Sensitization of Human Aortic Endothelial Cells to Lipopolysaccharide via Regulation of Toll-Like Receptor 4 by Bacterial Fimbria-Dependent Invasion

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Received 22 April 2005/Returned for modification 8 August 2005/Accepted 21 September 2005

Toll-like receptors (TLRs) are differentially up-regulated in response to microbial infection and chronic inflammatory diseases such as atherosclerosis. Epidemiological data support the idea that periodontal disease may be a risk factor for acceleration of atherosclerosis. Porphyromonas gingivalis, the etiological agent of periodontal disease, invades endothelium, has been detected in human atheromatous tissue, and accelerates atheroma formation in apolipoprotein E−/− mice with concurrent induction of TLRs in the aorta. As endothelial cells can present antigen via TLRs and play an important role in the development of atherosclerosis, we examined TLR expression in human aortic endothelial cells (HAEC) cultured with wild-type P. gingivalis, a fimbria-deficient mutant, and purified antigens. Following a wild-type P. gingivalis challenge, functional TLR2 and TLR4 activation was assessed by subsequent stimulation with TLR agonists Staphylococcus aureus lipoteichoic acid (SLTA; TLR2 ligand) and Escherichia coli lipopolysaccharide (LPS; TLR4 ligand). Unchallenged HAEC failed to elicit monocyte chemoattractant protein 1 (MCP-1) in response to LPS or SLTA but did so when cultured with wild-type P. gingivalis. P. gingivalis-induced TLR2 and -4 expression on HAEC functionally reacted to SLTA and E. coli LPS as measured by a further increase in MCP-1 production. Furthermore, MCP-1 expression elicited by E. coli LPS was inhibitable with TLR4-specific antibody and polymyxin B. These results indicate that invasive P. gingivalis stimulates TLR expression on the surface of endothelium and these primed cells respond to defined TLR-specific ligands.

Recent epidemiological reports suggest an association between chronic infectious diseases and an increased risk for cardiovascular disease. It is now recognized that there are responses to inflammatory components of the atherogenic process that are shared with similar disease processes in infectious and other acute and chronic diseases (4) and that the inflammatory responses during the atherogenic process (3, 39) in many ways parallel the inflammatory response to infections (4). Innate immunity is characterized by a natural selection of germ line-encoded receptors, which focus the host response to highly conserved molecular patterns (pathogen-associated molecular patterns; PAMPs) shared by many microorganisms (21, 23, 32). This immune response is not only responsible for first-line microbial clearance but also plays an instructive role in the adaptive immune response through release of inflammatory cytokines and expression of costimulatory molecules by antigen-presenting cells (24). Toll-like receptors (TLRs), a group of PAMP recognition receptors, play an important role in innate immune signaling in response to microbial infection. Specific TLRs have demonstrated functional pattern recognition of peptidoglycan and bacterial lipopeptides (TLR2), double-stranded RNA (TLR3), lipopolysaccharide (LPS; TLR4), flagellin (TLR5), and unmethylated CpG DNA motifs (TLR9) (22). Recent reports suggest that TLR expression may be altered in some disease states, including cardiovascular disease (6, 12, 20). A recent study with semiquantitative reverse transcription-PCR and immunohistochemical analysis demonstrated that the expression of TLRs, in particular TLR1, -2, and -4, is markedly augmented in human atherosclerotic lesions (12). Furthermore, this augmentation was associated with endothelial cells and macrophages in areas infiltrated with inflammatory cells (12).

The association between human periodontal disease, a chronic bacterial infection of the tissue that supports the teeth, and atherosclerosis has been suggested on the basis of epidemiological studies (1, 2, 16, 19, 26, 29, 30). Porphyromonas gingivalis, the primary infectious agent of adult periodontal disease, possesses a broad array of virulence factors, including LPS, hemagglutinins, proteases, capsular polysaccharide, and two types of fimbriae (41-kDa major fimbria and 67-kDa minor fimbria). In recent animal experimental studies, other groups...
have demonstrated that infection of heterozygous apolipoprotein E (ApoE) knockout mice with Porphyromonas gingivalis increased the mean area and extent of atherosclerotic lesions histologically relative to those in uninfected animals (7) and accelerated the progression of atherosclerosis (27). We also demonstrated that ApoE knockout (ApoE<sup>−/−</sup>) mice challenged with wild-type (WT) P. gingivalis presented with increased atherosclerotic plaque and expressed TLR2 and -4 in aortic tissue (15). Despite early detection of an invasion-impaired P. gingivalis fimbra-deficient mutant (FimA<sup>−</sup>) in the blood and in aortic arch tissue, ApoE<sup>−/−</sup> mice challenged with the FimA<sup>−</sup> mutant did not present with up-regulation of TLR expression or accelerated atherosclerosis (15).

