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Periodontal Disease and Gingival Innate Immunity – Who Has the Upper Hand?

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1. Introduction

Dental plaque is a complex microbial biofilm that forms at high cell density in the oral cavity by the successive accumulation of hundreds of different species of bacteria. Both host immune and bacterial factors are involved in the progression from healthy to diseased state in plaque biofilm, and in the oral cavity, gingival epithelial cells (GECs) are one of the first host cell types that encounter colonizing bacteria. As a consequence, GECs respond to the presence of bacteria through an elaborate signaling network, producing antimicrobial peptides (AMPs) and cytokines, leading to host innate immune responses. Periodontal disease is a consequence of the imbalance between the pathogenic potential of the biofilm and host immune defense properties, resulting in an inflammatory reaction of the periodontium. As a part of host defense mechanism, GECs secrete specific endogenous serine protease inhibitors to prevent tissue damage from excessive proteolytic enzyme activity due to inflammation. Recent studies showed GECs induced different serine protease inhibitors in the presence of non-pathogenic bacteria, but these protease inhibitors were attenuated by periopathogens, whose main virulence factors are proteases. Furthermore, periodontal patients with periopathogens present in their plaque exhibited significantly lower protease inhibitors in gingival crevicular fluid in comparison to healthy controls. The degradation of protease inhibitors by periopathogens may result in decreased host protective capacity, and the balance between cellular protease inhibitors and their degradation by periodontal pathogens may be an important factor in susceptibility to breakdown from chronic infection. In addition to bacterial infection, genetic and environmental factors contribute to occurrence and progression of periodontal disease. Recent studies suggest that the manifestation and severity of periodontal disease may be influenced by epigenetic factors. Many patients with the same clinical symptoms respond differently to the same therapy, suggesting the inter-individual variability observed as a clinical outcome of the disease is influenced by genetic as well as epigenetic factors.

In this chapter, we will closely examine the mechanisms gingival epithelia utilize in inducing AMPs in response to bacterial presence and assess future therapeutic potential of AMPs. We will also focus on the impact the balance between the proteases and protease inhibitors has on oral health and how epigenetic modifications brought on by exposure to periodontal pathogens affect the progression of periodontal disease.

2. Microbial biofilm and innate immune responses of gingiva

Dental plaque is a complex microbial biofilm that forms at high cell density on tooth surfaces in the oral cavity by the successive accumulation of over 500 different species of bacteria (Kolenbrander, Andersen et al. 2002; Rickard, Gilbert et al. 2003). The early colonizers of the tooth surface are mainly non-pathogens comprised of Gram-positive facultative organisms, including *Streptococcus gordonii*, *Streptococcus sanguis* and *Streptococcus oralis*. These initial colonizers adhere to salivary pellicle on teeth, leading to successive colonization of Gram-negative anaerobes such as *Fusobacterium nucleatum* and finally to pathogens such as *Porphyromonas gingivalis*. The formation of plaque has been linked to the human oral diseases, caries and periodontitis (Socransky, Smith et al. 2002; Socransky and Haffajee 2005), and both host immune and bacterial factors are involved in the progression from healthy to diseased state in plaque biofilm.

Periodontitis is one of most common inflammatory diseases and can be of inflammatory, traumatic, metabolic, developmental and/or genetic origin. In most cases, periodontal disease results in an inflammatory reaction of the periodontium to pathogenic microorganisms. Among various species of microorganisms making up oral biofilm that accumulates on the tooth surface adjacent to the gingiva, Gram-negative anaerobic bacteria *P. gingivalis*, *Tannerella forsythia* and *Treponema denticola* in particular have been strongly associated with periodontal disease (Socransky, Haffajee et al. 1998; Armitage 1999; Socransky and Haffajee 2003). Bacteria first form a supra-gingival biofilm attached to the tooth surface, and once they have passed the junctional epithelium, bacteria may enter the gingival crevice to form sub-gingival biofilm, which provides an optimal environment for anaerobic bacteria to colonize and reproduce (Socransky and Haffajee 2003). The number of Gram-negative anaerobic bacteria increases during development and maturation of the dental biofilm. Both host immune and bacterial factors are involved in the progression from healthy to diseased state in plaque biofilm, thus periodontal disease is the result of the imbalance between the pathogenic potential of the biofilm and host immune defense properties. In addition, genetic and/or environmental factors, such as smoking, contribute to occurrence and progression of periodontal disease (Michalowicz, Aeppli et al. 1991; Michalowicz, Diehl et al. 2000; Kinane and Hart 2003; Loos, John et al. 2005).

P. gingivalis is an aggressive pathogen and considered an etiologic agent of severe adult periodontitis. Colonization of the oral cavity by *P. gingivalis* is facilitated by adherence to various oral surfaces, including epithelial cells, the salivary pellicle that coats tooth surfaces, and other oral bacteria that comprise the plaque biofilm (Socransky and Haffajee 1992). However, *P. gingivalis* is considered a secondary colonizer of plaque and rarely colonizes the tooth surface until initial plaque bacteria, such as *S. gordonii*, establish an appropriate environment. Adhesion between *S. gordonii* and *P. gingivalis* is mediated by *S. gordonii* cell-surface protein SspB and *P. gingivalis* minor fimbriae (Chung, Demuth et al. 2000). In the oral cavity, gingival epithelial cells are one of the first host cell types that encounter colonizing bacteria. As a consequence, epithelial cells respond to the presence of bacteria through an elaborate signaling network, producing antimicrobial peptides and cytokines, and at times stimulating apoptotic cell death. This bacterial-host communication takes place via a number of signal transduction pathways, but different bacteria may induce different signals from the host. Conversely, various host immune responses may interfere with the way commensals and pathogens communicate to form biofilm, although this means of defense is poorly understood.

Periodontal disease is of importance not only in oral health, but also in general health because of its association with an increased risk of preterm births and low birth weight babies (Offenbacher, Katz et al. 1996; Buduneli, Baylas et al. 2005). Thus, it is of importance to understand how oral bacteria alter host innate immune responses and how periodontal disease is affected by protective factors induced by the host.

