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A role for gingipains in cellular responses and bacterial survival in *Porphyromonas gingivalis*-infected cells

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1. ABSTRACT

Porphyromonas gingivalis is one of the primary etiologic agents of adult periodontitis and is known to produce a unique class of cysteine proteinases, termed gingipains. They consist of Arg-gingipain (Rgp) and Lys-gingipain (Kgp) and exist in the cell-associated and secreted forms. In the current review, we summarize recent knowledge on the pathophysiological role of gingipains in the virulence of *P. gingivalis* including host cell responses to bacterial infection and its evasion from host defense mechanisms. Studies with various *P. gingivalis* mutants deficient in Rgp- and/or Kgp-encoding genes and proteinase inhibitors specific for each enzyme have demonstrated that both enzymes play a substantial role in disruption of host defense mechanisms by the bacterium and its survival *in vivo*. Gingipains are also important in the bacterium-mediated host cell responses and the subsequent intracellular signaling in the infected cells. *P. gingivalis* can evade the autophagic pathway and instead directly traffic to the endocytic pathway to lysosomes in the infected cells. In addition, gingipains play an important role in acquiring resistance against destruction of the bacterium in the lysosomal system. Furthermore, a major form of the cell-associated gingipain complex composed of the catalytic domains of both enzymes, their adhesin domains, phospholipids, and lipopolysaccharide has recently been isolated and shown to contribute the bacterial evasion of host defense mechanisms and the host tissue breakdown.

2. INTRODUCTION

Adult periodontitis is the most prevalent form of periodontal disease known to be characterized by massive accumulation of immune system cells, and profound destruction of periodontal tissues, including in bone resorption, formation of periodontal pocket and tooth attachment loss. Recent epidemiologic studies also demonstrate the association of the disease with systemic diseases such as cardiovascular diseases and preterm delivery. *P. gingivalis* is a Gram-negative anaerobic bacterium that is strongly implicated as a major causative pathogen in the initiation and progression of adult periodontitis. This bacterium produces a wide variety of virulence factors, including proteases, lipopolysaccharides (LPS), hemagglutinins, and fimbriae (1). Among these factors, a unique class of cysteine proteinases, termed gingipains composed of arginine-specific (Rgp) and lysine-specific (Kgp) proteases, is produced in both secretory and cell-associated forms (2-7) and are implicated in a wide range of both pathological and physiological processes of the bacterium. The cell-associated gingipains comprise the majority (~80%) of Rgp and Kgp activities and are thus considered to represent most of the virulence of these proteinases. Studies with various *P. gingivalis* mutants deficient in Rgp- and/or Kgp-encoding genes and proteinase inhibitors specific for each enzyme have demonstrated that both enzymes play a substantial role in virulence and survival of the bacterium within host cells

and suggested that inhibition of these enzymes may be useful approach to the treatment of periodontal diseases (8-16).

Increasing evidence also suggest that gingipains contribute to the disruption of host defense mechanisms by the bacterium through degradation of a wide variety of host proteins known to be involved in host defense, including IgG and IgA (4, 5) and inflammatory cytokines such as TNF-α and interleukin (IL)-6 (17-19). Gingipains were also found to effectively inhibit the generation of radical oxygen species from activated polymorphoneucelar leukocytes (PMNs) (4, 5) and degrade C3 and C3-derived opsonins (20), thereby leading to evasion of the bacterium from attacks by phagocytes. More recently, gingipains were shown to inactivate a cell surface component of *P. gingivalis,* which mediates TLR2- and TLR4-independent signaling, before being recognized by CD14, a co-receptor of TLRs (21). Consistent with this finding, gingipains were shown to degrade human monocyte CD14, resulting in LPS hyporesponsiveness (22). Increasing evidence also demonstrates the possible association of gingipains with the development and progression of various systemic diseases such as atherosclerosis (23-27). To date, a number of studies have shown that gingipains play a substantial role in the bacterial evasion from host defense mechanisms and the bacterial survival in infected host cells.

The present review thus summarizes recent progress in biochemical and molecular genetical studies on the role of gingipains in multistage processes important for cellular responses and disrupting host defense mechanisms after *P. gingivalis* infection.

3. PHARMACOLOGIC AND GENETIC INACTIVATION OF GINGIPAINS ABOLISHES THE VIRULENCE OF *P. GINGIVALIS*

We have previously shown with *P. gingivalis* mutants deficient in Rgp- and/or Kgp-encoding genes that both enzymes play a critical role in most of the virulence of the bacterium (8-12). These include the destruction of periodontal tissues, the disruption of host defense mechanisms, and the loss of the adhesion activity and viability of human fibroblasts and endothelial cells. We also found that, despite a significant increase in the expression of IL-8 and monocyte chemoattractant protein (MCP)-1 in *P. gingivalis*-infected cells, their intra- and extracellular protein levels were markedly decreased (13). The decrease was completely abolished by deficiency in both Rgp- and Kgp-encoding genes (13), demonstrating that *P. gingivalis* modulates the cytokine response in host cells. The activity levels of gingipains are likewise related to the decreased extracellular levels of various cytokines such as IL-8 and IL-6 (28, 29). In addition, the adhesion activity and viability of human endothelial cells was markedly decreased by the bacterial infection, most probably due to the cooperative action of Rgp and Kgp (12, 13, 30-32). *P. gingivalis* infection also induced a marked decrease in the bactericidal function of PMNs, which was also abolished by gene disruption of both gingipains (8, 10), thereby suggesting the importance of gingipains in disruption of the bactericidal function of PMNs. Furthermore, the mutants deficient in genes encoding both Rgp and Kgp (*rgpA rgpB kgp*-deficient triple mutant, KDP136) or lacking translation products of either protein showed the loss of physiologically important functions of the bacterium, such as the coaggregation (15), hemagglutination (8, 11), hemoglobin adsorption and heme accumulation (10), platelet aggregation in plasma (33), processing of the secretory and cell surface proteins of the bacterium (9, 29), and the acquisition of amino acids and heme required for bacterial growth. (11). Given that KDP136, unlike wild-type *P. gingivalis*, is structurally devoid of cell surface adhesin molecules such as fimbriae and functionally lacks abilities to coaggregate, bind hemoglobin, and induce hemagglutination (8, 10, 11, 15, 34), it is possible that the observed effects of deficiency in both Rgp- and Kgp-encoding genes may be due to such structural or functional defects for this mutant. To exclude this possibility, we had to develop proteinase inhibitors specific for Rgp and Kgp and examine their effects on the virulence of wild-type *P. gingivalis*.

