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Short communication

Adherence of *Porphyromonas gingivalis* to gingival epithelial cells: modulation of bacterial protein expression

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The protein profiles of *Porphyromonas gingivalis* (ATCC 33277 and W83) bound to KB gingival epithelial cells were analyzed by SDS-PAGE and immunoblotting. We found that a 51-kDa component was formed in bacteria that adhered to the KB cells, whereas 26- to 29-kDa bands were less intensive, in contrast to the protein profile of free bacteria. *P. gingivalis* ATCC 33277 incubated with protease-treated KB cells retained the profile of free bacteria. These results demonstrate the specificity of bacterial recognition of eukaryotic membrane components.

Key words: *Porphyromonas gingivalis*; gingival epithelial cells

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It is well documented that *Porphyromonas gingivalis* plays an important role in the initiation and progression of periodontal diseases (25, 29). This organism expresses a vast array of virulence factors including proteases, fimbriae, hemagglutinins, membrane vesicles, lipopolysaccharide and a capsule, each of which contributes to interactions with cells (13). A crucial step in successful colonization and, ultimately, periodontal tissue destruction by microbial pathogens is the ability to adhere to host surfaces. Bacterial outer membrane molecules are certainly required for attachment to epithelial cells. However, the structures involved in eukaryotic host recognition are not well known. Fimbriae likely facilitate the initial interaction between the bacteria and the host (10, 12, 14, 19). Several studies using anti-fimbria monoclonal antibodies have shown that this surface component is composed of polymerized 42-kDa fimbrillin subunits and minor 28-kDa polypeptidic subunits (5, 8). Fimbriae have been reported to form complexes with the HA-Ag2 hemagglutining adhesin, and 43- and 49-kDa polypeptides. Some structural homology between these molecules is strongly suspected (6). Many other molecules, like the family of cysteine proteinases first referred to as trypsin-like enzymes, may be involved in attachment to epithelial cells (15, 16, 21). Pavloff et al. (20) have shown that a cysteine proteinase, Arg-gingipain, is a polyprotein containing adhesion molecules involved in binding to erythrocytes. Various forms of the enzyme are produced by *P. gingivalis* (24). It has recently been suggested that these enzymes are associated with hemagglutinins and fimbriae (22, 27). These studies point out that a number of complex molecules are involved in the adherence of *P. gingivalis* to host cells. Another study reported that the expression of certain factors was regulated by environment stress factors (3). In this study, we looked at whether the presence of epithelial cells triggered the formation of protein complexes by *P. gingivalis* when the bacteria attached to the epithelial cell surface. We used an *in vitro* system, which allowed optimal bacterial proliferation and prevented internalization into the epithelial cells. Our findings showed that *P. gingivalis* that bound to epithelial cells specifically expressed proteins different from those of freely floating bacterial cells.

P. gingivalis (ATCC 33277 and W83) was grown in anaerobic Todd-Hewitt broth and, after a 1/10 dilution in the same medium, was incubated for 18 h until the bacteria reached the exponential phase. Just before the experiments, the bacteria were harvested by centrifugation at 10,000×*g* and washed in phosphate-buffered saline (PBS). The bacterial concentration was determined by taking spectrophotometric readings at 660 nm. Immunoblotting experiments were carried out using purified anti-fimbria and anti-HA-Ag2 monoclonal antibodies (8, 9), and with the

