[Frontiers in Bioscience 13, 966-984, January 1, 2008]

P. gingivalis interactions with epithelial cells

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1. ABSTRACT

Dental plaque, a microbial biofilm that accumulates on teeth and initiates periodontal disease, is composed of hundreds of different bacterial species within an organized structure. The biofilm bacteria and their byproducts irritate the gingival epithelium and induce an "inflammatory response". The perturbation of epithelial cells by bacteria is the first stage in the initiation of inflammatory and immune processes which eventually cause destruction of the tissues surrounding and supporting the teeth, and ultimately result in tooth loss. This review addresses the early bacterial-epithelial cell interactions and the subsequent responses of the epithelial cell. It includes discussion of how epithelial Toll-like receptors (TLRs) respond to different bacterial challenges, the variable antimicrobial peptides released and the host signaling responses which trigger release of these molecules and the overall fate of these cells in terms of survival, apoptosis, or cell lysis.

2. INTRODUCTION

The primary initiating agent in periodontal disease is dental plaque, a microbial biofilm that accumulates on teeth, composed of hundreds of different bacterial species within an organized structure (1-3). The bacteria in the biofilm and their byproducts accumulate in the gingival crevice and irritate the gingival, inducing an "inflammatory response" (4,5). This early stage in chronic inflammatory periodontal disease, when bacteria perturbate epithelial cells, constitutes the first step in the initiation of the immune response, and is part of the *innate immune* system, i.e. part of the inborn responses that require no prior learning or experience (6,7). These processes eventually lead to destruction of the tissues surrounding and supporting the teeth, including connective tissue and bone, which finally results in tooth loss. Thus, the etiopathogenesis of periodontal disease is complex and involves microbial perturbation of innate, inflammatory and adaptive immune systems and is characterized by

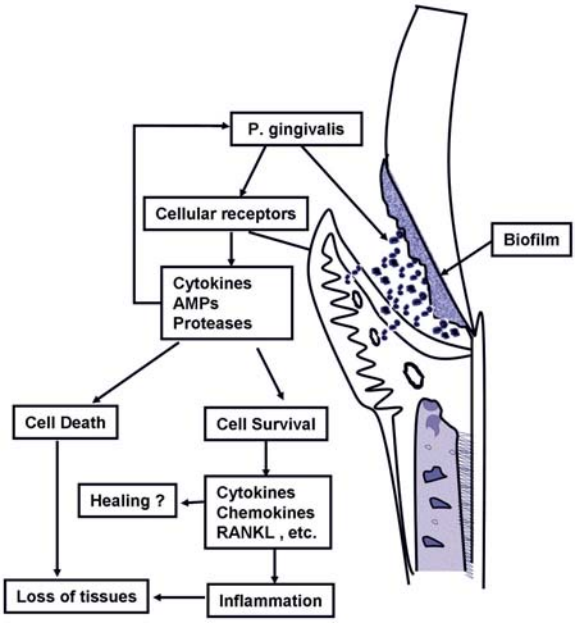

Figure 1. *P. gingivalis* in the dental biofilm interacts with epithelial cells through multiple receptors including the Toll-like receptors, G-protein coupled receptors and others to elicit various responses directed towards the destruction of bacteria (though antimicrobial peptides or AMPs), elimination of infected cells (by apoptosis) or invocation for help (through cytokines and chemokines).

lesions resembling chronic granulation tissue with a full range of inflammatory and immune cells and considerable destruction of structural cells and proliferation of epithelium and granulation tissue in a frustrated healing response (8). This review addresses the early bacterial-epithelial cell interactions and the subsequent responses of the epithelial cell. It includes discussion of the role of epithelial cell Toll-like receptors (TLRs) responding to different bacterial challenges, the variable antimicrobial peptides released in response to these insults and the host signaling responses which produce the cytokine and overall cellular response of these cells in terms of protein secretion, apoptosis or cell lysis (overview presented in Figure 1).

3. EPITHELIAL CELL FUNCTIONS

3.1. Epithelial cells in host defense

Epithelial cells line the body and have a vast array of functions including being a permeability barrier for ingress and egress of everything from ions and molecules to bacteria (9). The outer epithelial cell layer is also the habitat for a multitude of commensal bacteria and is the first physical barrier which pathogenic microbes encounter (9,10). Epithelial cells work in concert to mount inflammatory and antimicrobial responses and may be involved in triggering adaptive immune responses to microbes often through dendritic cells or Langerhans cells located within the epithelial cell layers (11,12). Epithelial cells of the oral mucosa are stratified squamous cells, grouped in layers or strata, and these cells work as a team in their defensive responses. This contrasts with the highly capable neutrophil and monocyte that utilize similar systems and additional receptors and molecules permitting them to function very effectively alone and with other cell types as "professional" defense cells (Figure 2). Thus, the autocrine effects of molecules released by epithelial cells for other epithelial cells are important. These molecules include the chemokines, which act as alarm or calling signals to recruit professional phagocytes and lymphocytes to the region. A multitude of complex activities are undertaken by epithelial cells from: 1) apoptosis, designed to limit the damage of cell lysis by programmed cell death rather than cytolysis where molecules would be released that might harm neighboring cells; to 2) initiation of inflammation and other host defensive responses by proinflammatory cytokine and chemokine release; and 3) immediate killing of microbes by production of antimicrobial peptides and; 4) release of molecules to increase membrane integrity and proliferation to cover potential gaps in the epithelial covering and; 5) induction of adaptive immune responses (against pathogens in conjunction with antigen presenting cells) or tolerance to microbes (commensals for example) that do not negatively affect the host (6,12,13,14).Epithelial cells are constantly bombarded by a vast array of stimuli; from hormones and enzymes to microbes and cytokines. The events that follow stimulation involve a labyrinthine course of upstream and downstream signals that determines whether a cell responds, does nothing, calls for help, mounts an antimicrobial response, proliferates or dies.

3.2. Epithelial innate immunity

It is important to discuss the typical innate immune responses of epithelial cells here and to introduce the important role of TLRs. Innate immunity is now recognized as crucial to the host response, is multi-faceted and may determine subsequent inflammatory and adaptive immune processes. Variability in these processes may explain some of the differences seen in subjects undergoing experimental gingivitis (15) and in susceptibility to chronic inflammatory periodontal disease (16).

