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Bacterial invasion of epithelial cells and spreading in periodontal tissue

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The composition of the subgingival bacterial microbiota is a critical determinant in the health status of periodontal tissues. Gram-negative anaerobes such as *Porphyromonas gingivalis* are well-established periodontal pathogens, and high numbers of these bacteria are found in the subgingival sulcus of patients with chronic periodontitis (43). However, a burgeoning pool of evidence indicates that these organisms have a far more complex relationship with the host than merely as pathogens. Gram-negative anaerobes are frequently present in the oral cavity of periodontally healthy individuals (23, 44, 92, 121, 127, 128, 170), and indeed health is the most common status of the human gingiva despite years of exposure to a large microbial burden. Periodontal organisms thus appear to have co-evolved with their host to maintain an ecologically balanced association whereby minimal harm is inflicted on, or by, either party. Disease will only ensue when this interaction becomes unbalanced, an event that has been termed an ecological catastrophe (83). Organisms such as *P. gingivalis* may thus be more accurately characterized as accidental, or host-adapted, pathogens.

In the subgingival compartment, epithelial cells represent a major host interface for colonizing organisms; hence, the interaction between gingival epithelial cells and periodontal bacteria will contribute to the success or failure of colonization, and to the maintenance of health or disease in the host. Undeniably, in the case of *P. gingivalis*, an intricate and multithreaded relationship exists between the organism and gingival epithelial cells, which, under optimal conditions, results in stable cohabitation, with both bacteria and host cells responding and adapting to the presence of their partner to maintain a state of health. In the event that this relationship becomes perturbed, for example because of an increase in bacterial burden or an inappropriate immune response, the periodontal disease process can be initiated (13).

The ability to adapt in response to the host environment is reflected in the genetic diversity found within many species of periodontal bacteria. Bacteria are masters of adaptation, and at the genetic level are able to rapidly modify and share DNA. For example, there are significant levels of genetic variation among *P. gingivalis* strains, and many studies have linked this genetic variability to virulence potential (7, 14, 18, 38, 67, 85, 108). Genetic variability among strains is common in bacteria with long-term carrier states, possibly arising from the co-evolutionary dynamic of host–pathogen interactions (62, 123). Genetic variation can produce lineages of bacteria with `good or evil' personalities, in that some are more virulent and associated with disease, whereas other strains of the same species behave in a more commensal manner. In this review, we will discuss the current understanding of pathogenic and commensal aspects of bacterial interactions with periodontal tissues, with a specific focus on *P. gingivalis* intracellular invasion and molecular modulation of host cells.

Interactions of periodontal bacteria with epithelial cells observed *in vivo*

Tissue destruction, mediated either by the host or by bacteria, is a hallmark of periodontal disease, and with the consequent loss of barrier function it is not surprising that periodontal bacteria are frequently detected within gingival tissues (1, 20, 41, 101, 116, 131–133, 152–

154, 157). While tissue invasion (intercellular invasion) is an almost inevitable corollary of the disease process, a number of oral bacteria have been observed to locate inside host cells (intracellular invasion) both in the presence and in the absence of disease. Fluorescence *in situ* hybridization, combined with confocal microscopy, has established that buccal epithelial cells from healthy individuals contain a polymicrobial intracellular microbiota that includes the periodontal bacteria P. gingivalis, Tannerella forsythia, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Prevotella intermedia, Eikenella corrodens and *Treponema denticola* (128, 129). Importantly, these colonized epithelial cells are not necrotic or apoptotic, but remain viable (127). In tissue samples from patients with periodontitis, electron microscopy has demonstrated the presence of periodontal bacterial within epithelial cells (165); and immunohistochemistry revealed that these intracellular bacteria include *P. gingivalis* (122). Similar microscopic techniques have shown P. gingivalis, T. forsythia, A. actinomycetemcomitans and *T. denticola* within gingival and buccal epithelial cells from both healthy individuals and patients with periodontitis (22).

Residence within host cells provides bacteria with a nutrient-rich, generally reducing environment that is partially protected from the host immune system. Accessing this secure niche may be critical in the early stages of sulcus colonization by periodontal bacteria, as low numbers of bacteria are particularly susceptible to clearance by immune mechanisms. While not immediately contributing to disease, invasive bacteria may use the intracellular locale to safely persist and replicate. In disease states, intracellular bacteria are less likely to be physically removed by scaling and root planing (61) and are more resistant to antibiotics (36). Furthermore, this intracellular population could constitute a reservoir of bacteria for the repopulation of treated subgingival sites. The ability to invade and persist in host cells is evidently an important factor in the overall disease process, and *P. gingivalis* strains isolated from disease sites possess greater invasion capabilities *in vitro* than strains from healthy sites (58). Collectively, these observations indicate that an intracellular location is an integral component of the lifestyle of many periodontal bacteria, whether in a healthy or diseased host, and probably contributes to the chronic nature of periodontal disease.

