INFECTION AND IMMUNITY, Jan. 2002, p. 268–276 Vol. 70, No. 1
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.1.268–276.2002

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

Hamdy Nassar,[1,2] Hsin-Hua Chou,[1,3,4] Mary Khlgatian,[1] Frank C. Gibson III,[1] T. E. Van Dyke,[5] and Caroline Attardo Genco[1]*

Department of Medicine, Boston University School of Medicine,[1] and Department of Oral Biology, Goldman School of Dental Medicine, Boston University,[5] Boston, Massachusetts; Department of Periodontics, Al-Azhar University, Cairo, Egypt[2]; and School of Dentistry, Taipei Medical University,[3] and Dental Department, Wan-Fan Hospital,[4] Taipei, Taiwan

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 antisera prior to the addition to HUVEC. Furthermore, treatment of *P. gingivalis*-infected HUVEC with cytochalsin 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. Coculture of HUVEC with a *P. gingivalis* mutant deficient in lysine-specific cysteine proteinase (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

* Corresponding author. Mailing address: Department of Medicine, Section of Infectious Diseases, Boston University School of Medicine, 650 Albany St., Boston, MA 02118. Phone: (617) 414-5305. Fax: (617) 414-5280. E-mail: caroline.genco@bmc.org.

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. Chemoattractant 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 proteinases (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 fimA* 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 (*rgpA*) and YPP2 (*kgp*) and the corresponding wild-type strain 33277 (39). The *P. gingivalis fimA* mutant (DPG3) and *rgpA* (YPP1) and *kgp* (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% N_2, 5% H_2, and 10% CO_2 for 3 to 5 days. For infection assays, *P. gingivalis* was transferred from plates into Schaedler broth (Difco, Detroit, Mich.) and grown for 24 h or until the optical density at 660 nm reached 1.0.

Preparation of heat-killed *P. gingivalis* whole cell extracts and *P. gingivalis* and *Escherichia coli* LPS. *P. gingivalis* whole cell extracts were prepared by heating a bacterial suspension (equivalent to 10^8 CFU) for 1 h at 60°C. *P. gingivalis* and *E. coli* lipopolysaccharide (LPS) extraction was prepared by a hot phenol-water technique (12, 46). LPS preparations were analyzed for protein contamination by electrophoresis by overloading a sodium dodecyl sulfate–12.5% polyacrylamide gel stained with Coomassie blue and silver nitrate. LPS samples were also examined on commercially prepared 10 to 20% gradient gels. LPS was further analyzed for protein contamination with the use of a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). For HUVEC (Cascade Biologics, Inc, Portland, Oreg.) stimulation assays, LPS samples were diluted, sonicated in HUVEC culture media, and added to HUVEC cultures (see below).

Fimbrillin peptides. Fimbrillin peptides based on the amino acid sequence of the native fimbrillin of *P. gingivalis* strain 381 and corresponding to sequences 61 to 80 (GKTLAEVKALTTELTAENQE) and 171 to 185 (DANYLTGSLTT FNGA) and control peptides corresponding to a scrambled version of each peptide to be tested were commercially synthesized (Bio Synthesis, Inc., Lewisville, Tex.). All peptides were determined to be free of contaminating endotoxin by high-performance liquid chromatography analysis as indicated by the manufacturer. Peptides were diluted and added to HUVEC cultures as described below.

Infection of HUVEC with *P. gingivalis*. HUVEC cultures were grown in Media-200 (Cascade Biologics, Inc., Portland, Oreg.) supplemented with low-serum-growth supplement (20 μl/ml). HUVEC were plated at a concentration of 10^5 to 10^6 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 10^8 cells per ml. Bacterial suspensions (1.0 ml) were added to confluent HUVEC monolayers (MOI = 100) and incubated at 37°C in 5% CO_2. 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% CO_2. 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 fimA* mutant (DPG3) or by preincubating *P. gingivalis* with fimbrillin peptide-specific antisera. We have previously established that preincubation of *P. gingivalis* with fimbria-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 (VVMANTAGAMELVGKTLAEVK) and 69 to 90 (ALTTELTAENQEAAGLIMTAEP) 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 cytochalsin 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* was pretreated with protease inhibitors. The following inhibitors in the solvent and at the final concentration indicated were used: leupeptin, 0.1 mM in distilled H_2O (dH_2O) (Sigma, St. Louis, Mo.); Z-FKck (benzylooxycarbonyl-Phe-Lys-CH_2OCO-2,4,6-*Z*-Phe-Lys-2,4,6-trimethylbenzoyloxymethylketone trifluoroacetate), 0.1 mM in dH_2O (BACHEM Bioscience Inc., King of Prussia, Pa.); and a cocktail of protease inhibitors containing aprotonin (2 μg/ml in dH_2O), 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 dH_2O 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 5×10^7 CFU per ml. HUVEC monolayers were infected with 1.0 ml of the *P. gingivalis* bacterial suspension (MOI = 100) and incubated at 37°C in 5% CO_2 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.

