Role for Fimbriae and Lysine-Specific Cysteine Proteinase Gingipain K in Expression of Interleukin-8 and Monocyte Chemoattractant Protein in *Porphyromonas gingivalis*-Infected Endothelial Cells

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Received 18 May 2001/Returned for modification 19 July 2001/Accepted 18 September 2001

Recent cross-sectional and prospective epidemiological studies have demonstrated an association between periodontal disease and atherosclerosis and human coronary heart disease. Previously, we have established that the periodontal pathogen *Porphyromonas gingivalis* is capable of invading aortic, heart, and human umbilical vein endothelial cells (HUVEC). Since atherosclerosis is a chronic inflammatory response initiated at the vascular wall, interactions of *P. gingivalis* with endothelial cells and the subsequent host cell response to infection may be important in the pathogenesis of atherosclerosis. In this study we examined the consequences of *P. gingivalis* infection of HUVEC on the expression of the chemokines interleukin-8 (IL-8) and monocyte chemotactic protein 1 (MCP-1). HUVEC were found to constitutively produce low levels of IL-8 and MCP-1. The addition of *P. gingivalis* fimbrillin-specific peptides, lipopolysaccharides (LPS), or heat-killed whole cell preparations to HUVEC stimulated modest IL-8 and MCP-1 responses. In contrast, coculture of HUVEC with live *P. gingivalis* strain A7436, 33277, or 381 abolished the IL-8 and MCP-1 responses. Inhibition of IL-8 and MCP-1 production was not dependent on bacterial adherence since similar results were obtained with the nonadherent *P. gingivalis fimA* mutant DPG3 or when *P. gingivalis* was preincubated with fimbrillin peptide antiserum prior to the addition to HUVEC. Furthermore, treatment of *P. gingivalis*-infected HUVEC with cytochalasin D, which prevented *P. gingivalis* invasion, also abolished the constitutive IL-8 and MCP-1 responses. Treatment of HUVEC with *E. coli* LPS stimulated robust IL-8 and MCP-1 responses that were abolished when stimulated cells were cocultured with live *P. gingivalis*. Analysis of *P. gingivalis*-infected HUVEC cultures by an RNase protection assay revealed an increase in the IL-8 transcript relative to uninfected HUVEC. Pretreatment of *P. gingivalis* with protease inhibitors prior to the addition to HUVEC prevented the inhibition of IL-8 and MCP-1 production in *P. gingivalis*-infected HUVEC, indicating that the inhibition was proteolytically mediated. Co-culture of HUVEC with a *P. gingivalis* mutant deficient in lysine-specific cysteine protease (gingipain K [Kgp]) resulted in an increase in both IL-8 transcription and protein expression relative to that observed in HUVEC cocultured with the *P. gingivalis* wild-type strain. These results indicate that *P. gingivalis* can temporally modulate the chemokine response in endothelial cells through both fimbriae and gingipain-mediated mechanisms.

An association between periodontal disease and chronic diseases such as atherosclerosis and coronary heart disease has been established on the basis of epidemiological studies (3, 4, 16, 27, 28, 32). These reports include case control studies, which demonstrated significant associations after correction for cholesterol, smoking, hypertension, social class, and body mass index (3–5, 47). Periodontal disease as a local persistent chronic infection may exert systemic effects by the interaction of specific periodontal pathogens with the host immune system. While it has generally been accepted that the innate host defense system functions by limiting the spread of *Porphyromonas gingivalis*, the primary etiological agent associated with periodontal disease (7, 43), mounting evidence argues that *P. gingivalis* may pass through the epithelial barrier (10, 15). The connective tissues of the periodontium are well vascularized, allowing invading microorganisms such as *P. gingivalis* to readily enter the blood stream. Indeed, *P. gingivalis* has been observed within gingival tissues in vivo, suggesting that as well as colonizing mucosal surfaces it may also invade deeper structures of connective tissues (41). *P. gingivalis* has also been reported to degrade epithelial cell-cell junction complexes, a process that could contribute to the spread of the organism (24).

Pathological studies have recently identified *P. gingivalis* in diseased atherosclerotic tissue by PCR (18). Furthermore, *P. gingivalis* infection of apoE mice has been demonstrated to increase the mean area and the extent of atherosclerotic lesions histologically relative to those in uninfected animals (6). While these studies support a role for *P. gingivalis* in the development and progression of atherosclerosis, the mechanisms by which *P. gingivalis* infection influences the initiation and progression of atherosclerotic plaque have not been identified. Since it is now apparent that atherosclerosis is an inflammatory
disease (30), the interactions of P. gingivalis with host cells and the subsequent host cell response to infection may be important in understanding the role of P. gingivalis in atherosclerosis initiation. We have previously demonstrated that P. gingivalis can actively invade aortic, heart, and vein endothelial cells (11). Endothelial cells, among other vascular wall cells, may have an important role both as local reservoirs of P. gingivalis and P. gingivalis components and as contributors to immunostimulation during P. gingivalis infection. However, it is not clear how active invasion of endothelial cells by P. gingivalis modulates the inflammatory response of these cells.