Models to study intracellular invasion

Epithelial tissues are structurally diverse and range from simple, single-layered gut or glandular epithelia to the complex, stratified epithelia that form the body surface, including the oral cavity. While all epithelia have features and functions in common, they also exhibit many tissue-specific properties. The gingival epithelium is a stratified and squamous tissue, and is composed of oral, sulcular and junctional epithelium. The periodontally relevant sulcular and junctional epithelia are neither keratinized nor terminally differentiated, unlike other oral epithelial cells (118). As the differentiation status and tissue of origin can affect bacteria–epithelium interactions (66, 81, 91, 117), the most relevant models for periodontal bacteria will involve cells derived from, or with characteristics of, junctional or sulcular epithelium. A number of *in vitro* tissue culture models have been developed to study periodontal bacteria–epithelium interactions, including primary gingival epithelial cells (73, 74), transformed epithelial cells (46) and multilayers (4, 110, 140). Primary gingival epithelial cells, obtained from gingival explants, express keratin and differentiation markers characteristic of the junctional epithelium, and are naturally senescent (105, 106). Transformed lines derived from gingival epithelial cells are also poorly differentiated and respond similarly to oral bacteria (46, 104), without potential confounding influences of patient to patient variability (64). The KB and HEp-2 cell lines were originally thought to be derived from human oral epidermal carcinomas, and have been used as model systems for studies of periodontal disease. However, these cell lines are now known to have arisen from HeLa cell contamination, and as such are less relevant for studies of the oral cavity.

Utilization of orally derived model systems has demonstrated that several species of periodontal bacteria can internalize within epithelial cells through a bacterially directed process. Invasive organisms include *P. gingivalis* (Fig. 1), A. actinomycetemcomitans, T. forsythia, F. nucleatum and *P. intermedia* (Table 1) (6, 33, 45, 54, 55, 72–77, 86, 87, 130, 136). In addition, consistent with the multispecies etiology of periodontal disease, bacteria can co-operate with one another to facilitate invasion. For example, *P. gingivalis* (or its outer membrane vesicles) enhance the invasion of *T. forsythia* into epithelial cells (55). *F. nucleatum* can transport noninvasive *Streptococcus cristatus* into epithelial cells through the formation of co-adhered dual-species consortia (35). *F. nucleatum* can also enhance the invasion of *P. gingivalis*, although in this case the synergistic effect results from the interaction of *F. nucleatum* with the host cells (135). These *in vitro* observations emphasize the ecological nature of periodontal disease, in which multiple species act in concert. Periodontal bacteria can also enhance the invasion of *Pseudomonas aeruginosa* into epithelial cells, which may provide a mechanistic basis for the epidemiological association between periodontal disease and respiratory tract infections (109).

The initial interaction with epithelial cells

Attachment or close physical association between bacteria and epithelial cells can be a prelude to internalization. Engagement of membrane receptors by bacterial surface ligands allows recalibration of the cellular machinery to mediate pathogen entry into these nonphagocytic host cells. Many invasive bacterial species manipulate host cell receptors to activate their uptake, and oral pathogens are no exception to this paradigm (37). The most intensively studied of the invasive oral bacteria is *P. gingivalis*, and this subgingival resident will be the focus of the remainder of this review.

The mechanisms of *P. gingivalis* adhesion to, and invasion of, epithelial cells are multifaceted and involve a number of effector molecules (Fig. 2). In terms of initial binding, the predominant adhesins are the major fimbriae (168, 174), which are composed of the FimA structural subunit protein along with minor proteins FimC, D and E (100). The FimA subunit directly engages $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins on the epithelial surface (93, 174), and this interaction initiates an integrin-associated signaling cascade that triggers bacterial internalization (174). The focal adhesion adaptor and signaling proteins paxillin and focal adhesion kinase (FAK) are recruited to sites of *P. gingivalis* attachment (176), and the resulting information flow converges on the cytoskeletal architecture. Both actin microfilament and microtubule structures are remodeled to accommodate the entry of *P. gingivalis* (73, 174, 176). Integrin-mediated internalization may take place in lipid raft entry platforms that signal through the Rho GTPase, Rac1 (156, 161).

The strength of the initial adherence between *P. gingivalis* and the host epithelial cell can vary. The major (long) fimbriae are the primary bacterial adhesins, and there are at least six alleles of the *fimA* gene (*fimA I*, Ib, II, III, IV and V) distributed among strains worldwide (3, 95). In studies of *P. gingivalis* isolates from healthy and diseased individuals, major fimbriae composed of type Ib, II or IV are more commonly associated with periodontal disease, whereas type I, III, or V fimbriae are more often found in *P. gingivalis* strains colonizing healthy patients (3, 89, 90). The *fimA* II allele has been shown to result in stronger adherence to epithelial cell receptor $\alpha_5\beta_1$ integrin, compared with other *fimA* types (94, 96).