RNase protection assay. Total RNA was extracted from HUVEC cultures with a Trizol reagent (Gibco BRL, Grand Island, N.Y.) according to the manufac-

turer's instructions and was quantified spectrophotometrically. Detection and semiquantification of IL-8 mRNA were performed with a multiprobe RNase protection assay system (Pharmingen, San Diego, Calif.). Briefly, a mixture of [^{32}P]UTP-labeled antisense riboprobes were generated from a multiprobe template set (hck-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 10^6 cpm of the ^{32}P-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 difference 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).

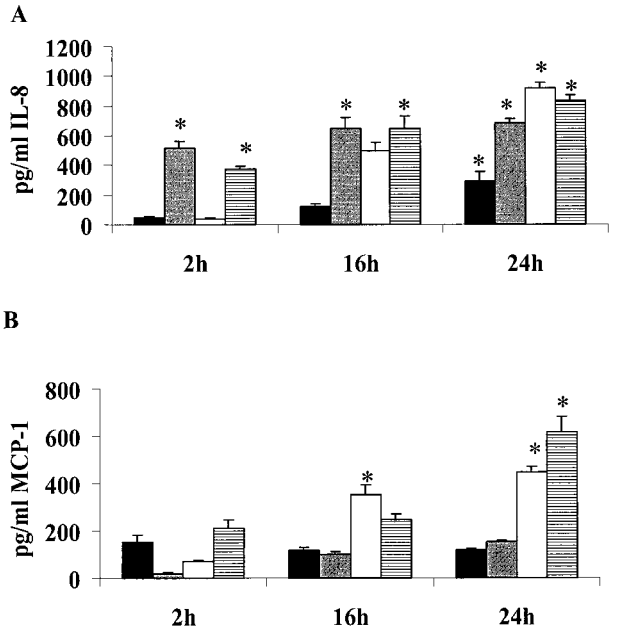

FIG. 1. *P. gingivalis* fimbrillin peptides and membrane components stimulate IL-8 and MCP-1 expression in endothelial cells. HUVEC were incubated with a *P. gingivalis* fimbrillin peptide corresponding to amino acid sequences 61 to 80 (10 and 100 μg/ml; black and gray bars, respectively), *P. gingivalis* A7436 LPS (10.0 μg/ml; open bars), or heat-killed whole cell preparations (corresponding to 10^8 CFU; horizontal line bars) at 37°C in 5% CO_2, and supernatant samples were collected at 2, 16, and 24 h postaddition. Supernatant samples were analyzed by ELISA for IL-8 (A) and MCP-1 (B). The data are the means ± standard deviations for at least three separate experiments performed in triplicate. *, *P* value of <0.01 compared to uninfected HUVEC at each respective time.

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.

