ORAL MICROBIOLOGY AND IMMUNOLOGY

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Porphyromonas gingivalis mediates the shedding and proteolysis of complement regulatory protein CD46 expressed by oral epithelial cells

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Introduction: Human cells express membrane-bound complement regulatory proteins to prevent complement-mediated autologous tissue damage. In this study, we hypothesized that *Porphyromonas gingivalis*, the major etiological agent of chronic periodontitis, causes the shedding or proteolysis of the complement regulatory protein CD46 expressed by oral epithelial cells.
Methods: Oral epithelial cells were treated with a culture of *P. gingivalis* before measurement of membrane-bound and shed CD46 by enzyme-linked immunosorbent assay (ELISA). The effect of soluble recombinant CD46 on secretion of interleukin-8 (IL-8) by epithelial cells was evaluated by ELISA. The susceptibility of soluble recombinant CD46 to proteolytic degradation by cells and purified Lys-gingipain of *P. gingivalis* was investigated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis/western immunoblotting analysis.
Results: Oral epithelial cells treated with a culture of *P. gingivalis* showed a lower reactivity with antibodies directed to CD46. ELISA revealed that such a treatment resulted in increased amounts of CD46 in the conditioned media suggesting that *P. gingivalis* caused the shedding of membrane-anchored CD46. Stimulation of epithelial cells with soluble recombinant CD46 induced IL-8 secretion in a dose-dependent manner. Whole cells and purified Lys-gingipain of *P. gingivalis* degraded recombinant CD46 in a dose-dependent manner.
Conclusion: This study showed the ability of *P. gingivalis* to induce the shedding/proteolysis of CD46 from the surface of oral epithelial cells. This may render host cells susceptible to the complement system and contribute to tissue damage and the inflammatory process in periodontitis.

Key words: CD46; complement regulatory protein; epithelial cells; gingipain; periodontitis; *Porphyromonas gingivalis*

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Periodontitis is an inflammatory disorder that results in the destruction of the supporting structures of the teeth, including the alveolar bone. It is the most important cause of tooth loss among adults and is initiated by an accumulation of predominantly anaerobic gram-negative bacteria in subgingival sites. Among the various bacterial species associated with the development of periodontitis, *Porphyromonas gingivalis* is suspected to be one of the most important causative agents

of the chronic form of this disease (29). *P. gingivalis* produces a broad spectrum of virulence factors, including outer membrane vesicles, adhesins, lipopolysaccharide, hemolysin and proteinases (5, 12). Arg-gingipain and Lys-gingipain cysteine proteinases are the main endopeptidases produced by *P. gingivalis* and are believed to contribute to host colonization, evasion of host defense mechanisms and destruction of periodontal tissues (14).

Bioactive molecules, which are linked to the human cell membrane by anchor structures, can be released from the cell surface to become soluble effectors (3). Matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) are usually involved in the shedding process (3). Surface-bound bioactive molecules can also be shed and/or degraded following contact with microorganisms or their products. More specifically, the shedding of the ectodomain of syndecan-1, a transmembrane coreceptor for various growth factors and matrix macromolecules expressed by epithelial cells (4), has been reported to be induced by several bacterial pathogens, including *P. gingivalis* (1, 2, 25, 26). The mechanisms involved are related to either activation of signaling pathways through contact with bacteria or direct action of bacterial proteinases.

To avoid autologous complement-mediated tissue injury, human cells express a number of membrane-bound complement regulatory proteins (22). The CD46 (membrane cofactor protein) complement regulator is highly expressed by epithelial cells (23) and consists of four complement control protein repeats, a serine/threonine/proline-rich region, a transmembrane domain and two types of cytoplasmic tails (22). It functions early in the complement cascade to dissociate self-cell-bound C3 convertases and promote the cleavage of covalently bound C3b and C4b fragments upon which the convertases assemble (18, 22, 27). In this study, we hypothesized that *P. gingivalis* causes the shedding/proteolysis of the complement regulatory protein CD46 expressed by oral epithelial cells.

Materials and methods

Bacteria and growth conditions

P. gingivalis HW24D-1 was grown in Todd–Hewitt broth (BBL Microbiology Systems, Cockeysville, MD) supplemented with hemin (10 μg/ml) and vitamin K (1 μg/ml). Cultures were incubated in an anaerobic chamber (80% N_2, 10% CO_2, 10% H_2) at 37°C.

