Disruption of epithelial barrier and impairment of cellular function by *Porphyromonas gingivalis*

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1. ABSTRACT

Porphyromonas gingivalis is a predominant periodontal pathogen that expresses a number of potential virulence factors involved in the pathogenesis of periodontitis. Gingival epithelial cells are spontaneously exposed to bacterial attacks and function to prevent invasion by bacteria into deeper tissues. *P. gingivalis* fimbriae are a critical factor for mediation of interaction of the organism with host tissues, as they promote both bacterial adhesion to and invasion of targeted sites. Fimbriae are capable of binding to human salivary components, extracellular matrix proteins, and commensal bacteria, while they also strongly adhere to cellular $\alpha5\beta1$-integrin. Following adhesion to $\alpha5\beta1$-integrin, *P. gingivalis* is captured by cellular pseudopodia, which enables invagination through an actin-mediated pathway. The invasive event has been reported to require host cellular dynamin, actin fibers, microtubules, and lipid rafts. Following passage through the epithelial barrier, the intracellular pathogen impairs cellular function. Fimbriae are classified into 6 genotypes (types I to V and Ib) based on the diversity of the *fimA* genes encoding each fimbria subunit, and intracellular *P. gingivalis* with type II fimbriae has been found to clearly degrade integrin-related signaling molecules, paxillin, and focal adhesion kinase, which disables cellular migration and proliferation. These events are considered to integrate the bacterial strategy for persistence in periodontal tissues.

2. INTRODUCTION

Epithelial cells that layer mucosal surfaces are spontaneously exposed to bacterial attacks and function to prevent invasion by bacteria into deeper tissues. In the oral cavity, this innate host defense system is postulated to act toward limiting the spread of oral bacteria by maintaining an intact epithelial barrier (1). *Porphyromonas gingivalis*, which has been characterized as a bona fide periodontal pathogen, has been detected in the gingival tissues of periodontitis patients (2), and also shown to invade human epithelial, endothelial, fibroblastic, and periodontal ligament cell lines *in vitro* (3, 4). These findings indicate that *P. gingivalis* has an ability to pass through the epithelial barrier.

P. gingivalis expresses a variety of adhesive components, such as major fimbriae, short fimbriae, proteases (gingipains), hemagglutinins, and lipopolysaccharide (LPS) (5). Among them, major fimbriae (hereinafter called fimbriae) have been shown to play an important role in the interactions between this bacterium and host cells. Fimbriae are thin, filamentous, and proteinaceous surface appendages found in many bacterial species, with *P. gingivalis* fimbriae first recognized on its outer surface by electron microscopic observations (6). Different strains of *P. gingivalis* possess numerous fimbria structures that have been observed as curly and single-stranded appendages, and arranged in a

Table 1. Specific constants of interactions between *P. gingivalis* fimbriae and host proteins

Proteins	*k*ass (1/Ms)	*k*diss (1/s)	*K*a (1/M)
Statherin	2.49×10^3	1.68×10^3	1.48×10^6
PRG	3.38×10^3	2.08×10^3	1.62×10^6
PRP	2.61×10^3	1.60×10^3	1.63×10^6
Laminin	3.62×10^3	1.68×10^3	2.15×10^6
Fibronectin	3.46×10^3	1.60×10^3	2.16×10^6
Fibrinogen	2.63×10^3	1.22×10^3	2.16×10^6
Thrombospondin	3.01×10^3	1.33×10^3	2.26×10^6
Hemoglobin	3.42×10^3	1.41×10^3	2.43×10^6
Type I collagen	3.04×10^3	1.10×10^3	2.76×10^6
Elastin	3.72×10^3	1.21×10^3	3.08×10^6
Vitronectin	4.16×10^3	1.10×10^3	3.79×10^6
Antifimbriae IgG	6.11×10^3	5.00×10^4	1.22×10^7

ass: association rate constant, *k*diss: dissociation rate constant, *K*a (=*k*ass/*k*diss): association constant, M: molar, s: second.

peritrichous manner and emerging from the cell surface of the bacterium. *P. gingivalis* fimbriae have been classified into 6 genotypes (I to V and Ib) based on the different nucleotide sequences of the *fimA* genes encoding FimA (a subunit protein of fimbriae), whose molecular masses vary from 41 to 45 kDa (3). These fimbriae allow *P. gingivalis* to adhere to oral surfaces and subsequently invade periodontal tissues (7). As an intracellular pathogen, the bacterium degrades cellular signaling molecules and disables cellular functions, such as adhesion, migration, and proliferation, which are crucial for the homeostatic, regenerative, and healing properties of periodontal tissues (4, 8, 9). In addition, localization allows the pathogen to penetrate deep into tissues (10). Thus, the adhesive/invasive abilities of *P. gingivalis* allows the pathogen to destroy periodontal tissues. In the present short review, the pathogenic role played by fimbriae of *P. gingivalis* is discussed.