Innate immunity represents the inherited resistance to microbial infection and includes specific responses directed and detected by pattern-recognition receptors (PRRs). PRRs are strategically located at the interface between the mammalian host and microbes, and have evolved to recognize conserved microbe-associated molecular patterns (MAMPs) (17). Toll-like receptors are important PRRs and play a central role in the induction of innate immune and inflammatory responses (18,19). Not surprisingly, TLRs are expressed predominantly in cells which mediate the first line of defense such as neutrophils, dendritic cells and monocytes/macrophages and in cells that are directly exposed to the outer environment such as epithelial cells. Distinct members of the TLR family respond to different types of MAMPs, endowing the innate response with a relative specificity (18,19). For example, TLR2 responds to lipoteichoic acid (LTA) and microbial lipoproteins, TLR4 to LPS, TLR5 to flagellin and TLR9 responds to bacterial CpG DNA (18,19). The discovery of TLRs and the identification of their ligand repertoire have prompted the "bar code" hypothesis of innate recognition

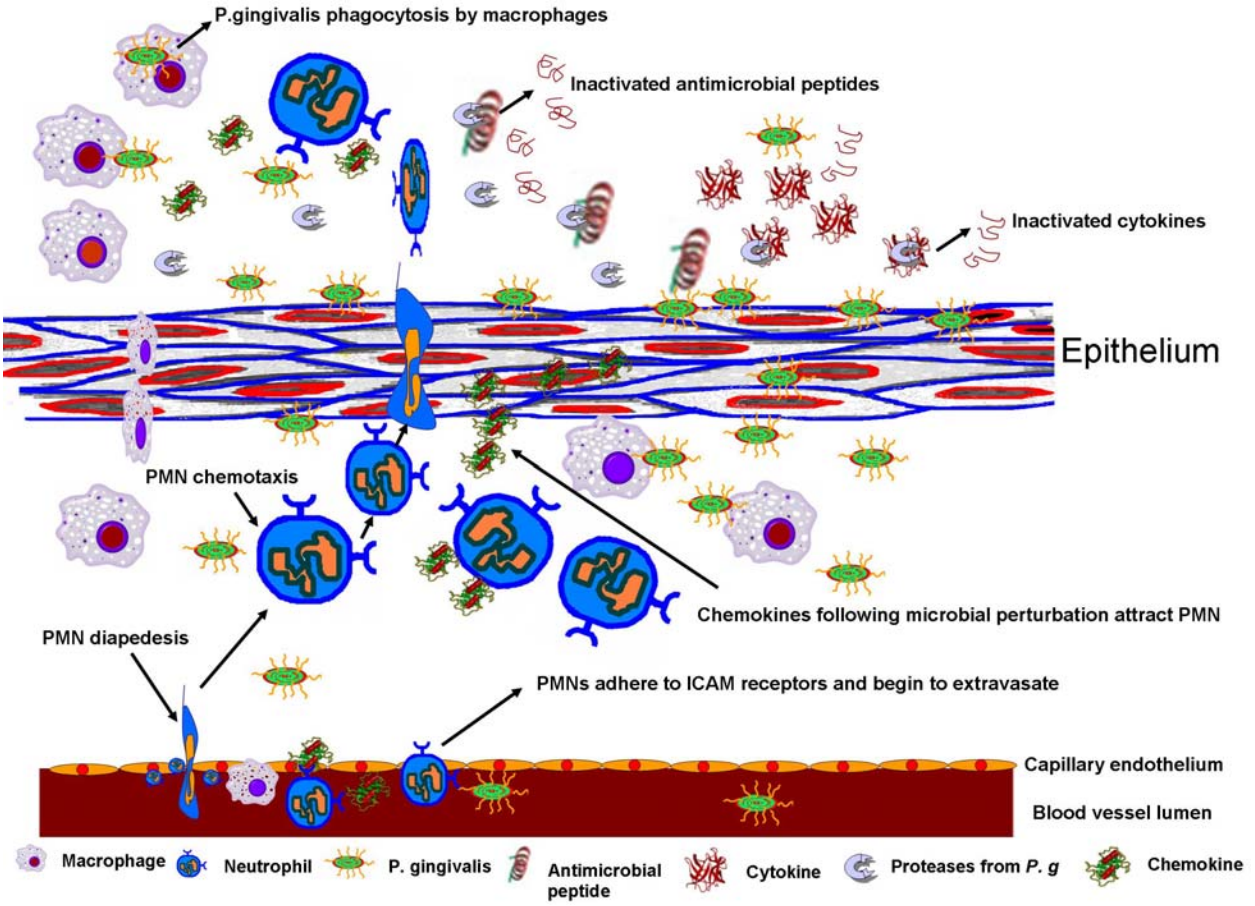

Figure 2. Epithelial cells trigger a cascade of events in response to microbial insult. These oral epithelial cells are stratified squamous layers that can be considered to work as a team in their defensive responses. Thus, the autocrine effects of molecules released by epithelial cells are important. These molecules include the chemokines, which act as homing beacons to recruit professional phagocytes, and lymphocytes to the region. The epithelial cells also produce cytokines and anti-microbial peptides which both trigger inflammation and kill *P. gingivalis*, respectively. Therefore, the balance between the microbial insults and the level of defensive responses by the epithelial cells may have an effect on the prognosis of periodontitis. The collective events happening at the cellular level manifest clinically as inflammation.

of microbes. According to this concept, TLRs read a "bar code" on microbes to tailor an appropriate innate response (20). For instance, simultaneous activation of TLR5 and TLR4 would be interpreted as infection with a flagellated gram-negative bacterium. Thus, the host immune system detects invading pathogens primarily through an array of pattern-recognition receptors which include, but are not limited to, the TLRs.

Lipopolysaccharide (LPS) is a major component of the outer cell wall of Gram-negative periodontal pathogens. It is a key factor in eliciting the inflammatory response that can lead to the diseased state (21) and is considered an important virulence factor in the pathogenesis of periodontal disease. The recognition of LPS by inflammatory cells and the transduction of LPS signal involve the toll-like receptors and several additional molecules, particularly the CD14 receptor and lipopolysaccharide-binding protein (LBP) (22,23). Recently, base-pair changes in both CD14 and TLR4 genes have been described in humans. Arbour *et al.* (24) demonstrated two co-segregating polymorphisms in the extra-cellular domain of the receptor of the human TLR4 gene (Asp299Gly and Thr399Ile). These mutations were found to be associated with decreased airway responsiveness after LPS stimulation and suggest a functional variability that may affect the host response to gram-negative infection. Meanwhile, a C to T polymorphism in the -159 position of the promoter region of the CD14 gene has also been reported (25) and associated with increased circulating soluble CD14 levels and a higher density of the CD14 receptor in monocytes (25,26). Agnese *et al.* (27) have found a significantly higher incidence of gram-negative infections among patients with the TLR4 polymorphisms, but no association between CD14 polymorphism and the incidence or outcome of infection. Periodontal infection is dominated by Gram-negative pathogens, so it is reasonable to hypothesize that any functional polymorphism in LPS-receptors may affect the inflammatory process and the clinical outcomes of

periodontal disease. A word of caution is needed however as there are a wide range of TLR and other receptors capable of responding to various microbial components. In addition, LPS is only one part of the microbial cell wall and numerous studies have shown that responses to whole bacteria are so much greater than to LPS alone, emphasizing this point (28). Furthermore, LPS extracted from different gram-negative bacteria activates different TLRs, and induces different responses (29,30). Recent data has indicated that TLRs may respond not only to bacterial but also non-bacterial challenges, such as oxidized low-density lipoprotein cholesterol (31,32). Thus, the host may respond through inflammation to a wide variety of challenges, ranging from a gram-negative bacterial infection to excess cholesterol (6). However, the nature of the response differs and its character will depend on specific receptors and signal transduction pathways.

Although TLR2 is primarily involved in the recognition of peptidoglycans and lipoteichoic acid of gram-positive bacteria (24,33), it is also involved in the recognition of LPS and other cell wall components of *Porphyromonas gingivalis* (34). Two functional single nucleotide polymorphisms (SNP) at positions 677 (Arg to Trp) and 753 (Arg to Gln) have been identified in the TLR2 gene. These SNPs have been reported to diminish the ability of TLR2 to mediate a response to bacterial components (35). TLR2 can thus respond to several *P. gingivalis* antigens and thus the polymorphic changes in the TLR2 structure may affect the course of periodontitis associated with *P. gingivalis* infection.

