and MCP-1 mRNA at every time-point compared with non-stimulated HAECs (Fig. 3A). RPS-stimulated HAECs expressed higher levels of ICAM-1, VCAM-1, and selectin mRNA at every time-point compared with non-stimulated HAECs (Fig. 3B). The maximal values of expression were observed at 2 h for VCAM-1 and selectin mRNA, and at 8 h for ICAM-1 mRNA.

Regarding TLR induction, RPS-stimulated HAECs expressed higher levels of TLR-1 and TLR-2 mRNA at 8 and 24 h compared with non-stimulated HAECs, and expression of TLR-2 mRNA was highest at 8 h

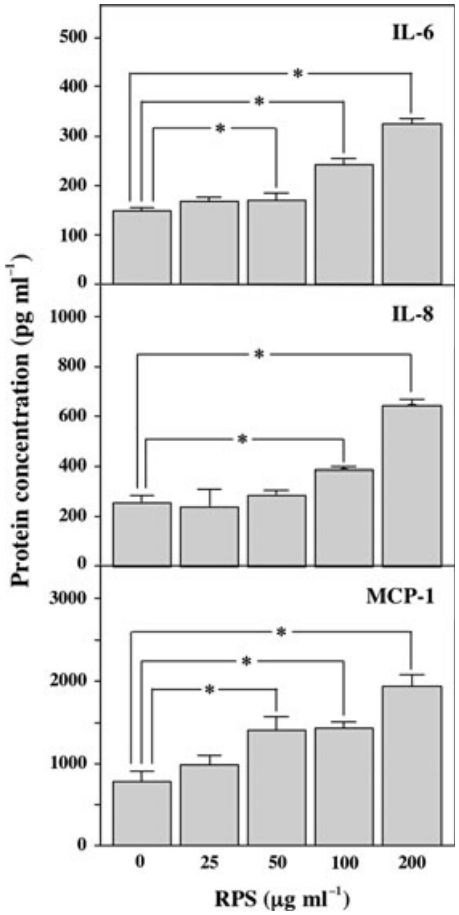

Figure 1 Cytokine production from human aortic endothelial cells (HAECs) stimulated with receptor polysaccharides (RPS). The HAECs were stimulated with several concentrations of RPS for 24 h. The culture supernatants were collected and the concentrations of cytokines were determined by enzyme-linked immunosorbent assay. The data are representative of three independent experiments, and values indicate the means ± standard deviations of triplicate assays. $^*P < 0.05$ compared with control (RPS 0 μg ml^{-1}) as determined using one-way analysis of variance followed by Dunnett's test.

(Fig. 3C). In contrast, RPS-stimulated HAECs did not induce TLR-4 mRNA expression compared with non-stimulated HAECs.

Pathways mediating changes in TLR-2 and cytokine induction by RPS

To investigate the contribution of the inflammatory intermediates p38 MAP kinase, JNK, and NF-κB to the induction of TLR-2 by RPS stimulation, HAECs were pretreated with inhibitors of these signal transduction intermediates before RPS stimulation.

