Effect of Cysteine Protease of *Porphyromonas gingivalis* on Adhesion Molecules in Gingival Epithelial Cells

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Received December 12, 1998 Accepted March 8, 1999

ABSTRACT—We examined the effect of cysteine protease of *Porphyromonas gingivalis* (P. gingivalis) on cell adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4) of human gingival epithelial cells. The cells were incubated for 48 hr with or without *P. gingivalis* protease. Their cell adhesion molecule expression levels were increased at 12 hr, but decreased at 18–48 hr. This result suggests that protease degrades cell adhesion molecules. After the stimulation with protease for 12 hr, *P. gingivalis* fimbrial binding to a monolayer of the cells was effectively inhibited by the addition of the cell adhesion molecules, suggesting the fimbrial binding to the cells occurred through cell surface adhesion molecules.

Keywords: *Porphyromonas gingivalis* protease, Adhesion molecule, Gingival epithelial

Periodontitis results in degradation of periodontal connective tissue and subsequent detachment of the tissue and is the main cause of tooth loss. Oral bacteria such as *Porphyromonas gingivalis* (P. gingivalis) are common in the subgingival plaque of adult periodontopathic bacteria, and these bacteria produce bioactive materials such as cytoplasm membranes, peptidoglycans, outer membrane proteins, lipopolysaccharide (LPS), capsules, fimbriae and protease. *P. gingivalis* proteases are a significant disease-causing factor and include exceedingly high concentrations of cysteine proteinases with trypsin-like activity. The protease of *P. gingivalis* as a major factor in progressive periodontal disease was reviewed recently by Yamamoto (1) and Travis et al. (2). Recently, two major cysteine proteinases responsible for trypsin-like activity were purified from the culture supernatant of various *P. gingivalis* strains, termed "Arg-gingipain (RGP)" and "Lys-gingipain (KGP)" (3, 4). These enzymes are found in gingival crevicular fluid collected from periodontal pockets in adult periodontitis patients, and they degrade basement membrane components such as collagens (types I and IV) (5, 6). In addition, these enzymes are associated with disruption of the normal host defense mechanisms through their ability to degrade immunoglobulins and to inhibit the bactericidal activity of polymorphonuclear leukocytes (5, 6). These findings suggest that proteases from *P. gingivalis* play an important role in the progression of periodontitis. More recently, Kontani et al. (7) have reported that *P. gingivalis* protease not only contributes to virulence by degrading host tissue, but represents a kind of adherence mediator or modulator for *P. gingivalis*. They also suggested that *P. gingivalis* fimbriae are involved in the adherence to host cells. In addition, it was demonstrated that *P. gingivalis* attached to and invaded gingival epithelial cells (8). Therefore, it is necessary to understand the cause and development of periodontal disease and to examine the relationship between *P. gingivalis* protease and cell adhesion molecules on gingival epithelial cells. In this study, we determined the effect of cysteine protease of *P. gingivalis* on cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4) of human gingival epithelial cells.

The whole envelope fraction of *P. gingivalis* 381 was prepared as described previously (8). Cysteine protease of *P. gingivalis* was prepared and purified according to the method of Kontani et al. (7). A protease that can hydrolyze Na-benzoyl-DL-arginine p-nitroanilide (BAPNA) (Wako Pure Chemical Industries, Ltd., Osaka) was obtained from *P. gingivalis* cells by sonication in phosphate-buffered 0.2% Triton X-100 and then purified by column chromatography (7). The protease was purified to homogeneity as assayed by Western immunoblotting
using anti-Arg-gingipain antibody, provided by Dr. M. Nishikata (Hokkaido University). The purified protease was observed on gels following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as a single band with a molecular weight of 47 kDa. Lipopolysaccharide contamination in the purified protease used in this study was not detected by the limulus test (Wako).