3. The role of Antimicrobial Peptides in periodontal health

3.1 Antimicrobial Peptides (AMPs)

In the presence of diverse environment of microbial consortiums, epithelia express several natural antimicrobial peptides (AMPs) which work synergistically with a broad spectrum of activity against both Gram-negative and Gram-positive bacteria, as well as against yeast and some virus to maintain balance between health and disease (Hancock and Chapple 1999; Lehrer and Ganz 2002; Premratanachai, Joly et al. 2004). AMPs are small cationic peptides with molecular weights typically ranging between 3,500 and 6,500 Da (Dale 2002). They adopt amphiphilic topologies, which allows them to interact and selectively disrupt microbial cell membranes (Som, Vemparala et al. 2008). In humans these antimicrobial peptides include defensins and a cathelicidin family member LL-37 in skin and oral mucosa and other epithelia (Hancock and Scott 2000; Lehrer and Ganz 2002; Selsted and Ouellette 2005). The human defensins include the alpha-defensins of intestinal and neutrophil origin, and the beta-defensins of skin and oral mucosa and other epithelia. Alpha-defensins are expressed in neutrophils as part of their non-oxidative antimicrobial mechanisms (Lehrer, Lichtenstein et al. 1993; van Wetering, Sterk et al. 1999). Alpha-defensins are also found in Paneth cells in the intestine (Selsted 1992; Ouellette 1999). They are synthesized as precursors that are proteolytically activated and released during inflammation (Rock 1998; Wilson, Ouellette et al. 1999). The human beta-defensins (hBDs) are small, cationic antimicrobial peptides made primarily by epithelial cells and expressed in all human epithelia tested to date (Dale 2002). The beta-defensins are secreted in biological fluids, including urine, bronchial fluids, nasal secretions, saliva and gingival crevicular fluid (Valore, Park et al. 1998; Cole 1999; Sahasrabudhe 2000; Diamond, Kimball et al. 2001). hBDs were first identified in tracheal epithelial cells and subsequently found in many epithelia including kidney and urinary tract, oral mucosa and skin (Diamond, Russell et al. 1996; Zhao, Wang et al. 1996; Krisanaprakornkit, Weinberg et al. 1998; Valore, Park et al. 1998).

The expression of the cathelicidin, LL-37, is found in human tongue, buccal mucosa and saliva following inflammatory stimulation (Frohm Nilsson, Sandstedt et al. 1999; Murakami, Ohtake et al. 2002). It is kept inactive until proteases cleave the conserved proregion (Zanetti, Gennaro et al. 2000). Immunohistochemistry studies found that LL-37, derived from neutrophils, was detected in the junctional epithelium (Dale, Kimball et al. 2001). The defensins and LL-37 are localized in different sites in gingiva, which suggests that they may play different roles in specific sites in which they are expressed (Dale, Kimball et al. 2001). Because these AMPs have synergistic effects, their presence in saliva may provide natural antimicrobial barrier (Tao, Jurevic et al. 2005). Different sites within the oral cavity where various AMPs are predominantly expressed are depicted in Figure 1.

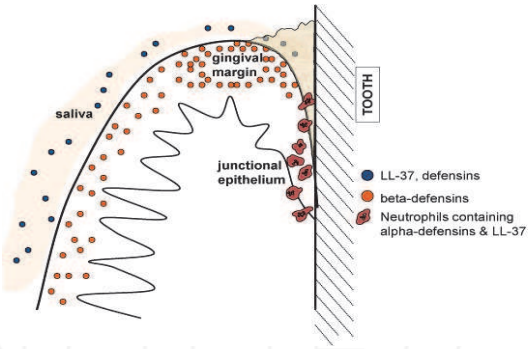

Fig. 1. Various sites in the oral cavity where different AMPs are predominantly expressed. Dale and Fredericks 2005; permission from Horizon Scientific Press

3.1.1 Alpha-defensins

Alpha- and beta-defensins are peptides with six disulfide-linked cysteines. Structurally, the difference between the two defensins lies within the length of peptide segments between the six cysteines and pairing of the cysteines (Bals and Wilson 2003). Six different human alpha-defensins have been identified so far, including four human neutrophil peptides, HNP1-4, and two others known as human defensins 5 and 6 (HD-5, HD-6) (Ganz, Selsted et al. 1985; Ganz and Lehrer 1994; Cunliffe 2003). Alpha-defensins are arginine-rich and localized in either neutrophil azurophilic granules or Paneth cells, which are the epithelia of the intestinal mucosa. During gingivitis, neutrophils dominate the lesion area, but the relative proportion compared to plasma cells and lymphocytes in neutrophils decreases during the transition to periodontitis (Kinane and Bouchard 2008; Nussbaum and Shapira 2011). Disorders in neutrophil production have been associated with destruction of periodontal tissue and eventual periodontal disease (Crawford, Wilton et al. 2000). Within neutrophils, human alpha-defensins are abundant and work together with the oral epithelium to provide a barrier to microbial colonization, particularly in the junctional epithelium of the tooth surface (Dale and Fredericks 2005). Studies have shown that two periodontal pathogens, *P. gingivalis* and *Aggregatibacter actinomycetemcomitans*, as well as non-pathogenic commensal bacteria *S. gordonii* are insensitive to alpha-defensin activity (Miyasaki, Bodeau et al. 1990; Zhong, Yang et al. 1998; Raj, Antonyraj et al. 2000). However, when extra amino acids were added to the N-terminus and C-terminus end of HNP2, an enhanced antibacterial activity against the same bacteria was shown, indicating the structural anatomy is a crucial determinant in this AMP's antibacterial activity (Raj, Antonyraj et al. 2000).

HNP 1-3 are detected in the junctional epithelium and the gingival crevicular fluid (GCF), and GCF from patients with aggressive and chronic periodontitis showed significantly elevated levels of HNP 1-3 compared to healthy patients (McKay, Olson et al. 1999; Dale, Kimball et al. 2001). Interestingly, the increased concentration of both alpha- and beta-defensins was correlated in patients with chronic periodontitis with the amount of periodontal pathogens *P. gingivalis*, *T. denticola*, and *T. forsythia* (Puklo, Guentsch et al. 2008). Recently, HNP1 and HNP2 were shown to decrease the response of pro-inflammatory cytokine IL-6, while enhancing antibody response to specific *P. gingivalis* adhesin in mice (Kohlgraf, Ackermann et al. 2010). Thus, alpha-defensins may play a key role as a mediator of innate immunity in gingiva against periopathogenic microbes.

