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Calprotectin Expression In Vitro by Oral Epithelial Cells Confers Resistance to Infection by *Porphyromonas gingivalis*

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Calprotectin, an S100 calcium-binding protein with broad-spectrum antimicrobial activity in vitro, is expressed in neutrophils, monocytes, and gingival keratinocytes. In periodontitis, calprotectin appears upregulated and is detected at higher levels in gingival crevicular fluid and tissue specimens. How calprotectin contributes to the pathogenesis of periodontal diseases is unknown. To isolate the effects of calprotectin, a calprotectin-negative oral epithelial cell line was transfected with calprotectin genes to enable expression. *Porphyromonas gingivalis* was permitted to bind and invade transfected cells expressing calprotectin and sham transfectants. Rates of invasion into both cell lines were compared using the antibiotic protection assay. Transfected cells expressing calprotectin showed 40 to 50% fewer internalized *P. gingivalis* than sham transfectants. Similarly, binding to calprotectin expressing cells was reduced approximately twofold at all time points (15, 30, 45, and 60 min) as estimated by immunofluorescence analysis. Independent of invasion, however, prolonged exposure to *P. gingivalis* induced epithelial cell rounding and detachment from the substratum. These morphological changes were delayed, however, in cells expressing calprotectin. Using *P. gingivalis* protease-deficient mutants, we found that Arg-gingipain and Lys-gingipain contributed to epithelial cell rounding and detachment. In conclusion, expression of calprotectin appears to protect epithelial cells in culture against binding and invasion by *P. gingivalis*. In addition, cells expressing calprotectin are more resistant to detachment mediated by Arg-gingipain and Lys-gingipain. In periodontal disease, calprotectin may augment both the barrier protection and innate immune functions of the gingival epithelium to promote resistance to *P. gingivalis* infection.

Calprotectin is a noncovalently complexed heterodimer of two small anionic proteins, MRP8 and MRP14 (20, 37). Constitutively expressed in the cytosol of neutrophils, monocytes (8), activated macrophages (35), and squamous mucosal epithelia (3, 50), calprotectin is a member of the S100 family of calcium binding proteins (24). With its broad spectrum of in vitro antimicrobial effects (28, 29, 46, 47), calprotectin has been proposed to play a role in innate host defense of epithelia (4, 13). In candidiasis, keratinocytes subjacent to the infected superficial layer exhibit intense staining of calprotectin, which appears as a limiting barrier to penetration by candida mycelia (13, 14). Similarly, increased calprotectin expression is also observed in herpes simplex virus-and Epstein-Barr virus-infected and neighboring oral keratinocytes (13).

Levels of calprotectin in body fluids, including plasma, saliva, and synovial fluid, elevate markedly in various infections and inflammatory diseases and serve as a marker for disease activity (6, 17, 38, 40). In periodontitis, calprotectin levels in gingival crevicular fluid (GCF) from diseased sites are significantly higher than those in GCF from healthy sites and show positive correlations with several clinical and biochemical markers, including probing depth, interleukin 1β, prostaglandin E2, collagenase, and aspartate aminotransferase activity (23, 30). In GCF, calprotectin probably originates from pocket epithelia and inflammatory cells, including neutrophils and monocytes. Calprotectin expression in pocket epithelia and oral epithelia is increased in periodontitis, as suggested by immunohistochemistry (43).

Porphyromonas gingivalis, a gram-negative anaerobe, is considered an important periodontal pathogen (52). In vitro, *P. gingivalis* invades gingival epithelial cells, pocket epithelial cells, and endothelial cells (11, 25, 41, 42). Invasion into epithelial cells is fimbria-dependent, mediating adherence of *P. gingivalis* to host cells (34, 49). *P. gingivalis* also expresses proteases which are able to degrade epithelial adhesion proteins and induce detachment of epithelial cells from the substratum (21, 22, 48). Together, these virulence factors may allow *P. gingivalis* to invade deeper in the connective tissue and contribute to tissue destruction in periodontal diseases.