The physiological and pathological importance of gingipains was corroborated by newly developed inhibitors specific for Rgp and Kgp (16). The loss of the adhesion activity and viability of human gingival fibroblasts (12) and human endothelial cells (13) induced by the culture supernatant of *P. gingivalis* was strongly inhibited by the Rgp-specific inhibitor KYT-1 and a combination of KYT-1 and the Kgp-specific inhibitor KYT-36, respectively. Consistent with these observations, the marked decrease in production of inflammatory cytokines such as IL-8 and MCP-1 by the bacterial infection was strongly inhibited by the combined use of KYT-1 and KYT-36, although a single use of KYT-1 significantly inhibited the decrease of their production (14). Several other virulence activities induced by the culture supernatant of *P. gingivalis*, including the extensive degradation of various host proteins such as type I collagen, IgG, IgA, fibronectin, and fibrinogen, disruption of the bactericidal activity of PMNs, and enhancement of the vascular permeability, were strongly inhibited by a combined action of both inhibitors (16). Besides the pathological processes, a wide range of physiological activities required for the bacterial growth and survival in the periodontal pocket, such as coaggregation with other oral bacteria, hemagglutination, and acquisition of amino acids and heme, were strongly inhibited by the combined action of KYT-1 and KYT-36 (16). Therefore, both pharmacologic and genetic inactivation studies have clearly demonstrated that gingipains play a substantial role in development and progression of periodontal disease and suggest that specific inhibitors specific for gingipains should provide a broader application in the treatment of periodontal disease and its related systemic diseases.

4. THE ROLE FOR GINGIPAINS IN RESPONSES OF HUMAN AORTIC ENDOTHELIAL CELLS TO *P. GINGIVALIS* INFECTION

Vascular endothelial cells represent the first line of defense against microbial protozoan pathogens invading the systemic circulation of the host, but the same time they

are the initial sites of host invasion. It has previously been shown that *P. gingivalis* can invade bovine and human endothelial cells and that cytoskeletal rearrangements, protein phosphorylation, energy metabolism and the bacterial proteases are essential for invasion (35). To further explore the effects of gingipains on the endothelial cells, Sheets *et al.* (31, 32) treated bovine coronary artery endothelial cells and human microvascular endothelial cells with gingipains and demonstrated that gingipains could induce endothelial cell detachment that was followed by apoptotic cell death. Furthermore, gingipains was shown to reduce the functional expression of CD99, an endothelial cell-associated adhesion molecule, on endothelial cells, leading to the disruption of adhesion molecule expression and of leukocyte recruitment to inflammatory foci (36).

To explore endothelial cell signaling and responses to *P. gingivalis* invasion, human aortic endothelial cells (HAECs, 5 x 10^3 cells/dish) were treated with the wild-type strain and its mutant lacking gingipains (KDP136) at a multiplicity of infection (MOI) of 10^2 in MCDB131 medium, the *P. gingivalis*-infected cells exhibited a biphasic effect on their proliferation, increasing the viable cells number for a 24-h incubation and reducing it thereafter (Figure 1). In contrast, HAECs infected with KDP136 showed a time-dependent increase in the viable cell number for a 24-h infection and then attained the plateau value for up to 72 h. To elucidate the underlying mechanisms of the increase or decrease in viable cells induced by bacterial infection, we determined the status of essential survival signaling events downstream of the phosphatidylinositol 3-kinase (PI3K), the phosphorylation of serine/threonine kinases Akt, extracellular signal-regulated kinases (ERK), the stress-activated protein kinases c-Jun N-terminal kinase (JNK) and p38 MAP kinase in the infected cells. Immunoblot analysis with specific phosphorylation antibody to Akt, ERK1/2, SAPK/JNK, and p38 kinase revealed that these enzymes were activated in HAECs infected with either the wild-type strain or KDP136 for the indicated times (Figure 2). Consistent with the results in Figure 1, *P. gingivalis*-infected HAECs showed a biphagic profile of their activation, increasing the phosphorylation of these kinases for up to 15-min infection and reducing it thereafter, whereas the levels of phosphorylation of these kinases of KDP136-infected cells were time-dependently increased for up to 1-h infection.

Previous studies have indicated that *P. gingivalis* infection affects the viability of gingival fibroblasts (12, 37), endothelial cells (13), neutrophils (38), and T cells (39). In contrast, human monocytes and macrophages exhibited resistance to apoptosis by *P. gingivalis* (40, 41). Intriguingly, primary gingival epithelial cells infected with *P. gingivalis* were shown to upregulate expression of the anti-apoptotic protein Bcl-2, which was accompanied by downregulation of expression of the proapoptotic protein Bax (42). A recent study has also reported that protein kinase/Akt is activated during infection of primary gingival epithelial cells with *P. gingivalis*, thereby contributing to survival of these cells (43). Furthermore, peritoneal macrophages treated with hemagglutinin B, a non-fimbrial adhesin expressed on the surface of *P. gingivalis*, showed an increased production of the proinflammatory cytokines, IL-12p40, IFN-γ, and TNF-α associated with the activation of ERK, JNK, and p38 MAP kinase (44). It is thus likely that apoptosis of *P. gingivalis*-infected cells is regulated by balancing apoptotic and survival systems through the MAPK signaling pathways. Given the biphasic effect of *P. gingivalis* and the monophasic effect of KDP136 on human endothelial cells, gingipains plays an important role in the regulation of the balance of apoptotic and survival systems depending on the number of the infected bacterial cells or the type of host cells infected. Endothelial nitric oxide synthetase (eNOS) also plays a critical role in the regulation of vascular tone and the maintenance of vascular integrity by production of NO. Although the expression of eNOS, as well as iNOS, in HAECs infected with either *P. gingivalis* or KDP136 at an MOI of 10^2 cells was not significantly changed over a period of 48 h, eNOS in the wild-type strain-infected HAECs was activated within 2 h of infection and greatly dephosphorylated after 16 h post-infection (Figure 3). In contrast, eNOS in the KDP136-infected cells was activated within 2 h and maintained the maximum level for up to 48 h postinfection.