3.3. Role of inflammation in the periodontal tissues

The primary etiologic agent initiating the periodontal diseases is the microbial plaque biofilm which accumulates in the gingival crevice. The biofilm bacteria irritate the gingiva and induce an "inflammatory response". The rapid, generalized inflammatory processes that occur in response to challenges constitute an early step in the initiation of the immune response, and are part of the *innate immune* system. Inflammation is a well-coordinated process that involves increased vascular permeability followed by migration of polymorphonuclear leukocytes, monocytes and lymphocytes into the lesion, and activation of cells to secrete inflammatory mediators that guide an amplifying cascade of biochemical and cellular events (36,37). Although inflammation was once considered a nonspecific arm of the immune response, current knowledge suggests remarkable specificity with a wide ranging repertoire of receptors and corresponding ligands are involved. The specific nature of inflammation allows rapid identification and a better tailored response to infection (38) or to other threatening external stimuli (7).

The primary role of the inflammatory cascade is to protect the host against bacterial invasion. The initial recruitment of leukocytes to the infected site is directed by chemoattractants or chemical mediators released from damaged cells. They migrate into the infected site, creating an "inflammatory infiltrate" within the periodontal tissues, close to the colonized tooth root surfaces (5). The adhesion molecules E-selectin and ICAM-1 are involved in the extravasation process of neutrophils out of the blood vessels and through the gingival tissues (39,40,41). In the tissue, the neutrophil follows the gradient of chemokines such as interleukin (IL)-8 produced by epithelial cells following microbial perturbation and crawl towards bacteria using ICAM receptors upregulated on fibroblasts and epithelial cells. Humoral factors, specific antibodies and complement, assist the neutrophils in the protection process, particularly in opsonization and phagocytosis.

This review predominantly focuses on the role of epithelial cells and thus the innate immune system, in host protection against bacteria: but it must be borne in mind that this is only one component of the immune system with the adaptive response also thought to play an important role. The relative contribution of the different arms *viz.* innate, inflammatory and adaptive immune responses is difficult to gauge with our present knowledge base and all must be considered. Recently, the innate and inflammatory systems have gained increasing attention and clearly have enormous influence on the subsequent course of chronic inflammatory periodontal disease. The innate immune system is the main arm available to epithelial cells in their role against bacteria and it contributes with other defensive cells to reduce the causative microbial irritation that initiates and perpetuates destruction of the periodontal apparatus.

Inflammation can be a double-edged sword. On one side, the inflammatory response is protective and aims to eliminate bacterial invasion into the tissues. Once the insult is eliminated, the inflammation resolves, and the subsequent immune reactions diminish. On the other side, in more chronic forms of inflammation, the persistence of excessive inflammatory mediators leads to destruction of the tooth supporting tissues and results in irreversible pathological changes such as those seen in periodontitis. Periodontitis can therefore be considered as one of the chronic inflammatory disorders which include inflammatory arthritis, inflammatory bowel disease and inflammatory skin diseases among others that share similar pathological features. These include inflammatory cell infiltration, granulation tissue formation and the loss of tissue architecture in the affected organ. In periodontal disease, the affected organs include the gingival epithelium and the dental attachment apparatus comprising the periodontal ligament, cementum, alveolar bone, fibers and contiguous connective tissues.

Progression of periodontal disease is due to a combination of environment, host-derived and genetic factors. Among these factors are pathogenic bacteria, high tissue levels of inflammatory cytokines, tissue destructive enzymes (including matrix metalloproteinases) and prostaglandins, and low levels of anti-inflammatory cytokines (40). High levels of inflammatory cytokines such as IL-1β and TNF-α have been described in diseased periodontal tissues (42) and are bioactive (4). These and other cytokines stimulate the production of many mediators that accelerate the inflammatory process. Uncontrolled production of these inflammatory cytokines may contribute to the pathogenesis of the disease. We have to bear in mind

that inflammatory cytokines are essential for clearing some bacterial infections (43): but the same beneficial enhanced response can also induce the tissue destruction observed in chronic inflammatory conditions such as periodontal disease.

4. MICROBIAL CHALLENGE

4.1. Biofilm interactions

Gingival epithelial cells are juxtaposed to the subgingival dental biofilm and thus form an important part of the host's defense against invasive and non-invasive bacteria. Recent research efforts have been geared towards understanding the mechanisms by which gingival epithelial cells modulate bacterial challenges. One bacterial species that has been extensively studied is *Porphyromonas gingivalis*, a gram-negative anaerobe that has been considered a periodontal pathogen for almost 20 years (44). *P. gingivalis* is a frequently isolated member of the unattached subgingival plaque - the most bioactive area of the tooth-biofilm environment (45). Elevated levels of various genotypes of this organism have been detected in periodontitis-affected areas and almost none in healthy sites (46). *P. gingivalis*, together with *Tannerella forsythia* and *Treponema denticola* comprises the red complex of oral bacterial species. Reviews by Socransky (2,47) on the microbial ecology of the periodontium are available for detailed information on this topic. However, the scientific reports on the fate of gingival epithelial cells that come in contact with *P. gingivalis* are few and are somewhat contradictory.

Periodontal disease arises from a polymicrobial infection of the gingival pocket by facultative and obligate anaerobic bacteria and the interactions among the different organisms in this biofilm likely influences the virulence of this microbial community. Diverse mechanisms utilized by *P. gingivalis* and *A. actinomycetemcomitans* modulate the host innate response and these mechanisms may function synergistically to facilitate colonization, persistence and virulence of multi-species microbial populations in the gingival pocket. For example, the reduced induction of IL-12 may also affect the activation of cytotoxic T cells or natural killer cells resulting in reduced clearance of host cells that have been invaded by *P. gingivalis* or *A. actinomycetemcomitans* (48,49). In addition, the antagonism of TLR4-mediated responses by specific isoforms of *P. gingivalis* LPS may function synergistically to reduce the inflammatory response induced by organisms such as *A. actinomycetemcomitans,* whose LPS is a strong agonist of TLR4-mediated signaling.

4.2. Signal transduction features relevant to host sensing of microbes

Agonist-induced activation of the TLR complex initiates a diverse array of intracellular signaling pathways that can dictate both qualitative and quantitative aspects of the host inflammatory response. The fundamental basis of this initial TLR-mediated signal transduction depends upon the association as well as the recruitment of various adapter molecules that contain the structurally conserved TIR-domain. To date, the best-described TIR-containing adaptor molecules that impart specificity to a given TLR signal transduction pathway include MyD88, TIRAP, TRIF, TRAM and SARM (50-53) (Figure 3). In turn, these adaptor molecules provide the necessary framework to recruit and activate downstream kinases and transcription factors that subsequently dictate the nature, magnitude, and duration of MyD88-dependent and MyD88-independent responses (50-53). Much attention has been focused on discerning the molecular differences between the MyD88-dependent and MyD88-independent pathways. It appears that a major mechanism modulating the nature and magnitude of the inflammatory response to a variety of TLR-agonists involves the recruitment and activation of specific kinase pathways. In this regard, most studies identifying and characterizing the regulatory processes that govern the inflammatory response to *P. gingivalis* or associated virulence factors have highlighted the importance of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways.