We hypothesize that calprotectin expression may protect epithelia against infection by *P. gingivalis*. In a previous study, we generated stable oral epithelial cell lines expressing calprotectin (KB-MRP8/14) and sham-control transfectants (KB-EGFP) from KB cells, a calprotectin-negative oral epithelial cell line (K. Nisapakultorn, K. F. Ross, and M. C. Herzberg, submitted for publication). Using these cell lines, we showed that calprotectin expression reduces invasion and binding of *Listeria monocytogenes* and salmonellae to epithelial cells (32; Nisapakultorn et al., submitted). The aim of this study was to assess the effect of epithelial calprotectin expression on an invasive periodontal pathogen, *P. gingivalis*, and to provide evidence for the role of calprotectin in innate host defense in periodontal disease. Expression of calprotectin appeared to reduce binding and invasion by *P. gingivalis* and protect keratinocyte monolayers from Arg- and Lys-gingipain protease-mediated cellular detachment.

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TABLE 1. *P. gingivalis* strains used in this study

Strain	Phenotype	Genotype	Source
FDC381	Wild type		C. A. Genco
DPG3	Fimbria-deficient mutant of FDC381	Δ*fimA*	C. A. Genco (26)
ATCC 33277	Wild type		American Type Culture Collection
KDP129	Kgp[a]-deficient mutant of ATCC 33277	Δ*kgp*	K. Nakayama (36)
KDP133	Rgp[b]-deficient mutant of ATCC 33277	Δ*rgpA* Δ*rgpB*	K. Nakayama (31)
KDP136	Kgp- and Rgp-deficient mutant of ATCC 33277	Δ*kgp* Δ*rgpA* Δ*rgpB*	K. Nakayama (45)

[a] Kgp, lysine-specific cysteine protease or Lys-gingipain.
[b] Rgp, arginine-specific cysteine protease or Arg-gingipain.

MATERIALS AND METHODS

Stable epithelial cell lines expressing calprotectin. KB epithelial cells expressing calprotectin (KB-MRP8/14) and KB sham-control transfectants (KB-EGFP) were generated from KB cells (ATCC CCL-17), a calprotectin-negative buccal carcinoma cell line, as previously described (Nisapakultorn et al., submitted). Briefly, KB cells were cotransfected with the mammalian expression vector, pIRES-EGFP (Clontech, Palo Alto, Calif.), containing MRP8 and MRP14 genes, and the selectable marker pSV2-neo, to generate KB-MRP8/14. A sham-control transfectant, KB-EGFP, was generated by cotransfection of insertless pIRES-EGFP and pSV2-neo. Cytosolic calprotectin (MRP8-MRP14) expression in transfected cells was verified both by sandwich enzyme-linked immunosorbent assay and indirect immunofluorescence using the MRP8-MRP14 heterodimer-specific (2) monoclonal antibody 27E10 (Bachem, King of Prussia, Pa.).

Epithelial cell culture. KB-EGFP and KB-MRP8/14 were maintained in modified Eagle medium supplemented with 10% fetal bovine serum and G418 sulfate (700 μg/ml; Geneticin; Mediatech, Herndon, Va.). To avoid an unwanted antimicrobial affect from G418 sulfate, KB transfectants were grown in media without G418 sulfate for 4 days before experiments.

Bacteria strain and culture conditions. *P. gingivalis* strains used in this study (Table 1) were grown anaerobically at 37°C in Todd-Hewitt broth (Difco, Detroit, Mich.) supplemented with hemin (5 μg/ml; Sigma, St. Louis, Mo.) and menadione (1 μg/ml; Sigma) or on Todd-Hewitt agar supplemented with hemin, menadione, and 5% defribinated sheep blood.