Recombinant CD46 protein and antibodies

Soluble recombinant human CD46 was prepared as described previously (6). Rabbit anti-human CD46 polyclonal antibody (H-294) and mouse anti-human CD46 monoclonal antibody (MEM-258) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) were purchased from Sigma-Aldrich Co (Oakville, ON, Canada).

Cultivation of human oral epithelial cells and identification of CD46

The immortalized human oral epithelial cell line GMSM-K, developed by V. Murrah (Department of Diagnostic Sciences and General Dentistry, University of North Carolina at Chapel Hill, NC) was used (10). This cell line showed an epithelial phenotype by electron microscopy and immunohistochemistry analyses (10). Cells were maintained and cultured as monolayers in tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine (HyClone Laboratories, Logan, UT), 10% heat-inactivated fetal bovine serum (FBS) (DMEM-FBS), penicillin (100 μg/ml) and streptomycin (50 μg/ml) in an atmosphere of 5% CO_2 at 37°C. Epithelial cells were harvested by gentle trypsinization (0.05% trypsin–ethylenediaminetetraacetic acid; Gibco-BRL, Grand Island, NY), washed once in DMEM-FBS, and suspended at a density of 4×10^4 cells/ml in DMEM-FBS, seeded in a six-well plate (8×10^4 cells/well in 2 ml), and cultured until confluence at 37°C in a 5% CO_2 atmosphere. The expression of CD46 by the GMSM-K epithelial cell line was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)/western immunoblotting analysis. Briefly, equal volumes of epithelial cell suspension (10^6 cells/ml) and denaturing buffer were mixed and boiled (10 min). Samples were loaded on a 12% polyacrylamide gel and subjected to electrophoresis under denaturing conditions (16). Proteins were then electrophoretically transferred onto a polyvinylidene fluoride membrane. The membrane was successively incubated with 50 mM phosphate-buffered saline with 0.1% Tween-20 (PBST) containing 5% fat-free milk (2 h), PBST containing 0.8% fat free milk and rabbit anti-CD46 antibody (1 : 1000) (16 h), and HRP-conjugated goat anti-rabbit IgG (1 : 20000) (1 h). CD46 was visualized using the Western Blot Chemiluminescence Reagent Plus (Perkin Elmer, Waltham, MA).

Treatment of human oral epithelial cells with *P. gingivalis*

Oral epithelial cells grown to confluence were treated for 2 h (37°C) with an overnight culture of *P. gingivalis* diluted 1 : 2 in DMEM with 1% FBS. Thereafter, culture medium supernatants were collected, subjected to centrifugation (10,000 ***g***, 5 min), concentrated 10-fold by ultrafiltration (molecular weight cut-off 10 kDa) and stored at −20°C until use. Cells incubated in M199-FBS:Todd–Hewitt broth were used as controls.

Determination of residual membrane-anchored CD46 and shed CD46

Following treatment of epithelial cells with *P. gingivalis*, residual membrane-anchored CD46 on epithelial cells was determined by a cell-based enzyme-linked immunosorbent assay (ELISA). After removing supernatants, cell surface CD46 was detected using rabbit anti-CD46 antibody (1 : 200) and HRP-conjugated goat anti-rabbit IgG (1 : 1000). The amount of shed CD46 in the 10-fold concentrated culture supernatant following treatment of oral epithelial cells with *P. gingivalis* was evaluated by sandwich ELISA using rabbit anti-CD46 antibody (1 : 200) as capture antibody. Bound CD46 was then detected using mouse anti-CD46 monoclonal antibody (1 : 200) and HRP-conjugated goat anti-mouse IgG (1 : 1000). All assays were performed in triplicate.

Treatment of epithelial cells with recombinant CD46 and interleukin-8 determination

Epithelial cells were treated with increasing concentrations of soluble recombinant CD46 (0, 300, 500 or 1000 ng/ml) and incubated at 37°C in 5% CO_2 for 24 h. Culture medium supernatants were collected and stored at −20°C until use. Cells incubated in culture medium without or with CD46 in the presence of polymyxin B (10 μg/ml) were used as controls. A commercial ELISA kit (R&D Systems, Minneapolis, MN) was used to quantify interleukin-8 (IL-8) in the cell-free culture supernatants according to the manufacturer's protocols. Cytokine concentrations were determined in triplicate.

