Role of Fimbriae in *Porphyromonas gingivalis* Invasion of Gingival Epithelial Cells

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***Porphyromonas gingivalis* is a periodontal pathogen capable of invading primary cultures of normal human gingival epithelial cells (NHGEC). Involvement of *P. gingivalis* fimbriae in the invasion process was examined. Purified *P. gingivalis* 33277 fimbriae blocked invasion of this organism into NHGEC in a dose-dependent manner. DPG3, a *P. gingivalis* fimbria-deficient mutant, was impaired in its invasion capability approximately eightfold compared to its parent, strain 381. However, adherence of the mutant was only 50% reduced compared to the parent. Biotin labeling of NHGEC surface proteins revealed that two fimbriated strains, but not DPG3, bound a 48-kDa NHGEC protein. Adhesin-receptor interactions, such as fimbriae binding to a 48-kDa NHGEC surface receptor, may trigger activation of eukaryotic proteins involved in signal transduction and/or provoke the generation of surface *P. gingivalis* molecules required for internalization.**

Porphyromonas gingivalis plays an important role in the initiation and progression of periodontal disease. An impressive armamentarium of both proteolytic and cytolytic properties is associated with this organism and is thought to contribute to tissue destruction (3). In order for *P. gingivalis* to express its wide range of virulence factors, it must first be able to colonize and multiply in the host's hostile microenvironment and circumvent host immune surveillance. Many bacterial pathogens that initiate infections at host mucosal surfaces utilize intracellular penetration as a major virulence factor (7, 9, 10, 27, 30, 38). Recently, we demonstrated that *P. gingivalis* can efficiently invade normal gingival epithelial cells (17, 20) using pathways characteristic of the bacterium-directed phagocytosis process described for enteric pathogens (33, 37). Others have corroborated the invasive capability of this organism by showing *P. gingivalis* invasion of multilayered pocket epithelium (36) and transformed cells (6). *P. gingivalis* fimbriae have been shown to mediate binding of this organism with human epithelial cells (13, 15), a perceived prerequisite for subsequent invasion. Our aim in this study was to investigate the role of *P. gingivalis* fimbriae in favoring an invasive event of this organism with normal human gingival epithelial cells (NHGEC).

To study the involvement of fimbriae in the *P. gingivalis* invasion processes of human cells, primary cultures of gingival epithelial cells were generated, cultured in monolayers, and reacted with *P. gingivalis* strains 33277, 381, and DPG3, as described previously (17, 20). Strain 33277 is the ATCC type strain, while strains 381 and DPG3, which were kindly provided by R. J. Genco (State University of New York), are a parent laboratory strain and a fimbria-deficient mutant, respectively. DPG3 was created by insertional inactivation of the *fimA* gene (26). Bacteria were grown anaerobically at 37°C in Trypticase soy broth supplemented with hemin and menadione (16). The invasion assay entailed incubation of the bacteria (10⁷) with the epithelial cell monolayer (10⁵ cells per well) at 37°C for 90 min followed by washing and killing extracellular bacteria with a combination of gentamicin and metronidazole for 1 h, as described previously (17). Controls for antibiotic killing were

included in all experiments. Following a second washing, epithelial cells were lysed with sterile distilled water, and the lysate was cultured anaerobically on blood plates supplemented with hemin and menadione, to determine numbers of intracellular bacteria. Invasion was expressed as the percentage of the initial inoculum recovered after antibiotic treatment and epithelial cell lysis. Isolated fimbriae were tested for their ability to block *P. gingivalis* invasion of NHGEC. Fimbriae, purified from *P. gingivalis* 33277 by the method of Yoshimura et al. (39), as described previously (16), were incubated, for 1 h, at various concentrations with monolayer cultures of NHGEC, after which invasion of *P. gingivalis* 33277 was assessed. Results depicting a dose-dependent inhibition of invasion of *P. gingivalis* 33277 by autologous fimbriae are shown in Fig. 1. Inhibition ranged from a low of 7% by 4 µg of fimbriae to a high of 63% by 16 µg of fimbriae. In comparison, 16 µg of bovine serum albumin (BSA) elicited no significant inhibition. Two-sided two-sample tests revealed that these results were statistically significant ($P \leq 0.05$). P values were adjusted for multiple comparisons by the method of Sidak (14). Comparable analyses were conducted for data presented in Fig. 2.

The invasive capacity of the respective *P. gingivalis* strains is shown in Fig. 2A. While strain 381 invaded half as efficiently as the type strain ($P < 0.005$), its fimbria-deficient mutant, DPG3, showed a reduction in invasion of almost eight times the level obtained for the parent strain: 0.8 versus 6.1% ($P < 0.005$). The result obtained for strain 33277 was comparable to that reported previously (17).