While FimA–integrin interactions constitute the predominant means of *P. gingivalis* adherence and entry into gingival epithelial cells, FimA binding to intercellular adhesion molecule (ICAM) 1 can also initiate invasion into HeLa cells (156), and this mechanism could also play a role in gingival cells. Furthermore, invasion occurs in the absence of

FimA, albeit less efficiently. Therefore, other invasins are operational, and these include the gingipain proteases (103, 119). The gingipains are a family of three arginine/lysine-specific proteases (RgpA, RgpB and Kgp) that are found in the outer membrane of the bacteria and are also secreted into the surrounding environment. The gingipain proteases have both enzymatic and structural functions that are integral to the successful adherence of *P. gingivalis* (19, 145). Gingipain protease activity has been shown to improve *P. gingivalis* binding to gingival cells by modifying matrix proteins and revealing epithelial surface cryptitopes (68, 69, 158), and protease-deficient mutants show diminished invasion efficiency (112). Structurally, the RgpA and Kgp gingipains possess hemagglutinin/adhesin domains that are involved in the attachment of *P. gingivalis* to epithelial cells (17, 114). The adhesin domain of RgpA associates with fibronectin and with the $\alpha_5\beta_1$ integrin receptor for fibronectin on gingival fibroblasts (142), which can lead to internalization and nuclear targeting of this gingipain protease (141). RgpA can interact with clatherin, and the purified protein can be internalized via a clathrin-dependent endocytosis pathway in HeLa (HEp-2) cells (12). As RgpA proteins are present on the surface of *P. gingivalis*, this molecule may act as an adhesin and allow internalization of *P. gingivalis* cells via an alternative pathway to that driven by FimA–integrin interactions.

Internalization

The interaction of *P. gingivalis* FimA fimbriae with epithelial cell surface integrins initiates a cellular response that recruits FAK and paxillin to the cytoplasmic membrane at the bacterial attachment site (174, 176). The resulting protein–protein interactions among integrin, FAK and paxillin produce a phosphorylation-regulated signaling scaffold that activates Rho-family GTPases, enzymes which play a central role in initiating downstream signaling cascades and regulating cytoskeletal dynamics (25, 47). The subsequent actin and microtubule remodeling, the recruitment of lipid raft components, and host-cell phosphorylation activity are all required for internalization of *P. gingivalis* (73, 75, 138, 161, 167, 176). The invasion process is complete in approximately 15 min and ultimately results in the perinuclear localization of the bacteria (11).

Entry of *P. gingivalis* into host cells results in the reprogramming of major host-cell signaling pathways. Consistent with activation by integrin-initiated Rho cascades, components of mitogen-activated protein kinase pathways are selectively targeted for regulation by internalized *P. gingivalis*. Extracellular signal-regulated kinase 1/2 and c-Jun N-terminal kinase activities are down-regulated and up-regulated, respectively (167). Kinase regulation occurs in a dose-dependent manner and requires metabolically active bacteria, implying that the regulation of host mitogen-activated protein kinases requires the production of bacterial effectors. Invasion by *P. gingivalis* also induces a transient increase in epithelial cell cytosolic calcium concentrations (10, 57). Calcium signaling can regulate a variety of cellular functions; and during invasion with bacterial pathogens, calcium levels can influence cytokine expression and modulate intracellular trafficking and cytoskeletal activities (159). Calcium levels impact *P. gingivalis* manipulation of host cells, as over-expression of the calcium-binding protein, calprotectin, in gingival epithelial cells inhibits invasion (99).

Although *P. gingivalis* does not possess the type III secretion machinery that injects bacterial invasion effectors directly into the host cell cytoplasm (56, 98, 134), it does secrete a distinct set of proteins upon encountering the epithelial cell environment (112). Among these is a HAD family serine phosphatase (SerB), that is active on host cell phosphoproteins and influences *P. gingivalis* entry and survival (48, 160). Microarray analysis found that SerB impacts the transcriptional profile of gingival epithelial cells, with pathways involving the actin cytoskeleton among those significantly overpopulated with differentially regulated

genes (48). Moreover, a SerB mutant of *P. gingivalis* is defective in actin remodeling and in internalization, and interaction between gingival epithelial cells and purified SerB protein results in actin re-arrangements, an increase in the F/G actin ratio, and disruption of microtubule dynamics. Thus, SerB can interact with signaling pathways that regulate gene expression, cytoskeletal dynamics and ultimately affect *P. gingivalis* internalization and survival. One could presume that the net effect of signaling pathway manipulation is alteration of the host cellular physiology, restructuring the cytoskeleton to direct bacterial uptake and perinuclear localization, and ultimately crafting a protected intracellular niche for these fastidious organisms.

Adaptation of *P. gingivalis* to the intracellular environment

It hardly bears mentioning that the mammalian intracellular environment is quite distinct from both bacterial culture medium and the subgingival sulcus. Intracellular bacteria will experience distinct nutritional and physical conditions and, as is facile for bacteria, will adapt to these conditions through gene and protein regulation. Global molecular approaches to examine bacterial transcriptional and proteomic changes have provided insights into this complex process.