3.1.2 Beta-defensins

Beta-defensins 1 and 2 (hBD-1 and hBD-2) are found in normal, uninflamed gingival tissues as part of the innate host defense mechanism (Krisanaprakornkit, Weinberg et al. 1998; Dale, Kimball et al. 2001). Furthermore, hBD-1 and hBD-2 are localized at the gingival margin where there is the most exposure to oral bacteria of the plaque on the tooth surface, but not in the junctional epithelium. Thus, the junctional epithelium is protected by alpha-defensins and LL-37 released from neutrophils, while the differentiated, stratified epithelia are protected by beta-defensins. Structurally a reduced hBD-1 differs from an oxidized hBD-1, and a reduction in the disulfide bridges of hBD-1 causes the peptide to become a potent AMP against opportunistic pathogen *Candida albicans* and *Lactobacillus* species (Schroeder, Wu et al. 2011). A structural modulation of hBD-1, dependent on the environment it exists in the oral cavity, could shield the healthy epithelium against colonization by commensal and periopathogenic bacteria. However, compared to other beta-defensins, hBD-1 only shows a minor effect against oral bacteria, such as *P. gingivalis*, *A. actinomycetemcomitans, Prevotella intermedia,* and *F. nucleatum* (Ouhara, Komatsuzawa et al. 2005).

In oral epithelia, the expression of hBD-2 is found in normal, uninflamed gingival tissues and is induced by various bacteria (Krisanaprakornkit, Kimball et al. 2000; Dale, Kimball et al. 2001; Chung and Dale 2004). The expression of hBD-2 after challenge from a commensal bacterium indicates that the normal oral epithelium is already at a heightened state to combat potentially harmful pathogens (Krisanaprakornkit, Kimball et al. 2000; Chung and Dale 2004).

hBD-3 has shown bactericidal activity against a wide range of oral bacteria, including periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis,* and cariogenic bacteria *Streptococcus mutans* (Maisetta, Batoni et al. 2003). Furthermore, both normal GECs and immortalized human oral epithelial cells showed an increase in hBD-3 levels upon exposure to *A. actinomycetemcomitans* (Feucht, DeSanti et al. 2003). Similar to HNPs, the peptide has also been detected in the GCF of healthy individuals, and a significant decrease in hBD-3 levels in GCF correlated with the stage of periodontitis, with a negative correlation between hBD-3 levels with the number of periopathogenic bacteria within the same site (Bissell, Joly et al. 2004; Brancatisano, Maisetta et al. 2011).

Studies on the regulation of the induction of beta-defensins reveal different ways gingival epithelia respond to the presence of pathogenic and non-pathogenic bacteria. Our group has reported the induction of hBD-2 by GECs in response to commensal bacteria like *F. nucleatum* and *S. gordonii* utilized p38 and JNK MAPK pathways, while in response to periopathogenic bacteria like *P. gingivalis* and *A. actinomycetemcomitans*, GECs utilized the NF-κB pathway in addition to the aforementioned MAPK (Krisanaprakornkit, Kimball et al. 2002; Chung and Dale 2008). Our group has further reported gingival innate immune response to *P. gingivalis* involves Protease-activated receptor-2 (PAR-2), a G-protein coupled receptor (Chung, Hansen et al. 2004; Dommisch, Chung et al. 2007). A study from another group reported mice given oral doses of *P. gingivalis* showed alveolar bone loss, but in PAR-2 deficient mice the amount of bone loss was significantly less, indicating PAR-2 may have a role in the inflammatory response against *P. gingivalis* (Holzhausen, Spolidorio et al. 2006). In addition, a recent study revealed that the expression of hBD-3 in response to another periodontal pathogen *T. denticola* is regulated via TLR2 (Shin, Kim et al. 2010). All these studies strongly suggest gingival epithelia are able to sense microbes, distinguish between commensal and periopathogenic bacteria, and regulate the appropriate responses for inflammation via regulation of AMPs.

3.1.3 Cathelicidin family – LL-37

Cathelicidin AMPs are heterogeneous and share similar characteristics with other AMPs, such as a basic residue, overall amphipathic nature, and a net positive charge at neutral pH (Dale and Fredericks 2005). LL-37, the only member in human cathelicidin family, is transcribed by *CAMP* (cathelicidin antimicrobial peptide) gene, which translates to an 18 kDa proprotein (Zanetti, Gennaro et al. 2000; Zaiou, Nizet et al. 2003). This AMP is detected and expressed in higher amounts within neutrophils that migrate through the junctional epithelium to the gingival sulcus (Dale, Kimball et al. 2001). This peptide is present in a different site than beta-defensins, suggesting they could serve different role in periodontium. The expression of LL-37 is detected in wide range of epithelia and other body sites, including junctional epithelium, inflamed epidermal keratinocytes, tongue, buccal mucosa and saliva following inflammatory stimulation (Frohm, Agerberth et al. 1997; Frohm Nilsson, Sandstedt et al. 1999; Dale, Kimball et al. 2001; Murakami, Ohtake et al. 2002; Howell 2007). Junctional epithelium also expresses IL-8, following a gradient that leads to directional migration of neutrophils into the gingival sulcus when exposed to bacteria (Tonetti, Imboden et al. 1994). Thus, it is plausible that neutrophil migration through the tissue may be the reason for the expression of LL-37 in gingival epithelium (Dale, Kimball et al. 2001; Dale and Fredericks 2005). LL-37 has shown antimicrobial activities against periodontal pathogen *A. actinomycetemcomitans* (Gomez-Garces, Alos et al. 1994), while is ineffective against some cariogenic bacteria, including *S. mutans*, *Streptococcus sobrinus* and *Actinomyces viscosus*, as well as periodontal pathogen *P. gingivalis* (Altman, Steinberg et al. 2006).