The host cytokine network plays a central role in the maintenance of both innate and acquired immunity. Chemotractant cytokines (chemokines) form a superfAMILY of closely related, secreted proteins, which specialize in mobilizing leukocytes to areas of immune challenge. Interleukin-8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) are potent chemokines in directing neutrophil migration and monocyte migration, respectively, to the site of infection (16, 17). Recruitment and adhesion of circulating leukocytes to endothelial cells are early steps in the inflammatory response characteristic of atherosclerotic lesions. To begin to define the mechanisms by which P. gingivalis infection influence the initiation and progression of atherosclerotic plaque, we have initiated studies to examine the inflammatory response of endothelial cells following P. gingivalis infection. In this study, we demonstrate that while P. gingivalis surface components including fimbrillin peptides can stimulate a chemokine response in human umbilical vein endothelial cells (HUVEC), live P. gingivalis abolishes the normal IL-8 and MCP-1 responses. Furthermore, this inhibition is not dependent on invasion and is mediated in part by the lysine- and arginine-specific cysteine protease (gingipain R and gingipain K) (1, 14, 23, 40, 45, 49).

MATERIALS AND METHODS

Bacterial strains and growth conditions. P. gingivalis wild-type strains A7436, 33277, and 381 (laboratory collection) were used in these studies and were maintained on anaerobic blood agar plates (Fisher Scientific Co., Springfield, N.J.). A P. gingivalis ficn4 mutant (DPG3) (28) and the corresponding wild-type strain 381 were used to define the role of fimbriae in chemokine expression in HUVEC as described below. Likewise, to examine the role of P. gingivalis gingipains in IL-8 and MCP-1 inhibition, we utilized the P. gingivalis mutants YPP1 (rgg4) and YPP2 (rgg2) and the corresponding wild-type strain 33277 (39). The P. gingivalis ficn4 mutant (DPG3) and rgg4 (YPP1) and rgg2 (YPP2) mutants were maintained on anaerobic blood agar containing erythromycin (10 μg/ml). All P. gingivalis cultures were incubated at 37°C in an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.) with 85% N2, 5% H2, and 10% CO2 for 16, 24, and 48 h. For infection assays, P. gingivalis was transferred from plates into Schaedler broth (Difco, Detroit, Mich.) and grown for 24 h or until the bacterial suspension (MOI = 100) and the corresponding wild-type strain 33277 (39).

Infection of HUVEC with P. gingivalis. HUVEC cultures were grown in Media-200 (Cascade Biologies, Inc., Portland, Oreg.) supplemented with low-serum growth supplement (20 μl/ml). HUVEC were plated at a concentration of 105 to 106 cells/ml, as determined by cell counting with a hemocytometer. For all experiments, six-well flat-bottom plates were used, with a volume of 2 to 4 ml/well. The multiplicity of infection (MOI) was calculated based on the number of HUVEC cells per well at confluence. P. gingivalis strains grown to an optical density of 1.0 were centrifuged, washed with phosphate-buffered saline (PBS), and resuspended in HUVEC growth medium to a final concentration of 105 cells per ml. Bacterial suspensions (1.0 ml) were added to confluent HUVEC monolayers (MOI = 100) and incubated at 37°C in 5% CO2. Supernatant samples were removed at 2, 16, 24, and 48 h postinfection. Control cultures were incubated with medium alone. Bacterial adherence and invasion were determined as previously described (11). To examine the effects of bacterial LPS, fimbrillin peptides, and P. gingivalis whole cell extracts on the chemokine response of HUVEC, various dilutions of these components were added to confluent HUVEC monolayers and incubated at 37°C in 5% CO2. Supernatant samples were removed at 2, 16, 24, and 48 h postaddition, filtered through a 0.22-μm pore-size low-protein-binding filter, and stored at −80°C. For all studies, viability of the endothelial cultures was monitored by either Trypan blue staining or with a Annexin V apoptosis detection kit (Vibrant Apoptosis; Molecular Probes, Eugene, Oreg.) and examined by fluorescence-activated cell sorting (FACS) analysis with a FACScan (Becton Dickinson, Sparks, Md.) flow cytometer.