5. THE ROLE FOR GINGIPAINS IN THE BACTERIAL TRAFFIC AND SURVIVAL IN HUMAN AORTIC ENDOTHELIAL CELLS INFECTED WITH *P. GINGIVALIS*

To evaluate whether and how gingipains are involved in the bacterial invasion process and persistence of the bacterium in HAECs, the intracellular trafficking and survival of *P. gingivalis* and KDP136 in the cells were investigated. Dorn *et al.* (45) previously described that the wild-type 381 strain was located within vacuoles morphologically resembling autophagosomes after infection into human coronary artery endothelial cells. These authors also demonstrated that *P. gingivalis*-containing autophagosome-like vacuoles were positive for the endoplasmic reticulum marker Bip and the lysosomal membrane-associated protein LGP120, but they were not positive for the lysosomal cysteine proteinase cathepsin L, thus suggesting that the bacterium evaded the endocytic pathway to lysosomes but instead trafficked to autophagosomes, in which the bacterium acquired a beneficial environment for its survival and growth. Autophagy is generally defined as a physiologically important process of sequestrating the cytoplasm and organelles into the lytic compartment, i.e. the lysosome system. In addition, autophagy is known to play an important role in the elimination of invading pathogens (46). Bacterial pathogens that invade cells through endocytosis are usually delivered to lysosomes and degraded there. However, *P. gingivalis* (45), *Brucella abortus* (47) and *Legionella pneumophila* (48) were shown to escape the host defense mechanism by blocking lysosomal fusion and acquisition of hydrolytic enzymes after entering the autophagic pathway. It has also been shown that autophagy is used to eliminate *Actinobacillus actinomycetemcomitans* (49), *Listeria monocytogenes* (50), and *Streptococcus pyogenes* (51), upon their escape from the phagosome into the cytosol, thus suggesting that

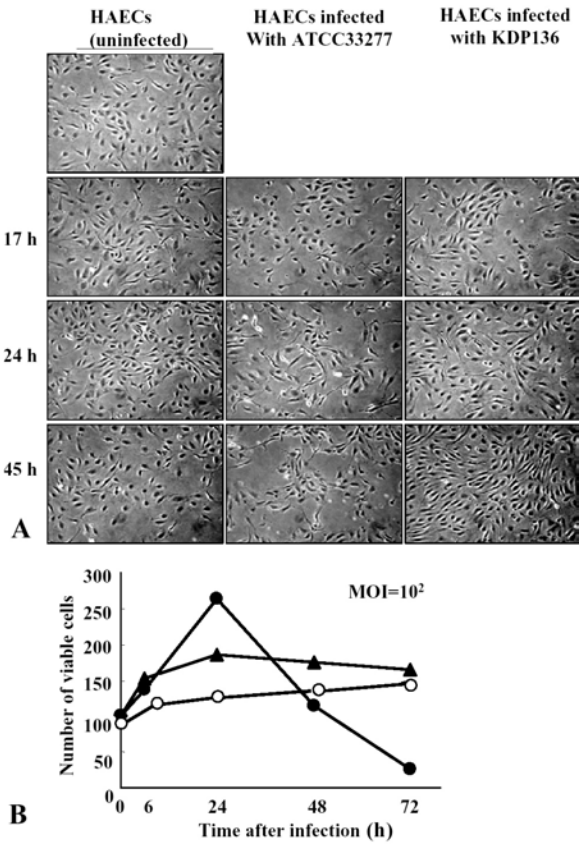

Figure 1. Proliferation of HAECs infected with *P. gingivalis* ATCC33277 and KDP136. (A) Phase contrast microscopy. HAEC were infected with vehicle (PBS), *P. gingivalis* ATCC33277 or KDP136 (M.O.I.=10^2) for the indicated periods, and then the morphologic observation was perforemd. (B) The number of the viable cells infected with the vehicle (open circle), ATCC33277 (closed circle), and KDP136 (closed triangle). The number of viable cells was quantified with Cell counting kit.

autophagy can act as an innate defense system against invading pathogens (51). More recently, we further explored whether and how the autophagic pathway was actually involved in the intracellular fate of *P. gingivalis* (52). Although autophagic events were found to be enhanced by infection with either the three different wild-type strains (ATCC33277, 381, and W83) or KDP136, more than 90% of intracellular wild-type strains and KDP136 were colocalized with the lysosomal marker cathepsin B, and only a few of the internalized cells were colocalized with the autophagosome marker LC3 during the 0.5- to 4-h postinfection period (Figure 4A). This was further confirmed by immunogold electron microscopic analysis (Figure 4B), thus implying that *P. gingivalis* can evade the autophagic pathway and instead directly traffics to the endocytic pathway to lysosomes. These data also suggest that gingipains have little or no effect on the intracellular trafficking in the infected cells. Given that the autophagic mechanism seems to function as an innate defense system against invading pathogens, the observed activation of autophagy by infection with either the wild-type strain or KDP136 is likely to act as a backup system for host defense mechanisms against invading pathogens.

Given that the endolysosome system play a substantial role in eliminating invading pathogens within cells, the efficiency of their sequestration and delivery to lysosomes is crucial for host defense. Thus, to address the question about how, and to what extent, the internalized bacteria are killed or survive after entering the phagolysosomal system, the number of the viable cells in HAECs after infection with the wild-type strains and KDP136 was scored by colony forming units (CFU) (52). The results indicated that the wild-type ATCC33277 and 381 strains were more resistant within the phagolysosomes than KDP136, although a time-dependent decrease in the extent of bacterial survival was observed with both the wild-type strains and KDP136. This finding was further substantiated by thin-section electron microscopy, in which most of the internalized *P. gingivalis* were enclosed and fused with single or double membranous structures with features characteristic of phagolysosomes and the bacterial double-membrane structures remained intact even at 6 h postinfection, whereas most of the internalized KDP136 lost the intact membrane structure in the entrapped or fused phagolysosomes at 2 h postinfection (52). These results strongly suggest that gingipains are necessary for the bacterium to acquire resistance against destruction within the phagolysosomes.

6. PHYSIOLOGICAL SIGNIFICANCE OF THE CELL-ASSOCIATED GINGIPAIN COMPLEX

The cell-associated gingipains comprise the majority of Rgp and Kgp activities and thus believed to be responsible for the virulence of the bacterium. However, compared to the well characterized effects of seceretory, monomeric gingipains, relatively little is known about the effects of the cell-associated forms of gingigains on host immune response. Recently, Bhogal *et al.* (6) reported the existent of a ~300 kDa cell-associated gingipains complex composed of both catalytic domains (PrtK15 and PrtK48) and seven C-terminal hemagglutinin/adhesin domains (PrtR44, PrtR15, PrtR17, PrtR27, PrtK39, PrtK15, and PrtK44) encoded by two genes, *prtR* (*rgpA*) and *prtK* (*kgp*), in the cell sonicate of *P. gingivalis*. More recently, we also isolated a 660-kDa cell-associated gingipain complex from *P. gingivalis* ATCC33277 strain after its efficient solubilization from the cell surface with sucrose monolaurate. The complex was found to exist as a homodimer of two catalytically active monomers which comprises the catalytic domains of Rgp (43 kDa) and Kgp (51 kDa) and C-terminal adhesin domains RgpA and Kgp (corresponding to the respective Hgp44, Hgp15, Hgp17, and Hgp27 subdomains) (7). Although there were no significant differences in several enzymatic properties including substrate specificity, optimum pH, thermal stability, and inhibitor profiles between the complex and the respective monomeric enzymes. Importantly, however, the proteolytic activities of the isolated complex toward type I collagen and elastin were much higher than those of the combined monomeric forms of Rgp and Kgp. The efficiency in the induction of the viability loss of host cells