3. INITIAL ADHERENCE OF *P. GINGIVALIS* IN THE ORAL CAVITY

3.1. Interactions of *P. gingivalis* fimbriae with host components

The longstanding battle of the defense system of periodontitis patients against *P. gingivalis* is thought to be initiated by bacterial adherence in the oral cavity (11, 12). Fimbriae enable *P. gingivalis* to interact with various host components, such as salivary proteins, hemoglobin, extracellular matrix (ECM) proteins, and periodontal cells, including gingival epithelial cells and fibroblasts (7). Oral surfaces including the teeth, gingival margin, mucosal membrane, and surface layer of plaque-forming bacteria are all coated with salivary fluid. Immediately upon entry into the oral cavity, *P. gingivalis* first interacts with saliva, with a number of salivary components reportedly capable of interacting with the bacterium. On the other hand, several components, such as fibrinogen (13), histatin (14), and fibronectin (15), have been suggested to inhibit bacterial adherence to periodontal tissue, and we previously speculated that the salivary components proline-rich proteins (PRPs), proline-rich glycoproteins (PRGs), and statherin act as salivary receptors that support the initial anchoring of *P. gingivalis* to oral surfaces (16).

Following its initial anchoring, *P. gingivalis* colonizes the gingival margin, which is likely to be mediated by the adhesive ability of fimbriae to host

proteins (11). We previously performed kinetic analyses of the interactions between fimbriae and those host component proteins using surface plasmon resonance technology with a biomolecular interaction analysis (BIACORE) system (17, 18), with the results shown in Table 1. The affinity constant (*K*a) was determined based on two kinetic constants; *k*ass, which determined the speed of the association, and *k*diss, which reflected the stability of the complexes of fimbriae and the host protein. The resulting *K*a values demonstrated that the interactions of fimbriae with host proteins were specific and the specificities were comparable to those of anti-fimbriae polyclonal antibodies. Further, these specific affinities suggest the functional significance of fimbriae as mediators of bacterial adherence to, and colonization and invasion of periodontal tissues.

3.2. Involvement of *P. gingivalis* fimbriae in biofilm formation

Dental plaque is a well studied model of the physiology of bacterial biofilm, which is a particularly crucial factor in the etiology of periodontal diseases. Biofilm formation is mediated by the cohesive interaction (coaggregation) of *P. gingivalis* with other bacteria (12), as the organism aggregates with various oral bacterial species and, in some cases, its fimbriae are ascribed to mediate coaggregation with other plaque-forming bacteria, such as *Actinomyces viscosus* (19)*, Treponema medium* (20), *Treponema denticola* (21), *and Streptococcus oralis* (22). We previously showed that the coaggregation of *P. gingivalis* with various streptococcal strains was clearly inhibited by purified fimbriae, recombinant fimbrillin protein, and some selected segment peptides of fimbrillin (22). These coaggregation activities were also inhibited by synthetic peptides analogous to the binding domain of salivary PRP to fimbrillin as effectively as recombinant fimbrillin protein (22, 23). Further, the peptide of the binding domain of PRP was engineered to be secreted from *S. gordonii* in a therapeutic design to block fimbriae-mediated adhesion to oral surfaces (24).