Role of fimbriae in the stimulation of HUVEC chemokine response. The role of bacterial adherence and invasion in P. gingivalis-mediated chemokine expression was also examined by using the P. gingivalis fimC4 mutant (DPG3) or by preincubating P. gingivalis with fimbrillin peptide-specific antisera. We have previously established that preincubation of P. gingivalis with fimbrinia-specific antisera inhibits P. gingivalis invasion of HUVEC (11). Likewise, Sojar et al. (44) have established that preincubation of P. gingivalis with specific anti-fimbrillin peptide sera inhibits P. gingivalis invasion of oral epithelial cells. Thus, to further define the role of fimbriae in the induction of chemokine expression, P. gingivalis was preincubated with rabbit polyclonal antisera to fimbrillin peptides corresponding to amino acids 49 to 68 (VVMANTAGAMELVGKTAEVK and 69 to 90 (ALTITLAEQNNoAGLMTAEP) of the mature fimbrillin protein (44) or a normal rabbit serum control (1:500 dilution) for 60 min prior to infection of HUVEC. To examine the effects of invasion on the chemokine response to P. gingivalis, we preincubated HUVEC with cytochalasin D (1 μg/ml in dimethyl sulfoxide) for 30 min prior to the addition of P. gingivalis 381 as previously described (12).

Preincubation of P. gingivalis with protease inhibitors. To determine the contribution of P. gingivalis proteases on chemokine expression in P. gingivalis-infected HUVEC, P. gingivalis whole-cell extracts were preincubated with the following inhibitors in the solvent and at the final concentration indicated were used: leupeptin, 0.1 mM in distilled H2O (dH2O) (Sigma, St. Louis, Mo.); Z-FKck (benzoylloxycarbonyl-Phe-Lys-CH2O2-C2,4, Z-Phe-Lys-2,4,6-trimethylbenzoxyloxyethylketone trifluoroacetate), 0.1 mM in dH2O (BACHEM Bioscience Inc., King of Prussia, Pa.); and a cocktail of protease inhibitors containing aprotonin (2 μg/ml in dH2O), phenylmethylsulfonyl fluoride (0.1 mM in methanol), pepstatin (0.7 μg/ml in methanol), and benzamidine (1 mM in methanol) (Sigma). P. gingivalis A7436 cultures grown to an optical density of 1.0 were centrifuged, washed with PBS, and resuspended in dH2O or treated with the different protease inhibitors for 1 h at 37°C under anaerobic conditions. P. gingivalis cultures were then washed and resuspended in HUVEC growth medium to a final concentration of 5 × 105 CFU/ml. HUVEC monolayers were infected with 1.0 ml of the P. gingivalis bacterial suspension (MOI = 100) and incubated at 37°C in 5% CO2 for 16, 24, and 48 h. Supernatant samples were removed at the designated times, filtered as described above, and stored at −80°C.

Chemokine expression. Supernatant samples from the HUVEC cultures were analyzed by a enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Woburn, Mass.) for IL-8, MCP-1, IL-1α, IL-1β, and tumor necrosis factor alpha (TNF-α). This assay was performed according to the manufacturer’s instructions, and the data were expressed relative to a standard curve prepared for each chemokine.

RNA protection assay. Total RNA was extracted from HUVEC cultures with a Trizol reagent (Gibco BRL, Grand Island, N.Y.) according to the manufac-
overnight at 56°C with 106 cpm of the 32P-labeled antisense riboprobe mixture.

[32P]UTP-labeled antisense riboprobes were generated from a multiprobe template set (hek-5; Pharmingen). The chemokine templates included lymphotactin (Ltn), RANTES, I-309, macrophage inhibitory protein 1 (MIP-1), IL-8, gamma interferon-inducible protein 10 (IP-10), and MCP-1. Two housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a human ribosomal protein (L32), were also included in the multiprobe templates to ensure equal loading of total RNA onto the gels. A total of 2 μg of RNA was hybridized overnight at 56°C with 106 cpm of the 32P-labeled antisense riboprobe mixture. After hybridization, the unprotected RNA was digested with a mixture of RNases A and T1. Nuclease-protected RNA fragments were resolved on a 6% polyacrylamide sequencing gel. After exposure to the Imaging-Screen K (Bio-Rad, Hercules, Calif.), the specific chemokine bands were identified on the basis of their individual mobilities compared with those of labeled standard probes. The band intensities shown in the Imaging-Screen K were digitized by scanning the images and analyzed with Quantity one software (Bio-Rad). The densitometric intensity was normalized with respect to the average intensities of the bands for the housekeeping genes GAPDH and L32.

**Data analysis.** Data were analyzed with the Instat computer software program (Graph Pad Software, Inc., San Diego, Calif.). All experiments were performed in duplicate or triplicate, and the significance of differences between the mean results for the different groups was determined by Student’s t test.