Enzyme activity was measured using the specific synthetic substrate of BApeNA as described previously (9). The hydrolysis of the substrate was started by the addition of 1 ml of a BApeNA (0.2 mM) substrate solution that contained 50 mM of Tris-HCl (pH 7.4) and 0.2 mM dithiothreitol (DTT) (Wako). The reaction was performed at 37°C for 10 min. The hydrolysis was monitored by the increase in absorbance at 410 nm. The protease showed trypsin-like activity with BApeNA. One unit (1 U) of enzyme activity was defined as the amount of enzyme required to release 1 μmol of p-nitroanilide under these conditions. The activity of P. gingivalis protease was completely suppressed by histatin, an inhibitor of enzyme activity (9).

Specimens of normal human gingival tissues were obtained from gingival biopsies and the tissue was cultured as described by Lamont et al. (8). The epithelial cells were cultured in α-Eagle’s minimum essential (α-MEM) medium (Gibco, Grand Island, NY, USA) with 10% calf serum (CS) (Gibco). The cells were cultured until they formed a confluent layer, and then they were trypsinized and subcultured. The cells were plated in a 100-mm dish (Costar, Cambridge, MA, USA) in α-MEM medium containing 10% CS. These cells were cultured over a 48-hr period using serum-free medium (ASF 301) (Ajinomoto Co., Kawasaki) that contained the protease (1 U per 1 x 10^6 cells) purified from P. gingivalis and 0.2 mM DTT. Serum starvation had no effect on cell viability. The cells attached to the plates or dishes were removed using a cell scraper (Nunc, Tokyo) and assayed.

The cells (1 x 10^5) were cultured in serum-free medium with or without protease over a 48-hr period. After centrifugation, the supernatants were discarded, and the pellets were then mixed with anti-ICAM-1 mAb, anti-VCAM-1 anti-VCAM-4 monoclonal antibody (mAb) or with an isotype-matched irrelevant Ab (pp100) (Chemicon International Inc., Temecula, CA, USA) as a control for 30 min at 37°C. After washing twice with PBS, the cells were counterstained with FITC-conjugated rabbit anti-mouse IgG1 (Organon Teknika Co., Durham, NC, USA) in phosphate-buffered Saline (PBS) on ice for 30 min. Thereafter, the cells were washed twice with cold PBS. Analysis of fluorescence staining was performed with a FACScan flow cytometer (FACScan; Becton Dickinson Electric Laboratories, Mountain View, CA, USA) and CELLQuest Software. Anti-ICAM-1 mAb, anti-VCAM-1 mAb and anti-VCAM-4 mAb were purchased from Ancel Co. (Bayport, MN, USA).

The purified P. gingivalis fimbriae and the binding of fimbriae to gingival epithelial cells were assessed according to the method of Kontani et al. (7). The cells (5 x 10^3) were cultured in serum-free medium with protease (1 U) for 12 hr. After being washed twice with PBS, fimbriae were detached from P. gingivalis 381 cells, and the purified fimbriae (10 mM) were incubated with 1.2 mg of biotin-N-hydroxysuccinimide (Calbiochem, La Jolla, CA, USA) in 11 ml of 100 mM NaH2CO3 (pH 8.0) at 25°C for 2 hr; then dialysis against PBS performed. The epithelial cells were grown to confluent culture in serum-free medium (ASF 301) (Ajinomoto Co.). Trypsin-EDTA-treated fibroblast cells were seeded in flat-bottomed culture plate wells at a concentration of 5 x 10^3 cells per well. After incubation at 37°C in a 5% CO2 atmosphere for 2 days, the monolayer of fibroblasts was washed twice with PBS, and biotinylated fimbriae (10 μM) were added to the wells in the presence of adhesion molecules (1 mM of ICAM-1, VCAM-1 and VLA-4) (Ancel Co., Bayport, MN, USA), in 100 μl of PBS. The biotinylated fimbria solution containing bovine serum albumin (BSA) was used as a control solution. The amount of bound fimbriae was determined colorimetrically with streptavidin-alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL, USA).