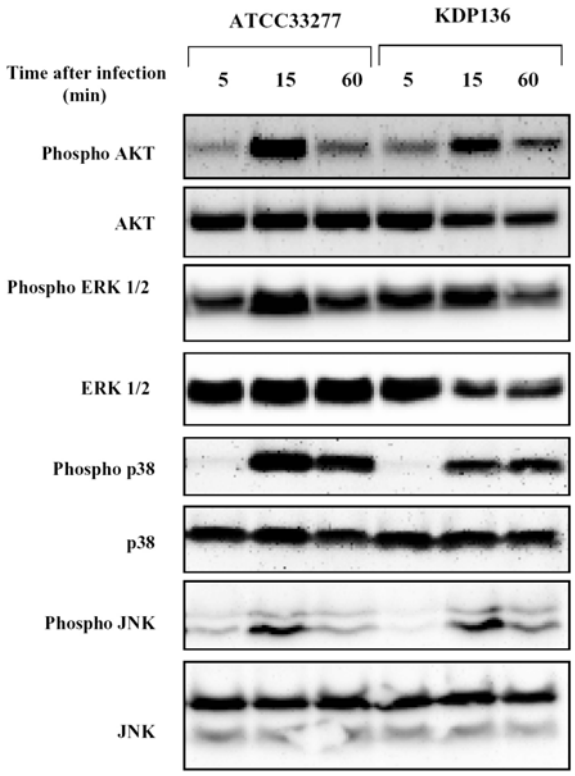

Figure 2. Activation of AKT, ERK, JNK and p38 MAP kinase in in HAECs infected with *P. gingivalis* ATCC33277 and KDP136. HAECs infected with either bacteria at M.O.I=10^2 were harvested and lysed at the indicated postinfection periods. The lysates were subjected to SDS-PAGE and immunoblot analysis for phosphorylation of the respective enzymes.

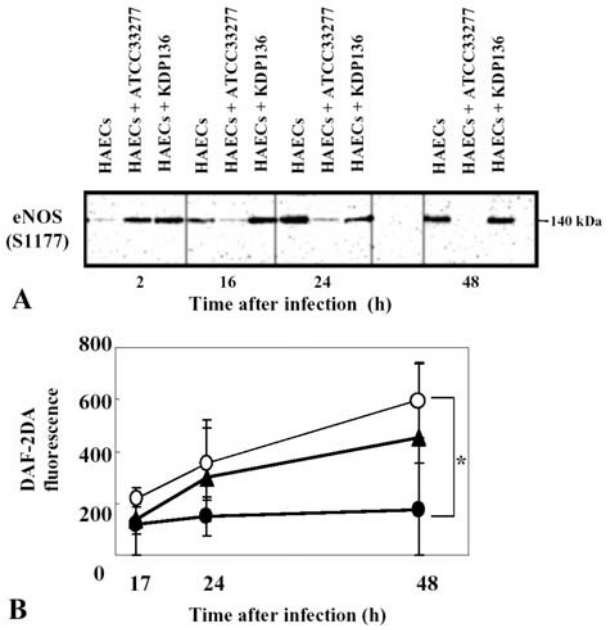

Figure 3. NO_2 production by HAECs infected with *P. gingivalis* ATCC33277 or KDP136. (A) Activation of eNOS (S1177) in the infected cells. HAECs infected with either bacteria at M.O.I=10^2 were harvested and lysed at the indicated postinfection periods. The lysates were subjected to SDS-PAGE and immunoblot analysis for phosphorylation of eNOS (S1177). (B) NO_2 production by HAECs after infection with vehicle (open circle), *P. gingivalis* ATCC33277 (closed circle) or KDP136 (closed triangle) was quantified with 4,5-diaminofluorescein diacetate (DAF-2DA) fluorescence at the indicated period postinfection periods. *p<0.05 versus the vehicle-treated cells.

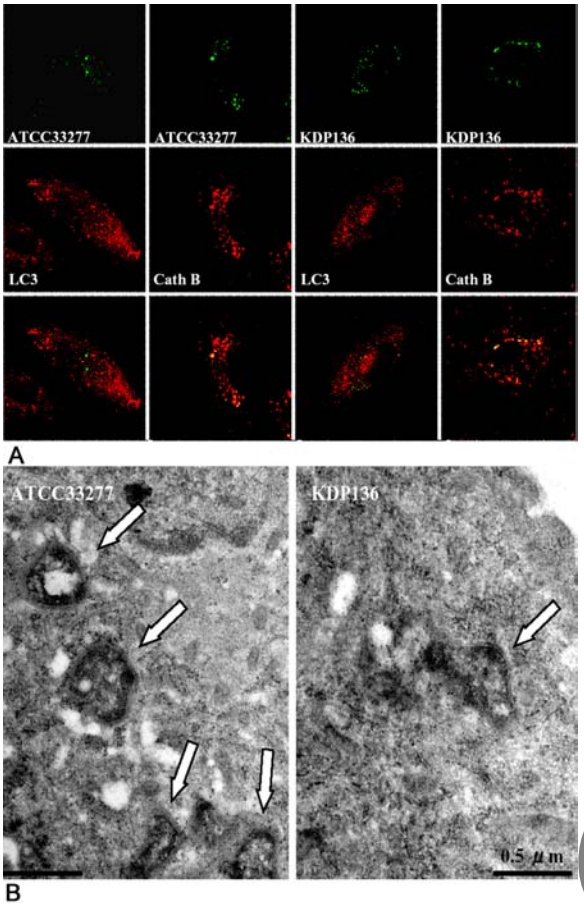

Figure 4. Intracellular localization of *P. gingivalis* ATCC33277 and KDP136 in the infected HAECs. (A) Confocal microscopic images of intracellular *P. gingivalis* ATCC33277 and KDP136 in the infected cells at 2 h postinfection. After infection with fluorescence-labeled *P. gingivalis* ATCC33277 and KDP136 for 20 min, HAECs were washed and further cultured for 2 h in the absence of extracellular bacteria, and then stained with antibodies against LC3 or cathepsin B. Localization of each bacterial cell type (green) and LC3 or cathepsin B (red), was analyzed by confocal microscopy. Both the internalized ATCC33277 and KDP136 were exclusively confined to cathepsin B-positive compartments, and only a few of them were colocalized with LC3. (B) Immunoelectron microscopy of *P. gingivalis*-infected HAECs. After infection with ATCC33277 and KDP136, HAECs were incubated in the absence of extracellular bacteria for 2 h. The localization of cathepsin B was examined by immunogold electron microscopy with anti-cathepsin B antibody. Arrows indicate intracellular bacteria.

such as gingival fibroblasts and umbilical vein endothelial cells by the complex was higher than that by the combined action of monomeric Rgp and Kgp. These results strongly suggest that the virulence of the bacterium is more efficiently exerted by the cell-associated form of gingipains compared with their secreted monomers.