3.2 Differential expression of AMPs in periodontal health and disease

How the expression of various AMPs varies during gingival and periodontal inflammation has been reported by various groups, and these studies show high inter-individual variability in both gene and protein expression of AMPs in patient samples (Dunsche, Acil et al. 2002; Lu, Jin et al. 2004; Dommisch, Acil et al. 2005; Lu, Samaranayake et al. 2005). Analyses of gene expression by RT-PCR showed hBD-1 and hBD-2 mRNA expression was less frequently detected in tissues with gingivitis than in healthy gingiva. In biopsies from patients with gingivitis, mRNA of hBD-1 and hBD-2 was detectable in 66 % and 86 % of samples, respectively, while 100 % of all gingivitis samples showed the expression of hBD-3 mRNA (Dunsche, Acil et al. 2002). In addition, compared to the samples from healthy subjects, the level of beta-defensin mRNA expression was lower and less frequently found in samples from periodontitis patients (Dunsche, Acil et al. 2002; Bissell, Joly et al. 2004). Similar results have also been reported testing mRNA level by *in situ* hybridization and protein level using immunohistochemistry (Lu, Jin et al. 2004; Lu, Samaranayake et al. 2005; Hosokawa, Hosokawa et al. 2006). These studies suggest a decrease in the expression of hBD-2 and hBD-3 in both patient groups of gingivitis and periodontitis. However, other studies suggest differential expression of beta-defensins in patients with specific periodontal diseases, highlighting inter-individual variability in the expression of these AMPs. In samples collected from gingivitis and periodontitis patients, the amount of hBD-2 mRNA was up-regulated compared to the ones from healthy subjects, while the quantity of hBD-3 mRNA was equivalent in healthy and gingivitis groups, but increased in periodontitis samples (Dommisch, Acil et al. 2005). Quantitative RT-PCR analyses of hBD-1 and hBD-2 expression levels in gingiva of patients with gingivitis, aggressive periodontitis and chronic periodontitis found a significantly higher level of hBD-1 in chronic periodontitis group

compared to gingivitis and aggressive periodontitis groups (Vardar-Sengul, Demirci et al. 2007). On the other hand, the expression level of hBD-2 was significantly higher in aggressive periodontitis group than in gingivitis and chronic periodontitis groups (Vardar-Sengul, Demirci et al. 2007). The localization of beta-defensin protein expression also varied among different patient groups. The protein expression of hBD-1 and hBD-2 was mostly found in the granular and spinous cell layer in healthy and diseased gingival tissue samples (Lu, Jin et al. 2004). On the contrary, the expression of hBD-3 was found in basal cell layer in healthy samples, while in the basal and spinous cell layers in samples from periodontal disease (Lu, Samaranayake et al. 2005).

The levels of AMPs in GCF are thought to be associated with periodontal disease, as demonstrated by Puklo et al. that the GCF HNP1–3 levels were higher in patients with aggressive or chronic periodontitis when compared to healthy controls (Puklo, Guentsch et al. 2008). Gingival tissue samples from chronic periodontitis patients showed elevated mRNA expression and higher immunostaining of LL-37 on neutrophils, while the LL-37 levels were also elevated in the GCF of periodontitis patients (Hosokawa, Hosokawa et al. 2006; Turkoglu, Emingil et al. 2009; Turkoglu, Kandiloglu et al. 2011). In addition, patients with morbus Kostmann syndrome, an inherited disorder that causes lower than normal levels of neutrophils, have been found to be more susceptible to periodontal disease, while those with a bone marrow transplant are not (Putsep, Carlsson et al. 2002). The patients with Kostmann syndrome lack LL-37 in saliva and have lower concentrations of HNP1-3, the latter of which is commonly found in patients with other neutrophil disorders (Ganz, Metcalf et al. 1988). However, when these patients receive a bone marrow transplant, normal concentration of LL-37 is found in their saliva (Putsep, Carlsson et al. 2002). Of interesting to note is when patients with Kostmann syndrome have their levels of neutrophils restored via treatment with recombinant granulocyte-colony stimulating factor, they still experience recurring periodontal infections (Putsep, Carlsson et al. 2002; Carlsson, Wahlin et al. 2006). All these studies suggest that the deficiency in salivary LL-37 is a likely reason for chronic periodontitis in patients with morbus Kostmann prior to bone marrow transplant and further suggests a potential protective role in host defense by LL-37.

All the studies presented in this section demonstrate that AMPs are differentially expressed in various stages of periodontal health and disease. These studies also suggest that there may be complex regulatory mechanisms involved in gingival innate immunity (Chung, Dommisch et al. 2007), and further suggest AMPs play a crucial role in the maintenance of gingival health and prevention of periodontal disease.

3.3 Potential therapeutic value of AMPs

How AMPs maintain the delicate balance between oral health and dental plaque containing microbial consortium is still a matter of conjecture. Some hypotheses include AMPs creating physical holes that cause cellular contents to leak out, fatal depolarization of normally energized bacterial membrane, or the activation of deadly processes such as the induction of hydrolases that degrade the cell wall (Som, Vemparala et al. 2008). Overall, the mode of antimicrobial activity of AMPs has been most commonly attributed to disruption of cell membranes (Ganz and Lehrer 1999; Hancock and Diamond 2000), but a recent study also reported that defensins can inhibit cell wall biosynthesis via binding and sequestering of lipid II, a building block of bacterial cell wall (Wilmes, Cammue et al. 2011).

Currently, a combination of antimicrobial and mechanical applications is used in treatment plans for periodontal disease, such as applying tetracycline or doxycline families in

conjunction to scaling and root planning. Recently, a sub-antimicrobial dose doxycyline has been introduced where low doses are given to block matrix metalloproteinases (MMP), which are capable of degrading extracellular matrix proteins (Tuter, Kurtis et al. 2007; Payne, Golub et al. 2011). Yet, antibiotic treatment for periodontal disease still poses a risk of developing antibiotic-resistant periodontal bacteria in the subgingival plaque (van Winkelhoff, Herrera Gonzales et al. 2000; Handal, Caugant et al. 2003; Maestre, Bascones et al. 2007; Ardila, Granada et al. 2010). AMPs have several advantages as therapeutics, including the broad spectrum of antimicrobial activity and do not appear to induce antibiotic resistance. AMPs as therapeutics against microbes would be promising because the target of AMPs are the bacterial membrane, thus to combat AMPs the bacteria would need to redesign its membrane, which would be a "costly" solution for most species (Zasloff 2002). The possibility of alleviating bacterial infections related to cystic fibrosis through increasing physiological levels of LL-37, or re-engineering human macrophages to express beta-defensins to enhance efficacy against *Mycobacterium tuberculosis* have been proposed (Bals, Weiner et al. 1999; Kisich, Heifets et al. 2001). However, limitations as an effective therapeutic are stalled by high production costs and the susceptibility to proteolytic degradation, a mechanism which microbial pathogens secrete proteases to counter-measure the target of AMPs (Peters, Shirtliff et al. 2010). Due to these limitations, a new pursuit has been made to construct synthetic mimics of AMPs, which would capture the important properties of AMPs but also eliminate problems related to drug therapy. Structurally these AMP mimics would maintain its amphiphillic topology to eventually depolarize the membrane potential and ultimately kill bacteria, but also possess a non-natural backbone without amide or ester function so the peptide will not undergo proteolytic degradation from bacterial enzymes (Tew, Liu et al. 2002; Tew, Clements et al. 2006; Hua, Scott et al. 2010). A recent study showed one mimetic called mPE was able to exhibit potency against biofilm cultures of *A. actinomycetemcomitans* and *P. gingivalis*, while also inhibiting IL-1B-induced secretion of IL-8 in gingival epithelial cells (Hua, Scott et al. 2010). The anti-inflammatory activity was followed with a reduced activation of NF-κB, suggesting that these AMP mimics could act as an anti-biofilm and anti-inflammatory agent. Furthermore, it has been shown in bacterial resistance studies that *Staphylococcus aureus* showed increased minimum inhibitory concentration (MIC) for conventional antibiotics, but no change was observed with MIC for mPE (Beckloff, Laube et al. 2007; Hua, Scott et al. 2010). However, a current limitation of mimetic is that it has been tested on single bacterium but not on complex biofilm structures.