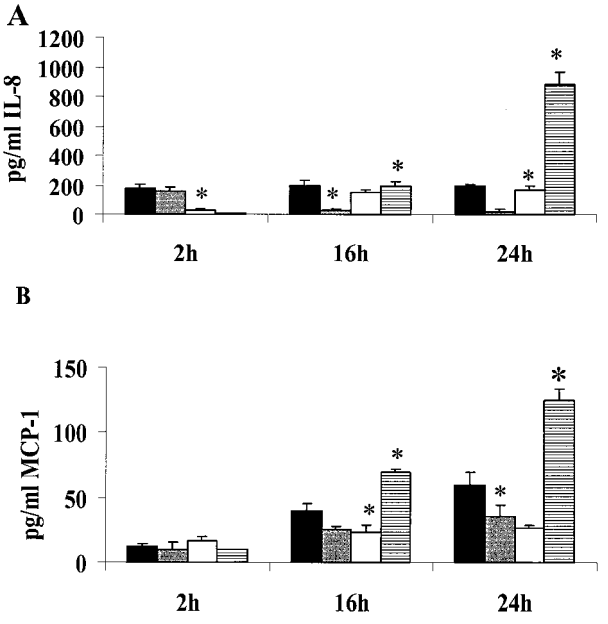

FIG. 2. Infection of HUVEC with *P. gingivalis* inhibits constitutive IL-8 and MCP-1 production. *P. gingivalis* strain A7436 (white bars) or 381 (gray bars) was added to the HUVEC monolayer (MOI of 1:100) and incubated at 37°C in 5% CO_2. *P. gingivalis* A7436 LPS (10.0 μg/ml; horizontal line bars) was used as a positive control. Black bars, uninfected HUVEC. At the designated time, supernatant samples were collected and analyzed by ELISA for IL-8 (A) and MCP-1 (B). The data are the means ± standard deviations for at least three separate experiments performed in triplicate. *, *P* value of <0.01 compared to uninfected HUVEC at each respective time.

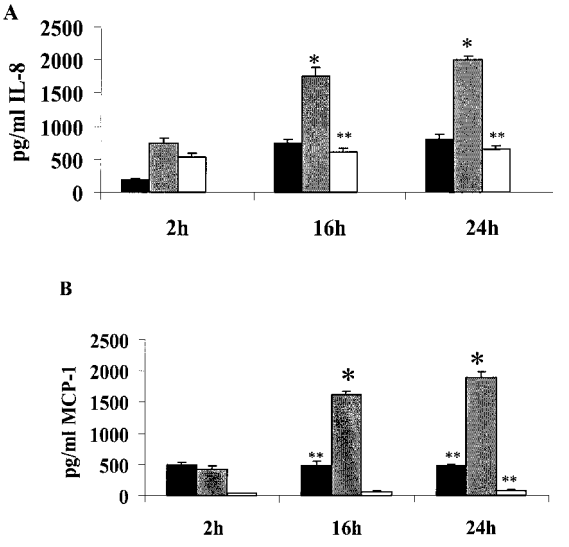

FIG. 3. *P. gingivalis* inhibits IL-8 and MCP-1 production by *E. coli* LPS-stimulated endothelial cells. *E. coli* LPS (0.1 μg/ml) and *P. gingivalis* strain A7436 (10^8 CFU) cultures were added to the HUVEC monolayer (10^6) and incubated at 37°C in 5% CO_2 (open bars). *E. coli* LPS (0.1 μg/ml) only was used as a positive control (gray bars). Black bars, uninfected HUVEC. At the designated times, supernatant samples were collected and analyzed by ELISA for IL-8 (A) and MCP-1 (B). The data are the means ± standard deviations for at least two separate experiments performed in triplicate. **, *P* value of <0.001 compared to uninfected HUVEC at each respective time. *, *P* value of <0.01 compared to *E. coli*-stimulated HUVEC at each respective time.

***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 fimbrillin peptide-specific antisera compared to that in uninfected cultures or to *P. gingivalis* cultures preincubated with normal rabbit serum. The ability of the fimbrillin 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 cytochalsin 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, bactericidal 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,