4. ADHESION TO AND INVASION OF EPITHELIAL CELLS BY *P. GINGIVALIS*

4.1. Adhesion to epithelial cells

The interaction of *P. gingivalis* fimbriae with gingival epithelial cells plays an important role in establishment of chronic periodontal infection (4, 12). *P.*
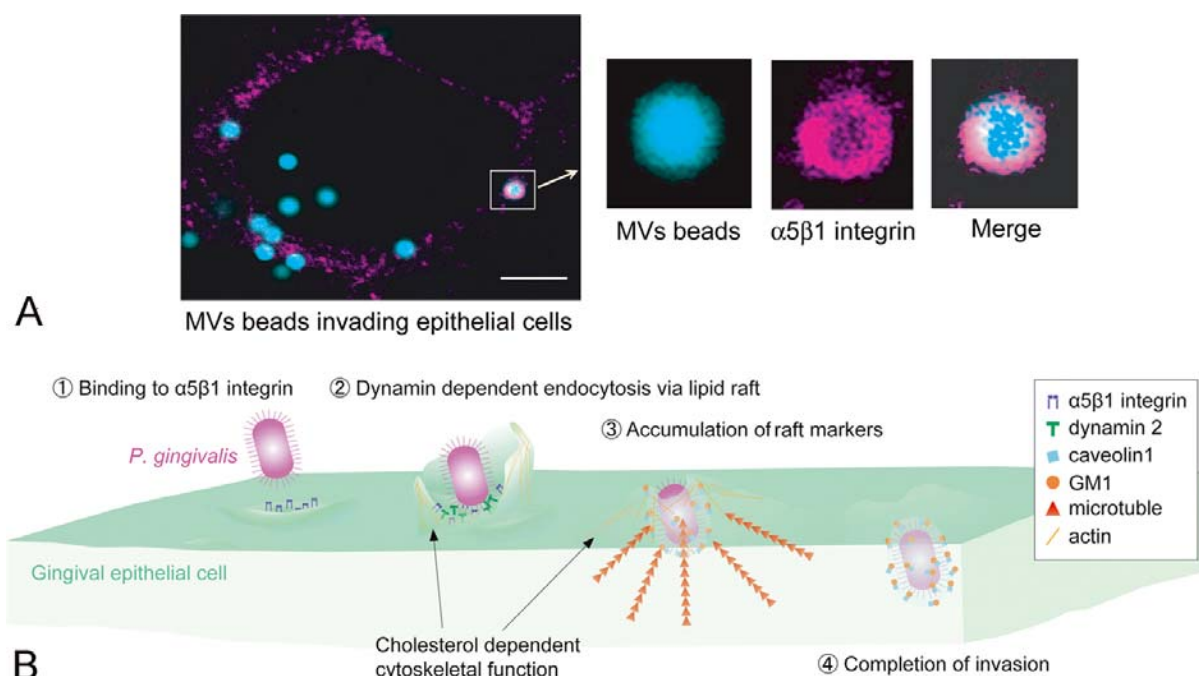
Figure 1. Invasion of epithelial cells by *P. gingivalis*. A. Invasion of HeLa cells by polystyrene fluorescent-beads conjugated with membrane vesicles (MVs) of *P. gingivalis*. The boxed area shows that α5β1-integrin (magenta) was recruited around the MV-coated beads (cyan) (Reproduced with permission from ref. 26). Bar=5 μm. B. Suggested scheme of the invasion mechanism of *P. gingivalis* with respect to host cellular endocytic machinery, cytoskeleton, and lipid rafts. *P. gingivalis* fimbriae adhere to α5β1-integrin, after which the bacterium is subsequently surrounded by α5β1-integrin and invaginated through the actin-mediated pathway controlled by phosphatidylinositol (PI) 3-kinase. The invasive event requires host cellular dynamin, actin fibers, microtubules, and lipid rafts. The dynamics of microtubule assembly and disassembly are essential for this process.

gingivalis fimbriae were shown to bind to cellular α5β1-integrin, which mediates bacterial adherence to host cells (26, 27). Cellular integrins, ubiquitously expressed heterodimeric receptors for ECM proteins, are intimately involved in cellular physiological processes that are related to cell activation, proliferation, differentiation, metabolism, and motility (28), and those cellular functions are highly dependent on the interaction between α5β1-integrin and its ligand fibronectin (29). In a study that utilized a human α5β1-integrin over-expressing Chinese hamster ovary cell line, it was shown that *P. gingivalis* fimbriae compete with fibronectin for α5β1-integrin and inhibit fibronectin/integrin-regulating cellular functions (30). Thus, fimbriae are likely to facilitate not only the adhesion of *P. gingivalis* to host cells, but also prevention of cellular homeostatic control.

4.2. Invasion of epithelial cells

Many bacterial pathogens are known to enter non-phagocytic cells and that invasion is mediated by cellular actin rearrangement, which is induced through specific interactions between bacterial ligands and cell surface receptors (31). In several studies, lipid rafts in the plasma membrane were suggested as bacterial entry sites (32, 33). Lipid rafts are membrane sub-domains rich in cholesterol, sphingolipids, and specific membrane proteins, such as glycosylphosphatidylinositol-anchored proteins (GPI-

APs) and caveolae, which are flask-shaped invaginations found in the plasma membrane of mammalian cells that are involved in cholesterol homeostasis, endocytosis, and cell signalling (34). It has also been suggested that lipid rafts act as platforms for protein sorting and signal transduction (33).