All data presented are means ± S.D. Student’s t-test was used and significance was set at P < 0.05.

Novel cell adhesion molecules, including ICAM-1, VCAM-1 and VLA-4 have been identified and cloned (10). In addition, there are now several examples of receptor-counterreceptor adhesion pairs that are implicated in signal transduction as well as in cell-cell adhesion (11). A previous study (12) on adhesion molecules and periodontal disease, which used immunohistochemical analysis of periodontal tissue, demonstrated that ICAM-1 is expressed on endothelial cells, adhesion cells, monocytes and fibroblasts. It has been suggested that in local inflammation, the expression of ICAM-1 on endothelial cells and fibroblasts increases, and leukocytes infiltrate and become fixed locally. To our knowledge, no studies have been published on the effect of protease on the adhesion molecules of oral cells.

As shown in Fig. 1, to clarify whether ICAM-1, VCAM-1 and VLA-4 are expressed on the surface of the gingival epithelial cells, we examined the levels of ICAM-1, VCAM-1 and VLA-4 expression in the cells by flow-cytometric analysis. ICAM-1, VCAM-1 and VLA-4 were expressed on the gingival epithelial cells (Fig. 1). As shown in Fig. 2, confluent monolayer cultures of gingival epithelial cells were incubated for 48 hr in the presence or absence of P. gingivalis protease (1 U). The mean fluores-
Fig. 1. Flow cytometric analysis of human gingival epithelial cells. Cells (1 × 10^6) were incubated with an isotype-matched irrelevant Ab (control), anti-ICAM-1 mAb, anti-VCAM-1 mAb or anti-VLA-4 mAb for 30 min, washed and developed with FITC-conjugated rabbit anti-mouse IgG1. The cells were washed twice and then analyzed by flow cytometry. The experiment was performed three times with similar results.

Fluorescence intensity (M.F.I.) of ICAM-1, VCAM-1 and VLA-4 on gingival epithelial cells treated with P. gingivalis protease is shown in Fig. 2. The M.F.I. increased significantly at 12 hr (P < 0.05) and decreased significantly at 18–48 hr (P < 0.05). From this result, we concluded that P. gingivalis protease can destroy ICAM-1, VCAM-1 and VLA-4. Also for the cells treated with protease at 0.01 and 1 U, the M.F.I. value was almost the same (data not shown). These results are supported by previous studies (13) demonstrating that P. gingivalis protease is a periodontopathic bacteria protease and can destroy periodontal tissue. This study also suggests that the cell-matrix and cell adhesion molecule degradation caused by P. gingivalis protease contributes to the progression of periodontal disease.