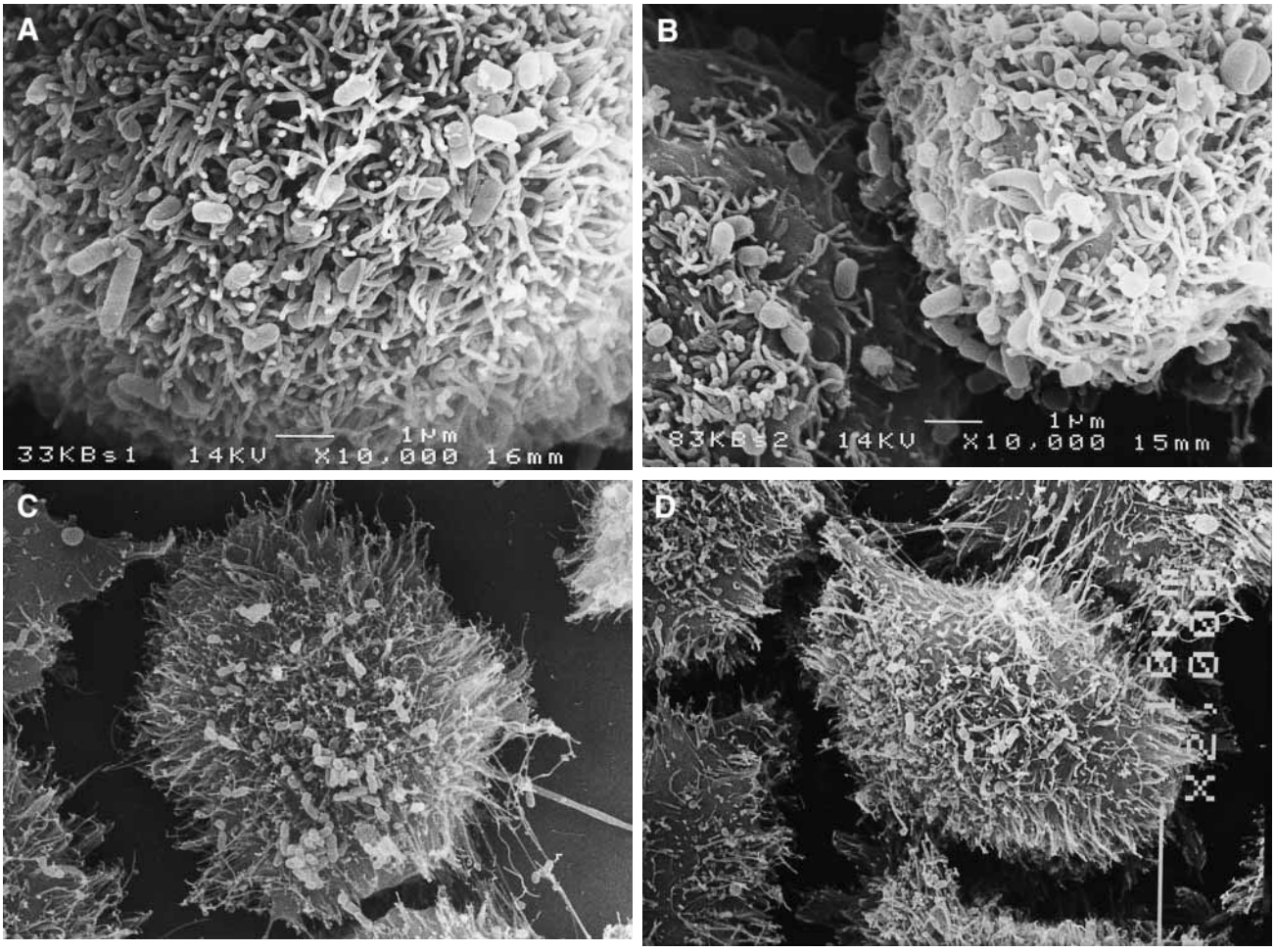

Fig. 1. Electron micrograph of *P. gingivalis* attached to the KB cell surface. ATCC 33277 and W83 were incubated in Todd-Hewitt broth under anaerobic conditions for 18 h at 37°C with glutaraldehyde-fixed KB cells in suspension (panels **A** and **B** respectively) or in monolayers (panels **C** and **D** respectively). Magnification: ×10,000 (A and B) and ×4000 (C and D).

culture medium supernatants that produced them, referred to as unpurified monoclonal antibodies.

The KB human gingival epithelial cells (ATCC CCL-17) were grown to confluence in RPMI1640 medium (Bio-Whittaker) supplemented with fetal calf serum (10%), L-glutamine (2 mM) and antibiotics (penicillin-streptomycin: 100 IU-100 μg/ml) in 75 cm^2 flasks or 35-mm dishes.

Cell monolayers were washed twice with PBS then incubated with 5 ml of 0.02% EDTA for 5 min. Cell detachment was stopped by adding 5 ml of culture medium. The cells were then collected by centrifugation, rinsed with PBS and counted. Suspended KB cells were fixed in PBS–2% glutaraldehyde for 1 h at room temperature with gentle agitation, and washed three times with PBS before being incubated at 37°C with bacteria (200 bacteria per cell) under anaerobic conditions in Todd-Hewitt broth. Microscopy experiments were carried out on undetached cell monolayers in 35-mm dishes. The monolayers were fixed with 2% glutaraldehyde and washed three times with PBS before the bacteria were added. The mean cell number value in triplicate unfixed dishes was used to calculate the number of bacteria to be incubated with the fixed KB cells.

After incubation, the cell suspensions were centrifuged (1200 rpm for 5 min), washed twice in PBS, and the pellets containing the cell-bound bacteria were recovered in 100 μl of PBS. The bacteria remaining in the supernatants, as well as those in the controls incubated without KB cells, were washed in PBS by centrifugation (10,000 rpm for 10 min) and recovered in 1 ml of PBS. This glutaraldehyde fixation procedure has previously been shown not to affect interactions between membrane receptors and their ligands (4). In our model, *P. gingivalis* strains were incubated under anaerobic conditions to provide them with optimal growth conditions.