Bacterial invasion assays. Bacterial invasion into KB transfected cells was determined by an antibiotic protection assay (12). KB-EGFP and KB-MRP8/14 (1.2×10^5 cells) were grown overnight in 24-well cell culture plates. *P. gingivalis* ATCC 33277 or FDC381 was added at a multiplicity of infection (MOI) of 100 and incubated for 1.5 h at 37°C in an atmosphere of 5% CO_2–95% air. The monolayers were washed twice with Dulbecco's phosphate-buffered saline (DPBS), and incubated with medium containing metronidazole (200 μg/ml; Sigma) and gentamicin (300 μg/ml; Sigma) for 1 h to kill extracellular bacteria. The monolayers were washed again with DPBS and lysed with distilled water for 15 min at room temperature. Released intracellular bacteria were diluted and plated with a spiral plater (Spiral Biotech, Bethesda, Md.). Internalized bacteria were enumerated by colony counting after 5 to 7 days. Each invasion assay was performed in triplicate wells and repeated in at least three independent experiments.

Bacterial adhesion assay. KB-EGFP and KB-MRP8/14 were grown to confluence on gelatin-coated coverslips. KB transfectants were incubated with *P. gingivalis* ATCC 33277 (1.2×10^7 CFU) for 15, 30, 45, and 60 min at 37°C. The monolayers were washed twice with DPBS and fixed for 10 min with 4% paraformaldehyde. Adherent bacteria were stained with rabbit anti-*P. gingivalis* serum (dilution, 1:10,000; kindly provided by A. Sharma, State University of New York at Buffalo), followed by goat anti-rabbit Alexa 568 (dilution, 1:500; Molecular Probes, Eugene, Oreg.). Since KB cells were not permeabilized, the antibody detected only adherent bacteria. At each time point, digital images from 10 random microscopic fields viewed at a × 200 magnification were captured with a Spot camera (Diagnostic Instruments Inc., Sterling Heights, Mich.), and the adherent bacteria in each field were counted.

Prolonged incubation with *P. gingivalis*. KB, KB-EGFP, and KB-MRP8/14 (3×10^5 cells/well) were grown in six-well cell culture plates for 48 h before assay. *P. gingivalis* were resuspended in modified Eagle medium and added to the monolayers at an MOI of 100. Strains used in the experiments included *P. gingivalis* FDC381, DPG3, ATCC 33277, KDP129, KDP133, KDP136, and heat-killed (60°C for 1 h) FDC381 (Table 1). The monolayers without *P. gingivalis* served as controls. Epithelial monolayers were monitored with a phase-contrast microscope (Nikon, Tokyo, Japan), and images of cell monolayers were taken at 0, 5, 9, and 24 h after exposure to *P. gingivalis*.

Statistical analysis. Data are presented as the means ± standard deviations (SD). Statistically significant differences between KB-EGFP and KB-MRP8/14 were determined by using a repeated-measure analysis of variance for bacterial invasion assay and two-way analysis of variance for bacterial adhesion assay.

RESULTS

Reduced *P. gingivalis* invasion into KB cells expressing calprotectin. To determine whether calprotectin expression affects *P. gingivalis* invasion, the numbers of internalized *P. gingivalis* in KB-EGFP and KB-MRP8/14 were compared using the antibiotic protection assay. Approximately 40 to 50% fewer viable intracellular *P. gingivalis* from each of the two strains was recovered from KB-MRP8/14 than was recovered from KB-EGFP (Fig. 1).

Reduced *P. gingivalis* binding to KB cells expressing calprotectin. By immunofluorescence analysis (Fig. 2), the number of *P. gingivalis* that bound to KB-transfected cells increased over time. At all time points, approximately twofold fewer *P. gingivalis* cells bound to KB-MRP8/14 than bound to KB-EGFP.

KB cells expressing calprotectin are resistant to cell detachment induced by prolonged exposure to *P. gingivalis*. Prolonged exposure to *P. gingivalis* has been shown to induce epithelial cell rounding (1), suggesting compromise of the gingival epithelial barrier in periodontitis. To determine whether

