The host–bacterial interaction that initiates periodontal disease takes place within the gingival crevice. The gingival epithelial cells that line the periodontal tissues are the first line of defense eliciting a wide array of responses to bacteria that includes cytokines and chemokines. Clinical studies have shown that proinflammatory cytokines and chemokines are present in the gingival crevicular fluid (GCF), both in health and disease, and are elevated in sites exhibiting clinical signs of inflammation (23). Interleukin-1β (IL-1β), a primary proinflammatory cytokine, was found in higher concentrations in the GCF of patients with periodontitis compared to that of healthy controls (7, 30). In contrast, IL-8, a secondary chemokine, was found in lower concentrations in the GCF of sites with unresolved defects compared to resolved defects following treatment (9).

The fact that primary and secondary cytokines are at different levels in the periodontal pocket is surprising because at the cellular level they are linked (5). In vitro, IL-1β has been shown to be secreted early after challenge, and so is characterized as ‘primary’; it then modulates secretion of IL-6, IL-8, and other so-called ‘secondary’ cytokines in an
autocrine manner (5). Inhibition of IL-1β secretion or blockage of the IL-1 receptor significantly reduces IL-6 and IL-8 production in different cell types, indicating that IL-1β is a key regulator of the inflammatory response (5). It would be expected that upregulation of IL-1β in the GCF would be followed by increased levels of IL-6 and IL-8. Since there is a disconnect between primary and secondary cytokines in vivo, we hypothesized that Porphyromonas gingivalis, a putative periodontal pathogen with powerful proteases, might modulate the inflammatory cytokine responses (10, 14).

Consistent with the published clinical data, our preliminary work reveals that gingival epithelial cells, challenged with live P. gingivalis, mount a primary cytokine response that is not followed by a secondary cytokine response. P. gingivalis possess several virulence mechanisms including the gingipains, a family of cysteine proteases that are either secreted or membrane-bound and arginine-specific or lysine-specific. The gingipains have numerous pathological actions such as dysregulation of clotting and fibrinolytic pathways, activation of the kallikrein/kinin pathway, modulation of cytokine networks, and activation of matrix metalloproteinases (12, 21, 28, 29). Our hypothesis is that the decreased level of secondary cytokines, despite an elevated primary response in vivo and in vitro, is the result of cytokine degradation by P. gingivalis gingipains. The aim of the current study was therefore to determine the rates of IL-1β, IL-6, and IL-8 degradation by P. gingivalis and identify the role of gingipains in this process.

Materials and methods

Cell isolation and culture

Gingival tissue biopsies were obtained with informed consent from periodontally healthy patients undergoing crown-lengthening procedures at the University of Louisville School of Dentistry Graduate Periodontics Clinic, according to an Institutional Review Board approval. The gingiva was treated with 0.025% trypsin and 0.01% ethylenediaminetetraacetic acid overnight at 4°C and human gingival epithelial cells (HGECs) were isolated as previously described (25). The authenticity of the HGECs was confirmed by immunohistochemistry with monoclonal antibody against human pankeratin (Dako, Carpinteria, CA) and histologically by cell morphology. The HGECs were seeded in 60-mm plastic tissue culture plates coated with type-I collagen (BD Biocoat, Franklin Lakes, NJ) and incubated in 5% CO₂ at 37°C using K-SFM medium (Invitrogen, Carlsbad, CA) containing 10 μg/ml of insulin, 5 μg/ml of transferrin, 10 μM 2-mercaptoethanol, 10 μM of 2-aminoethanol, 10 mM of sodium selenite, 50 μg/ml of bovine pituitary extract, 100 units/ml of penicillin/streptomycin, and 50 ng/ml of fungizone (complete medium). When the cells reached sub-confluence, they were harvested and sub-cultured as previously described (6).