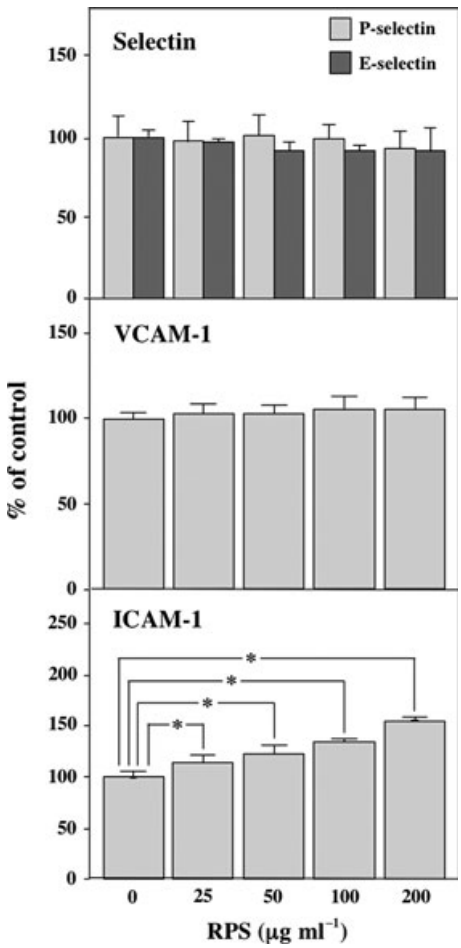

Figure 2 Adhesion molecule production from human aortic endothelial cells (HAECs) stimulated with receptor polysaccharides (RPS). The HAECs were stimulated with several concentrations of RPS for 2 h (E-selectin and P-selectin) or 24 h [intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1)], fixed with 0.1% glutaraldehyde and then the production of adhesion molecules was measured by cell enzyme-linked immunosorbent assay. The data are representative of three independent experiments, and values indicate the means ± standard deviations of triplicate assays. *$P < 0.05$ compared with control as determined using one-way analysis of variance followed by Dunnett's test.

SB202190 (an inhibitor of p38 MAP kinase) and Bay11-7082 (an inhibitor of NF-κB) strongly inhibited the TLR-2 mRNA induction by RPS compared with the control (without inhibitors) (Fig. 4A). However, SP600125 (inhibitor of JNK) showed no inhibitory effect on the TLR-2 mRNA induction. Simultaneously, we examined the effects of signal transduction intermediate inhibitors on cytokine production from HAECs. SB202190 strongly inhibited IL-6 and MCP-1 induction by RPS compared with the control (Fig. 4B). MCP-1 induction was also moderately inhibited by SP600125 and Bay11-7082. Interleukin-6 induction was weakly inhibited by Bay11-7082. However, no inhibitors reduced IL-8 induction.

Invasion of HAECs by *S. oralis*

To examine the role of RPS in the invasion of endothelial cells by *S. oralis*, HAECs were co-cultured with *S. oralis* ATCC 10557 and *S. oralis* TC2. Both strains were able to invade HAECs, but the invasive bacterial number (colony-forming units per well) was significantly different (3030 ± 254 for *S. oralis* ATCC 10557 and 8259 ± 571 for *S. oralis* TC2; mean ± standard deviation of three independent experiments) ($P < 0.05$ by Student's *t*-test). The confocal scanning laser microscopy revealed HAEC invasion by *S. oralis* ATCC10557 and TC2 (Fig. 5), though the quantitative analysis of invasive bacteria was difficult to perform. Intracellular bacteria are stained red, and the HAEC skeleton is stained green. Because extracellular bacteria are stained blue and red, they appear purple.

Cytokine induction by HAECs after stimulation by *S. oralis*

Cytokine production by the invaded HAECs was examined using ELISA. When non-invasive bacteria were killed after a 2-h co-culture period and invaded HAECs were further cultured for 2 or 24 h, HAECs invaded by *S. oralis* ATCC 10557 showed more MCP-1 production at 24 h and IL-8 production at 2 h than non-invaded HAECs and HAECs invaded by *S. oralis* TC2 (Fig. 6). In contrast, none of the antibiotics-killed *S. oralis* strains increased cytokine production from co-cultured HAECs compared with non-stimulated HAECs (Fig. S1).