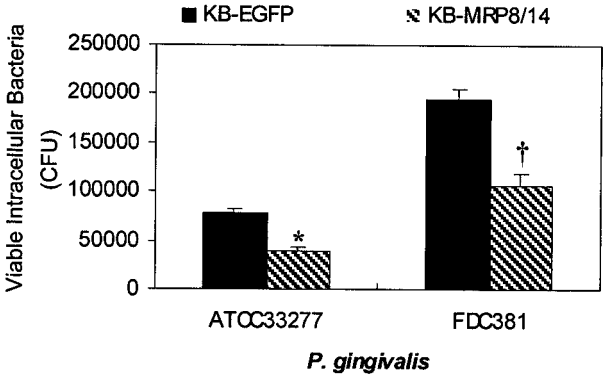

FIG. 1. *P. gingivalis* invasion into KB-EGFP and KB-MRP8/14. KB-EGFP or KB-MRP8/14 was incubated with *P. gingivalis* at an MOI of 100 for 1.5 h, followed by 1 h with antibiotics. The cell monolayers were then washed and lysed with distilled H_2O to release intracellular bacteria. Internalized bacteria were plated and counted. Values are means ± SD from a representative experiment. Each experiment was performed in triplicate wells. The experiments were repeated at least three times with similar results for each strain of *P. gingivalis*. The differences between KB-EGFP and KB-MRP8/14 from three experiments were statistically significant (*, $P < 0.05$; †, $P < 0.01$).

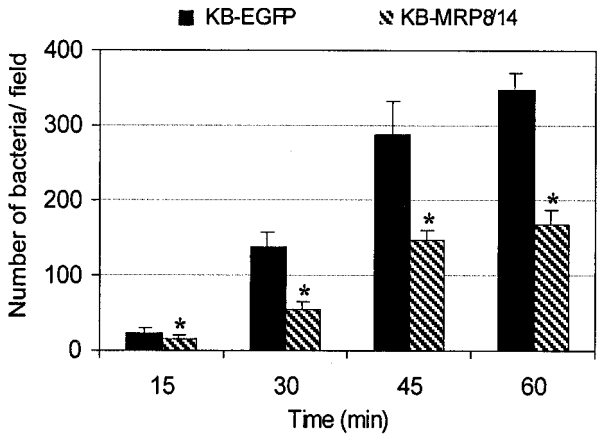

FIG. 2. Binding of *P. gingivalis* ATCC 33277 to KB-EGFP and KB-MRP8/14. KB-EGFP and KB-MRP8/14 cell monolayers were incubated with *P. gingivalis* for 15, 30, 45, or 60 min; washed; fixed; and stained for adherent *P. gingivalis* by indirect immunofluorescence. At each time point, images from 10 random microscopic fields at a magnification of ×200 were captured and the adherent bacteria in each field were counted. The differences between KB-EGFP and KB-MRP8/14 were significant (*, $P < 0.001$). Error bars, SD.

calprotectin expression affects epithelial cell responses to prolonged exposure to *P. gingivalis*, we incubated KB parental cells, KB-EGFP, and KB-MRP8/14 with *P. gingivalis* FDC381 and observed the morphological changes over time. KB-MRP8/14 showed a delay in cell rounding (Fig. 3). KB and KB-EGFP cells changed morphology from a flattened polygonal to round shape after 5 h of incubation. No change was observed in KB-MRP8/14 until 9 h of exposure to *P. gingivalis*. By 24 h, KB and KB-EGFP cells incubated with strain FDC381 were round, and some were detached from the culture plates (Fig. 4). In contrast, some KB-MRP8/14 cells did not become round and retained their polygonal morphology or appeared to be in the process of retraction. DPG3, a fimbria-deficient mutant that is unable to adhere to or invade KB cells (34), induced changes similar to those observed by the parental wild-type strain FDC381. Heat-killed *P. gingivalis* FDC381 had no effect on cell morphology (Fig. 4). Using trypan blue dye exclusion, more than 95% of detached cells were viable and able to attach and grow after subculture into fresh media (data not shown).