The endothelium, a continuous cellular monolayer lining the blood cells, has an enormous range of important homeostatic roles (42). When this homeostatic balance is disturbed, endothelial dysfunction develops and may contribute to the pathogenesis of atherosclerosis (42). We and others have previously demonstrated that P. gingivalis can actively invade aortic, heart, and vein endothelial cells and coronary artery smooth muscle cells (8–10) and that fimbriae are required for this invasion process. However, it is not clear how P. gingivalis infection of endothelial cells modulates the inflammatory response of these cells. In this study, we examined the expression of TLRs and chemokines in endothelial cells in response to P. gingivalis infection and determined the functional activity of TLRs on human aortic endothelial cells (HAEC). Our results indicate that invasive P. gingivalis bacteria, but not purified outer cell membrane components, up-regulate TLR expression on HAEC and stimulate HAEC to respond to TLR2- and -4-specific ligands.

**MATERIALS AND METHODS**

**Antibodies and reagents.** Monoclonal antibodies against human TLR2, -3, -4, -6, and -9 isotype-matched control antibodies and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibodies were purchased from BIOCARTA (Carlsbad, Calif.). Cytochalasin D and polymyxin B were purchased from Sigma-Aldrich (St. Louis, Mo.). For TLR functional assays, functionally active anti-human TLR4 monoclonal antibody and isotype-matched control antibody were purchased from eBioscience (San Diego, Calif.). Escherichia coli O111:B4 LPS (ultrapure grade; a TLR4 ligand), and Staphylococcus aureus lipoteichoic acid (SLTA; a TLR2 ligand) were purchased from InvivoGen (San Diego, Calif.).

**Bacterial strains and growth conditions.** P. gingivalis WT strains 381 and 33277 were maintained on anaerobic blood agar plates (BBL media; Becton Dickinson Co., Cockeysville, Md.). The P. gingivalis fimA (major 41-kDa fimbria)
mutant (DPG3) (31) and a 67-kDa minor fimbrial mutant (MF1) (Y. Takahashi et al., unpublished data) were constructed in WT strain 381. *P. gingivalis* fimbrial mutants (DPG3 and MF1) were cultured in the presence of erythromycin (10 μg/ml) and tetracycline (1 μg/ml) as required, respectively. All bacteria were grown at 37°C in an anaerobic environment containing 85% N₂, 5% H₂, and 10% CO₂ for 3 to 5 days. At 24 h prior to infection assays, *P. gingivalis* was transferred from plates into brain heart infusion broth (Difco, Detroit, Mich.) containing 0.5% yeast extract (Difco), 10 μg/ml hemin, 1 μg/ml vitamin K, and antibiotics as necessary and grown until the optical density at 660 nm reached 1.0.

**Primary HAEC cultures.** Primary HAEC (Cascade Biologics, Portland, Oreg.) were maintained and grown in M200 supplemented with low-serum growth supplement (Cascade Biologics) at 20 μl/ml at 37°C in 5% CO₂ in tissue culture flasks. Confluent second- to fourth-passage cells were used in all experiments. For infection studies, HAEC were plated at concentrations of 6 × 10⁵ to 8 × 10⁵ and 1.25 × 10⁶ to 1.66 × 10⁶ cells/well in 6- and 24-well flat-bottom plates, respectively.

**Preparation of heat-killed *P. gingivalis* and *P. gingivalis* fimbriae and LPS.** Heat-killed *P. gingivalis* was prepared by heating a bacterial suspension for 10 min at 60°C. Purification of the major fimbrial protein of *P. gingivalis* strain MF1, the minor fimbrial protein of *P. gingivalis* strain DPG3, and whole native fimbrial protein (major and minor fimbriae) of *P. gingivalis* strain 33277 was done by a modification of procedures described previously (41). The fimbrial preparations were analyzed for LPS contamination by electrophoresis by loading polyacrylamide gels stained with silver nitrate, and contamination of LPS was not detected.