DISCUSSION

We investigated the role of RPS from *S. oralis* in the induction of inflammatory responses in HAECs. The RPS-stimulated HAECs produced more cytokines (IL-6, IL-8, and MCP-1) and adhesion molecules (ICAM-1) than non-stimulated HAECs. The mRNA expression of all tested cytokines and adhesion molecules by RPS-stimulated HAECs increased markedly compared with that by non-stimulated HAECs. As for

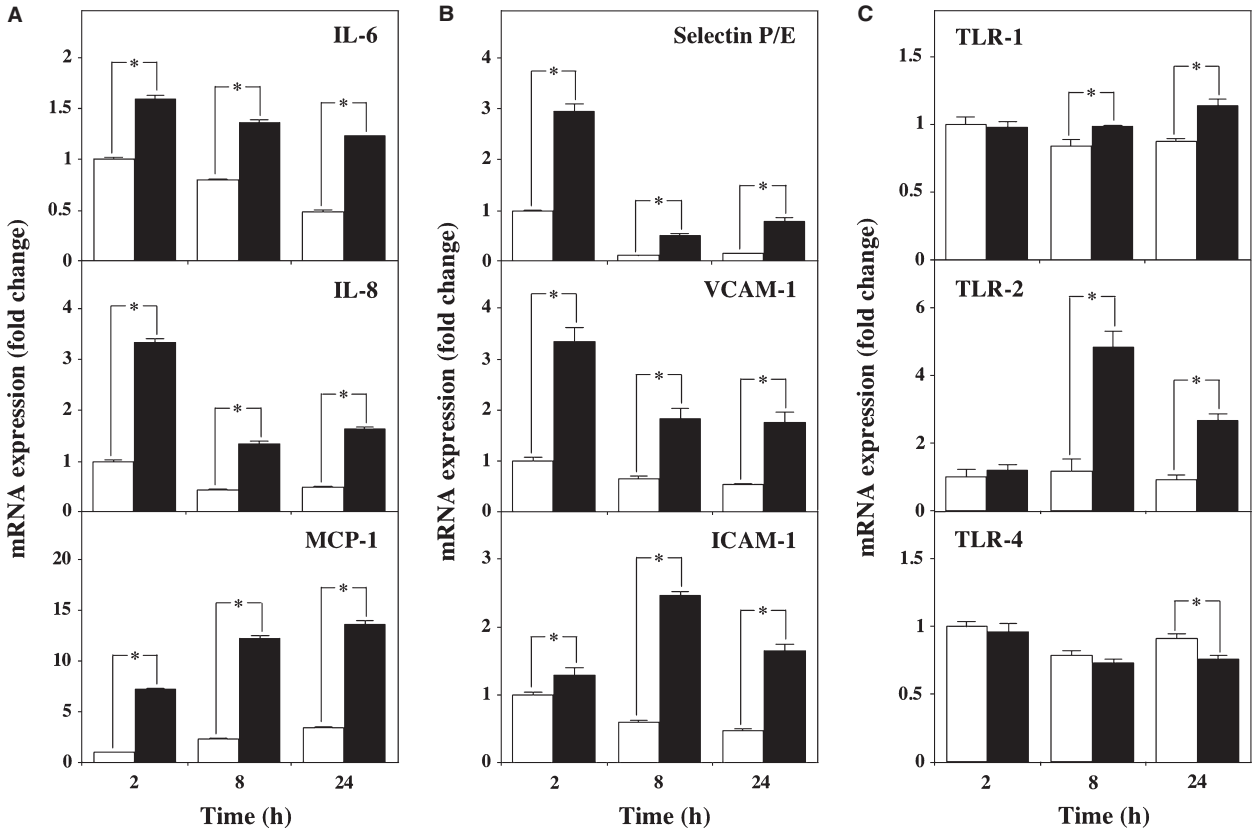

Figure 3 Cytokine (A), adhesion molecule (B), and Toll-like receptor (TLR) (C) gene expression in human aortic endothelial cells (HAECs) stimulated with receptor polysaccharides (RPS). The HAECs were stimulated with 200 μg ml^{-1} RPS for 2, 8 or 24 h and quantitative analysis of messenger RNA (mRNA) expression was performed with real-time reverse transcription-polymerase chain reaction. Open bars, non-stimulated HAECs (control); solid bars, HAECs stimulated with RPS. The data are representative of three independent experiments, and values indicate the means ± standard deviations of triplicate assays. *$P < 0.05$ compared with control at each time-point as determined by Student's *t*-test.