It is known that fimbriae are essential for the successful invasion of host cells by *P. gingivalis* (9, 35, 36), though the mechanisms involved in the invasive events remain to be elucidated. *P. gingivalis* releases membrane vesicles (MVs) extracellularly, which retain the full components of the outer membrane constituents of the cell wall including proteins, LPS, muramic acid, capsule, and fimbriae (37, 38). We previously used polystyrene fluorescent-beads conjugated with MVs as homogenous artificial intruders of epithelial cells to characterize the invasion mechanism of *P. gingivalis* with respect to the host cellular endocytic machinery, cytoskeleton, and lipid rafts (26). The beads adhered to and were surrounded by α5β1-integrin, then were invaginated through the actin-mediated pathway that is controlled by phosphatidylinositol 3-kinase (PI3K) (Figure 1). We also found that invasion by the beads required host cellular dynamin, actin fibers, microtubules, and lipid rafts. In addition, the dynamics of microtubule assembly and disassembly are essential to that process, whereas other bacteria usually employ

stable microtubules (39). Such a combination of cellular factors is not known to occur in the invasive events associated with other bacterial organisms, indicating the particular ability of *P. gingivalis* to become internalized in cells and the diversity of its bacterial entry mechanisms (33, 39).

5. IMPAIRMENT OF EPITHELIAL CELLULAR FUNCTION BY *P. GINGIVALIS* WITH SPECIFIC *FIMA* GENOTYPES

5.1. Relationship of 6 *fimA* genotypes with virulence of *P. gingivalis*

The fact that *P. gingivalis* can be present in periodontal pockets undergoing destruction as well as healthy gingival margins (40-43) suggests its clonal heterogenicity, with subpopulations with high and low pathogenicity. The diversities in virulence among clinical isolates and laboratory strains of *P. gingivalis* were first examined using animal models (44-47), among which mouse and guinea pig abscess models were extensively employed. Following subcutaneous infection of rodents with *P. gingivalis*, virulence was generally evaluated in relation to the size of the abscesses and/or eroded skin lesions that developed, along with cachexia and death. In those studies, many strains of *P. gingivalis* were classified as either virulent/invasive or avirulent/noninvasive (48, 49). Although encapsulated strains appeared to be more virulent and invasive, the factor(s) regulating the expression of virulence in *P. gingivalis* have not been clearly elucidated.

P. gingivalis fimA genes have been classified into 6 variants (type I to V and Ib) on the basis of their different nucleotide sequences (50-52). We developed a sensitive polymerase chain reaction (PCR) assay using *fimA* type-specific primer sets to differentiate the *fimA* genotypes found in the organisms present in saliva and dental plaque samples (53). The clonal distribution of the 6 *fimA* types was examined among *P. gingivalis* clones harbored by periodontitis patients and periodontal healthy adults using this PCR assay (54, 55). A majority of *P. gingivalis* organisms isolated from periodontitis patients were found to carry type II *fimA*. In contrast, in healthy adults, the most prevalent *fimA* type of *P. gingivalis* was type I and significant associations of specific *fimA* types with periodontal health status were demonstrated. Further, *P. gingivalis* with type II *fimA* was detected more frequently in deeper periodontal pockets (53). The significant association of type II *fimA P. gingivalis* with marginal periodontitis has also been reported by other studies conducted in other nations, i.e., Germany (56), Brazil (57), Switzerland (58), and China (59). In addition, our previous study of a mouse abscess model demonstrated that type II strains caused the most significant induction of acute general inflammation among the 6 types, while fimbriae-deletion type II mutants clearly lost their infectious abilities (46). These findings strongly suggest that clonal variations of fimbriae are related to

bacterial infectious traits that influence disease development and deterioration.

5.2. Impairment of cellular function by *P. gingivalis* with type II *fimA* gene
5.2.1. Study of different recombinant *fimA* genotypes with recombinant FimA proteins

We generated 5 recombinant FimA (rFimA) proteins corresponding to their clonal variants (types I to V), and characterized the capabilities of adhesion/invasion of these purified rFimA proteins with human gingival fibroblasts (HGF) and a human epithelial cell line (HEp-2 cells) (27). There were no significant differences in the adhesion ability of microsphere beads conjugated with these rFimAs to HGF. However, the adhesion of type II rFimA-beads to HEp-2 cells was significantly greater than those of the other rFimA types. It was also observed that the type II rFimA-beads markedly invaded epithelial cells and accumulated around their nuclei, with a similar accumulation also observed upon invasion of epithelial cells by viable *P. gingivalis* cells (35, 60). The adhesion/invasion activities of the type II rFimA-beads were abrogated by the addition of antibodies against type II rFimA or $\alpha5\beta1$-integrin (27).

5.2.2. Study of different *fimA* genotypes with membrane vesicles

The invasive efficiencies of native fimbriae with all 6 different *fimA* types were also compared using microsphere beads conjugated with *P. gingivalis* vesicles (61). Type II fimbriae were the most efficient human epithelial cell invaders among the types. Further, cell death was induced by beads coated with type II vesicles, though the incidence was reduced over time. Thus, invasion efficiency seems to be partially related to cell death.