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**FIG. 2.** The major fimbria of *P. gingivalis* is required for invasion-induced TLR expression by HAEC. HAEC were either left uninfected or infected with WT *P. gingivalis* 381 or *P. gingivalis* DPG3 at an MOI of 100 or 500 for the indicated periods. After infection, HAEC were stained with the indicated anti-TLR monoclonal antibody and analyzed with a FACSscan flow cytometer. Data are shown as the mean fluorescence intensity and standard deviation from four independent experiments. *, P < 0.05 compared with the uninfected control.
in these preparations, *P. gingivalis* LPS was prepared by the hot phenol-water technique (11, 44). LPS preparations were analyzed for protein contamination by a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.) and by electrophoresis by overloading polyacrylamide gels and staining them with Coomassie blue or silver nitrate.

**Invasion of HAEC by *P. gingivalis***. *P. gingivalis* invasion of HAEC monolayers was quantified by determining the number of CFU recovered following metronidazole treatment as described previously (36). To examine the effects of invasion on TLR expression in response to live invasive *P. gingivalis*, we preincubated HAEC with cytochalasin D (1 μg/ml in dimethyl sulfoxide [DMSO]), an inhibitor of actin polymerization, for 1 h as described previously (8). In preliminary experiments, cytochalasin D was assessed for toxicity for both *P. gingivalis* and HAEC viability and found to have no adverse effects at concentrations of up to 5 μg/ml. The multiplicity of infection (MOI) was calculated based on the number of endothelial cells per well in six-well flat-bottom plates at confluence.

**Fluorescence-activated cell sorter analysis**. Confluent endothelial cell monolayers incubated with *P. gingivalis* cells, purified fimbriae, or purified LPS were incubated with anti-human TLR2-, -3, -4, -6, and -9 mouse monoclonal antibodies or isotype-matched immunoglobulin G (IgG; 5 μg/ml) in accordance with the manufacturer’s instructions and labeled with fluorescein isothiocyanate-labeled goat anti-mouse IgG (1:100 dilution), and 10,000 events were analyzed by flow cytometry with a FACScan flow cytometer (Becton Dickinson). The viability and integrity of HAEC after infection were confirmed by the trypan blue exclusion method and microscopic morphological observation, as well as side and forward scatter signal determination by FACScan (data not shown).

**TLR functional assay**. *P. gingivalis* were added to confluent HAEC monolayers in 24-well culture plates at an MOI of 100 and incubated for 5 h. Nonadherent bacteria were removed by washing, and HAEC infected with *P. gingivalis* were stimulated with 10 ng/ml or 10 μg/ml of LPS (E. coli O111:B4 LPS (TLR4 ligand) or *E. coli* O111:B4 LPS (TLR4 ligand)) or 100 ng/ml of SLTA (TLR2 ligand) for 24 h. For blocking of TLR4 function or LPS activity, HAEC infected with *P. gingivalis* were also treated with 10 μg/ml of mouse anti-human TLR4 monoclonal antibody, an isotype-matched control IgG, or 10 μg/ml of polymixin B for 1 h before LPS stimulation; LPS was then added to HAEC in the presence of TLR4 monoclonal antibody, isotype-matched control antibody, or polymixin B, and HAEC were incubated for 24 h. The culture supernatant fluids were collected and stored at −20°C until enzyme-linked immunosorbent assays (ELISAs) were performed.

**Cytokine and chemokine assays**. The concentrations of monocyte chemotactic protein 1 (MCP-1), interleukin-1β (IL-1β), and tumor necrosis factor alpha (TNF-α) in cell culture supernatants were determined with commercially available ELISA kits (BD Biosciences, San Diego, Calif.) in accordance with the manufacturer’s instructions.

**Statistical analysis**. All statistical analyses were performed by one-way analysis of variance with the Tukey-Kramer multiple-comparison test. Differences in the data were considered significant when the probability was less than 5.0% (*P* < 0.05).