selectin and VCAM-1, RPS induced increased expression of mRNA but not at the protein level. This discrepancy seems to be the result of specific post-transcriptional and/or post-translational regulatory mechanisms (Marui *et al.*, 1993; Kacimi *et al.*, 1998). Regarding the specificity of the effects of *S. oralis* on HAECs, Hahn *et al.* (2005) reported that *S. oralis* ATCC 10557 induced the expression of interleukin-12, CD83, and CD86 from human mononuclear cells, though the virulence factors of the organism responsible for the induction were not examined. We used only HAECs to determine the inflammation-inducible effects of *S. oralis* RPS, and it is uncertain whether the effects are HAEC specific or not. Polysaccharide-containing bacterial components such as lipopolysaccharide (Tsoyi *et al.*, 2009; Zhang *et al.*, 2011) and peptidoglycan (Timmerman *et al.*, 1993) have been shown to induce inflammatory responses in human cells. These bacterial components are hetero-type complexes of sugars and lipids/proteins. Several reports have demonstrated that bacterial surface polysaccharides, composed of monosaccharide repeating units, such as staphylococcal capsular polysaccharide (Soell *et al.*, 1995a) and *S. mutans* RGP (Vernier *et al.*, 1996; Engels-Deutsch *et al.*, 2003) have inflammation-inducible effects on human endothelial cells. In this study, *S. oralis* RPS induced IL-6 and IL-8, as was seen by staphylococcal capsular polysaccharide and *S. mutans* RGP. The RPS purified in this study is composed of a distinct hexasaccharide repeating unit and each repeating unit contains galactose, *N*-acetylgalactosamine, rhamnose, and glucose (Yoshida *et al.*, 2006). Although the sugar compositions of staphylococcal capsular