Transcriptional profiling by microarrays has been adopted to examine global gene regulation during the process of *P. gingivalis* invasion. Hosogi & Duncan (50) found that during attachment to HeLa (HEp-2) cells, genes encoding oxidative stress-response components and heat shock proteins were up-regulated, indicating that *P. gingivalis* bacteria on the surface of host cells experience oxidative stress and produce heat shock proteins to maintain protein function and viability. A study of gene expression of *P. gingivalis* within endothelial cells (125), demonstrated that internalized bacteria regulate pathways relating to energy metabolism, protein synthesis and transport through the outer membrane. Differential display reverse transcription-polymerase chain reaction confirmed that inside epithelial cells *P. gingivalis* regulates the expression of membrane transporters, and that loss of the corresponding gene products impairs bacterial invasive ability (113). Although functional roles for these transporters have yet to be defined, it is likely that they will affect the import/export of cations and nutrients.

Protein expression has been compared between internal and external *P. gingivalis* using whole-cell quantitative proteomic analyses (169). Interestingly, several classical virulence factors, including FimA, RgpA/B and Kgp, show decreased expression in internalized *P. gingivalis*. While FimA is required for optimal adherence and to initiate invasion pathways, production of this protein is evidently superfluous once the bacteria reach the intracellular milieu. Tight control of gingipain production is also a prudent maneuver for bacteria attempting to establish an intracellular niche, as gingipains are potent proteases and their over-expression could result in excessive damage to the interior of the host cell. Internal *P. gingivalis* also down-regulate a number of hemin-acquisition systems, and thus iron may not be limiting in the intracellular environment.

Establishment of a stable relationship between the host cell and internalized bacteria is stressful for both host and pathogen. For *P. gingivalis*, the transition from extracellular to intracellular environments evidently requires a drastic overhaul of the proteins and enzymes required for survival. As is common for bacteria adapting to changing conditions, multiple systems exist to mediate the disposal of toxic products, degrade inactive proteins and to assist in the expression and folding of new proteins. Intracellular *P. gingivalis* up-regulates the production of stress-associated proteins such as peroxidases, components of the Clp family and heat shock proteins such as HtrA. Deletion of the *clpB* or *clpP* genes has a negative impact on bacterial survival in gingival epithelial cells (16, 177, 181), and mutation

of *htrA* results in an increased sensitivity to hydrogen peroxide and a decreased survival in animal infection models (126, 178).

In terms of bacterial metabolism, in internalized bacteria there is an increased abundance of proteins comprising the energy pathway leading from asparagine/aspartate amino acids to ATP. The pathway producing propionate shows an increased abundance of component proteins, while a tendency towards decreased abundance of proteins is observed for the pathway leading to butyrate production. As propionate is a less potent inducer of apoptosis than butyrate (84), this metabolic shift could also minimize damage to the host cells. The translational machinery, including ribosomal proteins and transfer RNA synthetases, shows a significant increase in expression, as do proteins responsible for transcription. In total, approximately 50% of the expressed proteome is differentially regulated by intracellular *P. gingivalis*. Overall, these results suggest that the intracellular environment, while initially stressful, is energy rich for *P. gingivalis* and consequently it is advantageous for the organism to undergo major adaptations that permit entry into, and use of the metabolic substrates available within, the host cell.

Intracellular localization

Intracellular bacteria must avoid host cell defenses located within the cytoplasm in order to establish long-term residence in the host cell. A primary defense organelle is the acid-containing lysosome, which is the `garbage disposal' system of the eukaryotic cell. Membrane-bound vacuoles containing cytoplasmic debris fuse with lysosomes, which degrade the material contained within the vacuole by exposure to lysosomal acids and enzymes. Once inside the cell, bacteria must act swiftly to prevent exposure to lysosomes. Two cytoplasmic membrane-trafficking systems converge on lysosomes: the autophagic pathway and the endosomal pathway. Intracellular pathogens use varying approaches to manipulate these membrane trafficking systems. Some pathogens remain within a membrane-bound vacuole and express effectors to block fusion with lysosomes. Other organisms escape from the vacuole and subsist freely in the cytoplasm. *P. gingivalis* is capable of invading multiple cell types (30), and accumulating evidence indicates that it uses different strategies to evade lysosomes in epithelial cells and endothelial cells.

Gingival epithelial cells are the main host tissue in contact with *P. gingivalis*, thus eons of host–pathogen contact and adaptation have resulted in a finely tuned relationship. *P. gingivalis* traversing the epithelial cell outer membrane must initially be encompassed within a compartment derived from the host membrane (34, 74, 139, 140). Once inside the cell, however, *P. gingivalis* escapes and survives unbound in the cytoplasm, where it remains viable for extended periods of time and even replicates (73, 74, 79, 111). Ultimately, these nonmotile bacteria localize to the perinuclear region of the host cell, an area densely packed with endoplasmic reticulum. One could hypothesize that the endoplasmic reticulum contents act as an excellent nutrient source for these proteolytic bacteria, and they may target this location to feed off proteins produced by the endoplasmic reticulum during host cell translation.