In addition to these protein components, the complex was composed of phospholipids and LPS (7). The complex-associated phospholipids consisted of phosphatidylglycerol, phosphatidylethanolamine, sphingomyelin, phosphatidylserine, and phosphatidylcholin. The complex showed significant binding to liposomes consisting of either phospholipid, whereas the monomeric Rgp and Kgp had little or no binding to any types of liposomes. Given that eukaryotic plasma membranes preferentially include phosphatidylcholine and sphingomyelin, the complex is likely to bind directly to membranous lipids of both host and bacterial cells, thereby facilitating the colonization, coaggregation, hemagglutination, and infection of host cells by the bacterium. Furthermore, two-dimensional gel electrophoresis and immunoblot analysis with the monoclonal antibody to lipid A in LPS revealed that the complex was modified with LPS through attachment to the protease and Hgp44 domains of Rgp and Kgp (7). Earlier studies using the monoclonal antibody which recognized sugar portion of LPS have demonstrated that the membrane-associated forms of RgpA, RgpB, and Hgp27 are posttranslationally modified by LPS (53, 54). Importantly, Paramonov *et al.* (55) have recently demonstrated that the monoclonal antibody MAb1B5 raised to the monomeric RgpA, which is immunoreactive with *P. gingivalis* LPS as well as the membrane associated forms of RgpA and RgpB but not with the soluble form of RgpB (53), also cross-reacts with a cell-surface anionic polysaccharide of the bacterium that is distinct from both LPS and serotype capsule polysaccharide. Intriguingly, the same group also suggests that RgpB is required for the normal posttranslational glycosylation of RgpA (56). LPS is known to stimulate host cells mainly via the Toll-like receptor (TLR) 4/MD-2 pathway. However, previous studies have demonstrated that unlike enterobacterial LPS, *P. gingivalis* LPS induces human and murine innate immune responses through TLR2 and TLR4 (57, 58) and suppresses the biological activity of TLR4 agonists (59, 60). Considering that the mutation of the *porR* gene, which has homology with genes encoding transaminase involved in biosynthesis of the sugar portions of LPS and aminoglycosides, resulted in the preferential distribution of Rgp and Kgp in the culture supernatant and a defect of hemagglutination (61), the modification by LPS is most likely to contribute to the anchorage of the complex to the cell surface. Accordingly, it is likely that the initial translation products of RgpA and Kgp are proteolytically processed, modified by LPS, and formed to the mature complex on the cell surface (or in the periplasm) after trafficking the inner and outer membranes. Interestingly, despite the existence of LPS in the cell-associated complex, it failed to induce the production of NO_2 by human and murine macrophages (7). However, the production of NO_2 was significantly increased when the complex was denatured by heating at 60 °C for 30 min (Table 1) or proteinase K treatment (7). Since inhibition of the proteolytic activities of gingipains in the complex by the class specific protease inhibitor leupeptin did not

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Table 1. NO_2 production by mouse macrophages stimulated by the cell-associated gingipain complex or *P. gingivalis* bacterial cells

Stimulant	NO_2 production (mM)	
	Native	Denatured
The complex	2.26 ± 0.20	10.83 ± 1.25[1]
The complex + leupeptin	2.50 ± 0.01	12.46 ± 0.46[1]
LPS derived from *P. gingivalis*	12.00 ± 0.79	n.d.
LPS derived from *P. gingivalis* plus monomeric Rgp and Kgp	11.75 ± 1.09	n.d.
P. gingivalis ATCC33277	2.40	4.32
P. gingivalis KDP136	6.62	3.64

Mouse peritoneal macrophages (5 X 10^4 cells) were incubated with the native or denatured (60 °C for 30 min) complex (4 ng), LPS (1 ng), *P. gingivalis* ATCC33277 or KDP136 (10^6 CFU, respectively) at 37 °C for 24 h. [1]$p<0.005$ compared with the value obtained with the corresponding native stimulants (Student *t*-test). n.d.: not determined.

Table 2. Production of pro-inflammatory molecules by mouse macrophages upon treatment with the native or denatured gingipain complex

Strain of mouse	The complex	NO2 (μM)	TNF-α (ng/ml)	IL-6 (ng/ml)
C57BL/6	Native	3.46 ± 1.09	1.12 ± 0.40	1.99 ± 0.35
	Denatured (60 °C, 30 min)	12.56 ± 0.60[2]	4.34 ± 0.36[2]	8.53 ± 0.99[2]
C3H/HeJ	Native	0.73 ± 0.41	0.09 ± 0.12	0.19 ± 0.22
	Denatured (60 °C, 30 min)	4.40 ± 1.21[1]	0.36 ± 0.27	0.61 ± 0.16

[1] $p<0.01$, [2]$p<0.005$, compared with the values obtained with the native gingipain complex.

affect the production of NO_2 by macrophages (Table 1), the proteinase activities of Rgp and Kgp was unlikely to be directly associated with the macrophage response. This was further confirmed by the experiments with LPS isolated from *P. gingivalis*. While the isolated LPS was able to induce the production of NO_2 by macrophages, the addition of the monomeric Rgp and Kgp to the isolated LPS did not affect the production of NO_2. Furthermore, the production of NO_2, TNF-α, and IL-6 by macrophages derived from C57BL/6 mice was more profound with the heat-denatured complex compared with the native complex (Table 2). However, no significant differences between the native and heat-denatured complex in the production of TNF-α and IL-6, but not NO_2, by macrophages derived from LPS-hyposensitive C3H/HeJ mice having a point mutation in the *tlr4* gene. Therefore, it has been strongly suggested that the functional domains in LPS associated with the cell-associated gingipain complex are conformationally masked by the protein components, thereby resulting in the escape from the recognition of TLRs. It is thus more likely that the cell-associated form of gingipains contributes to the suppression of the host immune response to recruit and localize neutrophils and macrophages to gingival inflammatory sites by bacterial infection through evasion of host LPS signaling systems.