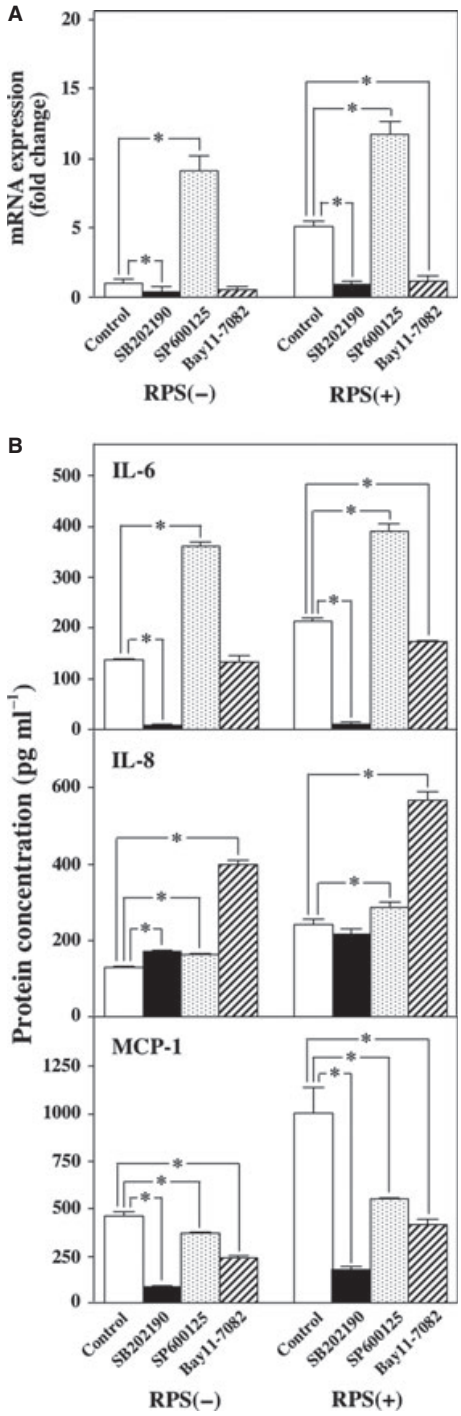

Figure 4 Effects of signal transduction intermediate inhibitors on the Toll-like receptor 2 (TLR-2) gene expression (A), and cytokine production (B) from human aortic endothelial cells (HAECs) stimulated with receptor polysaccharides (RPS). The HAECs were pretreated with inhibitors for 1 h and then incubated with or without RPS (200 μg ml^{-1}) for 24 h. The culture supernatants were collected and quantitative analysis of messenger RNA (mRNA) expression for TLR-2 was performed. The concentrations of cytokines were determined by enzyme-linked immunosorbent assay. RPS(−), without RPS; RPS(+), with RPS. The data are representative of three independent experiments, and values indicate the means ± standard deviations of triplicate assays. $^*P < 0.05$ compared with control (no inhibitors) as determined using one-way analysis of variance followed by Dunnett's test.

polysaccharides, *S. mutans* RGP, and *S. oralis* RPS differ from one another, all of the polysaccharides induced the same proinflammatory cytokines from endothelial cells. Other reports have demonstrated the immunomodulatory effects of polysaccharides that are composed of several monosaccharides (Karnjanapratum & You, 2011; Lin *et al.*, 2011; Liu *et al.*, 2011), and each polysaccharide induced an increase or a decrease in the cytokine production of different types from human cells. Therefore, it appears that the immunomodulatory effect of each polysaccharide on human cells differs depending on the differences in its sugar composition and polysaccharide structure.

We demonstrated that RPS induced TLR-2 mRNA expression in HAECs. The TLRs function as the recognition receptors and trigger signal transduction to initiate immune responses, including cytokine release. Induction of TLRs by bacterial components has been demonstrated. Lipopolysaccharide of gram-negative bacteria induces TLR-4 expression in human endothelial cells of coronary artery and aorta (Zeuke *et al.*, 2002; Yumoto *et al.*, 2005). TLR-2 is the primary host receptor involved in inflammatory responses to gram-positive bacteria and several bacterial components such as peptidoglycan and lipoteichoic acid have been proposed to be TLR-2 ligands (Dziarski & Gupta, 2005; Seo *et al.*, 2008). From our results, the RPS appears to be a TLR-2 inducer in HAECs; the p38 MAP kinase and NF-κB were required in the inflammatory activation process with RPS. Satta *et al.* (2008) reported that TLR-2 induction by lipopolysaccharide, tumor necrosis factor-α, and IL-1β depended on p38 MAP kinase, NF-κB, and JNK. Several reports also have shown that the induction of TLR-2 by lipopolysaccharide depends on p38 MAP kinase and NF-κB (An *et al.*, 2002; Fan *et al.*, 2003). Induction of TLR-2 in RPS-stimulated HAECs could be caused in the same manner. On the other hand, it is unclear whether RPS is recognized or not

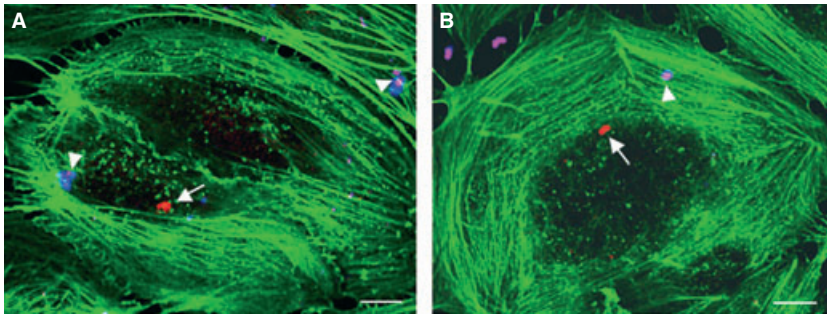

Figure 5 Invasion of bacterial cells into human aortic endothelial cells (HAECs) visualized by confocal scanning laser microscopy following dual labeling. The HAECs were co-cultured with bacteria at a multiplicity of infection (MOI) of 1000 for 2 h. Bacterial cells invading HAECs are stained red (indicated by arrows), whereas extracellular bacteria are stained purple (indicated by an arrow head). (A) *Streptococcus oralis* ATCC 10557, (B) *S. oralis* TC2. Bar represents 10 μm.