Bacterial adherence to the host cell is an essential step

Fig. 2. The effect of time on the expression level of the cell adhesion molecules (ICAM-1, VCAM-1 and VLA-4) of human gingival epithelial cells in flow cytometry. Cells (1 × 10^6) were treated with P. gingivalis protease (1 U) for 48 hr. The data is expressed as the mean ± S.D. of six cultures. The experiment was performed three times with similar results. *P < 0.05, compared to each M.F.I. (mean fluorescence intensity) at each control. ■ control, □ ICAM-1, ▪ VCAM-1, □ VLA-4.
in the initiation of infectious diseases, and the adhesive function of many pathogenic bacteria is well recognized and documented (14). It has been suggested that *P. gingivalis* fimbriae are involved in adherence to cultured gingival fibroblasts (7). Attachment to and penetration of gingival epithelial cells by *P. gingivalis* is an important factor in periodontal diseases. The mechanisms used to penetrate tissue are unknown, although it was suggested that *P. gingivalis* adhered to and invaded oral epithelial cells (8). Interestingly, as shown in Fig. 2, the M.F.I. increased significantly at 12 hr. Our results indicated an increase in the expression of cell adhesion molecules such as ICAM-1, VCAM-1 and VLA-4. From this result, we hypothesize that once the expression of an adhesion molecule on gingival epithelial cell is induced, the molecule binds to the immunocyte or bacteria. Therefore, next we examined by binding assay the relationship between *P. gingivalis* fimbriae and the cell adhesion molecules on gingival epithelial cells. As shown in Fig. 3 to test the effects of ICAM-1, VCAM-1 and VLA-4 in gingival epithelial cells, after stimulation with protease at 12 hr, the confluent monolayer cultures in the microtiter wells were washed, and then biotinylated fimbriae were added to the wells in the presence or absence of adhesion molecules (ICAM-1, VCAM-1 and VLA-4). The fimbrial binding to the cells was effectively inhibited by the addition of ICAM-1, VCAM-1 or VLA-4 in the assay mixture. Also the binding of fimbriae was inhibited in a dose-dependent fashion in the presence of these adhesion molecules (data not shown). This result suggests that fimbrial binding to the gingival epithelial cells occurred through cell adhesion molecules such as ICAM-1, VCAM-1 and VLA-4. However the binding was not completely inhibited by the adhesion molecules. It is possible that the *P. gingivalis* binds another cell surface matrix component such as collagen, fibronectin and laminin. Thus, once the expression of an adhesion molecule on gingival epithelial cell is induced, the adhesion molecule may bind to the immunocyte or bacteria. It is suggested that after their expression of cell adhesion molecules is initiated, the cell adhesion molecules might increase bacterial adhesion to the cells.

In the present study, we characterized the expression of ICAM-1, VCAM-1 and VLA-4 adhesion molecules that play a role in local inflammation on gingival epithelial cells in periodontal tissue and the effect of *P. gingivalis* protease on this process. Our findings suggest that *P. gingivalis* protease plays an important role in the progression of periodontitis. That there is a positive correlation between the protease activity and adherence of *P. gingivalis* is important: such as a relation was suggested by experimental findings wherein pretreatment of epithelial cells with trypsin, papain, chymotrypsin or neuraminidase significantly enhanced the adherence of *P. gingivalis* cells (15). More recently, it was reported that *P. gingivalis* protease enhanced binding of fimbriae to human fibroblasts (7). In vivo, protease is secreted by *P. gingivalis* in the plaque of periodontal pockets and also released by *P. gingivalis* invading the periodontal tissues (8). Therefore expression of adhesion molecules in *P. gingivalis* protease induced-gingival epithelial cells can occur either before or after *P. gingivalis* invades the periodontal tissue.

In conclusion, the results presented here support the concept that *P. gingivalis* protease not only contributes to virulence by degrading host tissues, but also represents a kind of adherence mediator or modulator for *P. gingivalis*. The effect of inflammatory mediators such as cell adhesion molecules might be changed by the periodontopathic bacteria protease in dental plaque that causes periodontal disease.

**Acknowledgments**

We are grateful to Dr. Katsuhide Sonoda (ILCS, Hokkaido University) and Dr. Katsuaki Sato (The Institute of Medical Science, Tokyo University) for valuable advice. Part of this study was performed at the Institute of Dental Research, Osaka Dental University.

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**Fig. 3.** Effect of the cell adhesion molecules on the binding of fimbriae to the monolayer of the cultured human gingival epithelial cells. After the stimulation with *P. gingivalis* protease (1 U) for 12 hr, the monolayered cells (3 x 10^5) in the microtiter wells were washed, and biotinylated fimbriae (10 μM) were added to the wells in the presence or absence of the cell adhesion molecules (1 mM of ICAM-1, VCAM-1 or VLA-4) in PBS for 30 min at 25°C. The amount of bound fimbriae was determined colorimetrically. The binding of biotinylated fimbriae in the presence of bovine serum albumin (BSA) was used as the control (100%). The data is expressed as the mean ± S.D. of six cultures. The experiment was performed three times with similar results.
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