Bacterial strains and conditions

P. gingivalis ATCC 33277 was purchased from the American Type Culture Collection (ATCC; Manassas, VA) and the derivative KDP128, an RgpA/RgpB/Kgp triple mutant (24), was kindly provided by Dr K. Nakayama (Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Tokyo). P. gingivalis W50 (ATCC 53978), and the derivative mutants E8, an RgpA/RgpB double mutant, and K1A, a Kgp mutant (1), were kindly provided by Dr M. Curtis (Barts and The London, Queen Mary’s School of Medicine and Dentistry, London, UK). All P. gingivalis strains at low passage were grown in GAM media (Nissui Pharmaceutical, Tokyo, Japan) under anaerobic conditions (85% N₂, 10% CO₂ and 5% H₂; Coy Laboratory, Grass Lake, MI) for 2 days. Aggregatibacter actinomycetemcomitans Y4 and Fusobacterium nucleatum 364 were kindly provided by Dr D. Demuth (University of Louisville School of Dentistry). A. actinomycetemcomitans was grown in brain–heart infusion (Difco Laboratories, Detroit, MI) supplemented with 40 mg NaHCO₃ per liter at 37°C in an atmosphere of 5% CO₂. F. nucleatum were grown in brain–heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 0.2% yeast extract (Difco Laboratories). 0.5 mg of L-cysteine hydrochloride, and freshly prepared 0.5% sodium bicarbonate under anaerobic conditions for 3 to 4 days. After cultivation, the bacteria were harvested by centrifugation, washed in phosphate-buffered saline (PBS; pH 7.4) and used immediately for the live cell challenge or heat-inactivated for 1 h at 60°C.

Gingipain inhibitors

For the gingipain inhibition assays, live P. gingivalis 33277 was incubated with gingipain inhibitors for 15 min at 37°C, just before the cytokine degradation assay. zFKck, a specific lysine gingipain (Kgp) inhibitor (20) was kindly provided by Dr J. Potempa (University of Georgia) and used at a final concentration of 10 μM. Leupeptin (Sigma, St Louis, MO), a specific arginine-gingipain (Rgp) inhibitor, was used at a final concentration of 100 μM. The final concentrations used were the maximum inhibitory doses that retained specificity, as determined by a dose-response using the enzymatic substate hydrolysis of N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA; Sigma), for Rgp activity, or acetyl-lysine-p-nitroanilide (ALNA; Bachem, King of Prussia, PA), for Kgp activity.

Bacterial challenge

HGEC cultures at the fourth passage were harvested and seeded at a density of 0.5 × 10⁵ cells/well in a six-well culture plate coated with type-I collagen, and maintained in 2 ml complete medium. When they reached confluence (approximately 10⁶ cells/well), the cells were washed twice with fresh medium and were challenged with live or heat-inactivated bacteria in antibiotic-free medium at a multiplicity of infection (MOI):100 (10⁸ bacteria/well) at 37°C in 5% CO₂ for 4 or 24 h. For each experiment the final concentration of the suspension was determined by measurement of absorbance at 600 nm (A₆₀₀) and appropriate dilutions were made to achieve the desired MOI. The bacterial number was confirmed by viable counting of colony-forming units (CFU) on blood agar plates incubated anaerobically at 37°C. The ratio of live to dead bacteria was determined using a Petroff-Hausser counting chamber. Each assessment confirmed that live bacteria comprised 95% of total bacterial cells counted.

Cytokine degradation assay

Culture supernatants produced after a 24-h incubation of HGECs with heat-killed P. gingivalis 33277 were incubated with 5 × 10⁵ live bacteria/ml of the various strains at 37°C. The reaction was stopped at 1 min, 30 min, 1 h, 2 h, and 4 h. Alternatively, solutions of recombinant human IL-1β, IL-6, and IL-8 (BD Biosciences, San Diego, CA) were also incubated with 5 × 10⁵ live bacteria/ml of the various strains at 37°C, and the reactions were carried out for the same length of time. After incubation, the enzymatic activity of lysine gingipain was terminated by addition of zFKck (10 μM), and those of arginine-gingipains were terminated by...
addition of leupeptin (100 μM). The initial concentration of the recombinant cytokine was determined by the concentration of the respective cytokine in the above culture supernatant. IL-1β, IL-6, and IL-8 were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (BD OptEIA; BD Biosciences) according to the manufacturer’s instructions. The absorbance was read at 450 nm.