Although the oral cavity is the natural home of *P. gingivalis*, these bacteria can disseminate intra-vascularly during the transient bacteremias that result from mastication or oral hygiene procedures. Under these circumstances, *P. gingivalis* will be in contact with the endothelial cells that line the vessels of the circulatory system. *P. gingivalis* can adhere to and invade endothelial cells, although at a lower frequency than gingival epithelial cells (29, 120). Variation between the frequencies of invasion into epithelial and endothelial cells may be related to differences in surface receptors and signal transduction pathways. Microscopic evidence illustrates differences in the intracellular localization route of *P. gingivalis* between

epithelial and endothelial cells. In endothelial cells, *P. gingivalis* ultimately traffics to autophagosomes, distinctive double-membraned vacuoles that are part of the autophagic pathway (31, 32). Once in these membrane compartments, the bacteria block fusion with lysosomes and probably use protein debris trafficked through the autophagic pathway as their nutrient source (9, 124). *P. gingivalis* persists in these autophagosome vacuoles, and inflammation and cell damage can result from the accumulation of high loads of these intracellular bacteria. It has been speculated that the invasion of coronary artery endothelial cells by oral bacteria may be a contributing factor to the link between periodontal disease and cardiovascular disease (28).

Phenotype of colonized cells

The stress associated with maintaining an intracellular bacterial burden results in significant phenotypic changes in the infected host cells. There is a spectrum of physiological and morphological outcomes that may result from bacterial invasion, with the ultimate destiny of the host cell being dependent on the characteristics of the invading *P. gingivalis* strain, the total bacterial burden and the host cell type. Perhaps one of the most critical bacterial characteristics is the production of gingipain virulence factors. Gingipain proteases are secreted to make protein nutrients available for the asaccharolytic *P. gingivalis*. High levels of these bacterial enzymes can damage host cells and connective tissue, and can induce apoptosis (145). Hence, strains of *P. gingivalis* that produce high levels of gingipains will be more cytotoxic (60, 102, 143, 144, 149, 166), whereas less proteolytic strains, and strains that can control protease production appropriately, are able to establish a more commensal relationship with the host by regulating apoptotic signaling pathways to prevent cell death (82, 97, 163, 172, 175).

Exposure of host cells to high-protease-secreting *P. gingivalis*, or to high numbers of bacteria, results in cell rounding and loss of attachment as a result of gingipain cleavage of cadherins and integrins (143). Gingipains can also penetrate the host cell (102), where gingipain protease activity is sufficient to activate pro-apoptotic molecules such as caspase-3, caspase-8, caspase-9, Bid and Bax (149). Additional damage to periodontal tissues can result from the activation of matrix metalloproteases by *P. gingivalis* gingipains (26, 27, 42, 115) and the destruction of paxillin and other focal adhesion components (49, 63, 96). Conversely, the anti-apoptotic phenotype induced by low-protease-secreting *P. gingivalis*, or by challenge with lower numbers of bacteria, is associated with activation of the phosphatidylinositol 3-kinase/Akt and Janus kinase/STAT pathways, up-regulation of anti-apoptosis genes Bcl-2 and survivin, and inhibition of cytochrome *c* release and of caspase-3 activity (Fig. 3) (82, 172). More recently, *P. gingivalis* has been shown to interfere with ATP-induced apoptotic pathways via the secretion of an ATP-hydrolyzing enzyme that is a homolog of nucleoside diphosphate kinase (175). ATP scavenging by *P. gingivalis* inhibits apoptosis by preventing ATP ligation of $P2X_7$ purinergic receptors (Fig. 3).

Bacterial interference with cellular physiology can also impact the host cell cycle, by either activating or inhibiting cell cycle progression. In gingival epithelial cells, invasion with *P. gingivalis* results in increased proliferation, which is associated with accelerated progression through the S-phase (Fig. 4) (70). Up-regulating the rate of cell division may be a mechanism to maintain a reservoir of bacterially infected cells, in response to the high rate of cell turnover in the junctional epithelium (13). Host cell division may also allow bacterial cells to replicate without creating an overwhelming intracellular bacterial burden. In disease states, loss of cell cycle control could impact wound healing in the periodontal pocket, thus facilitating bacterial penetration of the periodontal tissues.

Invasion of periodontal tissues

As discussed, subversion of host physiology is a complex and stressful process for both the host cell and the invading pathogen. Once a stable relationship is achieved, it is understandable that *P. gingivalis* would attempt to prolong the life span of its host cell by blocking apoptosis and stimulating proliferation. The host cell does not, however, necessarily achieve a state of immortality; ultimately, intracellular bacteria must have a strategy to access new environments. *P. gingivalis* has been detected in the periodontal connective tissue, implying that cell-to-cell spread of bacteria is a common event.