**RESULTS**

**IL-8 and MCP-1 responses to P. gingivalis fimbrillin peptides, heat-killed whole cells, and LPS.** To examine the ability of different P. gingivalis membrane components to stimulate HUVEC, we added different components to HUVEC and monitored the IL-8 and MCP-1 responses in culture supernatants over time. Uninfected HUVEC cultures were found to constitutively express low levels of IL-8 and MCP-1 (Fig. 1). The addition of a peptide within the N-terminal region of the mature P. gingivalis fimbrillin protein (amino acids [aa] 61 to 80) stimulated an IL-8 response in HUVEC which was dose-dependent and statistically significant compared to that in uninfected HUVEC cultures observed at 24 h (Fig. 1A). We did not observe an IL-8 or MCP-1 response in HUVEC incubated with a scrambled peptide control corresponding to aa 61 to 80 of the mature P. gingivalis fimbrillin protein (data not shown).

The addition of heat-killed P. gingivalis whole cells or P. gingivalis LPS stimulated the expression of IL-8 in HUVEC (Fig. 1A). The addition of the fimbrillin peptide at a higher concentration (100 μg) also stimulated the expression of MCP-1 in HUVEC (Fig. 1B). Stimulation of MCP-1 by heat-killed P. gingivalis whole cells or LPS followed a trend similar to that for IL-8, although higher levels of MCP-1 were observed with the heat-killed P. gingivalis whole cells and the fimbrillin peptides compared to the levels in LPS-stimulated cultures (Fig. 1B). We did not observe stimulation of IL-1α, IL-1β, or TNF-α in HUVEC incubated with P. gingivalis whole cells, LPS, or fimbrillin peptides (data not shown).

**Infection of HUVEC with P. gingivalis abolishes constitutive IL-8 and MCP-1 production.** The results described above indicated that P. gingivalis membrane components could stimulate the production of distinct chemokines in HUVEC. To examine the consequences of P. gingivalis infection of endothelial cells, we cocultured HUVEC confluent monolayers with P. gingivalis strain A7436 or 381 for various times and examined culture supernatants by ELISA for IL-8 and MCP-1 production. Interestingly, we observed a decrease in IL-8 production following infection of HUVEC with P. gingivalis A7436 compared to that in uninfected or unstimulated cultures (Fig. 2A). Similar to the results obtained with P. gingivalis A7436, we did not observe an increase in IL-8 production following a 24-h incubation period with P. gingivalis 381 (Fig. 2A). Inhibition of the MCP-1 response similar to that of IL-8 production was observed following infection of HUVEC with P. gingivalis A7436 and 381 (Fig. 2B). The invasion frequencies for P. gingivalis strains A7436 and 381 were confirmed by an antibiotic protection assay and were found to be similar to our previously reported results (11). As expected, an increase in IL-8 and MCP-1 was observed following stimulation with P. gingivalis LPS (Fig. 2).

To exclude the possibility that infection of HUVEC with P. gingivalis resulted in cell death such that the HUVEC cultures were no longer capable of mounting a chemokine response, we evaluated cell viability by trypan blue exclusion and Annexin V staining. Trypan blue staining revealed no obvious cytotoxic effects following coculture of HUVEC with P. gingivalis (data not shown). Annexin V staining revealed that the uninfected HUVEC cultures contained 8% apoptotic cells as determined following a 48-h incubation (data not shown). In HUVEC cultures incubated with P. gingivalis for 48 h, we observed a similar level of apoptotic cells. These results indicate that the inhibition of IL-8 and MCP-1 expression by P. gingivalis-infected HUVEC was not a result of cell death.
P. gingivalis inhibits IL-8 and MCP-1 production by E. coli LPS-stimulated HUVEC. The results described above indicated that infection of HUVEC with P. gingivalis abolished the IL-8 or MCP-1 response in unstimulated HUVEC. To determine if P. gingivalis could inhibit IL-8 and MCP-1 production by stimulated endothelial cells, we cocultured HUVEC with E. coli LPS and live P. gingivalis and monitored the IL-8 and MCP-1 responses. As expected, we observed a significant increase in IL-8 and MCP-1 levels following the addition of E. coli LPS as observed at both 16 and 24 h (Fig. 3). However, when HUVEC were cocultured with both P. gingivalis and E. coli LPS, the levels of both chemokines were significantly decreased relative to the levels in stimulated HUVEC samples. These results indicate that infection with live P. gingivalis abolishes the IL-8 and MCP-1 responses observed in stimulated HUVEC.

Inhibition of IL-8 and MCP-1 production by P. gingivalis is not dependent on fimbria-mediated adherence or bacterial invasion. To determine whether adherence and uptake of P. gingivalis by HUVEC were required for the inhibition of IL-8 and MCP-1, we performed a series of experiments in which we blocked the P. gingivalis adherence to and invasion of HUVEC and monitored the IL-8 and MCP-1 responses in HUVEC. We have previously established that preincubation of P. gingivalis with fimbria-specific antisera prior to culture with HUVEC prevents P. gingivalis adherence to and invasion of HUVEC (11). As shown in Fig. 4, we did not detect a significant increase in IL-8 or MCP-1 when P. gingivalis was preincubated with fimbriain peptide-specific antisera compared to that in uninfected cultures or to P. gingivalis cultures preincubated with normal rabbit serum. The inability of the fimbriain peptide-specific antisera to inhibit P. gingivalis invasion was confirmed by an antibiotic protection assay (data not shown).