Western blot analysis
Recombinant human (rh) IL-1β and rhIL-6 were incubated with live bacteria as described above and the samples were separated in a 15% polyacrylamide gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA). Briefly, the membranes were incubated for 1 h in the blocking buffer (5% non-fat milk in Tris-buffered saline–0.1% Tween-20) followed by the primary antibodies human IL-1β (Cell Signaling Technologies, Danvers, MA) and human IL-6 (R&D Systems, Minneapolis, MN) (1 : 1000) at 4°C overnight. Then the membranes were incubated in the secondary antibody anti-mouse immunoglobulin G (IgG) and anti-goat IgG, respectively (horseradish peroxidase-conjugated) (1 : 2000) for 1 h at room temperature. Both the primary and secondary antibodies were diluted in the blocking buffer, and Tris-buffered saline with Tween-20 was used for washing the membranes before, between, and after their incubation with the antibody solutions. Antibody-reactive proteins were detected using enhanced chemiluminescence reagents (ECL Plus kit; GE healthcare, Pittsburg, PA).

Quantitative real-time polymerase chain reaction
RNA was extracted from cultured cells using TRIzol (Invitrogen), and quantified by spectrometry at 260 and 280 nm. Ten micrograms from each RNA extract was used to perform first-strand complementary DNA (cDNA) synthesis using the High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) in a total volume of 100 μl. Real-time polymerase chain reaction (PCR) was performed using 100 ng cDNA with an ABI 7500 system (Applied Biosystems). TaqMan probes, sense and antisense primers for gene expression of human IL-1β, IL-6, IL-8, and GAPDH as an endogenous control were purchased from Applied Biosystems. Using a Universal PCR Master Mix (Applied Biosystems), the reactions were carried out according to the manufacturer’s protocol.

**Statistical analysis**
All data are expressed as the mean ± SD. Statistical analyses were performed by one-way analysis of variance (ANOVA) using the INSTAT program (GraphPad, San Diego, CA) with Bonferroni correction. Statistical differences were considered significant at the $P < 0.05$ level.

**Results**

HGECs challenged with live P. gingivalis appear unable to elicit a secondary cytokine response, while the primary cytokine response is elevated

HGECs were challenged with live or heat-killed P. gingivalis 33277 at an MOI:100 for 4 or 24 h. Unchallenged cells were used as a negative control. Secreted IL-1β, IL-6, and IL-8 were measured in the supernatant by ELISA. At 24 h, HGECs challenged with heat-killed bacteria elicited a secondary cytokine (IL-6) and chemokine (IL-8) response, while the primary cytokine (IL-1β) response remained at a low level, comparable to that of the unchallenged control (Fig. 1), controlled possibly by a negative feedback mechanism. Surprisingly, almost no IL-6 and IL-8 were detected in the supernatant of the HGECs challenged with live bacteria, while IL-1β was increased more than four-fold (Fig. 1). This phenomenon was only observed with live P. gingivalis, because HGECs challenged with other putative periodontal pathogens under the same conditions elicited a normal primary and secondary response (Fig. 1).

HGECs challenged with live P. gingivalis upregulate secondary cytokines at the transcriptional level

To identify if the downregulation of secondary cytokines after challenge with live P. gingivalis happens at the transcriptional or translational level, IL-1β, IL-6, and IL-8 messenger RNA (mRNA) levels were measured by quantitative real-time PCR (Fig. 2). HGECs were challenged with live P. gingivalis 33277 at an MOI:100 for 4 or 24 h and unchallenged cells were used as a negative control. The mRNA for all three cytokines was elevated up to two-fold after the 24-h challenge compared to the unchallenged negative control, indicating

that the lack of IL-6 and IL-8 in the final supernatant (Fig. 1) was not the result of downregulation of these molecules at the transcriptional level.