In a recent study, Yilmaz et al. (173) presented a new in vitro model system to study bacterial cell-to-cell transmission. In this system, *P. gingivalis* bacteria (labeled with red fluorescence) are allowed to invade cultured gingival epithelial cells (labeled with blue fluorescence). After 24 h of co-culture, the infected blue gingival cells are mixed with uninfected green gingival cells. Using fluorescence microscopy, the investigators were able to clearly demonstrate transmission of bacteria from infected to noninfected host cells. Transmission from cell to cell is mediated by a membranous projection with a structural scaffold composed of actin filaments. Initiation of the spreading mechanism occurs at the highest frequency after 24 h of invasion, indicating that cell-to-cell transmission is a late-stage strategy of invasive *P. gingivalis* (171). This intracellular transmission does not appear to affect host cell viability and may be a prominent mechanism for the spread of invasive bacteria in the `stealth' mode. By moving deeper into the epithelial layers, *P. gingivalis* can ensure access to viable, nonshedding epithelial cells.

In a three-dimensional cellular model for bacterial dissemination, Andrian et al. (4) demonstrated that *P. gingivalis* can spread through the upper layers of gingival epithelial cells and can also penetrate the basement membrane into connective tissues. *P. gingivalis* gingipain proteases are capable of degrading matrix and tight junction components, destroying the physical barriers formed by extracellular connective tissue and cellular adhesion (2, 4, 146, 147). A *P. gingivalis* gingipain mutant was also able to invade the upper gingival layers, but was unable to access the connective tissue layer (4). The ability to disseminate beyond the initial site of infection is a characteristic of pathogenic bacteria in general, and we can anticipate that *P. gingivalis* strains able to penetrate the basement membrane and approach the alveolar bone may be more likely to exacerbate the bone loss associated with periodontal disease.

Impact on innate immune surveillance

An expected advantage of residing in an intracellular niche is avoidance of the host immune response. However, during adherence and invasion bacteria are exposed to innate immune-surveillance systems. Gingival epithelial cells express toll-like receptors and other surface pattern-recognition receptors along with intracellular recognition systems such as NODs (150). A robust proinflammatory cytokine and chemokine response would thus be expected following an interaction between the gingival epithelium and the periodontal bacteria. In many instances, such as with *F. nucleatum*, this is indeed the case (164). *P. gingivalis* can also induce the expression of proinflammatory immune mediators from gingival epithelial cells (5, 71, 137); however, the inflammatory phenotype of *P. gingivalis* is much more subtle and nuanced. *P. gingivalis* often suppresses or evades various components of innate immunity, a feature that has led to its characterization as a stealth-like pathogen (24, 40). For example, gingival epithelial cells do not express CD14 (a co-receptor for toll-like receptor 2) on the surface and thus respond poorly to *P. gingivalis* FimA (39), which may limit inflammatory responses to fimbriated, invasive *P. gingivalis*. A more pro-active role for *P. gingivalis* in dampening innate immune responses can be seen from its ability to

suppress transcription of the interleukin-8 gene in gingival epithelial cells, and thus inhibit expression of this chemokine (51–53, 59, 164). Moreover, *P. gingivalis* can antagonize interleukin-8 secretion following stimulation of epithelial cells with other common plaque constituents, a phenomenon known as localized chemokine paralysis (24). A reduction in interleukin-8 levels, along with the down-regulation of intercellular adhesion molecule-1 (52, 80) will impair neutrophil infiltration of gingival tissues, and consequently debilitate local innate immunity and eventually disrupt the ecological balance between the host and the subgingival microbiota, contributing to the initiation of disease activity.

Mechanistically, invasive *P. gingivalis* inhibit the activity of the transcription factor NF-κB through the SerB-mediated disruption of signaling pathways (48). In addition, *P. gingivalis* proteases impair inflammatory responses through the degradation of cytokines, chemokines and their receptors (8, 15, 88, 107, 148, 155, 179, 180). However, consistent with the bipolar personality of *P. gingivalis*, gingipain RgpA–Kgp complexes can penetrate the gingival connective tissue and stimulate the secretion of proinflammatory mediators (102). Moreover, the activation of protease-activated receptors PAR-1 and PAR-2 by *P. gingivalis* proteases can both down-regulate the production of interleukin-8 (162) and up-regulate the production of interleukin-6 (78). Clearly the pro- or anti-inflammatory status of gingival tissues in the presence of *P. gingivalis* is highly context dependent.

Gingival epithelial cells can protect themselves against microbial challenge by the production of antimicrobial peptides, such as human beta-defensins (40), and intracellular antimicrobial compounds, such as calprotectin (99). As with some cytokine responses, there is no obvious trend for human beta-defensin regulation by *P. gingivalis*. In various studies, human beta-defensins 1, 2 and 3, which are produced by gingival epithelial cells, have been found to be all up-regulated, variously up-regulated or not regulated (21, 40, 151, 164). While bacterial strain differences and heterogeneity in epithelial cell receptor expression (65) may account for some of these differences, it is also possible that the bacterial load plays an important role. At a high number of *P. gingivalis* (possibly corresponding to advanced disease), the secreted proteases may overwhelm host cells and obscure the biological activity of other molecules, whereas at lower bacterial numbers (more equivalent to gingival health), the full range of *P. gingivalis* host physiology subversion may be observed.