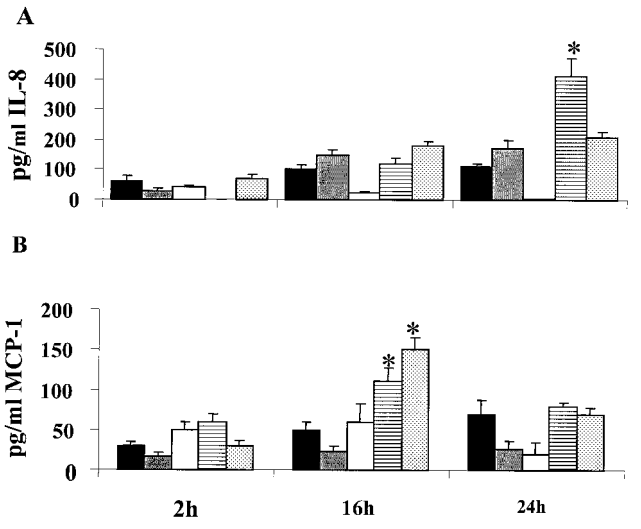

FIG. 4. Inhibition of IL-8 and MCP-1 production by *P. gingivalis* is not dependent on fimbria-mediated adherence or bacterial invasion. *P. gingivalis* strain 381 was preincubated with rabbit polyclonal anti-fimbrillin peptide sera (open bars) or a normal rabbit serum control (stippled bars) at a 1/500 dilution under anaerobic conditions prior to infection of HUVEC. Untreated or treated *P. gingivalis* strain 381 or DPG3 (10^8 CFU [gray bars]) was then added to the HUVEC monolayer (10^6) and incubated at 37°C in 5% CO_2. Black bars, uninfected HUVEC. To examine the effects of invasion on the chemokine response to *P. gingivalis*, we preincubated HUVEC with cytochalsin D (1 μg/ml in dimethyl sulfoxide) for 30 min prior to the addition of *P. gingivalis* strain 381 (horizontal line bars). At the designated times, supernatant samples were collected and analyzed by ELISA for IL-8 (A) and MCP-1 (B). The data are the means ± standard deviations for at least two separate experiments performed in triplicate. *, *P* value of <0.01 compared to uninfected HUVEC at each respective time.

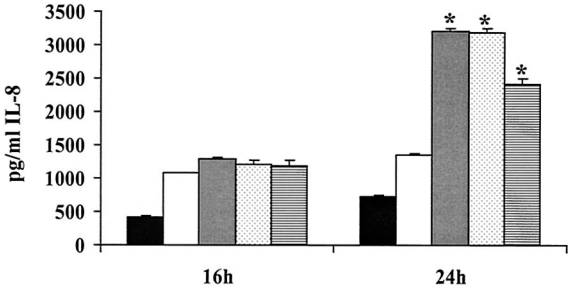

FIG. 5. Pretreatment of *P. gingivalis* with protease inhibitors stimulates IL-8 in *P. gingivalis*-infected endothelial cells. *P. gingivalis* A7436 cultures were incubated with protease inhibitors for 1 h at 37°C under anaerobic conditions, washed, and resuspended in HUVEC growth media. HUVEC monolayers were infected with 1.0 ml of the *P. gingivalis* bacterial suspension (MOI of 1:100) and incubated at 37°C in 5% CO_2 for 16 and 24 h. Supernatant samples were collected and analyzed by ELISA for IL-8. Gray 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; black bars, uninfected HUVEC; open bars, *P. gingivalis* preincubated with dH_2O. The data are expressed relative to the IL-8 expressed by HUVEC infected with *P. gingivalis* that was preincubated with dH_2O, and are the means ± standard deviations for at least two separate experiments performed in duplicate. *, *P* value of <0.1 compared to a control culture of *P. gingivalis* preincubated with dH_2O only.

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 dH_2O 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 dH_2O.

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 dH_2O (1.0×10 versus 1.1×10^4, 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 dH_2O (8.9×10^5 versus 1.6×10^6, 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 *P. gingivalis*-infected HUVEC.

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 quanititated. 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 (YPP1), a *kgp* mutant (YPP2), and the corresponding wild-type strain (33277). In agreement with previous observations for epithelial cells (39), we found that *P. gingivalis* strains YPP1 and YPP2 were not as invasive for

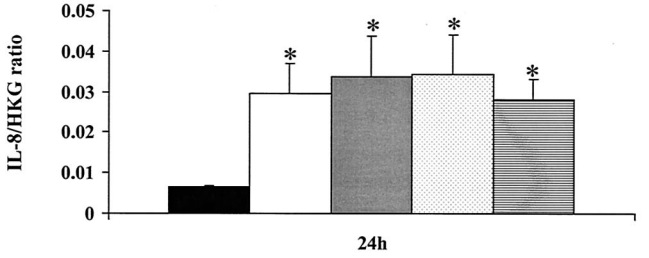

FIG. 6. *P. gingivalis* infection of endothelial cells stimulates IL-8 transcription. *P. gingivalis* A7436 cultures were incubated with protease inhibitors for 1 h at 37°C under anaerobic conditions, washed, and resuspended in HUVEC growth medium. HUVEC monolayers were infected with 1.0 ml of the *P. gingivalis* bacterial suspension (MOI of 1:100) and incubated at 37°C in 5% CO_2 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*; gray 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.