4.3 Adherence and invasion by *P. gingivalis*

It is estimated that over 700 different species are capable of colonizing the oral cavity (Aas *et al.* 2005) and subgingival plaque serves as a niche for about 300-400 bacterial species; however, less than 50 species are considered to be putative periodontal pathogens (54). Both subgingival residents, *Actinobacillus actinomycetemcomitans* and *P. gingivalis* are considered as major putative periodontopathic bacteria (3). Another 10–20 species are thought to play a role in the pathogenesis of destructive periodontal disease (1) . Among the oral bacteria, *P. gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *A. actinomycetemcomitans* have been reported to efficiently invade oral epithelial cells *in vitro* (55-63). Cell invasion is a well-orchestrated strategy that is considered an essential feature of the pathogenesis of *P. gingivalis* induced periodontal disease. Invasion, however, is preceded by cell adherence. The different factors that play essential roles in the both adherence and invasion processes range from inherent virulence factors like bacterial capsule and fimbriae to the host cell's membrane receptors. How invasion leads to loss of tissue is unknown, although 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 (64). Invasion may be a means of microbial persistence in the gingiva creating a reservoir from which the bacteria can exert detrimental effects through the host defensive responses; however, due to an inability to culture half of the of the bacteria present in the oral cavity, there is still uncertainty as to which bacteria are essential in causing periodontitis and the degree to which the disease process is polymicrobial.

4.4. *P. gingivalis* capsule hydrophobicity and adherence ability

Several polysaccharide capsular serotypes, designated as K-antigen serotypes and K (-) strains are known for *P. gingivalis* (65,66). The prevalence of the capsular serotypes differs across populations (67-69). The

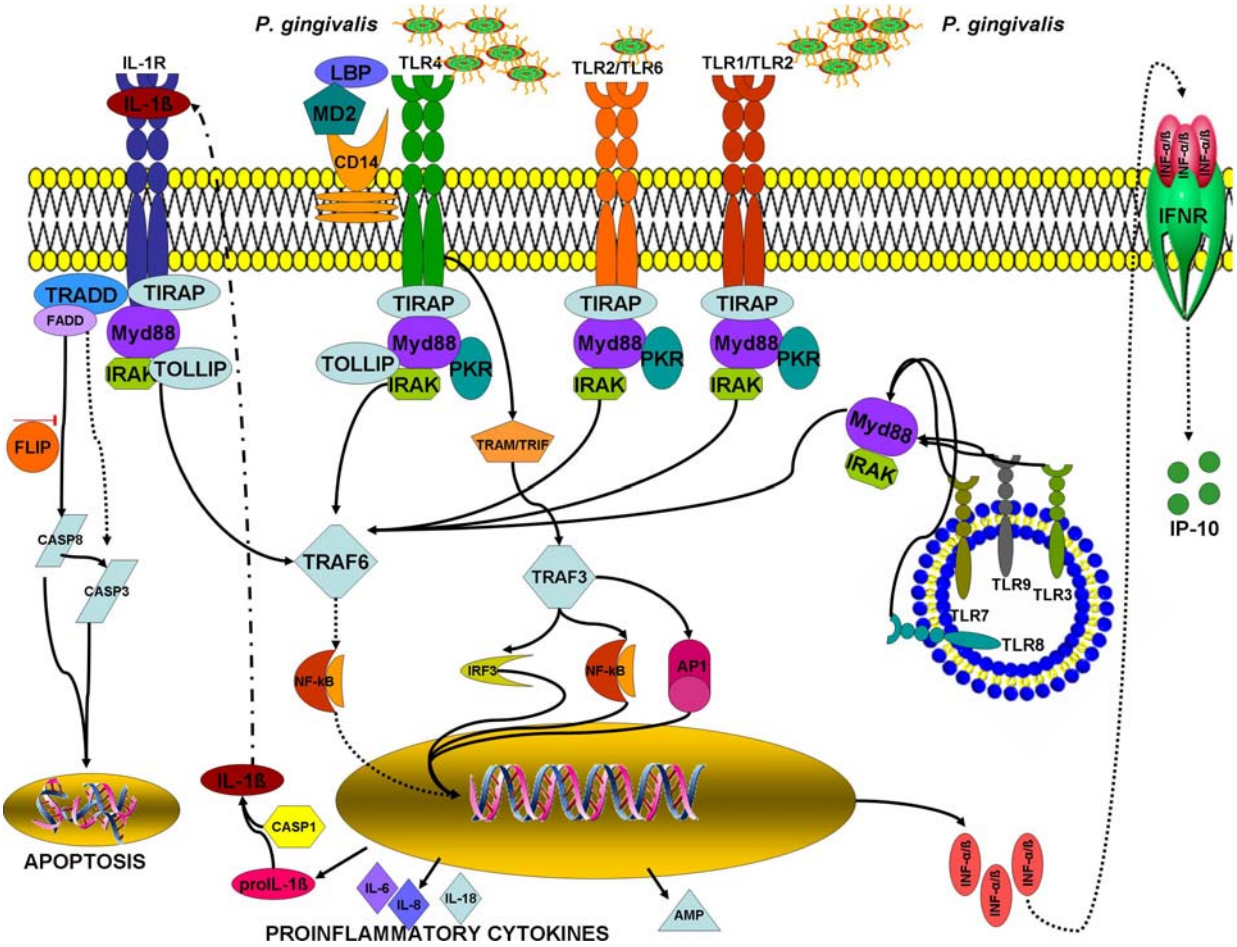

Figure 3. TLR Pathway - TLRs signal after *P. gingivalis* perturbation by MyD88 –dependent and –independent pathways. After sensing their microbial ligand they combine with their adaptor proteins and trigger a series of signals that result in a variety of actions. Primary cytokines like Interleukin-1b are then produced and are released to bind with their specific receptors. This results in the production of secondary cytokines like IL-6 and chemokines like IL-8 or in the activation of the caspase-dependent apoptosis pathway. When the cells survive because of FLIP (FLice/Caspase 8- Inhibitory Protein) activation, the cytokines and chemokines produced can perpetuate their function and the wider spectrum of immunological activity comes into action.

capsular serotypes have been shown to influence the adhesion capacity of *P. gingivalis* in *in vitro* studies. Dierickx *et al.* (70) reported that the non-encapsulated strains adhered more than the capsulated variants. They attributed their results to the hydrophobicity of the non-encapsulated strains that adhere and aggregate better than the capsulated strains (65). However, hydrophobicity varies depending on the surface characteristics of the cell. Various amounts of lipopolysaccharides, different types and numbers of cell surface appendages and capsular material may alter the cell's hydrophobic properties (71-76). Growth conditions and the expression of fimbriae based on the phase condition also influence the overall degree of hydrophobicity (77-79).