Live P. gingivalis induce cytokine degradation and the rate of IL-6 and IL-8 degradation is higher than that of IL-1β

HGECs were challenged with heat-killed P. gingivalis 33277 for 24 h, the supernatant was collected and subsequently
incubated with live *P. gingivalis* 33277 for 1 min, 30 min, 1 h, 2 h, and 4 h. Supernatant alone was used as a negative control. IL-1β, IL-6, and IL-8 were measured in the final supernatant by ELISA. IL-6 and IL-8 degradation began immediately and reached 100% after 30 min of exposure to *P. gingivalis* (Fig. 3). In contrast, there was no IL-1β degradation up to 1 h after exposure to *P. gingivalis*, while degradation reached 40 and 90% after 2 and 4 h, respectively. The slower rate of IL-1β degradation, compared to those for IL-6 and IL-8, could explain the persistence of IL-1β and the absence of IL-6 and IL-8 previously observed in the culture supernatant 24 h after challenge with live *P. gingivalis* (Fig. 1).

Lower rate of IL-1β degradation is not linked to mode of secretion

The observed differences in cytokine degradation rates could be an artifact resulting from the different mode of secretion for IL-1β, which is in vesicles and not as a free molecule (15). To test this hypothesis, the previous experiment was repeated using purified recombinant IL-1β, IL-6, and IL-8 in single cytokine solutions. IL-1β, IL-6, and IL-8 were measured in the final solution by ELISA (data not shown) and Western blotting (Fig. 6). The results were similar to those of the previous experiment, confirming that cytokine degradation is mainly the result of Lys-gingipain and that the lower degradation rate of IL-1β is not an artifact linked to mode of secretion.

Discussion

We demonstrate that live *P. gingivalis* reduce the secondary cytokine and chemokine responses of primary HGECs while the primary cytokine response is elevated at 24 h. We further demonstrate that the lack of secondary response is caused by direct cytokine degradation by *P. gingivalis*’ proteases and that the rate of IL-6 and IL-8 degradation is significantly higher than that of IL-1β. The *P. gingivalis* protease, lysine gingipain, is particularly implicated in this secondary cytokine degradation.

In the present study, primary HGECs challenged with live wild-type *P. gingivalis* were able to elicit a high primary (IL-1β) response (Fig. 1). IL-1β is considered a ‘primary’ cytokine, because it has been previously shown to be secreted within the first minutes of challenge (27) and mediates the release of other ‘secondary’ proinflammatory cytokines, such as IL-6 and IL-8, in an autocrine manner (5). However, in our study, when live *P. gingivalis* were used to challenge HGECs, the secondary cytokine response was not elevated, despite the elevated primary response. The lack of secondary response was not observed at the transcriptional level because both IL-6 and IL-8 mRNA were elevated compared to the unchallenged control (Fig. 2). The fact that IL-6 and IL-8 were at higher levels indicates that the primary cytokine IL-1β is effective in enhancing the inflammatory response in an autocrine manner and that this may function to increase vascular permeability (IL-6) and to enhance leukocyte recruitment to the region (IL-8). Furthermore, HGECs challenged with heat-killed bacteria significantly upregulated IL-6 and IL-8 secretion (Fig. 1), indicating that the HGECs used are capable of mounting both a primary and a secondary response, in accordance with our previous publication (5). These data indicate that the lack of secondary cytokines after challenge with live *P. gingivalis* does not result from signal transduction, transcription, or translation defects in the specific HGECs but may be the result of protease activity by *P. gingivalis* postsecretion.

To test the hypothesis that secondary cytokine levels are reduced because of extracellular degradation, HGECs were challenged with heat-killed bacteria for 24 h and the supernatant was subsequently incubated with live wild-type *P. gingivalis* for up to 4 h (Fig. 3). In addition, purified recombinant human IL-1β and IL-6 were incubated with live *P. gingivalis* for up to 4 h (Fig. 6) to estimate degradation. IL-6 and IL-8 degradation began immediately and reached 100% after 30 min of exposure to live *P. gingivalis*, while there was no IL-1β degradation during the first hour of exposure (Fig. 3). These results are consistent with other reports (8, 26). The much lower rate of IL-1β degradation, compared to IL-6 and IL-8, explains the persistence of IL-1β and the absence of IL-6 and IL-8 in culture supernatants after challenge with live *P. gingivalis* (Fig. 1), as well as the discrepancies between different cytokines in the GCF of patients with periodontitis (3, 7, 9, 13, 16, 19, 22, 30).