by TLR-2 in HAECs. The components of RPS are carbohydrates. Many TLR ligands contain carbohydrate moieties, but the non-carbohydrate portion seems important for recognition and activation of TLRs (Jin *et al.*, 2007). Further studies on the interaction between carbohydrates and TLR-2, especially the structure/activity relationship, may provide advanced insights into the mechanism of RPS-induced TLR-2 expression. Regarding cytokine production from RPS-stimulated HAECs after treatment with signal transduction intermediate inhibitors, the results showed that MCP-1, IL-6, and IL-8 were clearly not co-regulated. The MCP-1 induction from RPS-stimulated HAECs was reduced by three inhibitors of p38 MAP kinase, JNK, and NF-κB, and the IL-6 induction was reduced by an inhibitor of p38 MAP kinase. However, no inhibitors reduced the IL-8 induction. These results suggest that other signal transduction pathways including janus kinase/signal transducer and activator of transcription are involved in the regulation of IL-6 or IL-8 transcription (Legendre *et al.*, 2003; Gharavi *et al.*, 2007) in RPS-stimulated HAECs. In addition, the inhibitor of JNK enhanced the induction of IL-6 and the inhibitor of NF-κB increased IL-8 both in the presence and absence of RPS. The inhibitors used in this study themselves might have stimulated certain signaling molecules to induce IL-6 or IL-8.

The antibiotics protection assays and the confocal scanning laser microscopy revealed that *S. oralis* ATCC 10557 (an RPS-possessing wild-type strain) and TC2 (an RPS-defective mutant) had the capacity to invade HAECs, as similarly demonstrated using *S. oralis* ATCC 35037 in a previous report (Nagata *et al.*, 2011). In the confocal scanning laser microscopy, quantitative analysis of invasive bacteria was difficult to perform because the bacterial number was low compared with number of HAECs. Therefore, the antibiotics protection assay seems suitable for the count of invasive bacteria. Interestingly, *S. oralis* TC2 showed greater invasion into HAECs than *S. oralis* ATCC 10557. Capsular polysaccharide-deficient mutants of group B streptococci (Gibson *et al.*, 1993) and the RGP-deficient mutant of *S. mutans* (Tsuda *et al.*, 2000) exhibit enhanced invasion of human cells. Lack of RPS on the cell surface of *S. oralis* might have exposed adhesive ligands, allowing recognition of target cells and resulting in the greater invasion of HAECs by the RPS-defective mutant.

To examine the cytokine induction from *S. oralis*-stimulated HAECs, we used HAECs invaded by bacteria. In preliminary experiments, we observed morphological changes of HAECs after a co-culture period of more than 8 h with *S. oralis* strains at an MOI of 1. Therefore, we used invaded HAECs to stimulate for a longer period of 24 h. The invasive bacterial numbers were approximately 3×10^3 to 8×10^3 colony-forming units per well, and we think this is biologically significant because several streptococcal strains induced cytokine production at the same level of invasive bacterial numbers (Nagata *et al.*, 2011). The HAECs invaded by the RPS-possessing wild-type strain produced more cytokines than those invaded by the RPS-defective mutant, though the bacterial number of invasive RPS-possessing strain was fewer than that of the RPS-defective strain. This result reflects the cytokine-inducible capacity of RPS. To determine cytokine production