Conclusions

Periodontitis presents with a wide spectrum of clinical severity, and this complex disease phenotype is produced by variations in host susceptibility as well as variations in the composition and virulence of the oral bacterial microbiota. Investigation of the host-pathogen interaction at the cellular level has begun to reveal the behaviors of periodontal bacteria that contribute to the disease process. For many oral bacteria, the ability to invade host cells and establish an intracellular niche is a critical survival mechanism. As exemplified by *P. gingivalis*, this initially innocuous relationship with a host cell can potentially shift to a more sinister one.

From the host–bacteria interactions described here, it may seem as if *P. gingivalis* is suffering from multiple-personality disorder. However, this pathogenic variability is consistent with the genetic diversity of *P. gingivalis*. Strains with more potent combinations of virulence factors are capable of causing cell damage or death, whereas other, less virulent, strains behave in a more commensal manner. Thus, the specific virulence attributes of the invading bacterial strain are key to determining the final host cell outcome. Further investigation of these virulence traits will facilitate the development of the next generation of periodontal therapies.

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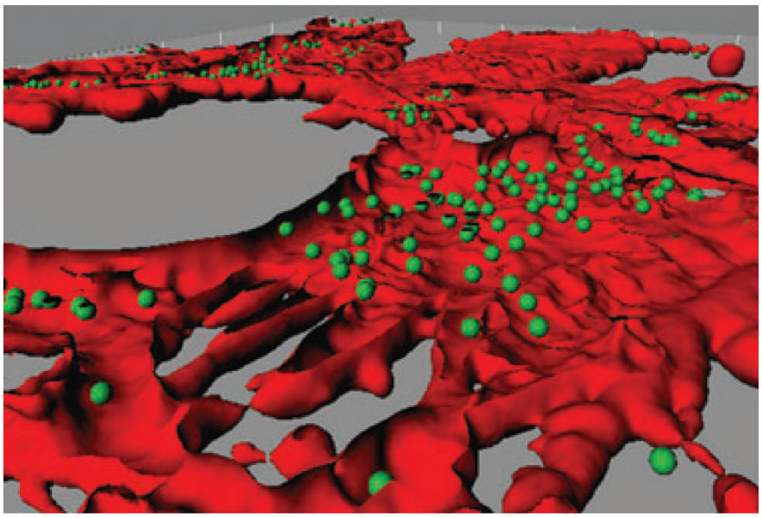

Fig. 1.

Invasion of human epithelial cells with *Porphyromonas gingivalis*. Confocal image of gingival epithelial cells [stained with TRITC-phalloidin (red)] infected with *P. gingivalis* [stained with FITC (green)]. The image was analyzed using Imaris version 5.0.1 software. A Z-stack of the *x*–*y* sections was converted to composite images using the iso surface and spot detection functions of the surpass option. The section view in the *x* and *y* axes was created using the clipping function. The image was generated by Masae Kuboniwa, Osaka University, Japan.

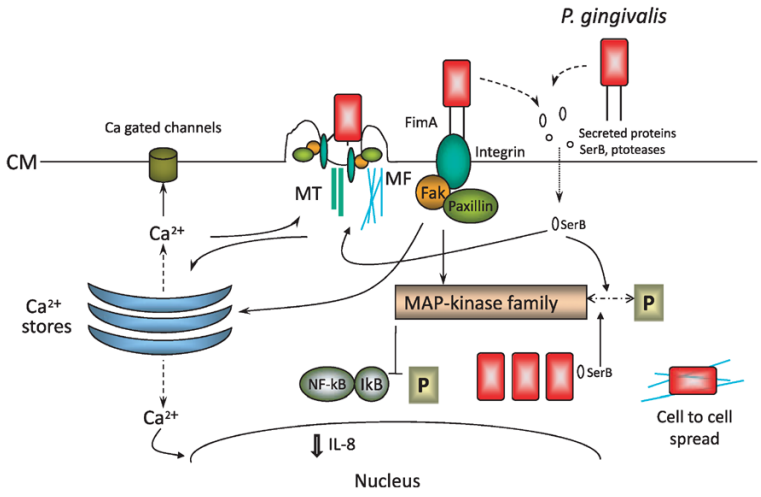

Fig. 2.

Model of interactions between *Porphyromonas gingivalis* and gingival epithelial cells that are associated with internalization. Proximity to gingival epithelial cells induces *P. gingivalis* to secrete proteins such as the SerB serine phosphatase. SerB enters gingival epithelial cells where it dephosphorylates target proteins, including mitogen-activated protein kinase family members, which in turn prevent NF-κB activation. SerB activity culminates in a reduction of interleukin-8 production and in the remodeling of microfilament and microtubule cytoskeletal architecture. Adhesion of *P. gingivalis* is mediated by the long (FimA) fimbriae that engage integrins and induce the formation of focal adhesin complexes and integrin-dependent signaling. Calcium ions (Ca^{2+}) are released from intracellular stores, a signaling event that also funnels through the cytoskeletal structure, and the cytoskeletal re-arrangements allow *P. gingivalis* to enter the host cell. *P. gingivalis* cells rapidly locate in the perinuclear area where they replicate and utilize microfilaments to spread to adjacent gingival epithelial cells. CM, cytoplasmic membrane; IL-8, interleukin-8; IκB, inhibitor of κB; MAP, mitogen-activated protein kinase; MF, micro-filament; MT, microtubule; NF-κB, nuclear factor-κB; P, phosphate.