## RESULTS

**Invasive *P. gingivalis*** bacteria stimulate TLR2-, -3, -4, -6, and -9 expression on the surface of HAEC. Our previous studies established that the 41-kDa major fimbria, encoded by the *fimA* gene, is required for invasion of human umbilical vein endothelial cells (HUVEC) and HAEC (8; Takahashi et al., unpublished). Additionally, we recently reported that invasive, but not noninvasive, FimA-deficient *P. gingivalis* bacteria stimulate the expression of TLR2 and -4 on the surface of HAEC (15). We thus next examined the stimulation of additional TLR expression following *P. gingivalis* infection by fluorescence-activated cell sorter. Uninfected primary cultures of HAEC expressed low levels of TLR2 and -4 on the cell surface; however, we did not detect expression of TLR3, -6, and -9 (data not shown). Following infection with invasive *P. gingivalis*, we observed increased expression of TLR2, -3, -4, -6, and -9 on HAEC at 2 and 6 h following infection with WT *P. gingivalis* (Fig. 1). Following a 24-h incubation with invasive *P. gingivalis*, the levels of TLR expression in HAEC declined to the baseline (Fig. 1). These results indicate that invasive *P. gingivalis* bacteria are capable of stimulating HAEC to rapidly express TLR on their surface. Similar results were obtained with HUVEC (data not shown).

**Up-regulation of TLR expression after infection of HAEC with invasive *P. gingivalis* is dose dependent**. To determine if the TLR expression in HAEC was *P. gingivalis* dose dependent, we infected HAEC with invasive *P. gingivalis* 381 at an MOI of 100 or 500 and assessed TLR expression. As shown in Fig. 2, we observed a both dose- and time-dependent increase in TLR2, -3, -4, -6, and -9 expression following infection of HAEC with *P. gingivalis* 381. The increased expression of all of the examined TLRs remained elevated 24 h postinfection when an MOI of 500 of *P. gingivalis* 381 was used (Fig. 2). These results suggest that the level of TLR expressed on HAEC, elicited by invasive *P. gingivalis* infection, is dose dependent.

**Role of *P. gingivalis* fimbriae in TLR expression in *P. gingivalis*-infected HAEC**. To further assess the contribution of fimbria-mediated TLR expression in response to *P. gingivalis* infection, we cultured HAEC monolayers with WT *P. gingivalis* 381 or the *fimA* mutant. As expected, we did not observe increased expression of TLRs following infection of HAEC with noninvasive *P. gingivalis* DPG3 mutants at an MOI of 100 (Fig. 2). However, at an MOI of 500, infection with noninvasive, fimbria (fimA)-deficient *P. gingivalis* resulted in increased expression of TLR2, -3, -4, and -6 on HAEC. The levels of TLRs increased within 2 h following infection with the noninvasive strain, reached a maximum at 6 h after infection, and were still elevated after 24 h of infection (Fig. 2). The observations of TLR up-regulation were consistent with the ability of the *P. gingivalis* FimA− mutant to invade HAEC when a high MOI (500) was used (Table 1).

To determine if TLR expression induced in response to WT *P. gingivalis* is dependent on bacterial viability, we challenged HAEC with heat-killed *P. gingivalis* 381. Low doses (MOI =

### Table 1. Invasion of HAEC by *P. gingivalis* strains

<table>
<thead>
<tr>
<th><em>P. gingivalis</em> strain</th>
<th>MOI</th>
<th>Invasion efficiency (%)</th>
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<tr>
<td></td>
<td></td>
<td>1 h</td>
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<tr>
<td>381</td>
<td>100</td>
<td>0.134 ± 0.028</td>
</tr>
<tr>
<td>DPG3</td>
<td>100</td>
<td>0.00018 ± 0.00007</td>
</tr>
<tr>
<td>381</td>
<td>500</td>
<td>0.622 ± 0.211</td>
</tr>
<tr>
<td>DPG3</td>
<td>500</td>
<td>0.000011 ± 0.000004</td>
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</tbody>
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* Percentage of the inoculum (*P. gingivalis*) protected from metronidazole killing after the infection period. Values are the means of triplicate independent determinations from a typical experiment ± the standard error measurement.
100) of heat-killed \( P. \) gingivalis 381 did not result in stimulation of TLR expression on HAEC and HUVEC (data not shown). We did, however, observe a slight increase in TLR expression on HAEC between 2 and 6 h postinfection with heat-killed \( P. \) gingivalis 381 at high doses (MOI = 500) (data not shown). These results suggest that high doses of heat-killed, fimbriated \( P. \) gingivalis can still slightly increase TLR expression on HAEC and that this response may be due to some unidentified heat-resistant outer membrane component of \( P. \) gingivalis.