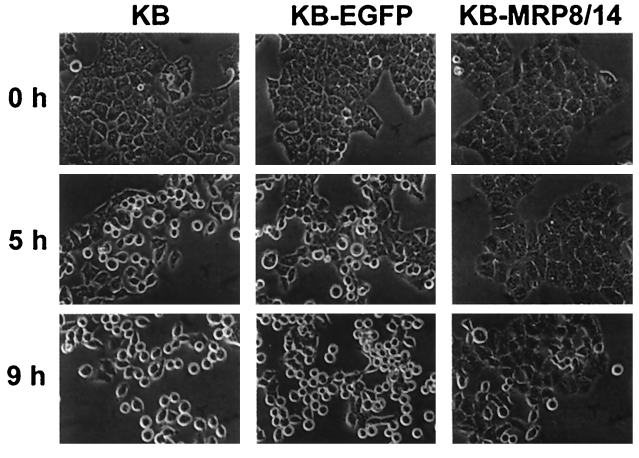

FIG. 3. Changes in epithelial cell morphology after exposure to *P. gingivalis* FDC381 for 0, 5, and 9 h. KB, KB-EGFP, or KB-MRP8/14 was incubated with *P. gingivalis* FDC381 at an MOI of 100. Changes in epithelial cell morphology over time were observed by phase-contrast microscopy. Magnification, ×850.

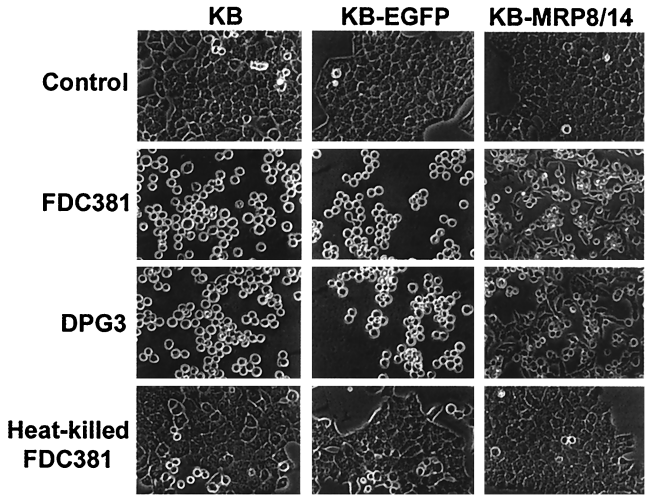

FIG. 4. Changes in epithelial cell morphology after 24 h of exposure to *P. gingivalis* FDC381, DPG3, or heat-killed FDC381. Epithelial cells that were not exposed to *P. gingivalis* served as controls. Magnification, ×800.

Arg-gingipain and Lys-gingipain induced epithelial cell detachment. *P. gingivalis* proteases have been shown to induce epithelial cell detachment from monolayers (21). To identify specific proteases involved in this process, we incubated epithelial cell lines with protease-deficient mutants. After 24 h, the Rgp-deficient mutant (KDP129) and the Kgp-deficient mutant (KDP133) induced KB-EGFP cell rounding, but the effect was less than that observed with the wild-type parental strain ATCC 33277 (Fig. 5). Similar results were observed with KB cells (data not shown). Neither Rgp-deficient nor Kgp-deficient mutants induced rounding of KB-MRP8/14 cells. The Rgp and Kgp double mutant (KDP136) was unable to induce cell rounding of the epithelial cell lines.

DISCUSSION

Periodontal diseases are infections of tooth-supporting tissues. *P. gingivalis*, a key putative periodontal pathogen, can invade the gingival tissue in advanced periodontitis (19, 39) and invade cultured epithelial cells in vitro (25, 41). *P. gingivalis* also produces enzymes that degrade a broad spectrum of host proteins, including epithelial adhesion molecules (22, 48) and extracellular matrix proteins (44). The ability of *P. gingivalis* to invade epithelial cells, disrupt the epithelial barrier, and penetrate deeper in connective tissue may contribute to tissue destruction and chronicity of periodontal diseases.