5.2.3. Study of different *fimA* genotypes with representative strains

Cellular integrins provide a physical linkage between the extracellular environment and intracellular cytoskeleton via focal adhesions (62), which are intimately involved in cellular anchorage and directed migration, as well as in signal transduction pathways, which control wound healing and regeneration, and tissue integrity (63). During these events, paxillin and focal adhesion kinase (FAK) play important roles. The phosphorylation of FAK is a central regulator of cell migration during integrin-mediated control of cell behavior (64). Paxillin is localized in cultured cells, primarily in sites of cell adhesion to the extracellular matrix (i.e., focal adhesions), and its activation is a prominent event upon integrin activation for actin-cytoskeleton formation as well as the recruitment of FAK to robust focal adhesions (65). It was previously reported that *P. gingivalis* invaded epithelial cells, and subsequently degraded paxillin and FAK, resulting in impaired cellular function (8).

We compared the efficiencies of *P. gingivalis* strains with distinct fimbria types in regard to their
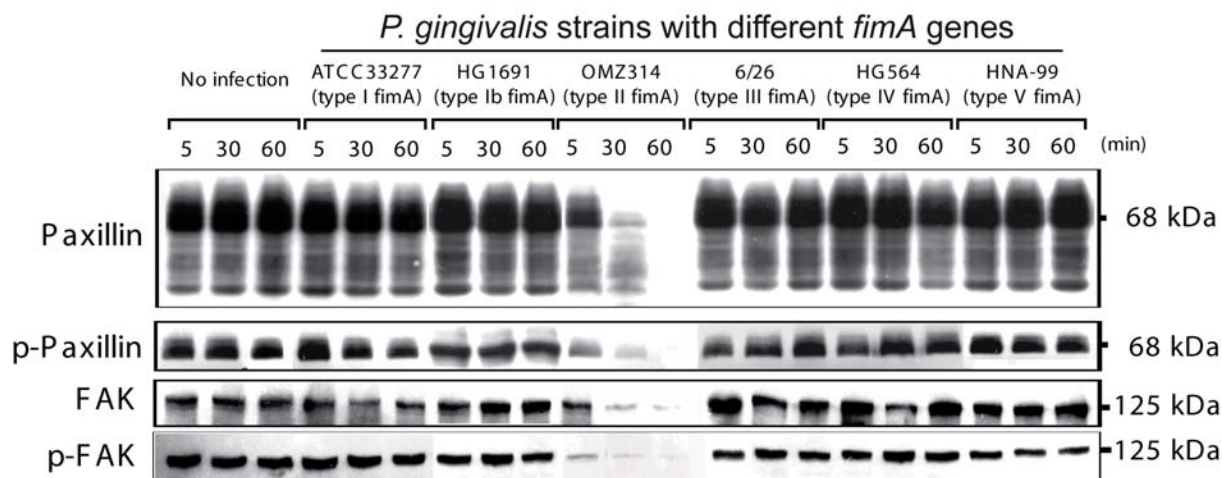
Figure 2. Degradation of cellular paxillin and focal adhesion kinase (FAK) by *P. gingivalis* with type II *fimA*. HeLa cells were infected with *P. gingivalis* with distinct fimbria types for 5, 30, and 60 minutes. The type II *fimA* clone (strain OMZ314) significantly adhered to and invaded the cells, as compared to the other types (Reproduced with permission from ref. 36). Immunoblotting of cellular lysates showed that paxillin and FAK were markedly degraded at 30 minutes after infection with type II *P. gingivalis*, which was associated with the disappearance of phosphorylated proteins, whereas negligible degradation was induced by infection with the other types.

Table 2. Infectious inflammation caused by *fimA*-substituted mutants

Original strain	Mutant	Body wt.	Spleen wt.	Maximum values of IL-1β	Maximum values of IL-6
		(%)	(mg/g)	(pg/ml)	(pg/ml)
ATCC 33277	WT (I)	102.4±1.7	4.70±0.39	121±14	837±192
	fimA (II)	96.5±0.7[1]	5.73±0.35[1]	317±82[1]	1623±371[1]
	ΔfimA	101.3±0.7	4.46±0.2	80±17	490±192
OMZ314	WT (II)	95.5±0.8	6.19±0.45	349±109	1782±440
	fimA (I)	101.7±1.2[1]	4.91±0.19[1]	109±29[1]	857±222[1]
	ΔfimA	102.2±1.9[1]	4.25±0.35[1]	77±19[1]	632±242[1]
No infection		102.3±0.8	4.10±0.25	10±5	25±9

[1] Significant difference compared to WT (*P*<0.01). Reproduced with permission from reference 9.