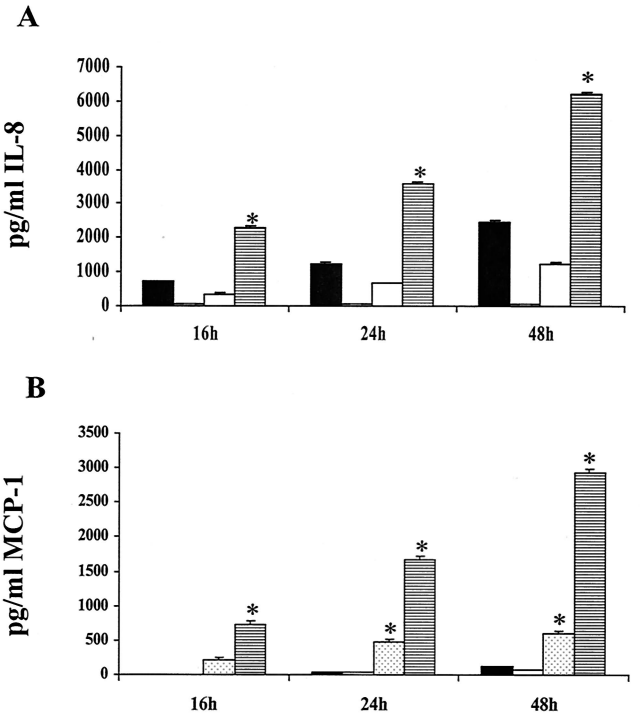

FIG. 7. Infection of endothelial cells with *P. gingivalis kgp* and *rgpA* mutants stimulates IL-8 and MCP-1 production. *P. gingivalis* strain 33277 or the corresponding *rgpA* (YPP1) or *kgp* (YPP2) mutant was added to the HUVEC monolayer at a MOI of 1:100 and incubated at 37°C in 5% CO_2. At the designated 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.

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 *rgpA* (YPP1) mutants resulted in an 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 *rgpA* 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.

DISCUSSION

In this study we have established that *P. gingivalis* outer membrane components, including peptides corresponding to the mature fimbrillin 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 *fimA* mutant, (ii) when *P. gingivalis* was preincubated with anti-fimbrillin peptide sera prior to coculture with HUVEC, and (iii) in cytochalsin 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

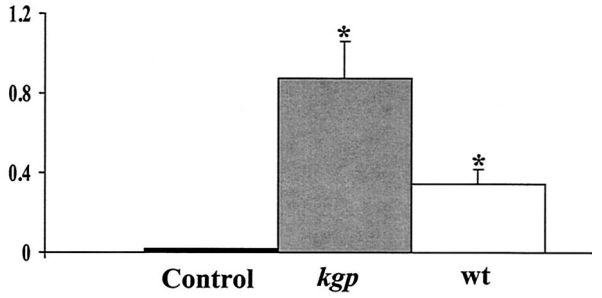

FIG. 8. Infection of endothelial cells with a *P. gingivalis kgp* mutant upregulates IL-8 transcription. *P. gingivalis* strains 33277 (wt) or YPP2 (*kgp*) was added to the HUVEC monolayer at a MOI of 1:100 and incubated at 37°C in 5% CO_2. Control, uninfected HUVEC. Samples were removed at 24 h postinfection, and RNA was extracted from HUVEC. 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. The data are the means ± standard deviations for at least two separate experiments performed in duplicate. *, *P* value of <0.05 compared to HUVEC cultures challenged with the *P. gingivalis* wild-type strain 33277.

endothelial cells have not yet been identified. Preincubation of *P. gingivalis* with antisera to fimbrillin 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 via fimbriae. Under these conditions, we did not observe the induction of a chemokine response. Furthermore, the ability of *P. gingivalis* fimbrillin peptides to stimulate IL-8 and MCP-1 responses suggests that the initial 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* fimbriae have a variety of immunobiological properties. *P. gingivalis* fimbriae 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 fimbrillin induce IL-8 expression in fibroblasts (35). Although *P. gingivalis* fimbriae themselves can trigger cell activation, they may also function to dock 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 fimbriae 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 uninfected 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 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 fimbriae. Under these conditions we would expect to see an early chemokine response. This possibility is supported by our results in which *P. gingivalis* fimbrillin 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 fimbriae includes the expression of IL-8 and MCP-1. We have also recently demonstrated that *P. gingivalis* fimbrillin 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 fimbriae 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 fimbriae 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 PO1DE13191 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 fimbrillin peptide-specific antisera.