These results were further confirmed following infection of HUVEC with the nonadherent, noninvasive P. gingivalis fimA mutant (DPG3). A similar lack of IL-8 and MCP-1 production was observed for both P. gingivalis DPG3 and the corresponding wild-type strain 381 (Fig. 4). Furthermore, when we prevented P. gingivalis invasion of HUVEC by using cytochalasin D as previously described (11), we observed only a modest increase in IL-8 and MCP-1 compared to that in uninfected cultures. These results indicate that uptake of P. gingivalis by HUVEC is not required for the inhibition of IL-8 and MCP-1 production observed with live P. gingivalis.

Pretreatment of P. gingivalis with protease inhibitors stimulates IL-8 in P. gingivalis-infected HUVEC. A recent report has demonstrated that membrane bound forms of the P. gingivalis cysteine proteases (gingipains) can degrade IL-8 (29). The arginine-specific gingipains (HRgpA and RgpB) and the lysine-specific gingipain (Kgp) exhibit activity against a wide range of host proteins, including immunoglobulins, extracellular matrix proteins, bacterioidal proteins, collagen, fibronectin, fibrinogen, and TNF, and proteins involved in the complement, coagulation, and kallikrein-kinin cascades (14, 45). We reasoned that the inhibitory activity observed with both invasive and noninvasive P. gingivalis strains could be due to the degradation of these chemokines by gingipains. We have previously demonstrated that inactivation of cysteine proteinases,
including gingipains R and gingipain K, with leupeptin, Z-FKck, or a cocktail of protease inhibitors prior to infection in mice resulted in a decrease in P. gingivalis virulence in the mouse chamber model (14). To address the role of gingipains in the inhibitory activity of P. gingivalis for IL-8, we preincubated P. gingivalis with several protease inhibitors specific for gingipains prior to the addition to the HUVEC monolayer. The viability of all cultures prior to HUVEC infection was confirmed (data not shown). As shown by the results in Fig. 5, we observed an increase in the levels of IL-8 produced by HUVEC following a 16-h incubation with P. gingivalis cultures which were preincubated with the protease inhibitors or in P. gingivalis cultures preincubated with dH2O only. At 24 h postinfection, we observed a significant increase in the IL-8 produced in HUVEC infected with P. gingivalis cultures which were preincubated with the protease inhibitors compared to that in P. gingivalis cultures preincubated with dH2O only. In agreement with our previous observations (11), we found that preincubation of P. gingivalis with protease inhibitors also inhibited bacterial invasion. In HUVEC infected with P. gingivalis that had been preincubated with protease inhibitors, we observed a 3-log reduction in the intracellular bacteria compared to that in HUVEC infected with P. gingivalis cultures preincubated with dH2O (1.0 \times 10^8 versus 1.1 \times 10^6, respectively). However, the numbers of extracellular bacteria observed at 24 h in the protease inhibitor-treated P. gingivalis cultures was similar to that observed for the P. gingivalis cultures preincubated with dH2O (8.9 \times 10^7 versus 1.6 \times 10^6, respectively). These results indicate that inhibition of P. gingivalis protease activity, including the activity of gingipain R and gingipain K, results in the stimulation of IL-8 production in HUVEC infected with P. gingivalis.

**Infection of HUVEC with P. gingivalis does not inhibit IL-8 transcription.** To determine whether P. gingivalis infection of HUVEC exerted inhibitory effects on IL-8 transcription, RNA was extracted from P. gingivalis-infected HUVEC cultures and IL-8 RNA was quantitated. We observed a significant increase in the IL-8 transcript in HUVEC cocultured with P. gingivalis strain A7436 compared to that in uninfected HUVEC (Fig. 6). Similar levels of IL-8 transcript were detected in P. gingivalis cultures that had been preincubated with protease inhibitors (Fig. 6). We did not detect an MCP-1 transcript in either uninfected HUVEC or P. gingivalis-infected HUVEC sampled at 24 postinfection (data not shown). Transcription of MCP-1 is typically observed prior to this time (22), and the absence of the MCP-1 transcript at 24 h was an expected observation. We did not observe any differences in the transcript levels of the other templates used in the RNase protection assay in HUVEC cocultured with P. gingivalis strain A7436 compared to that in uninfected HUVEC (data not shown).