Purification of Lys-gingipain

Lys-gingipain was purified from the cell envelope extract of *P. gingivalis* KDP112 by affinity chromatography on arginine Sepharose 4B (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada) as described previously (11). KDP112, an Arg-gingipain-deficient mutant of *P. gingivalis* ATCC 33277, was kindly provided by K. Nakayama (Nagasaki University, Nagasaki, Japan). Lys-gingipain activity was quantified using *N-p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide as substrate. One unit of enzyme activity was defined as the amount required to release 1 nM *p*-nitroaniline per hour.

Susceptibility of recombinant CD46 to degradation by *P. gingivalis* cells and purified Lys-gingipain

Degradation of soluble recombinant human CD46 (0.4 μg per 100 μl) by *P. gingivalis* was tested by adding increasing concentrations of bacteria (10^3–10^8 cells/ml) to 50 mM PBS containing 60 mM dithiothreitol. Samples were incubated at 37°C for 4 h and then boiled for 10 min. Degradation of CD46 by a preparation of Lys-gingipain was also tested by adding various amounts of proteinase (0.25–8 milliunits/ml). Cleavage of CD46 was monitored by SDS–PAGE/western immunoblotting analysis, as described above. The effect of 1 mM tosyl-Lys-chloromethylketone (TLCK) on the degradation of recombinant CD46 by the Lys-gingipain preparation was evaluated.

Results

The expression of the complement regulatory protein CD46 by the GMSM-K oral epithelial cell line was evaluated. As shown in Fig. 1, a major band with a molecular weight of approximately 60 kDa was detected in epithelial cell lysate using a specific antibody to CD46. Although much less important, a lower molecular weight band (52 kDa) was also detected.

Treatment of oral epithelial cells with an overnight culture of *P. gingivalis* decreased by approximately 80% the amount of membrane-anchored CD46 (Fig. 2). CD46 shedding from the cell surface of treated oral epithelial cells was investigated by ELISA. Basal levels of soluble CD46 were detected in the culture supernatants of untreated epithelial cells while a significant increase was observed in the culture supernatants of epithelial cells treated with *P. gingivalis* (Fig. 3). More specifically, soluble CD46 increased approximately 3.5-fold compared with basal levels.

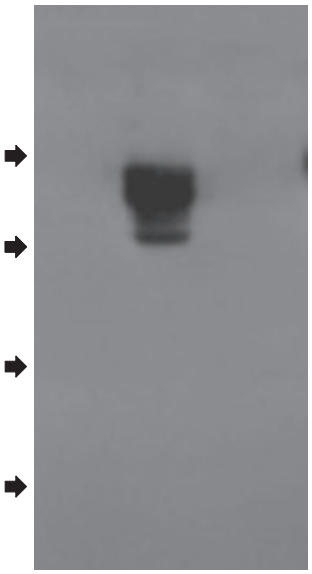

Fig. 1. Identification of CD46 in lysate of oral epithelial cells GMSM-K by sodium dodecyl sulfate–polyacrylamide gel electrophoresis/western immunoblotting analysis. Molecular weight markers were from top to bottom: bovine serum albumin (97.3 kDa), ovalbumin (50.4 kDa), carbonic anhydrase (37.2 kDa) and soybean trypsin inhibitor (29.2 kDa).

Thereafter, the effect of stimulating epithelial cells with soluble recombinant human CD46 on the secretion of the chemokine IL-8 was investigated. Epithelial cells secrete basal levels (42 ± 4 μg/ml mean ± standard deviation) of IL-8 (Fig. 4). Stimulation of cells with recombinant CD46 induced IL-8 secretion in a dose-dependent manner. When used at 1000 ng/ml, CD46 increased by six-fold the amounts of IL-8 secreted. Adding polymyxin B during stimulation of epithelial cells with recombinant CD46 had no effect on IL-8 secretion, indicating that the effect observed was not related to LPS contaminants (data not shown).