To quantitate the levels of adherence of the *P. gingivalis* strains to confluent NHGEC monolayers, the latter were treated with 50 mM sodium azide to block *P. gingivalis* invasion (17). This treatment was previously shown to completely block *P. gingivalis* invasion of NHGEC (17). Furthermore, microscopic examination following light staining with crystal violet demonstrated that azide treatment did not noticeably affect the cells' ability to act as a binding substrate for the bacteria. [³H]thymidine-labelled bacteria (10⁸) were reacted with epithelial cell monolayers (10⁵ per well) at room temperature for 1 h, as previously described (17). Following multiple washings, epithelial cultures were trypsinized and adherent *P. gingivalis* was quantitated by scintillation spectroscopy. Strains 33277 and 381 bound comparably to NHGEC monolayers, while

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FIG. 1. Dose response inhibition of *P. gingivalis* 33277 invasion of NHGEC by autologous fimbriae. Percent inhibition was the reduction in invasion in the presence of fimbriae or BSA, compared to the control with no exogenous protein. Error bars represent standard errors ($n = 4$). $P \leq 0.05$ for 16 versus 4 μg and for 8 versus 4 μg by the method of Sidak (14).

strain DPG3 showed a 50% reduction in binding compared to the parent strain ($P < 0.05$) (Fig. 2B).

To identify suggestive biotinylated epithelial cell membrane proteins binding to *P. gingivalis*, monolayer epithelial cell cul-

tures were first surface labelled with *N*-hydroxysuccinimidobiotin (5 mg/ 10^7 cells) on ice for 30 min. Following multiple washes with chilled phosphate-buffered saline (PBS), pH 7.4, cells were removed from the culture plates, collected by centrifugation ($10,000 \times g$, 5 min) and lysed by sonication. Soluble biotinylated material was recovered in the supernatant following centrifugation of the lysate. Respective *P. gingivalis* strains (10^9 cells in PBS) were incubated with a 1:20 dilution of this biotinylated NHGEC extract, with gentle agitation, at room temperature for 1 h. After four washes ($10,000 \times g$, 5 min each), the final pellet was resuspended in 600 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and 10- μl aliquot samples were subjected to SDS-PAGE, followed by electroblotting onto nitrocellulose, as described previously (19). Biotinylated NHGEC surface components that bound to *P. gingivalis* were visualized by developing with avidin-peroxidase (1:20,000), incubated at room temperature for 30 min. Following a second series of washes, the nitrocellulose was developed in 0.1 M Tris-HCl (pH 7.6), containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. Fimbriated *P. gingivalis* 33277 and 381 bound to a 48-kDa surface NHGEC protein, whereas DPG3 reacted poorly with this epithelial-cell molecule (Fig. 3, lanes 2 and 3 versus lane 4). On the other hand, a 25-kDa protein bound to DPG3 but was less reactive with the type and parent strains (Fig. 3). These epithelial-cell proteins could be candidate receptors for either activation or inhibition of invasion, respectively. All three strains tested interacted with a 110-kDa NHGEC protein.

The present results implicate fimbriae in *P. gingivalis* invasion of normal gingival epithelial cells. Fimbriae purified from *P. gingivalis* were capable of blocking invasion of NHGEC. In addition, a fimbria-deficient mutant was impaired in its inva-

FIG. 2. (A) Invasion of *P. gingivalis* 33277, 381, and its fimbria-deficient mutant, DPG3. Invasion was calculated from CFU recovered intracellularly as a percentage of total bacteria reacted with NHGEC. Error bars represent standard errors ($n = 4$). $P \leq 0.005$ for the three strains compared to each other by the method of Sidak (14). (B) Adherence of *P. gingivalis* strains 33277, 381, and its fimbria-deficient mutant, DPG3, to confluent NHGEC. Sodium azide-pretreated NHGEC (10^5 per well) and [^3H]thymidine-labeled bacteria (10^6) were incubated at room temperature for 1 h. Adherence is presented as the number of bacteria bound to 10^5 NHGEC, calculated from the specific activity of the bacteria. Error bars represent standard errors ($n = 4$). $P \leq 0.05$ for strain 33277 versus DPG3 and strain 381 versus DPG3 by the method of Sidak (14).