Blocking \( P. \) gingivalis invasion of HAEC with cytoskeleton inhibitors inhibits up-regulation of TLR expression by invasive \( P. \) gingivalis infection. To further define the role of \( P. \) gingivalis invasion and increased TLR expression, we treated HAEC with cytochalasin D, an inhibitor of actin polymerization and cytoskeleton rearrangements. Treatment of HAEC with cytochalasin D at 1.0 \( \mu \)g/ml inhibited \( P. \) gingivalis invasion of HAEC by 96% (Fig. 3A). However, low numbers of \( P. \) gingivalis 381 bacteria were capable of invading cytochalasin D-treated HAEC. \( P. \) gingivalis-induced expression of TLRs was decreased in cytochalasin D-treated HAEC compared to HAEC without added cytochalasin D (Fig. 3B); however, TLR expression was not completely diminished. These results may

FIG. 3. Cytochalasin D inhibits \( P. \) gingivalis invasion of HAEC and the up-regulation of TLRs induced by invasive \( P. \) gingivalis infection. HAEC were treated with cytochalasin D (1 \( \mu \)g/ml in DMSO) for 1 h, followed by infection with invasive \( P. \) gingivalis 381 at an MOI of 100 for 2 h at 37°C. (A) After infection, the invasion efficiency of \( P. \) gingivalis was determined. Percent invasion, shown on the y axis, is relative to the invasion efficiency with DMSO (control; 100%), and each value is the mean and standard deviation obtained from three independent experiments. Asterisks indicate significant differences (*, \( P < 0.05; **, P < 0.01)). (B) After infection, HAEC were stained with the indicated anti-TLR monoclonal antibody and analyzed with a FACSscan flow cytometer. Data are shown as the mean fluorescence intensity relative to the intensity for each TLR with DMSO (control; 100%). The data shown are the mean and standard deviation obtained from three independent experiments. Asterisks indicate significant differences (*, \( P < 0.05; **, P < 0.01)).
FIG. 4. Purified LPS and fimbriae of *P. gingivalis* have no effects on TLR expression on HAEC. (A) HAEC were stimulated or infected with various concentrations of purified fimbriae (1 or 10 μg/ml, as indicated) (A) or LPS (0.1, 1.0, or 10 μg/ml, as indicated) (B) from *P. gingivalis* or live invasive *P. gingivalis* 381 at an MOI of 100 for the indicated periods. After stimulation or infection, HAEC were stained with the indicated anti-TLR monoclonal antibody and analyzed with a FACScan flow cytometer. The data shown are the mean fluorescence intensity and standard deviation from four independent experiments. *, *P* < 0.05 compared with the uninfected control.
be explained by the observation that the invasion efficiency of invasive *P. gingivalis* 381 with cytochalasin D was still 17-fold higher than that observed with *fimA*-deficient *P. gingivalis* strain DPG3 and suggest that very low invasion frequencies are sufficient to alter TLR expression in HAEC.

*P. gingivalis* fimbriae and LPS do not stimulate TLR expression. To determine if purified *P. gingivalis* fimbriae contribute to increased TLR expression in HAEC, we stimulated these cells with purified *P. gingivalis* major or minor fimbriae or whole fimbral preparations containing both the major and minor fimbriae and examined TLR expression. When HAEC were stimulated with purified *P. gingivalis* major, minor, or whole native fimbriae (1.0 or 10 μg/ml), we failed to detect up-regulation of TLR expression on HAEC throughout the 24-h incubation period (Fig. 4A). To determine if other *P. gingivalis* surface-expressed antigens stimulate TLR expression on HAEC, these cells were cultured with purified *P. gingivalis* 381 LPS (0.1, 1.0, or 10 μg/ml) for 2, 6, or 24 h. As with *P. gingivalis* fimbriae, we did not observe an increase in TLR expression on HAEC with *P. gingivalis* LPS. As another source of bacterial antigen, LPS (0.1 or 1.0 μg/ml) from *E. coli* O111:B4 also failed to up-regulate TLR expression on HAEC, even after a 24-h incubation period (data not shown). These results suggest that *P. gingivalis* major and minor fimbriae in purified form, as well as *P. gingivalis* and enterobacterial LPSs, do not influence TLR expression on the surface of HAEC.