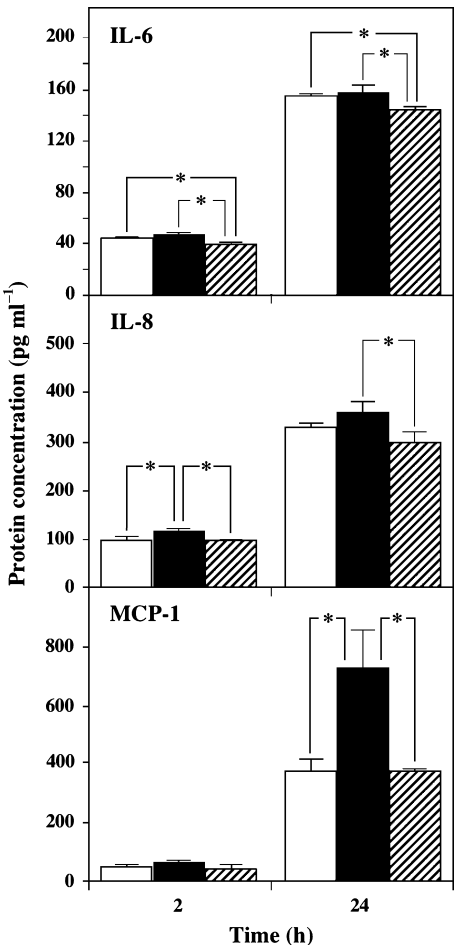

Figure 6 Cytokine production from human aortic endothelial cells (HAECs) invaded by *Streptococcus oralis* ATCC 10557 and TC2. Cells were co-cultured with bacteria at a multiplicity of infection (MOI) of 1000 for 2 h. After killing the non-invasive bacteria, HAECs invaded by bacteria were incubated in medium for 2 or 24 h. The concentrations of cytokines in culture supernatants were determined by enzyme-linked immunosorbent assay. Open bars, non-invaded HAECs (control); solid bars, HAECs invaded by *S. oralis* ATCC 10557; hatched bars, HAECs invaded by *S. oralis* TC2. The data are representative of three independent experiments, and values indicate the means ± standard deviations of triplicate assays. $^*P < 0.05$ as determined using one-way analysis of variance followed by Bonferroni's test.

from invaded HAECs, we performed gentamicin/penicillin treatment to kill non-invasive external bacteria. It is possible that the penicillin treatment would release cell wall components of bacteria including RPS and they could stimulate HAECs extracellularly. Therefore, we prepared a gentamicin/penicillin-killed bacterial suspension and examined its effect in stimulating HAECs. It could not induce cytokine production from co-cultured HAECs, suggesting that the observed cytokine production from invaded HAECs was due to intracellular bacteria. In addition, there is a possibility that RPS is recognized by receptors located on the surface of HAECs. Soell *et al.* (1995b) demonstrated that activation of human monocytes by *S. mutans* RGP is mediated by CD14 antigen on the monocytic cells. Detection of receptors for RPS on the surface of HAECs might provide further insight to clarify the inflammation-inducing mechanism of RPS.

Oral bacteria frequently enter the bloodstream following tooth extraction, scaling, and even daily oral practices such as tooth brushing or flossing, and most of the prevalent bacteria detected in blood specimens belong to the genus *Streptococcus* (Roberts *et al.*, 1997; Lockhart *et al.*, 2008; Olsen, 2008). Once oral bacteria enter the bloodstream, they are thought to cause mediator expression and initiate a cascade of inflammatory responses in the target tissues (Fowler *et al.*, 2001). We demonstrated that *S. oralis* RPS induces inflammatory responses in HAECs, in which increased production of cytokines, adhesion molecules, and TLR-2 is observed. Oral care practices are routine daily activities throughout life, and it is possible that aortic endothelial cells are exposed to frequent challenges by oral viridans group streptococci including *S. oralis*, and the RPS may facilitate the activation of inflammatory responses in aortic endothelial cells. Our results indicate that *S. oralis* RPS may be an important contributor to the pathogenesis of cardiovascular diseases such as infective endocarditis and atherosclerosis.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. Effect of antibiotics-killed bacteria on the cytokine production from human aortic endothelial cells.

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