7. CONCLUSION

Gingipains are produced as secreted or cell-associated forms on the cell-surface, although the cell-associated forms comprise the majority of gingipain activities. Pharmacologic and genetic inactivation of both Rgp and Kgp demonstrates that these two enzymes cooperatively or independently contribute to pathological and physiological processes executed by *P. gingivalis*. Gingipains also play a substantial role in responses of various host cells, such as endothelial cells, gingival fibroblasts, and macrophages, to the bacterium and the subsequent intracellular signaling. Within the infected cells, *P. gingivalis* evades the autophagic pathway and instead directly traffics to the endocytic pathway to lysosomes. Although gingipains have little or no effect on the intracellular trafficking in the infected cells, they are important for the bacterium to acquire resistance against destruction within the phagolysosomes. We also described the physiological significance of the existence of the cell-associated gingipain complex composed of the catalytic domains of Rgp and Kgp, the C-terminal adhesin domains of their translation products (Hgp44, Hgp15, Hgp17, and Hgp27 subdomains), phospholipids, and LPS. The findings demonstrate that the complex play a role in the suppression of the host immune response to bacterial infection through evasion of host LPS signaling systems.

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8. ACKNOWLEDGEMENT

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9. REFERENCES

1. Holt, S.C., L. Kesavalu, S. Walker, & C.A. Genco: Virulence factors of *Porphyromonas gingivalis*. *Periodontology*, 20: 168-238 (2000)
2. Tsutsui, H., T. Kinouchi, Y. Wakano, & Y. Ohnishi: Purification and characterization of a protease from *Bacteroides gingivalis* 381. *Infect. Immun.*, 55: 420-427 (1987)
3. Pike, R., W. McGraw, J. Potempa, & J. Travis: Lysine- and arginine-specific proteinases from *Porphyromonas gingivalis*. Isolation, characterization, and evidence for the existence of complexes with hemagglutinins. *J. Biol. Chem.* 269: 406-411 (1994)
4. Kadowaki T, M. Yoneda, K. Okamoto, K. Maeda, & K. Yamamoto: Purification and characterization of a novel arginin-specific cysteine proteinase (argingipain) involved in the pathogenesis of periodontal disease from the culture

supernatant of *Porphyromonas gingivalis*. *J. Biol. Chem*. 269: 21371-21378 (1994)
5. Abe, N., T. Kadowaki, K. Okamoto, K. Nakayama, M. Ohishi, & K. Yamamoto: Biochemical and functional properties of lysine-specific cysteine proteinase (Lys-gingipain) as a virulence factor of *Porphyromonas gingivalis* in periodontal disease. *J. Biochem.* 123: 305-312 (1998)
6. Bhogal, P.S., N. Slakeski, & E.C. Reynolds: A cell-associated protein complex of *Porphyromonas gingivalis* W50 composed of Arg- and Lys-specific cysteine proteinases and adhesins. *Microbiol.* 143: 2485-2495 (1997)
7. Takii, R., T. Kadowaki, A. Baba, T. Tsukuba, & K. Yamamoto: A functional virulence complex composed of gingipains, adhesins, and lipopolysaccharides shows high affinity to host cells and matrix proteins and escapes recognition by host immune systems. *Infect. Immun*. 73: 883-893 (2005)
8. Nakayama, K., T. Kadowaki, K. Okamoto, & K. Yamamoto: Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipains)-deficient mutants of *Porphyromonas gingivalis*. Evidence for significant contribution of Arg-gingipain to virulence. *J. Biol. Chem.*, 270: 23619-23626 (1995)
9. Kadowaki, T., K. Nakayama, F. Yoshimura, K. Okamoto, N. Abe, & K. Yamamoto: Arg-gingipain acts as a major processing enzyme for various cell surface proteins in *Porphyromonas gingivalis*. *J. Biol. Chem.*, 273: 29072-29076 (1998)
10. Okamoto, K., K. Nakayama, T. Kadowaki, N. Abe, D.B. Ratnayake, & K. Yamamoto: Involvement of a lysine-specific cysteine proteinase in hemoglobin adsorption and heme accumulation by *Porphyromonas gingivalis*. *J. Biol. Chem.*, 273: 21225-21231 (1998)
11. Shi, Y., D.B. Ratnayake, K. Okamoto, N. Abe, K. Yamamoto, & K. Nakayama: Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. Construction of mutants with a combination of *rgpA*, *rgpB*, *kgp*, and *hagA*. *J. Biol. Chem.*, 274: 17955-17960 (1999)
12. Baba, A., N. Abe, T. Kadowaki, H. Nakanishi, M. Ohishi, T. Asao, & K. Yamamoto: Arg-gingipain is responsible for the degradation of cell adhesion molecules of human gingival fibroblasts and their death induced by *Porphyromonas gingivalis*. *Biol. Chem.*, 382: 817-824 (2001)
13. Baba, A., T. Kadowaki, T. Asao, & K. Yamamoto: Roles for Arg- and Lys-gingipains in the disruption of cytokine responses and loss of viability of human endothelial cells by *Porphyromonas gingivalis* infection. *Biol. Chem.*, 383: 1223-1230 (2002)
14. Kadowaki, T., & K. Yamamoto: Suppression of virulence of *Porphyromonas gingivalis* by potent inhibitors specific for gingipains. *Curr. Prot. Pep. Sci.*, 4: 451-458 (2003)
15. Abe, N., A. Baba, K. Takii, K. Nakayama, A. Kamaguchi, Y. Shibata, Y. Abiko, K. Okamoto, T. Kadowaki, & K. Yamamoto: Roles of Arg- and Lys-gingipains in coaggregation of *Porphyromonas gingivalis*: identification of its responsible molecules in translation products of *rgpA*, *kgp*, and *hagA* genes. *Biol. Chem.*, 385: 1041-1047 (2004)
16. Kadowaki, T., A. Baba, N. Abe, R. Takii, M. Hashimoto, T. Tsukuba, S. Okazaki, Y. Suda, T. Asao, & K. Yamamoto: Suppression of pathogenicity of *Porphyromonas gingivalis* by newly developed gingipain inhibitors. *Mol. Pharmacol.*, 66: 1599-1606 (2004)
17. Calkins, C.C., K. Platt, J. Potempa, & J. Travis: Inactivation of tumor necrosis factor-α by proteinases (gingipains) from the periodontal pathogen, *Porphyromonas gingivalis*. *J. Bacteriol.*, 273: 6611-6614 (1998)
18. Banbula, A., M. Bugno, A. Kuster, P.C. Heinrich, J. Travis, & J. Potempa: Rapid and efficient inactivation of IL-6 by gingipains, lysine- and arginine-specific proteinases from *Porphyromonas gingivalis*. *Biochem. Biophys. Res. Commun.*, 261: 598-602 (1999).
19. Mežyk-Kopeć, R., M., Bzowska, J.,Potempa, M. Bzowska, N. Juna, A., Sroka, R.A. Black, & J. Bereta: Inactivation of membrane tumor necrosis factor alpha by gingipains from *Porphyromonas gingivalis*. *Infect. Immun*., 73: 1506-1514 (2005)
20. Wingrove, J.A., R.G. DiScipio, Z. Chen, J. Potempa, J. Travis, & T.E. Hugli: Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from *Porphyromonas gingivalis*. *J. Biol. Chem.*, 267: 18902-18907
21. Kishimoto, M., A. Yoshimura, M. Naito, K. Okamoto, K. Yamamoto, D.T. Golenbock, Y. Hara, & K. Nakayama: Gingipains inactivate a cell surface ligand on *Porphyromonas gingivalis* that induces TLR2- and TLR4-independent signaling. *Microbiol. Immunol.*, 50: 315-325 (2006)
22. Sugawara, S., E. Nemoto, H. Tada, K. Miyake, T. Imamura, & H. Takada: Proteolysis of human monocyte CD14 by cysteine proteinases (gingipains) from *Porphyromonas gingivalis* leading to lipopolysaccharide hyporesponsiveness. *J. Immunol*., 165: 411-418 (2000)
23. DeStefano, F., R.F. Anda, H.S., Kahn, D.F., Williamson, & C.M., Russell: Dental disease and risk of coronary heart disease and mortality. *Br. Med. J.*, 306: 688-691 (1993)
24. Mattila, K.J., V.V. Valtonen, M. Nieminen, & J.K. Huttenen: Dental infection and the risk of new coronary events: prospective study of patients with documented coronary artery disease. *Clin. Infec. Dis.* 20: 588-592 (1995)
25. Beck, J.D., J. Pankow, H.A., Tayroler, & S. Offenbacher: Dental infections and atherosclerosis. *Am. Heart J.*, 138: 528-533 (1999)
26. Miyakawa, H., K. Honma, M. Qi, & H.K. Kuramitsu: Interaction of *Porphyromonas gingivalis* with low-density lipoproteins: implications for a role for periodontitis in atherosclerosis. *J. Peroodontal. Res.*, 39: 1-9 (2004)
27. Hashimoto, M., T. Kadowaki, T. Tsukuba, & K. Yamamoto: Selective proteolysis of apolipoprotein B-100 by Arg-gingipain mediates atherosclerosis progression accelerated by bacterial exposure. *J. Biochem.*, 140: 713-723 (2006)
28. Steffen, M.J., S.C. Holt, & J.L. Ebersole: *Porphyromonas gingivalis* induction of mediator and