In a study on cultured periodontal pocket epithelium cells, the morphological changes associated with cell death occurred faster for mono-layers inoculated with non-encapsulated strains of *P. gingivalis* (80). The authors concluded that dead pocket epithelial cells harbor more *P. gingivalis* cells, with the non-encapsulated strain associating in higher numbers. Consequently, the damage they caused to the host cell occurred faster than in the encapsulated strain. In another study, *P. gingivalis* strains which showed high adherent activity had higher numbers of peritrichous fimbriae on the surface, whereas, fimbriae on the strains showing low adherent ability were barely apparent (81). There is a consensus among the past and the recent studies that the hydrophobicity of *P. gingivalis* leading to adherence on host cells is more likely related to fimbrial expression rather than capsular structure itself (63, 81-85).

4.5. *P. gingivalis* fimbriae, genotypes and varying adhesion/invasion efficiency

Fimbriae are important in the adherence and colonization of *P. gingivalis* (86-89). To date, six *P. gingivalis* fimbrial genotypes (I–V and Ib) have been identified according to the different nucleotide sequences of the *fimA* genes encoding the major fimbriae, FimA (62,

90-93). The *fimA* gene is present in all fimbriated strains but not in afimbriated strains (94) and recombinant fimbriae is less adherent than endogenous fimbriae (95). There is strong evidence supporting a crucial role for FimA in *P. gingivalis* adhesion in mammalian cell types; indeed, non-fimbriated mutants of *P. gingivalis*, constructed by inactivation of the *fimA* gene, displayed reduced adhesion and invasion of epithelial cells compared to wild type *P. gingivalis* (96). A major epithelial cell binding domain of *P. gingivalis* fimbriae is the amino-terminal domain corresponding to amino acid residues 49 to 90 of the fimbrillin protein (88). The other type of fimbriae, the short fimbriae, are not as extensively studied as the long fimbriae (FimA) but they are required for cell to cell interaction and are important in the formation of biofilm (97,98).

The different genotypes of fimbriae relate to the differences in *P. gingivalis* virulence and pathogenecity as evidenced by their varying capacity to adhere and invade the host cell. Among the six variants, *fimA* genotypes I and II seem to possess the greater adhesive ability and invasive efficiency; but whether their invasive efficiency correlates with their adhesive ability still remains unclear. Nakagawa *et al.* (83) examined the abilities of various recombinant FimAs to adhere to human pharyngeal carcinoma epithelial HEp-2 cells and to invade these cells over the course of 6 hours. They found that adhesion and invasion peaked after 4 hours of incubation. In addition, type II rFimA-microspheres adhered most prominently to HEp-2 cells and invaded significantly more than the other strains. A strong conclusion was drawn from the study, stating that *P. gingivalis* strain with type II *fimA* had the greatest abilities to adhere to and invade epithelial cells. Recently, however, Umeda *et al* (85) reported that P. gingivalis type II fimbriae's adhesive ability did not necessarily correlate with its invasive efficiency. *P. gingivalis* strain 33277, a FimA I carrier, was not as adhesive but it was more highly invasive than KdII 865 (FimA II). The authors suggested that other factors beyond adherence promoted by major fimbriae influence the ability to invade epithelial cells.

To test the virulence of *P. gingivalis* Type II fimbriae in epithelial cells, mutants were generated by substituting type I fimA with type II fimA and vice-versa using plasmid vectors (99). The substitution of type I fimA with type II enhanced bacterial adhesion/invasion to epithelial cells, whereas substitution with type I fimA resulted in diminished efficiency. Furthermore, type II clones swiftly degraded cellular paxillin and focal adhesion kinase, and inhibited cellular migration, whereas type I clones and failed to do so. The results show that type II fimA determines the virulence of P. gingivalis.

Epithelial cells function as a physical barrier and in immune surveillance through their ability to elicit an innate immune response. Human gingival epithelial cell (HGECs) express pattern recognition receptors (PRRs) including Toll-like receptors (TLR) 1, 2, 4 and 6 that respond to *P. gingivalis* (100) and in fact possess mRNA for all TLRs except TLR8. *P. gingivalis* FimA is detected by PRRs resulting in the activation of monocytes/macrophages (101,102). In HGECs, however, FimA was not able to elicit a response even in the presence of CD14, an essential coreceptor for TLR-2 mediated response (28). This reflects the different tactics used by *P. gingivalis* when interacting with different host cell types or a host strategy to limit inflammation.

Yilmaz and colleagues (103) reported that *P. gingivalis* fimbriae promote adhesion to gingival epithelial cells through interaction with β1 integrins. Integrins are major adhesion receptors that make transmembrane connections to the cytoskeleton and activate many intracellular signaling pathways (104). The fimbriae-integrin association is thought to represent a key step in the induction of the invasive process and the subsequent cell responses to *P. gingivalis* infection (103).

Studies on bacterial fimbriae offer a much clearer understanding on the role of bacteria and their components in the pathogenesis of periodontitis. However, a certain degree of prudence must be observed in designing research methodologies for growing bacteria. A screening study of *P. gingivalis* clinical isolates revealed that about 60% of the 21 strains studied had fimbriae (105); but whether this actually represents the true percentage before *in-vitro* propagation of the bacteria is not known. Several important environmental factors like growth temperature, absence versus presence of glucose and static versus shaking broth may enhance or repress fimbrial synthesis or influence the degree of fimbriation by up to 90% (106-110). Thus, the outcome of *in-vitro* bacterial culture, regardless of it being a clinical isolate or a laboratory strain, may have an effect on bacterial invasion. As discussed above, bacterial strains with high number of peritrichous fimbriae were more adherent, a virulence factor that is important in the invasion process. Research studies should attempt to closely mimic the oral environment that permits the "normal", uninterrupted growth of bacteria and researchers must relate results within the context of our best knowledge of the actual scenario pertaining in the periodontium. Although the technique for bacterial propagation has not changed extensively over the years, there is a need to describe in detail the methods used in all studies that utilize bacterial cultures. This way, other groups wishing to replicate the studies may religiously follow the materials and methods previously employed. This will minimize conflicting results that could restrict research advances on bacterial interaction with oral epithelial cells.

4.6. Other factors mediating adhesion and invasion efficiency

The interaction of *P. gingivalis* with the oral epithelium triggers the release of a vast array of proteins that mediate adherence or invasion. Proteases have been implicated in the pathogenicity of *P. gingivalis* and gingipain, a predominant member of the proteinase family, is secreted on the surface of *P. gingivalis* (111) and can cripple or negate host defense proteins by various mechanisms (111-115). Gingipain genes *rgpA* and *rgpB* encode Arg-gingipains (Rgp) A and B, respectively and these enzymes possess arginine-specific amidolytic activity, while a third gene, *kgp*, encodes an enzyme with lysine-

Table 1. *Porphyromonas gingivalis* and apoptosis studies

Cell type	Pg strain[1]	Apoptosis	Pathway	References
Oral Epithelial cells	ATCC 33277 ATCC 49417 A7A1-28 W83	Inhibition	JAK/Stat Phosphatidylinositol 3-Kinase/Akt Bcl-2	139 138 137
	ATCC 33277	Induction	NFκB/Fas–FasL	14
	W83 FLL33 V2296	Induction	Not reported	116
Endothelial cells	W83 FLL32	Induction	Caspase-3	119
	FDC 381	Induction	Not reported	129
	FDC381	Induction	Not reported	130
Fibroblasts	ATCC 33277	Induction	Caspase-3	128
	ATCC 53977	Induction	Not reported	127
	A7436	Induction	Not reported	126
Cardiac myoblasts	ATCC 33277	Induction	p38, ERK	215
PMN	W50	Inhibition	Not reported	135
	HG-184 A7A1-28 FDC 381	Late Induction	Not reported	136
	FDC 381	Inhibition	Not reported	134
Lymphocytes	W50	Induction	Not reported	132
Monocytes	ATC33277	Inhibition	ERK- and MAPK-dependent expression of p21	133