4. Proteases vs. protease inhibitors in periodontal health and disease

4.1 Various classes of protease inhibitors in gingival epithelia

Serine protease inhibitors play a critical role in host tissue homeostasis, as gingival epithelia secrete these protease inhibitors as a way to protect the tissue from excessive damage by proteases, which can be of pathogenic bacteria or of neutrophil origin. Thus, the balance between proteases and their inhibitors contributes to maintenance of tissue integrity (Magert, Drogemuller et al. 2005). These protease inhibitors include secretory leukocyte protease inhibitor (SLPI), elastase-specific inhibitor (ELAFIN) and squamous cell carcinoma antigen (SCCA). SLPI is found in a variety of mucous secretions, including in GCF from sites of periodontal disease (Minami 1999). This protease inhibitor protects tissues from destruction during an inflammatory response via regulating the activity of neutrophil

elastase (Giannopoulou, Di Felice et al. 1990). ELAFIN, also known as skin-derived anti-leukoproteinase (SKALP), is expressed in human epithelia of the tongue, palate, lingual tonsils, pharynx as well as gingiva (Molhuizen and Schalkwijk 1995). ELAFIN has been shown to inhibit neutrophil elastase and proteinase 3, thus has a role in protecting tissue from degradation by the neutrophil enzymes (Ying and Simon 1993; Zani, Nobar et al. 2004). ELAFIN and SLPI are chelonianin family of serine protease inhibitors and share 40% sequence identity (Ying and Simon 1993; Zani, Nobar et al. 2004; Guyot, Butler et al. 2008). Both SLPI and ELAFIN have antimicrobial activity against Gram-positive as well as Gram-negative pathogens (Sallenave, Cunningham et al. 2003; McMichael, Maxwell et al. 2005; King, Wheelhouse et al. 2009).

SCCA1 and SCCA2 are members of the ovalbumin-serpin and serve as a marker for certain inflammatory conditions. Within the mucous membranes lined with squamous epithelia, co-expression of SCCA1 and 2 plays an important role in the coordinated regulation of certain serine and cysteine proteases associated with both normal and transformed cells (Cataltepe, Gornstein et al. 2000). SCCA1 and SCCA2 share 91% homology at the amino acid level, and both are induced by IL-4 and IL-13 (Yuyama, Davies et al. 2002; Mitsuishi, Nakamura et al. 2005). However, their functions differ: SCCA1 inhibits cysteine proteases such as cathepsin K, while SCCA2 inhibits serine proteases such as cathepsin G and human mast cell chymase (Silverman, Bird et al. 2001).

These protease inhibitors are expressed by various epithelial cells and act as an anti-protease to protect against tissue damages caused during inflammation (Alkemade, Molhuizen et al. 1994; Pfundt, van Ruissen et al. 1996; van Wetering, van der Linden et al. 2000). In addition, other studies demonstrated anti-bacterial and anti-inflammatory activities of ELAFIN that are independent of anti-protease activity (Simpson, Maxwell et al. 1999; Meyer-Hoffert, Wichmann et al. 2003). In the context of the periodontium, these protease inhibitors produced by GECs might protect against bacterial proteases and limit tissue damage due to neutrophil proteases associated with inflammation. Thus, the balance between protease inhibitors and proteases may be a factor in the progression of disease.

4.2 Regulation of protease inhibitors by periodontal pathogens

The development of periodontal disease is characterized by the transition of the subgingival flora from Gram-positive complex, such as Streptococci, to a Gram-negative complex including the presumptive pathogen, *P. gingivalis* (Kolenbrander, Andersen et al. 2002). *P. gingivalis* gingipains are cysteine proteases with specificity for cleavage at either arginine (Rgp) or lysine (Kgp) (Potempa, Pike et al. 1995; Potempa and Travis 1996). Rgp activates cellular responses of both epithelial cells and fibroblasts via PAR2 and up-regulates inflammatory and innate immune responses (Lourbakos, Potempa et al. 2001; Holzhausen, Spolidorio et al. 2006). In addition to *P. gingivalis*, periodontal pathogens *T. denticola* and *T. forsythia* also have serine or cysteine proteases as their main virulence factors, and these proteases play a role in periodontitis (Fenno, Lee et al. 2001; van der Reijden, Bosch-Tijhof et al. 2006). *F. nucleatum* is a common microorganism within the periodontium in both healthy and diseased tissue and serves as a bridging organism between commensals and pathogens. Previous studies reported *F. nucleatum* as well as commensal bacterium *S. gordonii* have serine-type proteases which are involved in the degradation of collagen and/or fibronectin (Juarez and Stinson 1999; Bachrach, Rosen et al. 2004). In addition to bacterial proteases, neutrophils also release proteases. In the normal epithelium, neutrophils flow into the space between the tooth and soft tissue due to the

cytokine gradient. Although neutrophils serve as part of the continuous surveillance of the gingival sulcus, proteases released by neutrophils contribute to inflammation and tissue damage (Tonetti, Imboden et al. 1998; Nathan 2006).

Our laboratory previously showed that GECs exposed to *F. nucleatum* up-regulated expression of multiple protease inhibitors as well as antimicrobial peptides and other potentially protective factors (Table 1) (Yin and Dale 2007). Our data suggest that *F. nucleatum*, a bridging organism between non-pathogenic commensal and pathogenic bacteria, enhances expression of protease inhibitors that protect GECs in anticipation of virulent proteases secreted by pathogenic bacteria. Both host cell-derived proteases, such as neutrophil elastase, and pathogen-derived proteases, such as the gingipains, are targeted by these protease inhibitors, and therefore, the protease inhibitors may play an important role in maintaining the extent of inflammatory tissue damage (Into, Inomata et al. 2006; Williams, Brown et al. 2006; Yin, Swanson et al. 2010).