cytokine secretion by human gingival fibroblasts. *Oral Microbiol. Immunol.* 15: 172-180 (2000)
29. Bodet, C., F. Chandad, & D. Grenier: Modulation of cytokine production by *Porphyromonas gingivalis* in a macrophage and epithelial cell co-culture model. *Microbes Infect.* 7: 448-456 (2005)
30. Katz, J., Q-B. Yang, P. Zhang, J. Potempa, J. Travis, S.M. Michalek, & D.F. Balkovertz : Hydrolysis of epithelial jinctional proteins by *Porphyromonas gingivalis* gingipains. *Infect. Immun.* 70: 2512-2518 (2002)
31. Sheets, S.M., J. Potempa, J. Travis, C.A. Casiano, & H.M. Fletcher: Gingipains from *Porphyromonas gingivalis* W83 induces cell adhesion molecule cleavage and apoptosis in endothelial cells. *Infect. Immun.* 73: 1543-1552 (2005)
32. Sheets, S.M., J. Potempa, J. Travis, H.M. Fletcher, & C.A. Casiano: Gingipains from *Porphyromonas gingivalis* W83 synergistically disrupt endothelial cell adhesion and can induce caspase-independent apoptosis. *Infect. Immun.* 74: 5667-5678 (2006)
33. Naito, M., E. Sakai, Y. Shin, H. Ideguchi, M. Shoji, N. Ohara, K., Yamamoto, & K. Nakayama: *Porphyromonas gingivalis*-induced platelet aggregation in plasma depends on Hgp44 adhesin but not Rgp proteinase. *Mol. Microbiol*.59: 152-167 (2006)
34. Nakayama, K., F. Yoshimura, T. Kadowaki, & K. Yamamoto: Involvement of arginine-specific cysteine proteinase (Arg-gingipain) in fimbriation of *Porphyromonas gingivalis*. *J. Bacteriol.* 178: 2818-2824 (1996).
35. Deshpande, R.D., M.B. Khan, & C.A. Genco: Invasion of aortic and heart endothelial cells by *Porphyromonas gingivalis. Infect. Immun.* 66: 5337-5343 (1998)
36. Yun P.L.W., A.D. DeCarlo, & N. Hunter: Gingipains of *Porphyromonas gingivalis* modulate leukocyte adhesion molecule expression induced in human endothelial cells by ligation of CD99. *Infect. Immun.* 74: 1661-1672 (2006).
37. Wang, P.L., S. Shirasu, M. Shinohara, M. Daito, M. Oide, Y. Kawashi, & K. Ohura: Induction of apoptosis in human gingival fibroblasts by a *Porphyromonas gingivalis* protease preparation. *Arch, Oral Biol*., 44: 337-342 (1999)
38. Gamonal, J., M. Sanz, A. O'Conner, A. Acevedo, I. Suarezm A. Sanz, B. Martinez, & A. Silva: Delayed neutrophil apoptosis in chronic periodontitis patients. *J. Clin. Periodontol*., 30: 616-623 (2003)
39. Harris, J.I., R.R. Russel, M.A. Curtis, J. Aduse-Opoku, & J.J. Taylor : Molecular mediators of *Porphyromonas gingivalis*-induced T-cell apoptosis. *Oral Microbiol. Immunol.*, 17: 224-230 (2002)
40. Murray, D.A., & J.M. Wilton: Lipopolysaccharide from the periodontal pathogen *Porphyromonas gingivalis* prevents apoptosis of HL60-derived neutrophils *in vitro*. *Infect. Immun.*, 71: 7232-7235 (2003)
41. Ozaki, K., & S. Hanazawa: *Porphyromonas gingivalis* fimbriae inhibit caspase-3-mediated apoptosis of monocytic THP-1 cells under growth factor deprivation via extracellular signal-regulated kinase-dependent expression of p21 Cip/WAF1. *Infect. Immun.*, 69: 4944-4950 (2001)
42. Nakhjiri, S.F., Y. Park, O. Yilmaz, W.O. Chung, K. Watanabe, A. El-Sabaency, K. park, & R.J. Lamont: Inhibition of epithelial cell apoptosis by *Porphyromonas gingivalis*. *FEMS Microbiol. Lett.*, 200: 145-149 (2001)
43. Yilmaz, O., T. Jungas, P. Verbeke, & Ojcius: Activation of the phosphatidyl 3-kinase/Akt pathway contributes to survival of primary epithelial cells infected with the periodontal pathogen *Porphyromonas gingivalis*. *Infect. Immun.*, 72: 3743-3751 (2004).
44. Zhang, P., M. Martin, S.M. Michalek, & J. Katz: Role of mitogen-activated protein kinases and NF-κB in the regulation of proinflammatory and anti-inflammatory cytokines by *Porphyromonas gingivalis* hemagglutinin B. *Infect. Immun.*, 73: 3990-3998 (2005)
45. Dorn, B.R., W.A. Dunn Jr, & A. Progulske-Fox: *Porphyromonas gingivalis* traffics to autophagosomes in human coronary artery endothelial cells. *Infect. Immun.*, 69: 5698-5708 (2001)
46. Shintani T., & D.J. Klionsky: Autophagy in health and disease: a double-edged sword. *Science* 306: 990-995 (2004)
47. Pizzaro-Cerda, J., E. Moreno, & J.P. Gorvel: Invasion and intracellular trafficking of *Brucella abortus* in nonphagocytic cells (in process citation). *Microbes Infect.* 2: 829-835 (2000)
48. Swanson, M.S., & R.R. Isberg: Association of *Legionella pneumophila* with the macrophage endoplasmic reticulum. *Infect. Immun*. 63: 3609-3620 (1995)
49. Meyer D.H., J.E. Rose, J.E. Lippmann, & P.M. Fives-Taylor: Microtubules are associated with intracellular movement and spread of the periodontpathogen *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* 67: 6518-6525 (1999)
50. Rich K.A., C. Burkett, & P. Webster: Cytoplasmic bacteria can be targets for autophagy. *Cell. Microbiol.* 5: 455-468 (2003)
51. Nakagawa I., A. Amano, N. Mizushima, A. Yamamoto, H. Yamagichi, T. Kamimoto, A. Nara, J. Funao, M. Nakata, K. Tsuda, S. Hamada, & T. Yoshimori: Autophagy defends cells against invading group A *Streptococcus*. *Science* 306: 1037-1040 (2004)
52. Yamatake K., M. Maeda, T. Kadowaki, R. Takii, T. Tsukuba, T. Ueno, E. Kominami, S. Yokota, & K. Yamamoto: Role for gingipains in *Porphyromonas gingivalis* traffic to phagolysosomes and survival in human aortic endothelial cells. *Infect. Immun*., 75: in press (2007)
53. Curtis, M.A., A. Thickett, J.M. Slaney, M. Rangarajan, J. Aduse-Opoku, P. Shephield, N. Paramonov, & E.F. Hounsel: Variable carbohydrate modifications to the catalytic chains of the RgpA and RgpB proteases of *Porphyromonas gingivalis* W50. *Infect. Immun*., 67: 3816-3823 (1999)
54. Veith, P.D., G.H. Talbo, N. Slakeski, S.G. Dashper, C. Moore, R.A. Paolini, & E.C. Reynolds: Major outer membrane proteins and proteolytic processing RgpA and Kgp of *Porphyromonas gingivalis* W50. *Biochem. J*., 363: 105-115 (2002)
55. Paramonov N., M. Rangarajan, A. Hashim, A. Gallagher, J. Aduse-Opoku, J.M. Slaney, E. Hounsell, & M.A. Curtis: Structural analysis of a novel anionic polysaccharide from *Porphyromonas gingivalis* strain W50 related to Arg-gingipain glycans. *Mol. Microbiol.* 58: 847-863 (2005)
56. Rangarrajan, M., A. Hashim, J. Aduse-Opoku, N. Paramonov, E.F. Hounsell, & M.A. Curtis: Expression of Arg-gingipain RgpB is required for correct glycosylation