We previously reported that calprotectin expression is associated with reduced invasion and binding of intracellular pathogens, *L. monocytogenes* and salmonellae, to epithelial cells (32; Nisapakultorn et al., submitted). Since calprotectin expression is increased markedly in periodontal diseases (43),

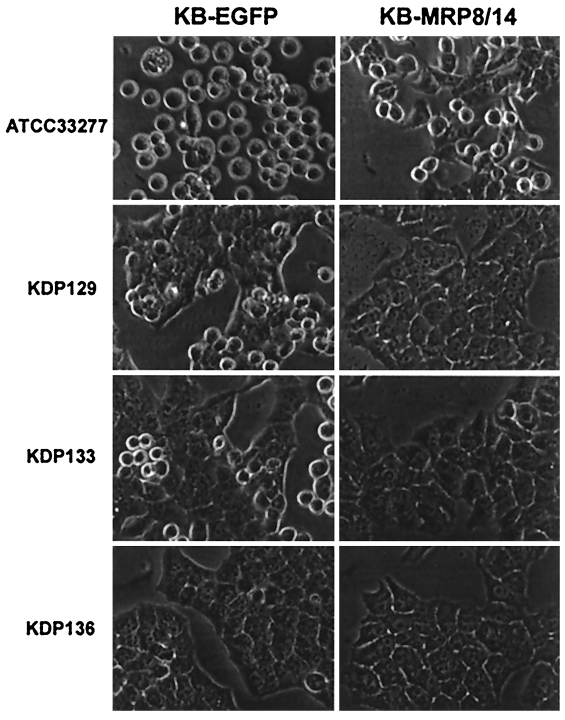

FIG. 5. Changes in epithelial cell morphology after exposure to *P. gingivalis* ATCC 33277 wild-type parental strain and protease-deficient mutants for 24 h. KDP129 is a Kgp-deficient mutant; KDP133 is an Rgp-deficient mutant; and KDP136 is a Kgp- and Rgp-deficient mutant. Magnification, ×1,000.

we tested whether epithelial calprotectin might promote resistance to *P. gingivalis* infection. To determine if calprotectin affects invasion and binding of the periodontal pathogen, *P. gingivalis*, the antibiotic protection assay was used. We recovered twofold fewer viable bacteria from epithelial cells that expressed calprotectin. However, the number of intracellular bacteria recovered after antibiotic treatment of extracellular bacteria is not absolute. In this study, extracellular *P. gingivalis* was treated with a combination of metronidazole (200 μg/ml) and gentamicin (300 μg/ml) for 1 h. This protocol has been widely used in studying *P. gingivalis* invasion (10, 11, 18, 25). To ensure that the antibiotic protocol actually killed all of the extracellular *P. gingivalis*, such that the viable counts could be attributed to only invaded intracellular bacteria, we determined the efficiency of killing in a broth assay. In this assay we carefully washed bacteria before plating to eliminate residual antibiotics and found that this amount of antibiotic killed 90 to 95% of the initial inoculum (10^8 CFU) after 1 h (data not shown). If we simply diluted the culture without washing the bacteria, we observed complete killing. Without washing the bacteria, the concentration of antibiotic present may still be sufficient to inhibit growth or kill bacteria on the plate. The findings suggested that drug carryover (33) can cause overestimation of killing and hence invasion in the antibiotic protection assay. This difficulty notwithstanding, the relative differences attributable to calprotectin are reliable.

The reduction in bacterial invasion may result from the well-known antibacterial effects of calprotectin. Bacterial invasion, however, is a multistep process involving binding of bacteria to the host cell surfaces and signaling between bacteria and host cells to induce cytoskeletal rearrangements which lead to translocation of the bacteria into host cells (15). Interference in any of these steps could reduce bacterial invasion. We observed twofold fewer *P. gingivalis* cells bound to cells expressing calprotectin. Reduced bacterial binding may, in part, explain reduced invasion into these cells. Host cell receptors that bind *P. gingivalis* to initiate internalization have not yet been identified. However, calprotectin-expressing cells bound fewer of each of three different bacterial species, *L. monocytogenes,* salmonellae, and now *P. gingivalis*, suggesting that common mechanisms probably exist. Calprotectin appeared to promote resistance to invasion by modulating cellular functions in addition to direct antibacterial activity.