H. Nassar and H.-H. Chou contributed equally to this work.

REFERENCES

1. **Abe, N., T. Kadowaki, K. Okamoto, K. Nakayama, M. Ohishi, and K. Yamamoto.** 1998. Biochemical and functional properties of lysine-specific cysteine proteinase (Lys-gingipain) as a virulence factor of *Porphyromonas gingivalis* in periodontal disease. J. Biochem. (Tokyo) **123:**305–312.
2. **Abraham, S. N., A. B. Jonsson, and S. Normark.** 1998. Fimbriae-mediated host-pathogen cross-talk. Curr. Opin. Microbiol. **1:**75–81.
3. **Arbes, S. J., Jr., G. D. Slade, and J. D. Beck.** 1999. Association between extent of periodontal attachment loss and self-reported history of heart attack: an analysis of NHANES III data. J. Dent. Res. **78:**1777–1782.
4. **Beck, J., R. Garcia, G. Heiss, P. S. Vokonas, and S. Offenbacher.** 1996. Periodontal disease and cardiovascular disease. J. Periodontol. **67:**1123–1137.
5. **Beck, J. D., S. Offenbacher, R. Williams, P. Gibbs, and R. Garcia.** 1998. Periodontitis: a risk factor for coronary heart disease? Ann. Periodontol. **3:**127–141.
6. **Chung, H., C. Champagne, J. Southerland, S. Geva, Y. Liu, D. Paquette, P. Madlanos, J. Beck, and S. Offenbacher.** 2000. Effects of *P. gingivalis* infection on atheroma formation in ApoE(+/−) Mice. J. Dent. Res. **79:**313.
7. **Culter, C., K. Patt, J. Potempa, and J. Travis.** 1995. Pathogenic strategies of the oral anaerobe, *Porphyromonas gingivalis*. Trends Microbiol. **3:**45–51.
8. **Darveau, R. P., C. M. Belton, R. A. Reife, and R. J. Lamont.** 1998. Local chemokine paralysis, a novel pathogenic mechanism for *Porphyromonas gingivalis*. Infect. Immun. **66:**1660–1665.
9. **Deghmane, A. E., S. Petit, A. Topilko, Y. Pereira, D. Giorgini, M. Larribe, and M. K. Taha.** 2000. Intimate adhesion of *Neisseria meningitidis* to human epithelial cells is under the control of the *crgA* gene, a novel LysR-type transcriptional regulator. EMBO J. **19:**1068–1078.
10. **Deshpande, R. G., M. Khan, and C. A. Genco.** 1998. Invasion strategies of the oral pathogen *Porphyromonas gingivalis*: implications for cardiovascular disease. Invasion Metastasis **18:**57–69.
11. **Deshpande, R. G., M. B. Khan, and C. A. Genco.** 1998. Invasion of aortic and heart endothelial cells by *Porphyromonas gingivalis*. Infect. Immun. **66:**5337–5343.
12. **Duncan, R. L., Jr., J. Hoffman, V. L. Tesh, and D. C. Morrison.** 1986. Immunologic activity of lipopolysaccharides released from macrophages after the uptake of intact *E. coli* in vitro. J. Immunol. **136:**2924–2929.
13. **Fletcher, J., K. Reddi, S. Poole, S. Nair, B. Henderson, P. Tabona, and M. Wilson.** 1997. Interactions between periodontopathogenic bacteria and cytokines. J. Periodontal Res. **32:**200–205.
14. **Genco, C. A., J. Potempa, J. Mikolajczyk-Pawlinska, and J. Travis.** 1999. Role of gingipains R in the pathogenesis of *Porphyromonas gingivalis*- mediated periodontal disease. Clin. Infect. Dis. **28:**456–465.
15. **Genco, R.** 1998. Periodontal disease and risk for myocardial infection and cardiovascular disease. Cardiovasc. Rev. Rep. **19:**34–40.
16. **Gerard, C., and B. J. Rollins.** 2001. Chemokines and disease. Nat. Immunol. **2:**108–115.
17. **Graves, D. T., and Y. Jiang.** 1995. Chemokines, a family of chemotactic cytokines. Crit. Rev. Oral Biol. Med. **6:**109–118.
18. **Haraszthy, V. I., J. J. Zambon, M. Trevisan, M. Zeid, and R. J. Genco.** 2000. Identification of periodontal pathogens in atheromatous plaques. J. Periodontol. **71:**1554–1560.
19. **Hedlund, M., B. Frendeus, C. Wachtler, L. Hang, H. Fischer, and C. Svanborg.** 2001. Type 1 fimbriae deliver an LPS- and TLR4-dependent activation signal to CD14-negative cells. Mol. Microbiol. **39:**542–552.
20. **Hedlund, M., C. Wachtler, E. Johansson, L. Hang, J. E. Somerville, R. P.**