Recombinant human CD46 was tested for its susceptibility to proteolytic degradation by *P. gingivalis* cells following a 4-h incubation (Fig. 5A). The results clearly indicated that CD46 was highly sensitive to degradation because complete hydrolysis of the protein was observed after incubation with 10^6 bacteria/ml.

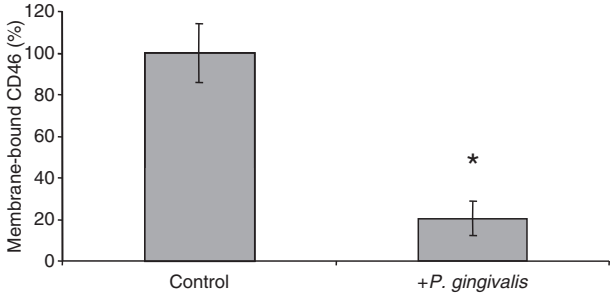

Fig. 2. Determination of residual membrane-anchored CD46 on oral epithelial cells treated or not with an overnight culture of *Porphyromonas gingivalis*. CD46 was determined by a cell-based enzyme-linked immunosorbent assay. A value of 100% was assigned to untreated epithelial cells.

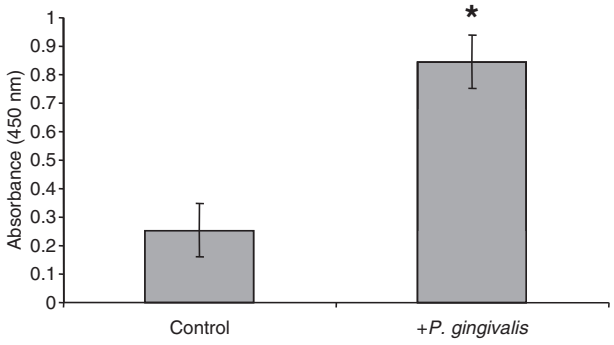

Fig. 3. Determination of shed CD46 in the cell-free culture supernatant (10-fold concentrated) of oral epithelial cells treated or not with an overnight culture of *Porphyromonas gingivalis*. CD46 levels were determined by enzyme-linked immunosorbent assay.

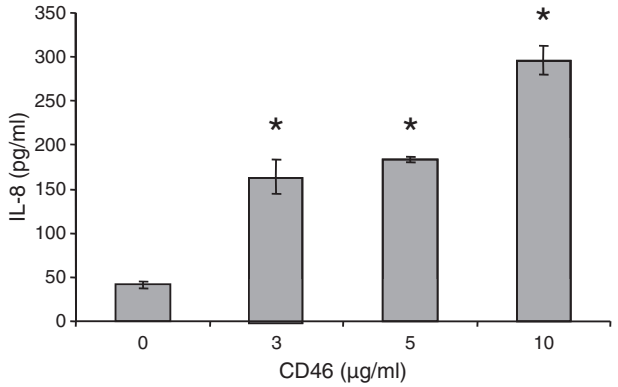

Fig. 4. Secretion of interleukin-8 (IL-8) by epithelial cells stimulated with various amounts of soluble recombinant CD46. IL-8 concentrations in cell-free culture supernatants were determined by enzyme-linked immunosorbent assay.

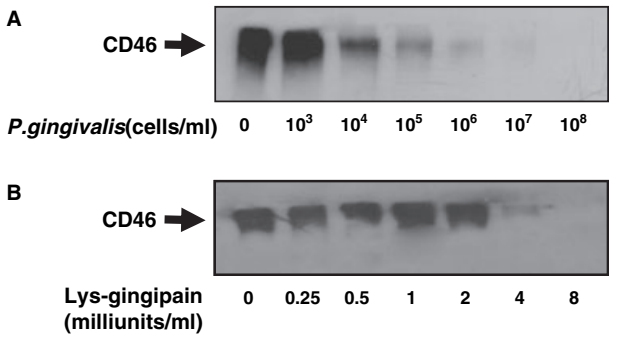

Fig. 5. Degradation of recombinant human CD46 by cells (A) and purified Lys-gingipain (B) of *Porphyromonas gingivalis*, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis/western immunoblotting analysis.