Analysis of the interaction of *P. gingivalis* with glutaraldehyde-fixed KB cells by scanning electron microscopy (SEM)

After an 18-h incubation at 37°C with bacteria, the glutaraldehyde-fixed KB cells (gf-KB cells) were washed twice with PBS, fixed in a 2.5% solution of glutaraldehyde in cacodylate buffer (0.2 M, pH 7.2) and dehydrated in ethanol. After critical point drying, the samples were metallized with palladium-gold and observed by SEM (JSM 6400).

No detectable differences in the cell morphologies of bacteria bound to suspended cells and bacteria bound to cells in monolayers were observed (Fig. 1).

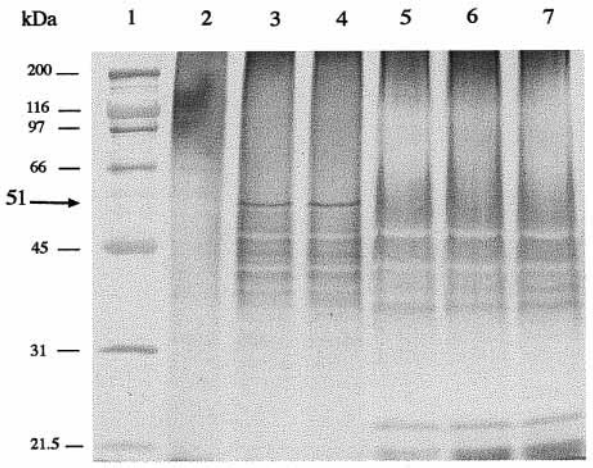

Fig. 2. Silver-stained SDS-polyacrylamide gel of W83 lysates. Bacteria were incubated under anaerobic conditions in Todd-Hewitt broth for 18 h at 37°C with or without gf-KB cells. Lane 1: molecular mass standards; lane 2: control gf-KB cells; lanes 3, 4: W83 bound to gf-KB cells; lane 5, 6: W83 from gf-KB cell supernatant; lane 7: W83 controls. Assays were carried out in duplicate. Molecular masses (kDa) of standards are shown on the left.

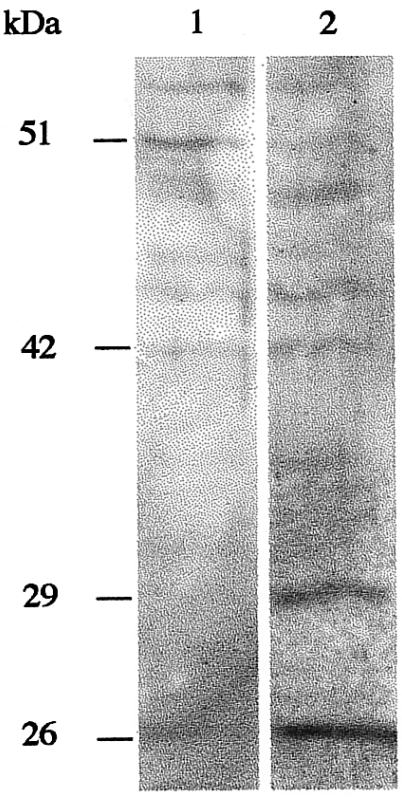

Fig. 3. Immunoblot of W83 lysates probed with the hybridome medium containing anti-fimbrial/anti-HA-Ag2 antibodies. The polyvinyl difluoride sheets (Millipore) were incubated with biotin-conjugated anti-mouse antibodies (Dako) (1/2000 in PBS containing 0.1% BSA, 0.1% gelatin and 0.05% Tween 20). After incubation with alkaline phosphatase-conjugated streptavidin (Dako), the PVDF sheets were developed with NBT/BCIP (nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) (Sigma Chemical Co.). Lane 1: W83 bound to gf-KB; lane 2: W83 controls. Assays were carried out in duplicate. The numbers on the left are molecular masses in kilodaltons.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis and immunoreactivity of bacteria lysates

After incubating *P. gingivalis* in the presence or absence of suspended gf-KB cells, the samples were solubilized in dissociating buffer (v/v) containing 2% SDS and 5% β-mercaptoethanol, and heated at 95°C for 5 min. The solubilized samples (5×10^8 bacteria) were electrophoresed in a 10% acrylamide gel as described by Laemmli (17). After electrophoresis, the polypeptides were stained using the potassium permanganate-silver nitrate procedure of Ansorge (2) or transferred to a polyvinyl difluoride membrane, (Immobilon-P, Millipore) for immunoblotting according to Towbin et al. (28). SDS-PAGE protein profiles of W83 incubated for 18 h in Todd-Hewitt medium in the presence of gf-KB cells differed significantly from those of the W83 controls (Fig. 2). The silver-stained protein profiles of gf-KB-bound *P. gingivalis* had an additional band of M_r=51,000 (Fig. 2, lanes 3, 4) compared to the profiles of bacteria from cell supernatants and the controls (Fig. 2, lanes 5, 6, 7). Furthermore, the protein profiles of bacteria attached to epithelial cells did not contain a 23-kDa band. The same results were obtained with strain ATCC 33277. After incubating *P. gingivalis* in the presence or absence of KB cells for 65 h, the protein profiles were similar and contained all bands (data not shown).