Darveau, and C. Svanborg. 1999. P fimbriae-dependent, lipopolysaccharide-independent activation of epithelial cytokine responses. Mol. Microbiol. **33:**693–703.
21. **Huang, G. T., D. Kim, J. K. Lee, H. K. Kuramitsu, and S. K. Haake.** 2001. Interleukin-8 and intercellular adhesion molecule 1 regulation in oral epithelial cells by selected periodontal bacteria: multiple effects of *Porphyromonas gingivalis* via antagonistic mechanisms. Infect. Immun. **69:**1364–1372.
22. **Jiang, Y., T. R. Russell, D. T. Graves, H. Cheng, S. H. Nong, and S. M. Levitz.** 1996. Monocyte chemoattractant protein 1 and interleukin-8 production in mononuclear cells stimulated by oral microorganisms. Infect. Immun. **64:**4450–4455.
23. **Kadowaki, T., M. Yoneda, K. Okamoto, K. Maeda, and K. Yamamoto.** 1994. Purification and characterization of a novel arginine-specific cysteine proteinase (argingipain) involved in the pathogenesis of periodontal disease from the culture supernatant of *Porphyromonas gingivalis*. J. Biol. Chem. **269:**21371–21378.
24. **Katz, J., V. Sambandam, J. H. Wu, S. M. Michalek, and D. F. Balkovetz.** 2000. Characterization of *Porphyromonas gingivalis*-induced degradation of epithelial cell junctional complexes. Infect. Immun. **68:**1441–1449.
25. **Khlgatian, M., H. Nassar, H.-H. Chou, F. C. Gibson III, and C. Genco.** 2001 Fimbria-dependent activation of cell adhesion molecule expression in *Porphyromonas gingivalis*-infected endothelial cells. Infect. Immun. **70:**257–267.
26. **Kiechl, S., G. Egger, M. Mayr, C. J. Wiedermann, E. Bonora, F. Oberhollenzer, M. Muggeo, Q. Xu, G. Wick, W. Poewe, and J. Willeit.** 2001. Chronic infections and the risk of carotid atherosclerosis: prospective results from a large population study. Circulation **103:**1064–1070.
27. **Loos, B. G., J. Craandijk, F. J. Hoek, P. M. Wertheim-van Dillen, and U. van der Velden.** 2000. Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of periodontitis patients. J. Periodontol. **71:**1528–1534.
28. **Malek, R., J. G. Fisher, A. Caleca, M. Stinson, C. J. van Oss, J. Y. Lee, M. I. Cho, R. J. Genco, R. T. Evans, and D. W. Dyer.** 1994. Inactivation of the *Porphyromonas gingivalis fimA* gene blocks periodontal damage in gnotobiotic rats. J. Bacteriol. **176:**1052–1059.
29. **Mikolajczyk-Pawlinska, J., J. Travis, and J. Potempa.** 1998. Modulation of interleukin-8 activity by gingipains from *Porphyromonas gingivalis*: implications for pathogenicity of periodontal disease. FEBS Lett. **440:**282–286.
30. **Noll, G.** 1998. Pathogenesis of atherosclerosis: a possible relation to infection. Atherosclerosis **140:**S3–S9.
31. **Offenbacher, S., P. N. Madianos, C. M. Champagne, J. H. Southerland, D. W. Paquette, R. C. Williams, G. Slade, and J. D. Beck.** 1999. Periodontitis-atherosclerosis syndrome: an expanded model of pathogenesis. J. Periodontal Res. **34:**346–352.
32. **Ogawa, T.** 1994. The potential protective immune responses to synthetic peptides containing conserved epitopes of *Porphyromonas gingivallis* fimbrial protein. J. Med. Microbiol. **41:**349–358.
33. **Ogawa, T., and S. Hamada.** 1994. Hemagglutinating and chemotactic properties of synthetic peptide segments of fimbrial protein from *Porphyromonas gingivalis*. Infect. Immun. **62:**3305–3310.