We previously showed that calprotectin expression is associated with increased long actin filament formation, increased α_3 integrin expression, and spreading cell morphology (Nisapakultorn et al., submitted). Increased expression of calprotectin and α_3 integrin is also observed in oral and pocket epithelia of periodontitis tissue specimens (9, 16, 43), suggesting that changes observed in calprotectin transfected cells probably occur in vivo as a response to infection. Indeed, these changes may contribute to reduced *P. gingivalis* invasion into calprotectin expressing cells. If host receptors for *P. gingivalis* are localized on basolateral surfaces of epithelial cells, as is the case for *L. monocytogenes* (27), increased cell-cell and cell-substrate adhesion mediated by $\alpha_3\beta_1$ integrin (5) may limit access to the receptors. Invasion by *P. gingivalis* is actin dependent (25). An alteration in actin cytoskeletal organization may also affect bacterial uptake. Finally, since calprotectin is a broad-spectrum antimicrobial, internalized *P. gingivalis* may be killed or its growth may be inhibited within epithelial cells.

In periodontitis, pocket epithelia are constantly exposed to a large number of bacteria in dental plaque, including *P. gingivalis*. Resistance to infection of the connective tissue depends in part on maintaining the integrity of the pocket mucosal epithelium. In vitro, prolonged exposure of gingival epithelial cells to *P. gingivalis* induced epithelial cell rounding (1). In this study, we showed that cells expressing calprotectin are resistant to cell rounding and detachment after prolonged exposure to *P. gingivalis*. To clarify whether cell rounding is mediated by *P. gingivalis* invasion (1) or by bacterial factors such as proteases (21) we compared changes in epithelial morphology after exposure to the wild type, FDC381, or the fimbria-deficient mutant, DPG3. The fimbria-deficient mutant, DPG3, shows no detectable adherence and invasion into KB cells (34). DPG3, however, induces cell detachment similar to the wild type, FDC381, suggesting that *P. gingivalis* invasion does not mediate cell detachment. In contrast, heat-killed *P. gingivalis* did not induce change in epithelial cell morphology, suggesting that heat-labile proteases may be responsible for detachment.

Cysteine proteases are major extracellular and cell-associated proteases of *P. gingivalis* and include an arginine-specific cysteine protease (Arg-gingipain, or Rgp) and lysine-specific cysteine protease (Lys-gingipain, or Kgp) (7). The Rgp has two isoforms, RgpA and RgpB, encoded by two genes, *rgpA* and *rgpB*, respectively. Kgp is encoded by the single gene *kgp. P. gingivalis* cysteine proteases have been implicated in induction of epithelial cell rounding and detachment from the substratum (21). Cell rounding and detachment appear to be medi-

ated by proteolysis of cellular adhesion molecules, including occludin, E-cadherin, β_1 integrin (22), intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and very late antigen-4 (48, 51).

Using protease-deficient mutants, we demonstrated that epithelial cell detachment after prolonged exposure to *P. gingivalis* is due to the activity of Kgp and Rgp. *P. gingivalis* mutants that do not express both Rgp and Kgp (KDP136 [Δ*rgpA* Δ*rgpB* Δ*kgp*]) were unable to induce cell rounding and detachment. The mutants that lack either Kgp (KDP129 [Δ*kgp*]) or Rgp (KDP133 [Δ*rgpA* Δ*rgpB*]) induced rounding in untransfected and sham-transfected cells but to a lesser extent than did the wild-type strain (ATCC 33277). Up to 24 h of incubation with these mutants was insufficient to affect transfected cells expressing calprotectin. Together, the data showed that cells expressing calprotectin are more resistant to *P. gingivalis* Kgp- and Rgp-mediated cell detachment. Increased α_3 integrin in calprotectin expressing cells may enhance cell-cell and cell-matrix adhesion, therefore limiting access and the time necessary for protease digestion and cell detachment from the monolayer.

In conclusion, we demonstrated that epithelial calprotectin expression may have a protective role in periodontal diseases. Reduced *P. gingivalis* binding and invasion into epithelial cells could reduce bacterial colonization and persistence during the infections. Calprotectin-mediated resistance to cysteine proteases appears to fortify the physical barrier role of epithelia and deter bacterial invasion into connective tissue matrix. Therefore, increased calprotectin expression during periodontitis may reflect complimentary innate mucosal host defense mechanisms in response to *P. gingivalis* infection.

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