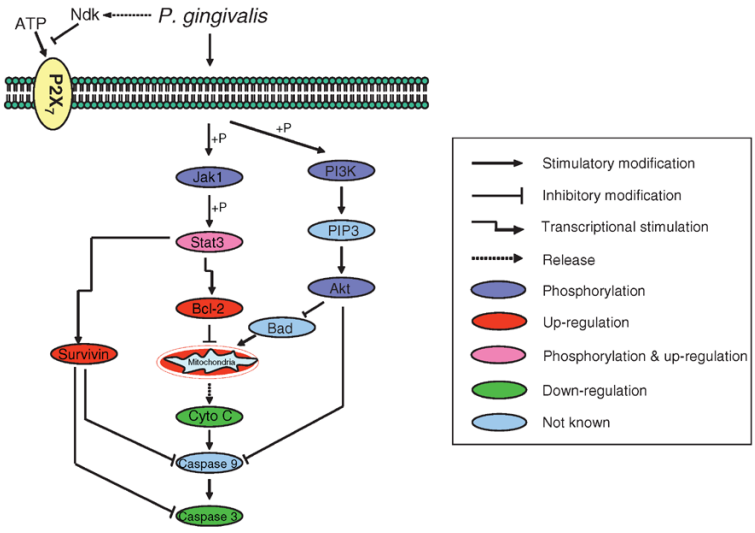

Fig. 3.

Summary of major apoptotic pathways modulated by *Porphyromonas gingivalis* in gingival epithelial cells to suppress apoptotic cell death. Jak1; P; PI3K; PIP3; Stat3.

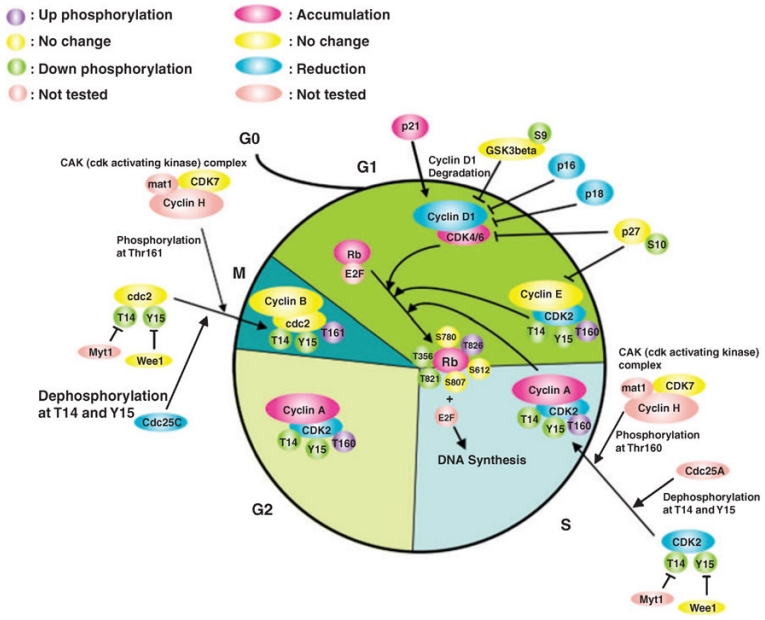

Fig. 4.

Porphyromonas gingivalis impacts cell cycle control in gingival epithelial cells. Schematic representation of cell cycle pathways modulated by *P. gingivalis* infection. The pointed arrow indicates molecular interactions resulting in activation; the flat arrow indicates molecular interactions resulting in inhibition. Reprinted with permission from *Microbes and Infection* 2008, 10:122-128, Copyright Elsevier (70).

Table 1

Invasive periodontal bacteria, invasion effectors and host cytoskeletal requirements for internalization

Species	Invasins	Cytoskeletal requirements
Porphyromonas gingivalis	Major fimbriae (FimA)	Actin polymerization
	Gingipains	Microtubule activity
	Phosphoserine phosphatase (SerB)	
Aggregatibacter actinomycetemcomitans	Fimbriae	Actin polymerization
	Membrane vesicles	
	Extracellular amorphous material	
	Api surface proteins/autotransporters	
Fusobacterium nucleatum	FadA surface protein	Actin polymerization
	Lam adhesin	Microtubule activity
Prevotella intermedia	Type C fimbriae	Actin polymerization
Tannerella forsythia	BspA leucine-rich surface protein	
	S-layer	