The lack of a secondary response after bacterial challenge is a unique characteristic of *P. gingivalis*, because it was not observed after challenge with other putative periodontal pathogens, such as *A. actinomycetemcomitans* and *F. nucleatum*. (Fig. 1). It has been previously suggested that cytokine degradation by *P. gingivalis* involves bacterial proteases and more specifically the gingipains (2, 11, 17, 18, 19).
Gingipains are cysteine proteases produced by *P. gingivalis* that are either secreted or membrane-bound and arginine-specific or lysine-specific. These proteases are unique to *P. gingivalis* and could be the causative agent of the fast cytokine degradation because other protease-producing bacteria, such as *F. nucleatum*, do not have a significant effect on cytokine degradation (Fig. 1). In the present study, the mechanism used by *P. gingivalis* to induce cytokine degradation was shown to be mainly Lys-gingipain-dependent. Gingipain-deficient *P. gingivalis* mutants lacking both Arg- and Lys-gingipains were unable to induce IL-1β, IL-6, and IL-8 degradation (Figs 4–6). More specifically, the Lys-gingipain-deficient mutant completely lacked the ability to degrade all cytokines, while the Arg-gingipain-deficient mutant induced cytokine degradation although at a slightly lower rate compared to the wild-type bacteria (Figs 5, 6). These results taken together indicate that cytokine degradation is mainly induced by Lys-gingipain and that the role of Arg-gingipains in the process is minimal.

The lower rate of IL-1β degradation compared to that of IL-6 or IL-8 could be due to a number of reasons. IL-1β may be degraded less because it can be secreted within vesicles, i.e. a protected mode of secretion (15); the variation in total number and spatial relationship of arginine and lysine residues might influence degradation; solvent accessibility to the molecules may differ; substrate specificity, optimal temperature, and pH may also affect degradation rates. The protected vesicle secretion of IL-1β does not appear to...
pertains here because purified recombinant human cytokines were assessed in the degradation assays (Fig. 6), giving results similar to the native cytokines found in the supernatants.

The possibility that the total number of arginine and lysine residues is lower for IL-1β compared to IL-6 and IL-8 is not correct because, according to the amino acid sequences, the number of predicted arginine cleavage sites is three for IL-1β, nine for IL-6, and five for IL-8. The number of lysine cleavage sites is 15 for IL-1β, 14 for IL-6, and 9 for IL-8 so although IL-1β has a lower number of arginine cleavage sites, the number of lysine cleavage sites is much higher and fails to explain the observed lack of IL-1β sensitivity to degradation by the lysine gingipain mutant (Fig. 5). Based on the crystal structure of IL-1β, IL-6, and IL-8, the arginine and lysine cleavage sites appear to be exposed and equally accessible to the solvent for all cytokines. However, the hydrolytic activity of lysine gingipain can be blocked when a lysine residue is followed by another lysine or arginine residue (4). Blockage of lysine gingipain activity in IL-1β as a result of its tertiary structure may be the reason for the observed differences in degradation rate between primary and secondary cytokines.

In conclusion, the present study provides evidence that Porphyromonas gingivalis can induce a primary proinflammatory response without an elevated secondary cytokine response, as these cytokines are reduced by direct degradation, mainly by lysine gingipain. This appears to be a virulence attribute of Porphyromonas gingivalis that subverts the protective host response; reduction in IL-6 will decrease the vascular and cellular inflammatory infiltrate and reduction in the chemokine IL-8 will reduce migration of phagocytes and subsequent bacterial phagocytosis and killing.

Acknowledgments

This work was supported by US Public Health Service, National Institutes of Health, NIDCR grant DE017364 to D.F.K. Publication costs of this article were defrayed in part by the payment of page charges. This article is therefore marked “advertisement” in accordance with 18 U.S.C. Section 1734, solely to indicate this fact.

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