[1]Live cells and/or cell extracts

specific amidolytic activity (Lys-gingipain [Kgp]) (116). The adhesin domains of gingipain are considered important for its virulence. Antibody raised against the recombinant adhesin domain of Arg-gingipain A blocked bacterial attachment to epithelial cells (117). In addition, gingipains, especially Kgp, are involved in the degradation of epithelial cell adherens junctions which may facilitate bacterial invasion through cell layers (112). The bacteria can also induce degradation of N-cadherin, a group of glycoproteins responsible for the calcium-dependent cell-to-cell adhesion (118). Gingipains from *P. gingivalis* W83 have also been shown to induce N- and VE-cadherin and integrin b1 cleavage in endothelial cells which cause cell detachment, disruption of cell adhesion and both caspase- dependent and caspase-independent apoptosis (119,120).

P. gingivalis exploits several other ways to fulfill epithelial cell adhesion and invasion. It has been shown to preferentially adhere to shorter chains of human glycolipids which might act as a first step in bacterial invasion process (121). Epithelial cytokeratins may also act as receptors for *P. gingivalis* fimbriae (89) . In addition, fimbriated *P. gingivalis* induces formation of integrin-associated focal adhesions with subsequent remodeling of actin and tubulin cytoskeleton for its accommodation inside the host cell (122) and can selectively target components of the mitogen-activated protein (MAP) kinase pathway, specifically c-Jun N-terminal kinase to complete the invasion process (123). MAP kinases are essential for cell signaling, cytokine responses and cytoskeletal reorganization among others (124).

4.7. Cell fate after *P. gingivalis* invasion

Gingival epithelial cells are among the first line of cellular defenses against *P. gingivalis* infection in the oral cavity. With such an important role, understanding their fate after bacterial invasion could shed more light on the pathogenesis of periodontitis, the most common cause of tooth loss among adults. As discussed above, *P. gingivalis* gains entry into the cell with the help of inherent virulence factors like fimbriae and proteases and through several other factors such as host cell receptors and surface glycolipids. However, the cascading events that happen after invasion of gingival epithelial cells are not as extensively explored as in other cells like macrophages and endothelial cells or in other tissues like the intestines. Despite limited scientific literature, the fate of epithelial cells after invasion could be dichotomized generally into inhibition or induction of apoptosis.

Kerr *et al* 1972 (125) first described the two forms of cell death, necrosis and apoptosis. Since then, the number of articles describing the two processes has significantly surged into a combined number of more than 320,000 (Pubmed search). The effect of *P.gingivalis* on apoptosis in several different cell types has been extensively studied (Table 1). Although the literature is consistent regarding apoptosis induced by whole bacteria or *Porphyromonas gingivalis* components in fibroblasts (126-128), endothelial cells (119,120,129,130), cardiac myoblasts (131), lymphocytes (132), monocytes (133) and PMNs (134-136), a controversy still exists regarding epithelial cells. In agreement with the studies in PMNs, monocytes and macrophages, Nakhjiri *et al* (137), Yilmaz *et al* (138) and Mao *et al* (139) have shown inhibition of apoptosis, while in agreement with the studies in fibroblasts, endothelial cells, cardiac myoblasts and lymphocytes, Chen *et al* (118) and Brozovic *et al* (14) have shown induction of apoptosis in epithelial cells.

Live *Porphyromonas gingivalis* ATCC 33277 at an MOI:100 and MOI:1000, was shown to induce transient DNA fragmentation at 2 hours but apoptosis was no more evident at 24 hours. Furthermore, infection with *Porphyromonas gingivalis* provided resistance to camptothecin-induced apoptosis. The anti-apoptotic molecule Bcl-2 and the pro-apoptotic Bax correlated with the results at the mRNA and protein level, (137). The expression of pro-apoptotic molecule Bax was transiently elevated but declined after 24 hours. In contrast, the anti-apoptotic molecule Bcl-2 was up-regulated by *P. gingivalis* only after 24 hours of stimulation. On the other hand, the

anti-apoptotic Bcl-x_l and the pro-apoptotic Bcl-x_s failed to show any correlation to the apoptosis detected by histone-associated DNA fragment ELISA. In another related study, the activation of phosphatidylinositol-3/Akt pathway, a known inhibitor of apoptosis, contributed to the survival of primary epithelial cells 24 hours post-infection with *P. gingivalis* 33277 at an MOI:100 and inhibition of staurosporine –induced apoptosis (138). SiRNA blocking of Akt and JAK1 abrogated the ability of *P. gingivalis* to block apoptosis suggesting that *P. gingivalis* can block apoptotic pathways in gingival epithelial cells through manipulation of the JAK/Stat pathway that controls the intrinsic mitochondrial cell death pathways. This effect on apoptosis was dose dependent and strain independent (139).

On the other hand, protease-active extracellular protein preparations from *Porphyromonas gingivalis* W83 and isogenic mutants with different levels of proteolytic activity were shown to induce apoptosis in human epithelial KB cells, as determined by microscopic observation of the apoptotic morphology. The percentage of apoptotic cells at 48 hours correlated with the proteolytic activity of the bacterial strain (118). Inaba *et al.* (140) investigated epithelial cell death and invasion using microspheres conjugated to *P. gingivalis* vesicles (vcMS). Apoptotic cell death was induced by vcMS at six hours in *P. gingivalis*-invaded cell. Heat-killed *Porphyromonas gingivalis* ATCC 33277 at an MOI:100 was also shown to induce apoptosis in primary human gingival epithelial cells in a time-dependent manner, as determined by DNA fragmentation and caspase-8 and -3 activation. Apoptosis was more than two-fold higher than the control at 24 hours, and it was induced through Fas-FasL upregulation (14). This was supported by a similar study on gingival fibroblasts that showed an activation of caspase-6, caspase-7, caspase-9 and caspase-3 starting at around 24 hours, coincident to apoptotic DNA damage (128). In addition, the invasion process needed a high MOI for optimal infection and plateaued at MOI 200. DUTP-biotin nick end labeling (TUNEL) kinetics showed that *P. gingivalis* apoptosis is a late process in infection and that mutant *P. gingivalis* is less proficient in this regard (128).