To determine whether adhesin structures were involved in the distinctive SDS-PAGE bands, we performed immunoblot assays using unpurified monoclonal antibodies that recognized epitopes corresponding to fimbrial subunits and hemagglutinin HA-Ag2 (5) (Fig. 3). Once again, a more intense band corresponding to a molecular mass of 51 kDa was obtained with extracts of *P. gingivalis* that had attached to gf-KB cells after an 18-h incubation, whereas the 26- and 29-kDa bands in control extracts were more intense. The 42-kDa polypeptide, which was identified by Chandad et al. (6) as corresponding to fimbriae, was expressed by bound bacteria at the same intensity as that of the bacterial controls. This suggested that fimbriae were probably not overexpressed.

Effect of protease treatment of KB cells on bacterial adhesin expression

To determine whether the differences in bacterial adhesin expression required that KB cells retain their plasma membrane integrity, the cells were treated with protease before the 18-h incubation with *P. gingivalis* ATCC 33277. KB cells were digested with different concentrations of dispase (a nonspecific protease, Sigma Chemical Co., St. Louis, MO) in PBS for 1 h at 37°C before being fixed with PBS–2% glutaraldehyde. This treatment modified the profile of the bacterial proteins recognized by the anti-fimbria and anti-HA-Ag2 monoclonal antibodies (Fig. 4). At a dispase concentration of 100 μg/ml, a slight shift in the 51-kDa band was noted (Fig. 4, lane 2). At a concentration of 200 μg/ml (Fig. 4, lane 3), the 51-kDa band became thinner and the surrounding protein profile changed, while at a concentration of 500 μg/ml the protein profile was similar to that of the bacterial controls (Fig. 4, lanes 4, 5). The induction of this component was thus specific to the presence of a eukaryotic substrate and not just due to environmental stress. The nature of the structure remains to be determined. The molecular mass may mean it is related to the 49-kDa hemagglutinin HA-

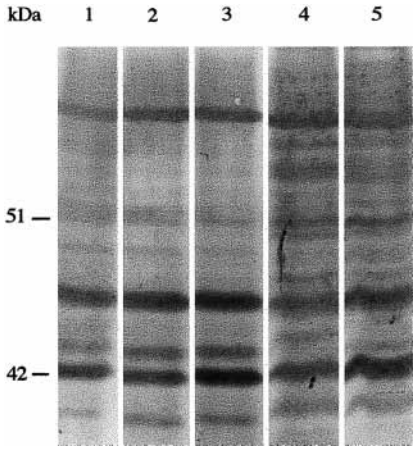

Fig. 4. Effect of protease treatment of KB cells on bacterial adhesin expression. ATCC 33277 bound to gf-KB cells were subjected to SDS-PAGE, immunoblotted, and probed with hybridome medium containing anti-fimbria/anti-HA-Ag2 antibodies. Lane 1: 0 μg/ml; lane 2: 100 μg/ml; lane 3: 200 μg/ml; lane 4: 500 μg/ml, lane 5: bacteria controls. The numbers on the left are molecular masses in kilodaltons.

Ag2 described by Chandad et al. (6). However, there is significant evidence relating adherence, coaggregation and protease activities to a single protein complex (18, 22, 26). Bacterial proteases have been shown at a genetic level to be associated with hemagglutinins (23). A 50-kDa arginine-specific cysteine protease, arg-gingipain, has been found in the culture supernatant of *P. gingivalis* (7), and a cysteine proteinase, lys-gingipain, has recently been reported to be composed of a single polypeptide with the same 51-kDa molecular mass as our component (1). It has also been shown that strains of *P. gingivalis* with high levels of trypsin-like protease activity adhered better to human erythrocytes and epithelial cells than strains with lower levels of such activity (11).

Our *in vitro* model demonstrated that a specific 51-kDa bacterial component was produced as early as the stage when bacteria adhered to epithelial cells. We are continuing to study the biochemical and functional features of this protein to better understand the first step in the attachment of *P. gingivalis* to epithelial cells.

Acknowledgments

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