Protease Inhibitor	Target Protease	Potential Function	Fold Change*
ELAFIN	Elastase, PMN	Innate immunity, antimicrobial	14.31
SERPINB1	Elastase, Cathepsin G	Innate immunity, inhibits PMN proteases	3.2
SERPINB2	Thrombin	Regulates extravascular plasminogen activation	2.2
SCCA1	Cathepsin S, K, L	Inhibits Cathepsin S, K, L and modulates host immune response	19.4
SCCA2	Cathepsin G	Inhibits mast cell proteases	8.6
SLPI	Elastase, Trypsin, Cathepsin B	Stimulates wound healing, inhibits PMN proteases	4.3
Cystatin B	Stefin B	Protection against intracellular proteases	2.0

*Fold increase after stimulation with *F. nucleatum* cell wall extract for 24h compared to unstimulated.

Table 1. Changes in the induction level of various protease inhibitors in gingival epithelial cells following stimulation with *F. nucleatum* (Yin and Dale 2007).

These protease inhibitors are also affected by perio-pathogenic organism *P. gingivalis*, whose main virulence factors are cysteine proteases. A protective effect of these protease inhibitors in gingival health is shown by our study that demonstrated pre-treatment of GECs with SLPI, SCCA1 or SCCA2 partially attenuated antimicrobial proteins hBD-2 and CCL20 mRNA expression in response to *P. gingivalis* (Yin, Swanson et al. 2010). However, the same study showed the presence of *P. gingivalis* disrupted the function of these serine protease inhibitors, suggesting that the presence of an organism colonizing oral plaque prior to the establishment by pathogens enhances expression of protease inhibitors that protect GECs, while *P. gingivalis* secretes proteases that degrade cellular protease inhibitors (Yin, Swanson et al. 2010). It is of interest to note that various periodontal pathogens which secrete proteases (*P. gingivalis*, *T. forsythia*, *A. actinomycetemcomitans*) were tested, but *P. gingivalis* was most effective at degrading protease inhibitors (Figure 2) (Yin, Swanson et al. 2010). The degradation of

protease inhibitors by *P. gingivalis* may result in decreased host protective capacity, and the balance between cellular protease inhibitors and their degradation by *P. gingivalis* and/or other periodontal pathogens may be an important factor in susceptibility to *P. gingivalis* infection. The dominance of *P. gingivalis* in the degradation of protease inhibitors is important to note, since during the formation of dental plaque, protease inhibitors may be induced as a host protective mechanism by the presence of non-pathogenic bacteria, but may become ineffective once protease-secreting pathogens are established.

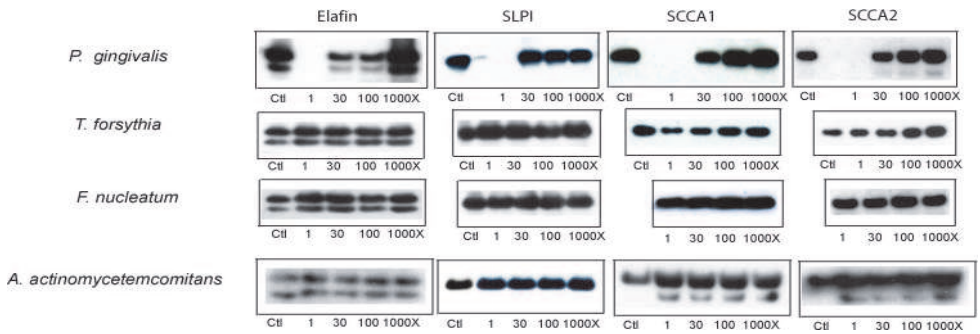

Fig. 2. Recombinant SLPI, ELAFIN, SCCA1, and SCCA2 are degraded by *P. gingivalis* supernatants *in vitro* in a dose-dependent manner. Western Blot analysis for each protease inhibitor using a constant concentration of recombinant protease inhibitor incubated with cell-free supernatants of oral bacteria for 15 min at RT. The undiluted supernatant (1) corresponds to MOI 100; increasing dilution factor is indicated below each protease inhibitor. Control: recombinant protein only. The controls shown with *P. gingivalis* also apply to the recombinant proteins treated with *T. forsythia* and *F. nucleatum* (Yin, Swanson et al. 2010). Permission from Co-Action Publishing.

In addition to exposure to proteases secreted by oral pathogenic bacteria, oral cavity may also be exposed to different neutrophil-derived serine proteases, such as human leukocyte elastase, cathepsin G and proteinase 3 (Sugawara, Uehara et al. 2001; Uehara, Muramoto et al. 2003). These neutrophil proteases may be secreted in response to the presence of oral bacteria, and thus oral cavity may be exposed to these neutrophil proteases prior to being exposed to proteases from periodontal pathogens. In addition, protease inhibitors are likely to have different effects on neutrophil proteases secreted by host vs. proteases secreted by periodontal pathogens: proteases secreted by pathogens may degrade host protease inhibitors; while proteases secreted by host neutrophils may maintain more natural balance in maintaining epithelial health. Proteases have to be at the right place at the right time to have an effect on the host, thus have to be tightly regulated by the host. Therefore, the balance between the proteases and protease inhibitors is crucial in the health of oral epithelia.

4.3 Changes in protease inhibitor levels in periodontitis

Periodontitis is a chronic inflammatory disease whose main etiologic agents include Gram-negative anaerobic bacteria and spirochetes (Haffajee and Socransky 1994). Among them, *P. gingivalis* in particular plays a significant role in the progression of chronic periodontitis (O'Brien-Simpson, Veith et al. 2004). Many virulence factors of this pathogen include proteases (gingipains), fimbriae and hemagglutinins (Amano 2003; Veith, Chen et al. 2004;

Into, Inomata et al. 2006). *P. gingivalis* gingipains have shown to degrade extracellular matrix components such as laminin, fibronectin, and collagen type III, IV, and V *in vitro* (Potempa, Banbula et al. 2000), and are thought to account for at least 85% of the general proteolytic activity displayed by *P. gingivalis* (Imamura 2003). Our previous *in vitro* study found that the secretion of SLPI and ELAFIN was significantly reduced in response to *P. gingivalis* and that *P. gingivalis* supernatants digested recombinant SLPI, ELAFIN, SCCA1 and 2 (Figure 2) (Yin, Swanson et al. 2010). These data suggest degradation of protease inhibitors by *P. gingivalis* may result in decreased host protective capacity and higher susceptibility to *P. gingivalis* infection (Yin, Swanson et al. 2010).