34. **Ogawa, T., Y. Kusumoto, H. Uchida, S. Nagashima, H. Ogo, and S. Hamada.** 1991. Immunobiological activities of synthetic peptide segments of fimbrial protein from *Porphyromonas gingivalis*. Biochem. Biophys. Res. Commun. **180:**1335–1341.
35. **Ogawa, T., H. Ogo, and A. Kinoshita.** 1997. Antagonistic effect of synthetic peptides corresponding to the binding regions within fimbrial subunit protein from *Porphyromonas gingivalis* to human gingival fibroblasts. Vaccine **15:** 230–236.
36. **Ogawa, T., H. Ogo, H. Uchida, and S. Hamada.** 1994. Humoral and cellular immune responses to the fimbriae of *Porphyromonas gingivalis* and their synthetic peptides. J. Med. Microbiol. **40:**397–402.
37. **Ogawa, T., H. Uchida, and S. Hamada.** 1994. *Porphyromonas gingivalis* fimbriae and their synthetic peptides induce proinflammatory cytokines in human peripheral blood monocyte cultures. FEMS Microbiol. Lett. **116:**237–242.
38. **Ogawa, T., H. Uchida, and K. Yasuda.** 1995. Mapping of murine Th1 and Th2 helper T-cell epitopes on fimbriae from *Porphyromonas gingivalis*. J. Med. Microbiol. **42:**165–170.
39. **Park, Y., and R. J. Lamont.** 1998. Contact-dependent protein secretion in *Porphyromonas gingivalis*. Infect. Immun. **66:**4777–4782.
40. **Potempa, J., R. Pike, and J. Travis.** 1995. The multiple forms of trypsin-like activity present in various strains of *Porphyromonas gingivalis* are due to the presence of either Arg-gingipain or Lys-gingipain. Infect. Immun. **63:**1176–1182.
41. **Saglie, F. R., A. Marfany, and P. Camargo.** 1988. Intragingival occurrence of *Actinobacillus actinomycetemcomitans* and *Bacteroides gingivalis* in active destructive periodontal lesions. J. Periodontol. **59:**259–265.
42. **Sandros, J., C. Karlsson, D. F. Lappin, P. N. Madianos, D. F. Kinane, and P. N. Papapanou.** 2000. Cytokine responses of oral epithelial cells to *Porphyromonas gingivalis* infection. J. Dent. Res. **79:**1808–1814.
43. **Socransky, S. S., and A. D. Haffajee.** 1992. The bacterial etiology of destructive periodontal disease: current concepts. J. Periodontol. **63:**322–331.
44. **Sojar, H. T., Y. Han, N. Hamada, A. Sharma, and R. J. Genco.** 1999. Role of the amino-terminal region of *Porphyromonas gingivalis* fimbriae in adherence to epithelial cells. Infect. Immun. **67:**6173–6176.
45. **Travis, J., R. Pike, T. Imamura, and J. Potempa.** 1997. *Porphyromonas gingivalis* proteinases as virulence factors in the development of periodontitis. J. Periodontal Res. **32:**120–125.
46. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharide: extraction with phenol water and further applications of the procedure. Methods Carbohydr. Chem. **5:**83–91.
47. **Wu, T., M. Trevisan, R. J. Genco, J. P. Dorn, K. L. Falkner, and C. T. Sempos.** 2000. Periodontal disease and risk of cerebrovascular disease: the first national health and nutrition examination survey and its follow-up study. Arch. Intern. Med. **160:**2749–2755.
48. **Xie, H., G. S. Cook, J. W. Costerton, G. Bruce, T. M. Rose, and R. J. Lamont.** 2000. Intergeneric communication in dental plaque biofilms. J. Bacteriol. **182:**7067–7069.
49. **Yamamoto, K., T. Kadowaki, K. Okamoto, N. Abe, and K. Nakayama.** 1997. Biological roles of a novel class of cysteine proteinases from *Porphyromonas gingivalis* in periodontal disease progression. IOS Press, Amsterdam, The Netherlands.

Editor: E. I. Tuomanen