Some degradation was also observed when CD46 was incubated with 10^4 bacteria/ml. The susceptibility of human CD46 to degradation by various concentrations of purified Lys-gingipain was also investigated (Fig. 5B). The purified Lys-gingipain was found to extensively degrade the CD46. This degradation was inhibited by adding 1 mM TLCK, an inhibitor of Lys-gingipain, to the assay mixture (data not shown).

Discussion

The complement system plays an important role in host defense. However, if not properly regulated, activated complement can cause significant damage to host tissues. To prevent complement-mediated autologous tissue damage, human cells express a number of membrane-bound complement regulatory proteins (22). CD46 is a widely distributed complement regulator which protects host cells from complement injury by acting as a cofactor for proteolytic inactivation of C3b and C4b by the serine protease factor I (18, 27). The cleavage of C3b produces the fragment C3bi whereas hydrolysis of C4b generates the fragments C4c and C4d, all of which are unable to support further complement activation. Our laboratory previously reported that *P. gingivalis* can mediate the shedding of important cell surface molecules: CD14 from human macrophages (7) and syndecan-1 from oral epithelial cells (1, 2). In this study, we investigated the ability of *P. gingivalis* to cause the shedding/proteolysis of the complement regulatory protein CD46 from the surface of oral epithelial cells.

The GMSM-K oral epithelial cell line used in this study was found to express CD46 as a major band at 60 kDa and a minor band at 52 kDa. This is consistent with a previous study reporting that up to four major CD46 isoforms (BC1, BC2, C1, C2), with molecular weights from 51 to 68 kDa, may be expressed per cell type (17). The size difference can be attributed mainly to the variations in the amount of *O*-linked sugars on the isoforms.

Our results clearly showed that *P. gingivalis* mediates the shedding of membrane-anchored CD46 in epithelial cells. First, treated cells showed only residual membrane-anchored CD46 after such treatment. Second, the amount of soluble CD46 in the cell-free culture supernatant increased significantly following treatment. The decrease of surface CD46 upon infection by *P. gingivalis* may render the cells more susceptible to lysis by complement, as previously demonstrated *in vitro* (8, 28). This is also supported by the fact that CD46 is naturally shed from the surface of apoptotic and necrotic cells, allowing their efficient removal by phagocytes (8).

Shed CD46 can have detrimental effects in periodontal disease. We showed that soluble CD46 can induce IL-8 secretion by epithelial cells. IL-8 is a potent chemokine that directs the migration of polymorphonuclear leukocytes, monocytes and macrophages to the site of infection. Increased levels of IL-8 are found in the gingival crevicular fluid of inflamed periodontal sites compared with healthy sites (13, 21, 30). Periodontal therapy reduces immune cell numbers in the infiltrate and the levels of IL-8, suggesting a relationship between this chemokine and periodontal status (9). Once present in the extracellular environment in the soluble form, CD46 could also interact with microorganisms. The capacity of human bacterial pathogens to bind the complement regulatory protein CD46 on their cell surface may confer on these microorganisms a capacity to resist serum killing. Although such a phenomenon has been previously reported for *Neisseria meningitidis* and group A streptococci (15, 19, 24), there are no data available regarding the capacity of oral pathogens to bind CD46 on their surface.

Gingipain cysteine proteinases of *P. gingivalis*, which are both secreted and cell-bound, may contribute to the pathogenic process of periodontitis by disrupting host defense mechanisms and degrading tissue components (14). These proteolytic enzymes may participate in the shedding and proteolysis of cell surface molecules. Our results clearly indicated that CD46 was highly sensitive to degradation by whole cells of *P. gingivalis*. The recombinant CD46 possesses thrice as many lysine residues as arginine residues in its amino acid sequence (7.8 and 2.5%, respectively) (20), so we tested its susceptibility to purified Lys-gingipain. SDS–PAGE/western immunoblotting analysis showed that the Lys-gingipain preparation caused a complete degradation of CD46.

In conclusion, our study showed that infection of oral epithelial cells with

P. gingivalis reduces membrane-anchored CD46, a phenomenon that may render cells susceptible to the complement system. Shed CD46 may either trigger cell signaling pathways leading to secretion of IL-8 or be cleaved by Lys-gingipain produced by *P. gingivalis*.

Acknowledgments

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