ability to invade epithelial cells, as well as degradation of the cellular focal adhesion components paxillin and FAK. Among the 6 representative fimbria-type strains tested, *P. gingivalis* with type II fimbriae (type II *P. gingivalis,* strain OMZ314) adhered to and invaded epithelial cells at significantly greater levels than the other strains (36) (Figure 2). There were negligible differences in gingipain activities among the 6 strains, however, type II *P. gingivalis* apparently degraded intracellular paxillin in association with a loss of phosphorylation at 30 minutes after infection. Further, cellular focal adhesions with macro-aggregates of green fluorescent labeled-paxillin were clearly destroyed, which was associated with cellular morphological changes. Degradation was blocked with cytochalasin D or in infected *fimA*-disrupted mutants, which indicated that the degradation was dependent on fimbria-mediated bacterial invasion of cells. Paxillin was degraded by a Lys-gingipain-disrupted mutant, which was prevented by inhibition of Arg-gingipain activity by TLCK, and we considered that the degradation was mainly due to the activities of Arg-gingipain. In an *in vitro* wound closure assay, type II *P. gingivalis* significantly inhibited cellular migration and proliferation, as compared to the other types. These results suggest that type II *P. gingivalis* efficiently invades epithelial cells and degrades focal adhesion components with Arg-gingipain, which causes cellular impairment during wound healing and periodontal tissue regeneration.

5.2.4. Study of *fimA*-substituted mutants.

In addition to fimbriae, *P. gingivalis* has a number of potential virulence factors such as LPS and gingipains that may contribute to the pathogenesis of periodontitis. Thus, other virulence factors may contribute to the varied pathogenicity that exists among the 6 distinct fimbria types. To ascertain whether fimbria variations have a marked influence on the virulence of *P. gingivalis*, we generated mutants in which *fimA* was substituted with different genotypes to elucidate the various levels of virulence, especially that of type II fimbriae (9). Using plasmid vectors, type I *fimA* of strain ATCC33277 was substituted with type II *fimA*, and that of OMZ314 (type II strain) with type I *fimA* (Figure 3A). The substitution of type I *fimA* with type II enhanced bacterial adhesion/invasion to
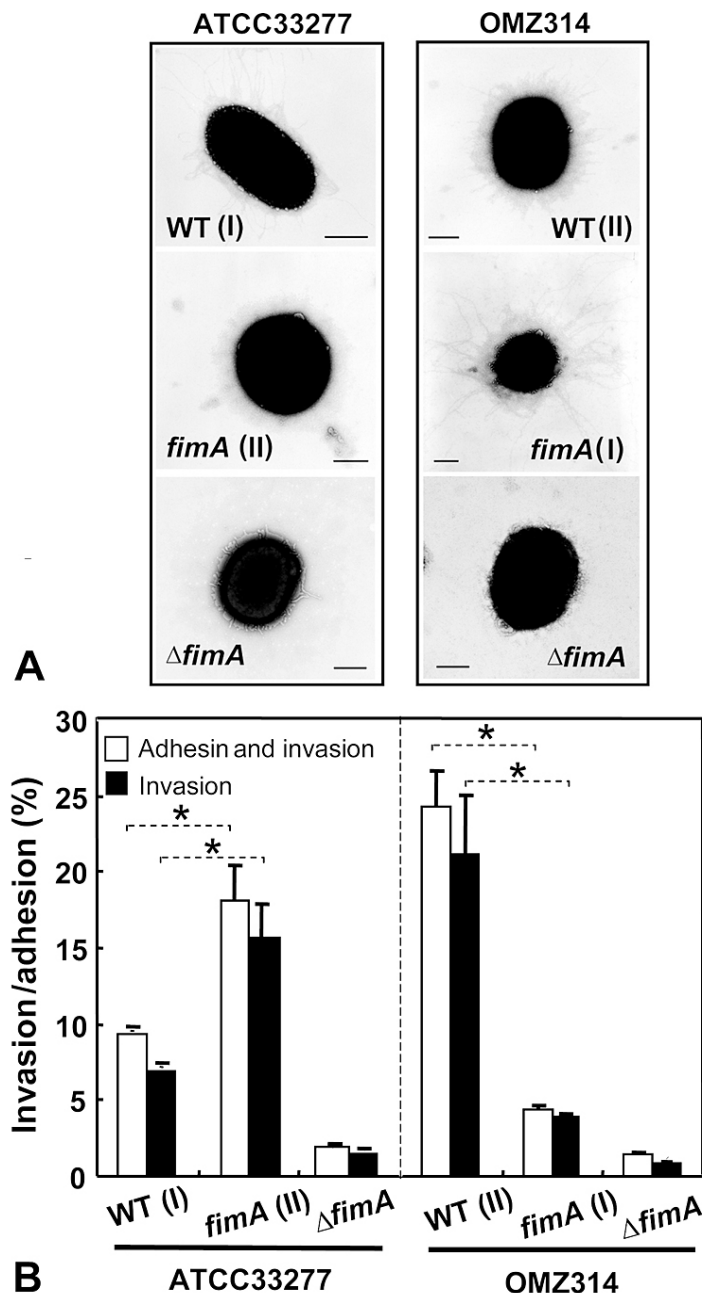
Figure 3. Phenotypic characterization of *fimA*-substituted mutants of *P. gingivalis*. A. Transmission electron microscopic analysis of *fimA*-substituted mutants. The *fimA* of ATCC33277 (type I strain) was substituted with type II *fimA*, and that of OMZ314 (type II strain) with type I *fimA*. The wild type of type I and II strains, WT (I) and WT (II), respectively, were also tested. *fimA* (I) and *fimA* (II) denote *fimA*-substituted mutants with types I and II *fimA* genes, respectively. *ΔfimA*, *fimA*-disrupted mutant. The type I fimbriae of WT (I) appeared long and willowy, while those of the type II fimbriae of WT (II) were shorter and had the appearance of a sunflower. Interestingly, these characteristics were also seen in the *fimA*-substituted mutants. However, the *fimA*-disrupted mutants apparently lack fimbria structures on their surfaces. B.Adhesion/invasion to gingival epithelial cells by *fimA*-substituted mutants. The substitution of type I *fimA* with type II enhanced bacterial adhesion/invasion to the cells, whereas substitution with type I *fimA* resulted in diminished adhesion/invasion efficiency (Reproduced with permission from ref. 9).