and stability of monomeric Arg-gingipain RgpA from Porphyromonas gingivalis W50. *Infect. Immun*. 73: 4864-4878 (2005)
57. Hirschfield, M., J.J. Weis, V. Toshchakov, C.A. Salkowski, M.J. Cody, D.C. Ward, N. Qureshi, S.M. Michalek, & S.N. Vogal: Signaling by Toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. *Infect. Immun*., 69: 1477-1482 (2001)
58. Martin, M., L. Katz, S.N. Vogel, & S.M. Michalek: Differential induction of endotoxin tolerance by lipopolysaccharides derived from *Porphyromonas gingivalis* and *Esherichia coli*. *J. Immunol.* 167: 5278-5285 (2001)
59. Coats, S.R., R.F. Reife, B.W. Bainbridge, T-T.T. Pham, & R.P. Darveau: *Porphyromonas gingivalis* lipopolysaccharide antagonizes *Esherichia coli* lipopolysaccharide at Toll-like receptor 4 in human endothelial cells. *Infect. Immun*., 71: 6799-6807 (2003)
60. Yoshimura, A., T. Kaneko, Y. Kato, D.T. Golenbock, & Y. Hara: Lipopolysaccharides from periodontopathic bacteria *Porphyromonas gingivalis* and *Capnocytophaga ochracea* are antagonists for human Toll-like receptor 4. *Infect. Immun.,* 70: 218-225 (2002)
61. Shoji, M., D.B. Ratnayake, Y. Shi, T. Kadowaki, K. Yamamoto, F. Yoshimura, A. Akamine, M.A. Curtis, & K. Nakayama: Construction and characterization of a nonpigmented mutant of *Porphyromonas gingivalis*: cell surface polysaccharide as an anchorage for gingipains. *Microbiology*, 148: 1183-1191 (2002)

Abbreviations: CFU, colony forming unit; ERK, extracellular signal-regulated kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; KDP136, *rgpA*, *rgpB kgp*-deficient triple mutant; Kgp, Lys-gingipain; KYT-1, Rgp-specific inhibitor; KYT-36, Kgp-specific inhibitor; LPS, lipopolysaccharide; MOI, multiplicity of infection; NOS, nitric oxide synthetase; PI3K, phosphatidylinositol 3-kinase; PMN, polymorphonuclear leukocyte; Rgp, Arg-gingipain; TNF, tumor necrosis factor; TLR, Toll-like receptor

Key Words: Autophagy, Arg-Gingipain, Host Defense, Lipopolysaccharide, Lys-Gingipain, Periodontitis, *Porphyromonas gingivalis*

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