FIG. 3. SDS-PAGE biotin-avidin blot analysis disclosing biotinylated NHGEC membrane proteins bound to *P. gingivalis*. Lanes: 1, total biotinylated NHGEC proteins; 2, *P. gingivalis* 33277; 3, *P. gingivalis* 381; 4, *P. gingivalis* fimbria-deficient mutant, DPG3; 5 to 7, control strips of *P. gingivalis* 33277, 381, and DPG3 alone. Molecular sizes (in kilodaltons) are indicated on the left.

sion capability, albeit this mutant was the result of insertional inactivation, which conceivably could affect other, as yet unidentified genes that may be involved in invasion. Fimbriae would appear to be important in either initial attachment-and/or a later invasion-associated pathway. The importance of fimbriae in *P. gingivalis* adherence and invasion is further corroborated by the work of Njoroge et al. (29). This study found that fimbria-deficient mutants were unable to adhere to or invade KB cells and that antibodies to the major fimbriae blocked adherence to the same cells.

P. gingivalis binds to a variety of host substrates, both inert, such as saliva-coated hydroxyapatite (4), as well as eukaryotic cells, such as normal and transformed gingival epithelial cells (6, 36). In addition, *P. gingivalis* binds to antecedent oral bacteria such as *Streptococcus gordonii* and *Actinomyces viscosus* (18, 24). In all these interactions, *P. gingivalis* fimbriae have been identified as important mediators (11, 15, 16, 21). However, fimbriae are not considered exclusive mediators of adherence, i.e., without which the respective interactions could not occur. Indeed nonfimbrial outer-membrane-associated adhesins have been identified in *P. gingivalis* (2, 12, 19, 22, 32). The results presented herein demonstrate that a fimbria-deficient mutant of *P. gingivalis* retains the capability to adhere to normal gingival epithelial cells, albeit approximately 50% less than the parent strain. These results are consistent with those reported by Hamada et al. (13) which demonstrated that adherence of a fimbria-deficient mutant of *P. gingivalis* to a gingival epithelial cell line was reduced by about 65%. In contrast, invasion of the fimbria-deficient mutant was reduced almost eightfold compared to the parent. Thus, it would appear that there is not a strict correlation between quantitative adherence and invasion of *P. gingivalis*. This concept is further supported by the finding that there was no significant difference in the adherence levels of strains 381 and 33277, although 33277 invaded more efficiently than 381.

While invasion and quantitative adherence do not appear to be directly related, there may be an association between certain adhesin-receptor interactions and subsequent invasion. In particular, the fimbria-dependent component of *P. gingivalis* adherence could play a major role in subsequent invasion of the organism. The fimbria-deficient mutant strain was found to be specifically unable to interact with a 48-kDa NHGEC protein. Although this protein is, as yet, uncharacterized, labelling with *N*-hydroxysuccinimidobiotin (which is unable to penetrate eukaryotic membranes [23]) indicates that it is surface exposed. It would appear probable, therefore, that the 48-kDa protein is the NHGEC cognate receptor for the fimbriae. Binding of *P. gingivalis* fimbriae to this protein may initiate the

signal transduction pathways that ultimately result in bacterial internalization.

Intracellular invasion of human epithelial cells is a key pathogenic property for a number of bacterial species found in a variety of genera and associated with both acute and chronic infections (7, 9, 10, 27, 30, 38). Common to these events is the need for the bacteria first to attach to the epithelial cell membrane and then to induce a series of structural and biochemical changes in either the organism or the host cell or both, culminating in bacterial penetration (1, 8, 33). Many of the eukaryotic signaling pathways have been shown to involve protein kinase activity (5, 34, 35). Recent reports have documented that *P. gingivalis* fimbriae can specifically induce a protein kinase-mediated phosphorylation of a protein in mouse peritoneal macrophages and in human monocyte-like cells (28). The fimbria-induced protein was inhibited by staurosporine, a potent inhibitor of protein kinase C (28). Furthermore, *P. gingivalis* fimbriae can stimulate differentiation of monocyte/macrophage progenitors by a mechanism that also requires protein kinase C activity (31). It is tempting to speculate, therefore, that fimbria-mediated binding of *P. gingivalis* triggers the invasion pathways. For example, binding could either induce the activation of eukaryotic proteins involved in signal transduction and/or provoke the generation of surface molecules of *P. gingivalis* required for internalization.

What has evaded the scrutiny of periodontal microbiologists over the years has been the inability to associate periodontopathogenic properties of microorganisms with the vagaries of a disease presenting clinically as a perpetual state of latent infection with periods of inflammatory exacerbations leading to tissue destruction. *P. gingivalis* appears to be adapted to life within gingival epithelial cells. The organism is found in the cytoplasm, not confined by a membranous vacuole, and is capable of intracellular replication (17). Recently, Madianos et al. showed that *P. gingivalis* can persist within KB epithelial cells over an 8-day period (25). Persistence within, and recrudescence from, cells of the periodontal tissues could thus begin to explain characteristics of latency and exacerbation in periodontal disease.

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