As a follow-up to this *in vitro* study, an *in vivo* study from our group correlated the amount of *P. gingivalis* in subgingival plaque of patients with chronic periodontitis with the level of protease inhibitors in GCF of healthy and periodontitis patients. Significantly lower levels of SLPI and ELAFIN were detected in subjects with periodontitis and *P. gingivalis* present in their plaque compare to healthy controls (Kretschmar, Yin et al. 2011). The level of SLPI was also decreased in GCF of periodontal patients without detectable level of *P. gingivalis* in their subgingival plaque. And an inverse correlation was observed between the ELAFIN and SLPI concentrations and the number of *P. gingivalis* present in subgingival plaque. Our findings showed that host-derived protease inhibitors SLPI and ELAFIN, which are secreted as a response to environmental and microbial stimuli, are decreased in concentration in periodontal pockets with *P. gingivalis.* The reduced concentrations of these protective protease inhibitors may contribute to the loss of host defense capacity and increase susceptibility to breakdown from chronic infection.

Similarly, a separate study reported when SLPI concentrations in GCF from active periodontitis patients and periodontitis patients in maintenance were compared, SLPI was significantly reduced in the group with high amount of *P. gingivalis* (Into, Inomata et al. 2006). The proteolytic activity of *P. gingivalis* gingipain isoform RgpA is thought to be responsible for this observation (Into, Inomata et al. 2006). Although the overall bacterial load in these samples was not specified, the data from this study is in agreement with our previous study utilizing *P. gingivalis* mutant strains lacking various gingipains (Yin, Swanson et al. 2010). In addition to the role RgpA may play in the degradation of SLPI, the bacterial biofilm may also play a role in the degradation of protease inhibitors, such as increased neutrophil elastase level as a result of high bacterial load in dental plaque.

5. Epigenetic regulation and its implication on periodontal disease

Epigenetics is heritable and reversible changes in gene expression without altering DNA sequence. Chromatin structure is made up of eight histone molecules (two each of H2A, H2B, H3 and H4) and DNA which winds around these proteins. Histones are subject to a number of post-translational modifications, such as acetylation, methylation, phosphorylation and ubiquitination (Hansen, Tse et al. 1998; Strahl and Allis 2000). The mechanisms of epigenetic modifications include histone acetylation, histone methylation and DNA methylation, and these modifications provide a way to control the expression of genes involved in various cellular functions as well as in cancer (Egger, Liang et al. 2004; Rodenhiser and Mann 2006). Enzymes involved in these epigenetic mechanisms are: histone acetyltransferases (HATs); histone deacetylases (HDACs); histone methyltransferases (HMTs); and DNA methyltransferases (DNMTs) (Figure 3). Modifications on chromatin structure can occur in response to diet, inherited polymorphisms in certain genes and to environmental toxins

(Sutherland and Costa 2003; Luch 2005; Rodenhiser and Mann 2006). When histones are acetylated, transcription factors can access DNA, leading to gene transcription, while deacetylated histones lead to condensed (or closed) chromatin structure, making DNA inaccessible to transcription factors and preventing gene expression (Figure 3). In addition, methylation of cytosine residues at CpG sites in DNA inhibits binding of transcription factors, leading to gene silencing. Methylation of gene promoter region is one of the most common epigenetic mechanisms in silencing tumor suppressor genes, and over-expression of DNMTs in humans is associated with a variety of cancers (Rodenhiser and Mann 2006). Furthermore, decreased methyltransferase activity and hypo-methylated DNA have been associated with autoimmune diseases (Richardson 2003; Oelke and Richardson 2004), and changes in the histone acetylation in central nervous system has been linked to cognitive decline in a mouse model (Peleg, Sananbenesi et al. 2010). Although various epigenetic mechanisms work in concert to produce long-term and stable regulation of gene expression, not much is known on how these processes are linked and how specific patterns of epigenetic modification are inherited.

A recent study reported that oral squamous cell carcinoma showed epigenetic changes associated with SERPINE1 expression (Gao, Nielsen et al. 2010), while other studies suggest that epigenetics play a critical role in regulating inflammatory responses and that the manifestation and severity of periodontal disease may be influenced by epigenetic factors (Bobetsis, Barros et al. 2007; Offenbacher, Barros et al. 2008). Many patients with the same clinical symptoms respond differently to the same therapy, suggesting the inter-individual variability observed as a clinical outcome of the disease is influenced by genetic (Schenkein 2002; Feinberg 2007) as well as epigenetic factors (Offenbacher, Barros et al. 2008). Epigenetic modifications alter patterns of gene expression, which in turn leads to various clinical outcomes. Furthermore, variations in epigenetic status will likely elicit diverse inflammatory responses. A new study from our group focused on finding answers to how epigenetic modifications brought on by exposure to oral bacteria, including periodontal pathogens, affect host innate immune responses and susceptibility to subsequent infections (Yin and Chung 2011).

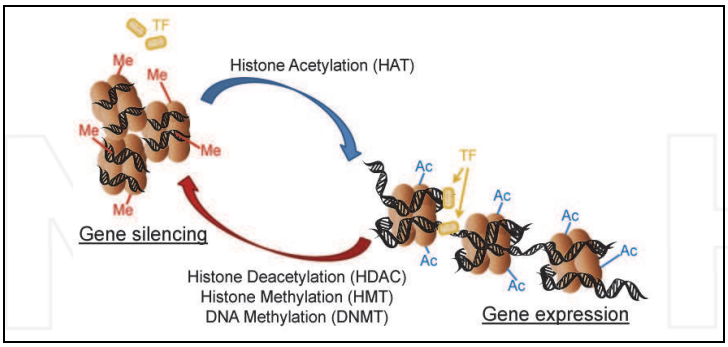

Fig. 3. Histone acetylation allows open chromatic structure, and transcription factors can access DNA. Deacetylation of histones as well as histone methylation and DNA methylation result in closed chromatin, thus transcription factors cannot access DNA, which results in gene silencing. Epigenetic modifications of histones and/or DNA via methylation lead to altered gene expression. TF: transcription factors; Ac: acetylation; Me: methylation; HAT: histone acetyltransferase; HDAC: histone deacetylase; HMT: histone methyltransferase; DNMT: DNA methyltransferase.