**Infection of HUVEC with P. gingivalis kgp and rgpA mutants stimulates IL-8 and MCP-1 production.** To further define the role of P. gingivalis gingipains in the inhibition of IL-8 and MCP-1 production by P. gingivalis-infected HUVEC, we examined the chemokine response following challenge with a P. gingivalis rgpA mutant (YPPI), a kgp mutant (YPPII), and the corresponding wild-type strain (33277). In agreement with previous observations for epithelial cells (39), we found that P. gingivalis strains YPPI and YPPII were not as invasive for
HUVEC as was strain 33277 (data not shown). However, for both strains, the numbers of extracellular bacteria observed at 24 h were similar to that observed for the wild-type strain 33277 (2.8 × 10^6 for YPP1 and YPP2 and 3.0 × 10^6 for 33277). As observed for P. gingivalis strain A7436 and 381 (see above), we did not observe an increase in the IL-8 and MCP-1 levels in HUVEC cultured with the wild-type P. gingivalis strain 33277 compared to the levels in uninfected HUVEC cultures at all times examined (Fig. 7). In contrast, infection of HUVEC with the P. gingivalis kgp (YPP2) or kgpA (YPP1) mutants resulted in a significant increase in IL-8 and MCP-1 as observed following 24 and 48 h of infection compared to that observed in the P. gingivalis wild-type strain 381-infected HUVEC cultures. Interestingly, the P. gingivalis kgp mutant was found to induce the expression of much higher levels of both IL-8 and MCP-1 than did the kgpA mutant at all times (Fig. 7). These results suggest that gingipain K is the major protease involved in the degradation of IL-8 and MCP-1 in P. gingivalis-infected HUVEC.

To determine whether gingipain K exerted effects on IL-8 transcription, RNA was extracted from P. gingivalis 33277 and YPP2-infected HUVEC cultures and IL-8 RNA was quantitated. In HUVEC cocultured with P. gingivalis strain 33277, we observed a significant increase in the IL-8 transcript compared to that in uninfected HUVEC (Fig. 8). Interestingly, we observed a significant increase in the IL-8 transcript in HUVEC cocultured with P. gingivalis YPP2 compared to that in HUVEC cocultured with the wild-type strain 33277 (Fig. 8). Taken together, these results indicate that the inhibition of IL-8 production in HUVEC is mediated via both transcriptional and posttranscriptional events due in part to gingipain K.

FIG. 6. P. gingivalis infection of endothelial cells stimulates IL-8 transcription. P. gingivalis A7436 cultures were incubated with protease inhibitors for 1 h at 37°C under anaerobic conditions, washed, and resuspended in HUVEC growth medium. HUVEC monolayers were infected with 1.0 ml of the P. gingivalis bacterial suspension (MOI of 1:100) and incubated at 37°C in 5% CO₂ for 24 h. Then, the RNA was extracted. Two micrograms of total RNA was hybridized to the probe template set. Nuclease-protected RNA fragments were analyzed on a polyacrylamide gel, which was subsequently exposed to Imaging Screen-K. The band intensities were determined with Quantity-one software, and the mRNA expression levels for IL-8 were normalized with respect to the average intensities of the bands of the housekeeping genes (HKG) GAPDH and L32. Black bars, uninfected HUVEC; open bars, HUVEC infected with P. gingivalis preincubated with ZFKck; stippled bars, HUVEC infected with P. gingivalis preincubated with leupeptin; horizontal line bars, HUVEC infected with P. gingivalis preincubated with the protease inhibitor cocktail. The data are the means ± standard deviations for at least two separate experiments performed in duplicate. * , P value of <0.2 compared to uninfected HUVEC.

FIG. 7. Infection of endothelial cells with P. gingivalis kgp and rggA mutants stimulates IL-8 and MCP-1 production. P. gingivalis strain 33277 or the corresponding rggA (YPP1) or kgp (YPP2) mutant was added to the HUVEC monolayer at a MOI of 1:100 and incubated at 37°C in 5% CO₂. At the designated times, supernatant samples were collected and analyzed by ELISA for IL-8 (A) and MCP-1 (B). Black bars, uninfected HUVEC; gray bars, HUVEC infected with P. gingivalis 33277; open bars, HUVEC infected with P. gingivalis YPP1; horizontal line bars, HUVEC infected with P. gingivalis YPP2. The data are the means ± standard deviations. * , P value of <0.05 compared to HUVEC cultures challenged with the P. gingivalis wild-type strain 33277.