HAEC that have been preincubated with live invasive *P. gingivalis* become primed to respond to TLR2- and TLR4-specific ligands. To determine if invasive *P. gingivalis* can prime HAEC to respond to a TLR-specific ligand, HAEC were either cultured in medium alone or challenged with WT *P. gingivalis*. Confluent HAEC monolayers were infected with invasive *P. gingivalis* 381 or DPG3 at an MOI of 100 for 5 h at 37°C. Nonadherent and uninvaded bacteria were removed by washing with phosphate-buffered saline. LPS from *E. coli* O111:B4 (10 ng/ml; A) or SLTA (100 ng/ml; B) was then added, and HAEC were incubated for an additional 24 h. At the end of this stimulation, the culture supernatant was collected for ELISA. To determine the effects of LPS through TLR signaling in HAEC, HAEC were pretreated with 10 μg/ml of anti-human TLR4 monoclonal antibody (Ab) or isotype-matched control antibody (C) or with 10 μg/ml of polymyxin B (D) for 1 h before LPS stimulation. The controls consisted of monolayers incubated in medium alone. The representative findings shown are the mean and standard deviation from three independent experiments. Asterisks indicate significant differences (*, *P* < 0.05; **, *P* < 0.01; ***. *P* < 0.001). NS, no significant difference.

**FIG. 5.** HAEC that have been preincubated with invasive live *P. gingivalis* become primed to respond to TLR-specific ligands. Confluent HAEC monolayers were infected with invasive *P. gingivalis* 381 or DPG3 at an MOI of 100 for 5 h at 37°C. Nonadherent and uninvaded bacteria were removed by washing with phosphate-buffered saline. LPS from *E. coli* O111:B4 (10 ng/ml; A) or SLTA (100 ng/ml; B) was then added, and HAEC were incubated for an additional 24 h. At the end of this stimulation, the culture supernatant was collected for ELISA. To determine the effects of LPS through TLR signaling in HAEC, HAEC were pretreated with 10 mg/ml of anti-human TLR4 monoclonal antibody (Ab) or isotype-matched control antibody (C) or with 10 μg/ml of polymyxin B (D) for 1 h before LPS stimulation. The controls consisted of monolayers incubated in medium alone. The representative findings shown are the mean and standard deviation from three independent experiments. Asterisks indicate significant differences (*, *P* < 0.05; **, *P* < 0.01; ***. *P* < 0.001). NS, no significant difference.
TLR4 functional activity was monitored by measuring MCP-1 in HAEC for 24 h following E. coli LPS (10 ng/ml) stimulation of HAEC previously cultured in medium or cultured with P. gingivalis 381. HAEC cultured in medium produced low levels of MCP-1. We observed increased expression of MCP-1 in HAEC infected with P. gingivalis 381 at 24 h following the removal of nonadherent bacteria. Stimulation with E. coli LPS superinduced the production of MCP-1 in HAEC infected with P. gingivalis 381 but not in uninfected control HAEC cultures and P. gingivalis DPG3-infected HAEC (Fig. 5A). Unexpectedly, E. coli LPS at 1 or 10 µg/ml did not superinduce the production of IL-1β and TNF-α in HAEC preinfected with the same number of bacteria (data not shown). To determine if the TLR2 response was also functionally able to elicit the superinduction of MCP-1 production, similar experiments were performed. HAEC were cultured with SLTA following a P. gingivalis challenge. We also observed that SLTA elicited superinduced MCP-1 production (Fig. 5B). These results demonstrate that P. gingivalis-infected HAEC are primed to respond to defined TLR agonists. Furthermore, these results suggest that the pathway of cellular signal transduction for MCP-1 expression is different from that for IL-1β and TNF-α expression in HAEC.