epithelial cells, whereas substitution with type I *fimA* resulted in diminished adhesion/invasion efficiency

(Figure 3B). Following bacterial invasion, the type II clones swiftly degraded cellular paxillin and focal
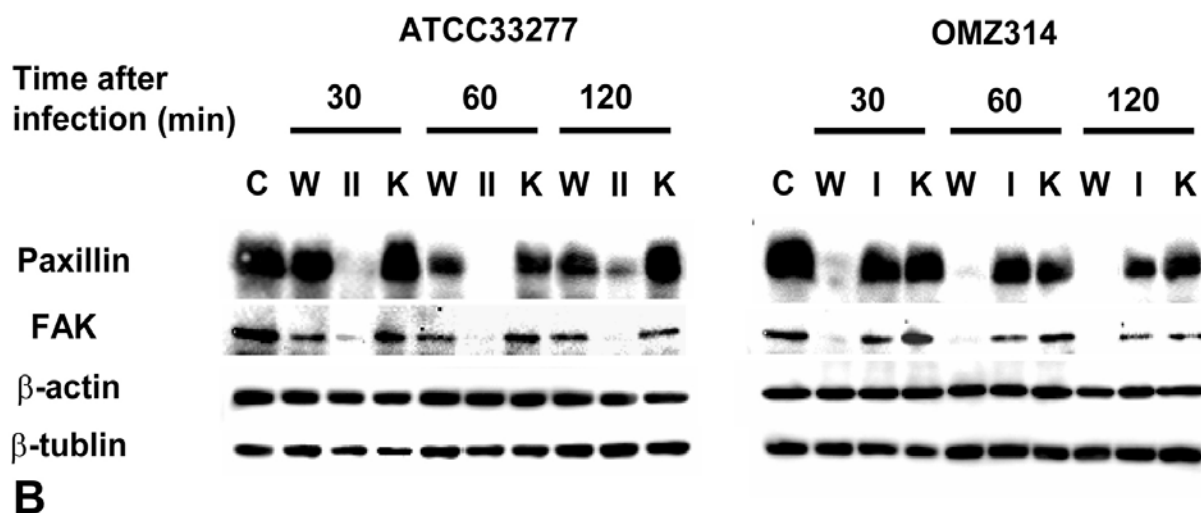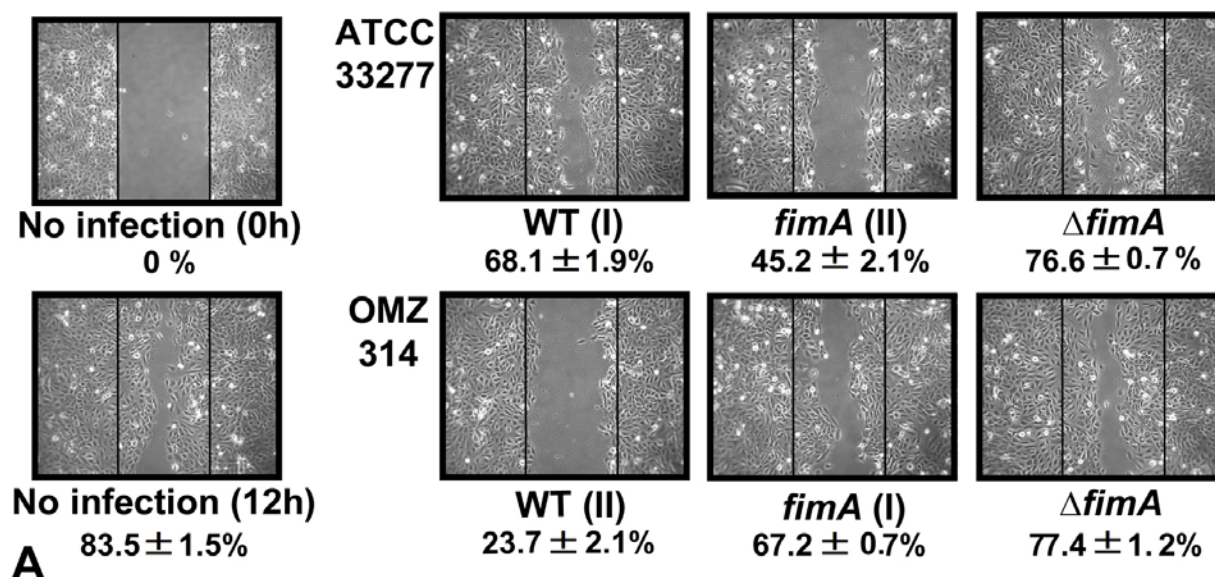
Figure 4. Impairment of cellular function by *P. gingivalis* with type II *fimA*. A. Inhibition of cellular migration and proliferation by *fimA*-substituted mutants. Gingival epithelial cell layers were scratched with a plastic tip, and cells subsequently migrated to and filled in the wounded scratch areas in a time dependent manner. *P. gingivalis* WT (I) and the *fimA* (I) mutant slightly inhibited scratch closure, while the *fimA* (II) mutant impaired the cellular wound closure process as significantly as WT (II). That inhibitory effect was not seen with the *ΔfimA* (I) and (II) mutants. These results show that type II fimbriae are a critical factor in the cellular functional impairment caused by *P. gingivalis*. The abbreviations used are the same as in Figure 3. B. Degradation of paxillin and FAK associated with invasion of gingival epithelial cells by *fimA*-substituted mutants. WT (I) did not clearly degrade paxillin and FAK, whereas the *fimA* (II) mutant markedly degraded both at 30 minutes after infection of gingival epithelial cells. In contrast, *fimA*-substitution from type II to type I caused a loss in degradation activities, though neither β-actin nor β-tublin were degraded, even when infected by type II clones. Type II fimbriae efficiently mediated the selective degradation of paxillin and FAK, which caused cellular impairment of migration and proliferation, following the bacterial invasion of epithelial cells. C; control (no infection), W, wild type strain (type I *fimA* strain, ATCC33277; type II *fimA* strain, OMZ314); I, *fimA* (I); II, *fimA* (II); and K, KO-mutant (*ΔfimA*). Reproduced with permission from ref. 9.