DISCUSSION

In this study we have established that P. gingivalis outer membrane components, including peptides corresponding to the mature fimbriae protein, can stimulate IL-8 and MCP-1 production in HUVEC. In contrast, infection of HUVEC with live P. gingivalis abolishes the normal IL-8 and MCP-1 responses. Inhibition of chemokine expression is not dependent on adherence and invasion since similar results were obtained (i) with the noninvasive fmaA mutant, (ii) when P. gingivalis was preincubated with anti-fimbrial peptide sera prior to coculture with HUVEC, and (iii) in cytochalasin D-treated HUVEC cocultured with P. gingivalis. We also found that treatment of HUVEC with E. coli LPS stimulated robust IL-8 and MCP-1 responses, which were abolished when similarly challenged cells were cocultured with P. gingivalis.

The interactions of P. gingivalis with endothelial cells appear to involve a two-stage process of initial and intimate attachment to the endothelial cell surface (10, 11). We have previously demonstrated that the initial attachment of P. gingivalis to the endothelial cell is mediated via the major fimbriae (11); this attachment is followed by the intimate attachment to the surface of the endothelial cell and bacterial engulfment. The proteins required for the tight adherence of P. gingivalis to
endothelial cells have not yet been identified. Preincubation of *P. gingivalis* with antisera to fimbriin peptides, as well as the use of the *fimA* mutant in this study, enabled us to block the step of adherence of *P. gingivalis* to the endothelial cell mediated by fimbrins. Under these conditions, we did not observe the induction of a chemokine response. Furthermore, the ability of *P. gingivalis* fimbrin peptides to stimulate IL-8 and MCP-1 responses suggests that the interactions of *P. gingivalis* with the endothelial cell have a stimulatory effect. Interestingly, contact of *P. gingivalis* with epithelial cells has been reported to repress the secretion of gingipains (39). If a similar phenomenon occurs with endothelial cells, physically blocking adherence of *P. gingivalis* to endothelial cells would enable the organism to continue to express the gingipains, which would function to degrade chemokines expressed by the endothelial cell.

Our study, together with other published reports (32–34, 36–38), indicate that *P. gingivalis* fimbrinae have a variety of immunobiological properties. *P. gingivalis* fimbriniae have been reported to elicit the production of several proinflammatory cytokines, such as IL-1, TNF, IL-6, and IL-8, in human peripheral blood monocytes and macrophages (37). Furthermore, our results of immunostimulation in endothelial cells are in agreement with those of recent studies which demonstrated that peptides corresponding to aa 69 to 80 of fimbrininduce IL-8 expression in fibroblasts (35). Although *P. gingivalis* fimbriniae themselves can trigger cell activation, they may also function to lock the organism to sites at which host receptors are expressed and may influence the host response by presenting other bacterial components, such as LPS, to these receptors (2, 19, 20). A recent study has shown that the host recognizes LPS and other microbial products not as purified molecules but as complexes and that fimbriniae determine the molecular context in which LPS is presented to host cells (19).

Initial interactions between pathogenic bacteria and target cells are crucial events in cell infection. Several studies have documented that contact of bacteria with host cells can induce cross talk (2). In *Neisseria meningitidis*, transient induction of the gene *pilC1* encoding a pilus-associated protein key to the initial attachment of meningococcal to target cells is observed upon cell contact (9). During the second step, intimate adhesion, the expression of *pilC1* is decreased to its basal level. It has been postulated that repression of *pilC1* might be necessary for bacterial adhesion to progress further into intimate adhesion. The latter may occur by the unmasking of structures involved in intimate adhesion. If a similar system for cross talk exists in *P. gingivalis*, one would expect a decrease in *fimA* expression during the second intimate adhesion step. A recent study has documented that the interaction of *P. gingivalis* with *Streptococcus* in a biofilm setting reduced *fimA* promoter activity (48). Thus, in addition to transcriptional control mediated by contact with bacteria in a biofilm setting, *P. gingivalis* *fimA* expression could be modulated following the interaction of *P. gingivalis* with host cells.

Our studies also point to a major role for the gingipains in the degradation of IL-8 and MCP-1. Studies using the *P. gingivalis kgp* mutant point to a role for gingipain K in both transcriptional and posttranscriptional inhibition of IL-8 in *P. gingivalis*-infected HUVEC. Interestingly, although the *P. gingivalis kgp* mutant still expresses functional gingipain R, we observed strong IL-8 and MCP-1 responses in HUVEC infected with this strain. These results suggest that cleavage of IL-8 by gingipain K may make IL-8 more susceptible to cleavage by gingipain R and are in agreement with our results obtained with the *rgpA* mutant, as well as recent in vitro studies (29). These in vitro studies also reported that membrane bound gingipains are active against IL-8 degradation in vitro (29). In contrast, soluble gingipains initially convert IL-8 to a more potent species truncated at the amino terminus. It has been proposed that this division of enhancing and inactivating activity between membrane and soluble gingipains can cause the compartmentalization of pro- and anti-inflammatory reactions to distal and proximal positions from bacterial plaque. Furthermore, it was proposed that this could explain why despite the massive neutrophil accumulation at periodontitis sites, there is no elimination of infection (29).