The later studies suggesting induction of apoptosis in epithelial cells by *P. gingivalis* are in agreement with several *ex vivo* studies in which apoptosis has been detected *in situ* in human gingival tissue at sites of chronic bacterially induced inflammation (141,142). The majority of the apoptotic activity is evident in the superficial layers of the junctional epithelium (142) and within the connective tissue (141,142), and is expressed in epithelial cells, fibroblasts and inflammatory cells. Caspase-3 and -7, two major effector caspases associated with apoptosis, were also shown to be activated to a higher extent in tissue homogenates from patients with chronic periodontitis than in healthy tissue (143) and this increased caspase activation was detected *in situ* in inflamed gingival biopsies. A considerable number of cells in the gingival epithelium and connective tissue also revealed active caspases, whereas in healthy tissue almost no caspase activation was observed. These results suggest that caspase activation may be functionally involved in periodontitis-associated tissue damage. To correlate their findings with the role of *P. gingivalis* in apoptosis, the authors co-incubated HaCat cells with *P. gingivalis* strain MccM 527. A 20 to 30 percent reduction in cell viability was seen, but the characteristic membrane blebbing or chromatin condensation seen in apoptotic cells was only noted after *P. gingivalis*-infected mononuclear cells were seeded into the culture wells.

The fate of gingival epithelial cells after bacterial invasion presents a challenging topic that still needs further investigation. Periodontitis is a chronic disease that initiates after a long-standing and continuous inflammatory insult to the tissues that has overcome the immune system. Different studies employ different research methodologies, but the peculiar environment that favors the propagation of *P. gingivalis* allowing it sufficient time and continuity to infect cells should be kept in mind.

5. EPITHELIAL DEFENSES

5.1. Anti-microbial peptides

To maintain health, the mucosal secretions of oral epithelia contain multiple anti-microbial proteins that act as early host-defense factors in response to microbial challenges. Saliva is a complex mixture of protein exudates from salivary glands and contains several antimicrobial peptides. Recently, Abiko *et al* (144) have reviewed defensins in saliva and salivary glands and speculated that the majority of antimicrobial peptides that are present in saliva are also secreted by oral keratinocytes. Over 800 eukaryotic antimicrobial peptides (AMP) have been identified and are accessible in antimicrobial peptide databases. Defensins typically share a few key amino acids that are needed for overall structure, but otherwise, they vary greatly between the different family members ranging from 5 to 50 kilo Daltons with a net positive charge (145,146). It is thought that this diversity of antimicrobial peptides allows the innate immune system to respond effectively to a wide range of microorganisms. Moreover, antimicrobial resistance may be less likely to develop when the host responses involves multiple antimicrobial proteins to a single pathogen.

Recent studies indicate that numerous antimicrobial proteins like neutrophil defensins, beta defensins, lysozyme, BPI, BPI-like proteins, histatins, proline-rich proteins, cathelicidin LL-37, cystatins, mucins and secretory leukoproteinase inhibitors (SLPI) are found in the oral cavity (147-156). These antimicrobial protein families represent a variety of antimicrobial functions including membrane permeabilization, cell wall degradation, bacterial oxidation and others (157,158). Several models have been proposed for the mechanism of action of these antimicrobial peptides. The cationic peptides interact with negatively charged phospholipid groups on the outer membrane of the microbial target cells via electrostatic attraction (159). According to "Shai-Matsuzaki-Huang model", defensins destroy microbes by disrupting the membrane integrity (159). Apart from bacteria, human defensins have been shown to have activity against fungi, viruses and protozoa (160,161). Recent

evidence suggests defensins also have chemoattractant properties for cells expressing the chemokine receptor CCR-6, such as dendritic cells (162). This suggests that defensins play a crucial role bridging innate and adaptive immune systems (163,164).

Cationic proteins constitute a large group of antimicrobial proteins that represent different antimicrobial activities. Their importance is illustrated by the fact that depletion of cationic proteins in body fluids also removes antibacterial activity (165). The different antimicrobial functions of cationic peptides are represented by: i) defensins, cathelicidin and PLUNC that can interact directly with the bacterial cell membrane leading to its permeabilization (166); ii) lysozyme, which cleaves the peptidoglycans of bacterial cell walls leading to membrane rupture and; iii) the antifungal histatins that bind to a fungal cell membrane receptor to enter the cells and block mitochondrial function (167).

Both the overexpression and the lack of antimicrobial peptides have been linked with the development of oral diseases. Defensin expression is induced by oral bacteria (168) and the levels of defensin-1 are significantly higher in patients with oral inflammation than in normal controls (169). A typical example to illustrate the important role of AMP is morbus Kostmann disease, a congenital neutropenia that is associated with recurrent infections and periodontal disease and deficiencies in LL-37 and α-defensins HNP1-3 (170). The finding that one morbus Kostmann patient who had undergone bone marrow transplant had normal levels of LL-37 and no periodontal disease underscores the potential role of antimicrobial peptides in host defense of the oral cavity (170). *P. gingivalis*, however, has been shown to be resistant to human derived LL-37 and dhvar4a antimicrobial peptides (171). LL-37 peptide is more sensitive to bacteria as it has higher protein binding potential and lower hydrophobicity compared to hBD-2 (172).

Evaluating the levels of antimicrobial peptides may have diagnostic value. Low levels of HNP1-3 correlated with high caries incidence in children. However, other antimicrobial peptides (e.g. hBD-3, LL-37) did not correlate with caries incidence (173). While these single protein deficiencies are linked to specific diseases, they are different from other conditions like xerostomia in Sjögren's syndrome where rampant oral infections and dental decay occur as a result of the depletion of all salivary proteins. This difference emphasizes the importance of complementarity in the mucosal innate immune defense (158). With a more comprehensive understanding of the antimicrobial proteins that act in the oral cavity, it is possible that protein expression signatures ("fingerprints") can be developed for the diagnosis of individual oral diseases or the identification of at-risk individuals, prior to the development of the disease. Several families of antimicrobial proteins have been extensively studied and can be reviewed elsewhere (157,167,174-176).

Synthetic antimicrobial peptides hBD-1, 2, 3 and LL-37 were tested against oral pathogens and cariogenic bacteria (177). *F. nucleatum* was found to be highly susceptible to hBD-2 and -3 while *S. mutans* was highly susceptible to hBD-3. In another related study, aerobes were 100% susceptible to HBD-2 and HBD-3, whereas only 21.4 and 50% of the anaerobes were susceptible to HBD-2 and HBD-3, respectively (178). Antimicrobial activity of synthetic human BD-2 against *A. actinomycetemcomitens, P. gingivalis* and *S. mutans* were tested by antibacterial broth assay and diffusion assay (179). It was observed that the antimicrobial activity of hBD-2 was approximately equal to that of minocycline at equimolar concentrations. The hBD-3 was shown to have bactericidal activity on oral bacteria such as *S. mutans, S. sanguinis, S. sobrinus, L. acidophilus, A. actinomycetemcomitens* and *P. gingivalis* (180).

Recently, adrenomedullin, a peptide expressed in oral epithelial cells was also claimed to be antimicrobial in function (181,182). Adrenomedullin was also found to be present in gingival crevicular fluid in the range of 1-2 µg/mL (183). However, the concentration of this antimicrobial peptide and its role in periodontal disease is still debatable and the lack of sensitivity to adrenomedullin may enable *P. gingivalis* resistance (184). Other peptides like cathelicidins (SMAP29 and CAP18) also have been shown to exhibit antimicrobial activity against *P. gingivalis* (185). Cysteine proteases, cystatin and cystatin derived peptides also displayed antimicrobial properties (186).