adhesion kinase, and inhibited cellular migration, whereas the type I clones and *fimA*-deletion mutants (*ΔfimA*) did not (Figure 4). Further, BIACORE analysis results demonstrated that type II fimbriae possess greater adhesive abilities for α5β1-integrin than those of type I. In a mouse abscess model, the type II clones significantly induced serum IL-1b and IL-6, as well as other infectious symptoms (Table 2). Together, these

results indicate that type II fimbriae are a critical determinant of *P. gingivalis* virulence.

6. CONCLUSION

P. gingivalis fimbriae are able to interact with various host components and promote bacterial colonization in the periodontal margin. The bacterium

invades epithelial cells via the binding of fimbriae with cellular α5β1-integrin, thereby disrupting the epithelial barrier, while the subsequent invasion requires host cellular dynamin, actin fibers, microtubules, PI3K, and lipid rafts. Following invasion, the intracellular pathogen impairs cellular function. Notably, *P. gingivalis* with type II fimbriae efficiently invades epithelial cells and degrades focal adhesion components with Arg-gingipain, which causes cellular impairment during wound healing and periodontal tissue regeneration.

Genomic variations of the fimbria structures of *P. gingivalis* are likely to be closely related to the initiation and progression of human marginal periodontitis. Additional studies will be carried out at the molecular level to examine if the virulence traits of type II *P. gingivalis* directly contribute to the development of periodontitis and associated deterioration. In addition, it would be important for periodontal therapy and assessment of prognosis if disease contributing strains could be differentiated based on clonal variations of the *fimA* gene.

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