The ability of *P. gingivalis* to inhibit IL-8 accumulation from gingival epithelial cells has recently been reported (21). These investigators reported that the inhibition was associated with a decrease in mRNA for IL-8. However, we detected a significant increase in the IL-8 transcript in *P. gingivalis*-infected HUVEC compared to that in uninjected HUVEC cultures. The differences observed in these studies may be related to intricate differences in epithelial versus endothelial cells, to the *P. gingivalis* inoculum used, and to the length of exposure of *P. gingivalis* to the various cell types. A separate study (8) has reported that at low concentrations, the noninvasive *P. gingivalis* strain DPG3 was unable to antagonize IL-8 accumulation in gingival epithelial cells. However, using an inoculum similar to that used in our studies (10^8 CFU), these investigators did observe inhibition of IL-8 accumulation. These investigators suggested that whether a lesion was acute or chronic could be influenced by the dose of *P. gingivalis*. Others have also documented that the size of the microbial inoculum could affect the expression of a given chemokine (13).
Other studies have demonstrated that P. gingivalis infection stimulates a strong cytokine and chemokine response in KB cells and primary cultures of pocket epithelium (42). These investigators correlated the ability of infecting strains to invade these cells with the increase in the cytokine response. The differences observed by these investigators and in our studies may be due to differences in the growth of the bacteria used for the infection assays and to differences in epithelial versus endothelial cells, as well as the length of time that cells were exposed to P. gingivalis cultures. It is important to stress that in our studies were intended to mimic a chronic infection in which P. gingivalis was present throughout the incubation period. Continual exposure of endothelial cells to membrane-bound gingipains from viable bacteria is most likely responsible for the observation of IL-8 inhibition reported here. In the study by Sandros et al. (42), P. gingivalis was incubated with epithelial cells for 90 min; thus, we would assume that this study was concerned with the early events of P. gingivalis host cell interactions, i.e., initial attachment to the host cell mediated via fibrinect. Under these conditions we would expect to see an early chemokine response. This possibility is supported by our results in which P. gingivalis fibrinectin peptides and membrane components were found to stimulate a chemokine response in endothelial cells. It is also important to point out that for the studies described here, P. gingivalis cultures were grown to the logarithmic phase in liquid broth. In contrast, in studies described by Sandros et al. (42), bacteria were grown on agar plates and presumably the majority of bacteria were in the stationary phase of growth. It has been reported that P. gingivalis gingipains are maximally expressed during logarithmic growth (14).

Recent cross-sectional and prospective epidemiological studies have demonstrated an association between periodontal disease and atherosclerosis and human coronary heart disease (3, 4, 15, 26, 27, 31). Furthermore, pathological studies have recently identified P. gingivalis in diseased atherosclerotic tissue by PCR (18). A hallmark of atherosclerosis is the accumulation of blood-borne leukocytes into the inflamed tissues in response to antigenic stimulation. This process is initiated with the binding of leukocytes to the activated endothelium via induced expression of adhesion molecules (30). Leukocyte chemotaxis and migration across the endothelium are modulated by several chemokines, including IL-8 and MCP-1. The results obtained in this study indicate the initial response following attachment of P. gingivalis to the endothelial cell mediated via fibrinect includes the expression of IL-8 and MCP-1. We have also recently demonstrated that P. gingivalis fibrinectin peptides can induce cell surface-associated adhesion molecule expression including intracellular adhesion molecule 1, vascular cell adhesion molecule 1, and E- and P-selectin in endothelial cells (25). Furthermore, active P. gingivalis invasion of HUVEC was also found to result in the stimulation of these cell adhesion molecules (25). We propose that the chemokine and cell adhesion molecule response induced by the initial attachment of P. gingivalis to endothelial cells mediated via fibrinect leads ultimately to the recruitment, activation, and firm adhesion of neutrophils to the endothelial cell. We propose that this host-mediated response to P. gingivalis may contribute to the early events associated with the atherosclerotic process.

In summary, our studies indicate that the interaction of P. gingivalis with endothelial cells and the subsequent activation of the proinflammatory response involve a complex series of events involving both fibrinect and gingipain-mediated mechanisms. The ability of P. gingivalis to temporally modulate the chemokine response in endothelial cells may serve as a means of productive chronic and symbiotic interactions with the host and may play an important role in the pathogenesis of systemic chronic diseases associated with this organism including atherosclerosis.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant PO1DE11391 from the National Institute of Dental and Craniofacial Research to C.A.G.

We acknowledge Dana Graves and Salomon Amar for stimulating discussions and scientific advice. We also thank Hakim Sojar for fibrinect peptide-specific antisera. H. Nassar and H.-H. Chou contributed equally to this work.

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