5.2. BPI-like proteins

Several of the human Bactericidal/Permeability-Increasing or BPI-like proteins correspond to previously identified animal proteins including parotid secretory protein (PSP) (187), palate, lung and nasal epithelium carcinoma associated protein (PLUNC) (Weston *et al.*, 1999), bovine salivary proteins (BSP)30 (188) and von Ebner's minor salivary gland protein (VEMSGP) (Genbank# U46068). The human proteins have been termed the PLUNC family (189) or BPI-like proteins (190) BPI is an antibacterial protein with selectivity for Gram-negative bacteria. In addition, BPI binds lipopolysaccharides and exhibits anti-inflammatory activity by inhibiting the binding of LPS to LBP (191). The molecular structure has been elucidated and consists of two BPI domains, BPI1 and BPI2 (192). This structure formed the basis for the identification of the BPI-like proteins.

The BPI-like proteins are typically either about 250 amino acids in length or more than 450 amino acids. The predicted structure of these proteins is related to the BPI structure, containing one or two of the BPI domains. As an example, PSP and PLUNC are similar to the N-terminal BPI1 domain of BPI while the longer VEMSGP and BPIL2 contain both a BPI1 and BPI2 domains. As is the case in other families of antimicrobial proteins the sequence conservation among BPI-like proteins is poor, with the exception of two conserved Cys residues. These residues are also found in BPI where they form a disulfide bridge.

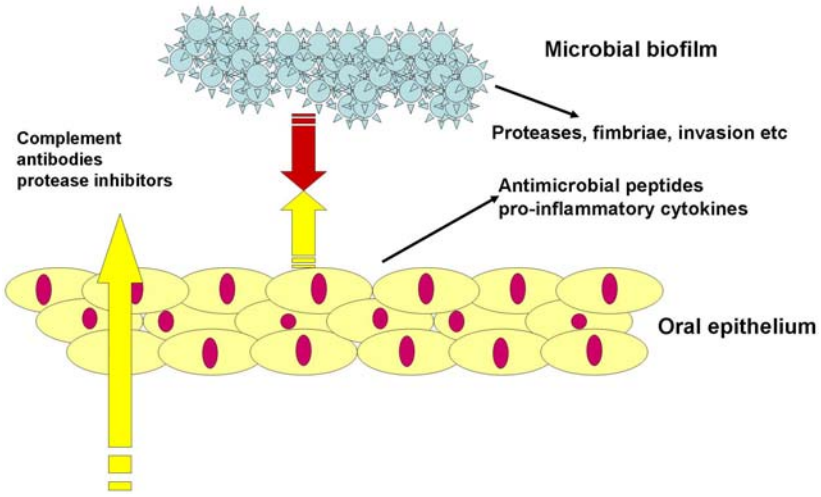

Figure 4. Model for microbial plaque and epithelial cell interactions in the initial stages. In later stages neutrophils, macrophages and adaptive immune responses predominate.

5.3. Expression of BPI-like proteins

The expression of BPI-like proteins in human gingival keratinocytes is influenced by oral bacteria such as *P. gingivalis* and pro-inflammatory cytokines such as TNFα and IL-1b (193). In the airways, PLUNC expression is upregulated by retinoic acid (194) and expression has been linked to epithelial injury, irritation and cancers (195-197). In contrast, PLUNC expression is downregulated in nasopharyngeal carcinoma (198), in some smokers (199) and in seasonal allergic rhinitis (200).

5.4. Function of BPI-like proteins

The differential expression of PLUNC in epithelial injury and irritation suggests that PLUNC or the other BPI-like proteins could play a role in inflammation. Consistent with this suggestion, PLUNC binds to LPS (201). Indeed, Geetha *et al* (202) found that peptides based on the predicted PSP structure block the binding of LPS to LBP and block the LPS stimulated secretion of TNFα from macrophages. In addition, intact PSP is antibacterial to *P. aeruginosa* and preliminary data suggest that PSP peptides are antibacterial to Gram negative but not Gram positive bacteria (203). Antibacterial and anti-inflammatory activity has also been reported for human PLUNC (204).

5.5. Mechanism of antimicrobial peptide induction

The molecular mechanism of antimicrobial peptide regulation in oral epithelia is not fully understood. The involvement of TLR4 in dendritic cells (205), TLR2 and TLR4 in intestinal epithelial cells (206) and IL-1R in human epidermal cultures (207) have been reported. It has also been demonstrated *that P. gingivalis* induces cytokine responses and antimicrobial peptide secretion (208). Recently, hBD-2 induction has been associated with the Mitogen Activated Protein Kinases (MAPKs) signaling pathway in gingival epithelial cells (209) with JNK in PC12 and mesangial cells (210,211). It has also been shown that different bacteria regulate the expression of antimicrobial peptide by differentially activating either NF-kB, p38 MAPK or JNK signaling pathways (212).

The periodontal disease niche is an environment in which the bacterial population is present as a biofilm adherent to the tooth substance within the gingival crevice, with constant exposure to serum and tissue derived gingival crevicular fluid. A model could be proposed based on current knowledge wherein the bacterial biofilm constantly produce proteases and the host produces locally antimicrobial peptides, and the whole area is bathed in serum transudate that contains a vast range of molecules including protease inhibitors (4,213,214) and other systemic host defense molecules such as antibodies and complement. Clearly there is balance between pathogen and the host as in the case of Crohn's disease (164) (Figure 4) but upsets in this balance could lead to exacerbations which would be manifested as local abscesses or periods of tissue breakdown and this is consistent with the episodic nature of periodontal destruction. The biofilm produces proteases ostensibly to help garner host tissue molecules for nutrition but also to disrupt host attack on the biofilm through antimicrobial peptides and systemically complement and antibodies. The release of cytokines by the epithelial cells as well as chemokines and chemotactic molecules produced by bacteria and from complement ensures the ingress of phagocytes to further attack the host biofilm. The professional phagocytes are a further important aspect, not however within the remit of this review, but must be borne in mind when considering the complete periodontitis lesion host microbe interaction.

The shedding of bacteria from the biofilm that then attach to the host epithelial barrier and undertake invasion, apoptosis, cell killing etc is a complex additional interaction that current knowledge implies we consider although no definitive *in vivo* proof currently pertains. Thus the initial outcome of the microbial-host conflict is dependent on the magnitude and nature of both the microbial biofilm challenge and the cytokine and antimicrobial peptide secretion by epithelial cells and in the later stages by phacocytes and adaptive immune responses.

6. CONCLUSION

The cellular and soluble defenses of the oral cavity are engaged dynamically in a continuous response to microbial challenges. *P. gingivalis* interacts with oral epithelial cells through TLRs and from there, various responses are elicited including: cytokine and antimicrobial protein secretion, apoptosis, necrosis and others. These responses are directed towards the microbial biofilm and trigger a plethora of local and systemic reactions (*viz.* recruitment and development of inflammatory and immune cells), directed towards eliminating the source of infection and minimizing and resolving cellular or structural loss. The epithelial cells, being part of the innate immune system, possess a natural ability to ignore, fight against offending microorganisms, and/or call for help via the cytokines and chemokines as needed.

7. ACKNOWLEDGEMENTS

This review was supported by a grant NIH (NIDCR) DE017384 to Dr. Kinane.

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Key Words: Epithelial Cell, innate, *P. gingivalis*, Toll-like Receptors, Apoptosis, Review

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