To confirm that the increase in MCP-1 expression was due to the interaction between E. coli LPS and TLR4 expression induced by invasive P. gingivalis infection, we treated cultures with anti-TLR4 antibody for blocking of TLR4 function or isotype-matched control antibody prior to E. coli LPS stimulation. Treatment of P. gingivalis-infected HAEC with anti-TLR4 antibody inhibited MCP-1 production elicited by E. coli LPS stimulation, but this inhibition was not observed with the isotype-matched control antibody (Fig. 5C). Moreover, to confirm that the enhanced responsiveness was due to LPS, we treated cultures with polymyxin B (10 µg/ml) to block LPS activity. Treatment with polymyxin B also resulted in an inhibition of MCP-1 production elicited by E. coli LPS stimulation (Fig. 5D). SLTA-blocking experiments were not performed, as a TLR2-blocking antibody has not been characterized. Taken together, these results indicate that P. gingivalis infection sensitizes HAEC to a subsequent E. coli LPS exposure by surface regulation of TLR4 and suggest that P. gingivalis infection can convert HAEC from an LPS-hyporesponsive state to an LPS-hyperresponsive state.

**DISCUSSION**

In this study, we demonstrate that live invasive P. gingivalis bacteria stimulate TLR2, -3, -4, -6, and -9 expression on the surface of HAEC. Our studies indicate that neither P. gingivalis major or minor fimbriae nor P. gingivalis LPS, as a purified component, stimulates TLR expression on the surface of HAEC, indicating that stimulation of TLR surface expression requires interaction of live, fimbriated P. gingivalis with HAEC. Importantly, we also demonstrated that the increased TLR2 and -4 on HAEC resulting from stimulation with invasive P. gingivalis was capable of functionally reacting with either SLTA as a TLR2-specific ligand or E. coli LPS as a TLR4-specific ligand. These results suggest that P. gingivalis bacteria modify the levels of TLRs expressed on the surface of HAEC and indicate that P. gingivalis infection can convert HAEC from TLR ligand-hyporesponsive cells to TLR ligand-hyperresponsive cells (Fig. 6). Our present results are in agreement with a recent report in which respiratory syncytial virus infection was demonstrated to sensitize airway epithelium to a subsequent LPS exposure by altering TLR4 expression and membrane localization of TLR4 expression and to convert these cells from LPS nonresponsive to LPS responsive (34). However, our studies have extended these studies by performing blocking of TLR4 and LPS with TLR4 monoclonal antibody and polymyxin B, respectively. In addition, our results are in agreement with our recent animal studies in which we demonstrated that ApoE−/− mice orally challenged with invasive...
P. gingivalis, but not noninvasive P. gingivalis, present with increased atherosclerotic plaque and increased expression of TLR2 and -4 in aortic tissue (15).

Recent reports have demonstrated that TLRs are selectively up-regulated following stimulation with microbial products and during infection and inflammation (13, 14, 28, 35). These studies are in disagreement with the results presented here, which demonstrate that LPS from P. gingivalis and E. coli O111:B4 fail to up-regulate TLRs on primary HAEC. Interestingly, other studies found that E. coli LPS inhibits the expression of TLR4 mRNA in mouse macrophage cells (37, 38). A separate study with outer membrane components of P. gingivalis demonstrated that TLR2 expression on THP-1 cells was significantly up-regulated by P. gingivalis LPS (17) but that expression of TLR4 on the same cells was slightly down-regulated by LPS-free recombinant fimbriilin (rFimA) (18). The divergence between our results and previous studies may reflect differences in cell types and LPS from different origins.

Inflammation-dependent induction of TLR2 and -4 expression in intestinal macrophages or epithelial cells from patients with Crohn’s disease, ulcerative colitis, or sigmoid diverticulitis has also been recently reported (6, 20). Recent reports have also demonstrated that TLRs are selectively regulated in murine macrophages and human epithelial cells following infection with Mycobacterium avium and nontypeable Haemophilus influenzae, respectively (40, 43). Most interestingly, increased TLR expression has also been associated with inflammatory activation in human atherosclerotic lesions. Xu et al. (45) reported on the preferential expression of TLR4 in lipid-rich and macrophage-infiltrated murine and human atherosclerotic plaques. Recently, two studies have reported that genetic deficiency of TLR4 and myeloid differentiation factor 88 (MyD88), which transduces TLR signaling molecule-specific knockout mouse response with TLR signaling molecule-specific knockout mouse models.

**ACKNOWLEDGMENT**

This work was supported by Public Health Service grant PO1 DE13191 from the National Institute of Dental and Craniofacial Research to Caroline A. Genco.

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Editor: V. J. DiRita

Vol. 73, 2005

P. GINGIVALIS INFECTION SENSITIZES ENDOTHELIUM VIA TLR

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