When any changes in the expression levels of enzymes involved in the epigenetic modification after GECs were exposed to oral bacteria were investigated, we found the expression of histone deacetylases and DNA methyltransferase changed in response to the presence of oral bacteria. Histone deacetylases (HDACs) remove acetyl groups from histone, leading to suppression of genes, while DNA methyltransferases (DNMTs) catalyze transfer of methyl groups onto DNA, which also leads to gene suppression (Figure 3). Quantitative real-time PCR analyses showed changes in the expression levels of these genes when GECs were treated with *P. gingivalis* or *F. nucleatum* at various multiplicities of infection for 1, 4, 24 and 48 h. The gene expression levels of DNMT1, HDAC1 and HDAC2 decreased in GECs treated with *P. gingivalis* or *F. nucleatum*, although the levels of decrease differed between bacterial species and/or exposure time (Yin and Chung 2011). As changes in the expression of enzymes catalyzing epigenetic modifications were observed, it was also of interest to note the changes in methylation levels of various genes in GECs after the cells were exposed to these oral bacteria. Studies utilizing methylation PCR Array showed a dose-dependent and statistically significant increase in methylation levels of the following genes after GECs were exposed to *P. gingivalis*: CD276, an immune regulator; elastase 2, a serine protease that plays a role in inflammatory diseases; INHBA, a tumor-suppressing protein; GATA 3, a putative tumor suppressor; TLR2; and IL-12A. Stimulation of GECs with *P. gingivalis* also resulted in hypo-methylation of ZNF287, a member of Zinc finger protein family. The up-regulation of Zinc finger proteins has been associated with cardiovascular disease (Dai and Liew 1999), thus this observation is in line with recent studies linking periodontal disease with increased risk of systemic disease (Persson and Persson 2008). GECs exposed to *P. gingivalis* also showed a decrease in the methylation of STAT5A, which mediates cellular responses to cytokines IL-2, IL-3, IL-7, GM-CSF and plays a role in progression of tumors. On the other hand, the methylation levels of elastase 2 and GATA3 decreased significantly after cells were stimulated with *F. nucleatum* (Yin and Chung 2011). Interestingly, only *F. nucleatum* induced hyper-methylation of MALT1 (Mucosa-associated lymphoid tissue lymphoma translocation gene) in GECs. MALT1 induces IKK catalytic activity, resulting in NFκB activation in immune cells (Schulze-Luehrmann and Ghosh 2006). The methylation of MALT1 is associated with silencing of MALT1 gene, which is consistent with our previous reports that *F. nucleatum* does not utilize the NFκB signaling pathway in the induction of innate immune responses (Chung and Dale 2004; Chung and Dale 2008). Taken together, these data suggest that epigenetic modification of genes, whose function is associated with growth control and inflammation, is differentially regulated by different oral bacteria (Yin and Chung 2011).

Modulations of chromatin structure play an important role in the regulation of transcription, and these modifications directly affect the accessibility of chromatin to transcription factors, thus on gene expression. When the changes in histone H3 level in GECs after exposure to periopathogen vs. non-pathogen were examined, the endogenous level of histone H3 that is tri-methylated at Lys4 was significantly decreased following stimulated with *P. gingivalis* compared to unstimulated control, while the level increased after exposure to *F. nucleatum* (Yin and Chung 2011). Our data suggest these two bacterial species, pathogen vs. non-pathogen, differentially regulate H3K4 methylation and further suggest bacterial infection in oral epithelia is associated with changes in H3K4 methylation (Yin and Chung 2011).

Gene promoter methylation is the most common epigenetic mechanism silencing tumor suppressor genes during oncogenesis. Almost all cancer-related signaling pathways are affected by methylation, and the number of genes affected in each major type of cancer is

still rapidly growing. However, even the most relevant genes have not yet been correlated to individual cancer types for development of DNA methylation biomarkers. Recent studies reported that particular histone modifications are correlated with certain types of cancers and that histone modifications will be useful biomarkers for cancer (Su, Lucas et al. 2009; Manuyakorn, Paulus et al. 2010; Svotelis, Gevry et al. 2010). Since our recent data strongly suggest presence of oral bacteria affects chromatin modification in GECs, it is plausible periodontal patients with high number of periodontal pathogens recovered from the oral cavity will show altered chromatin modifications. Further studies are needed to identify epigenetic factors involved in development and pathogenesis of periodontitis, contributing to better defining of epigenetic modifications as an indicator of periodontal disease. It would be of importance in a periodontal treatment plan to identify a certain species of bacteria which induce epigenetic changes and subsequently modify host responses. Furthermore, better understanding of specific bacteria that show capacity to induce epigenetic changes would be of importance in developing specific therapeutic strategies for each patient.

6. Conclusion

Periodontitis is a disease that is not only caused by one single bacterium, but by a number of different bacterial species, organized in a complex biofilm and thereby exhibiting various properties. Understanding how gingival epithelia response to the presence of different commensals and pathogens, leading to induction of appropriate innate immune responses, will provide significant new insights into this complex biological system in the oral epithelia. Furthermore, better understanding the role of other factors influencing periodontal health, such as the balance between proteases and protease inhibitors, and the role epigenetic status plays in health and disease, will have direct implications for new understanding of oral innate immune responses and the development of potential new and innovative therapeutic interventions for periodontal disease.

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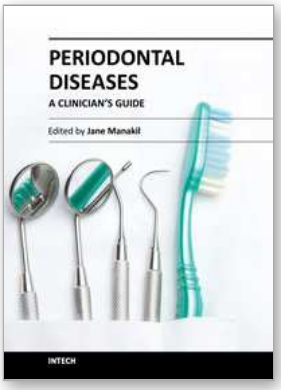

Periodontal Diseases - A Clinician's Guide

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"Periodontal diseases" is a web-based resource intended to reach the contemporary practitioners as well as educators and students in the field of periodontology. It is fully searchable and designed to enhance the learning experience. Within the book a description is presented of the current concepts presenting the complex interactions of microbial fingerprint, multiple genotypes, and host modulations. In addition, an overview is given of the clinical outcome of the disease's progression, as influenced by the epigenetic factors. Emerging concepts on periodontitis as a risk factor for various systemic diseases and as a bilateral modulating factor have been elucidated in detail as well.

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