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The Role of Antimicrobial Peptides in Periodontal Disease (Part I): an Overview of Human Defensins and Cathelicidin

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Abstract

The oral cavity is a warm and moist environment, suitable for microorganisms to colonize and live in harmony as a community, so-called biofilm. In this environment, antimicrobial peptides may play a critical role in maintaining normal oral health and controlling innate and acquired immune systems in response to continuous microbial challenges in periodontal disease. Two major families of antimicrobial peptides, found in the oral cavity, are defensin and cathelicidin. Members of the defensin family are cysteine-rich peptides, synthesized by plants, insects, and mammals. In the oral cavity, four alpha-defensins are synthesized and stored in neutrophil granules, which are converted into active peptides by proteolytic processing, while three human beta-defensins (hBDs), hBD-1, hBD-2, and hBD-3, are predominantly produced by oral epithelial cells. The only member of the cathelicidin family found in humans is LL-37, which contains 37 amino acids and begins with two leucines at its N-terminus. Clinically, differential expression of antimicrobial peptides has been reported in different types of periodontal disease, and their presence has been shown in saliva and gingival crevicular fluid. In the first part of our review article, basic knowledge of antimicrobial peptides will be discussed in detail.

Key word: Cathelicidin, Defensin, Periodontal disease

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Review Article

บทบาทของเพปไทด์ต้านจุลชีพต่อโรคปริทันต์ (ตอนที่ 1): ลักษณะทั่วไปของดีเฟนซินและแคทีลิไซดินของมนุษย์

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บทคัดย่อ

สิ่งแวดล้อมภายในช่องปากมีลักษณะชุ่มชื้นและอุณหภูมิอุ่นซึ่งเหมาะสมสำหรับจุลชีพในการตั้งถิ่นฐานและอาศัยอยู่อย่างกลมกลืนจนเป็นชุมชนที่เรียกว่าไบโอฟิลม์ ในสิ่งแวดล้อมนี้ เพปไทด์ต้านจุลชีพอาจมีบทบาทสำคัญในการคงสภาพของสุขภาพช่องปากให้เป็นปกติและยังควบคุมระบบภูมิคุ้มกันที่เป็นมาแต่กำเนิดและที่เกิดภายหลังเพื่อตอบสนองต่อสิ่งท้าทายที่เป็นจุลชีพก่อโรคปริทันต์อย่างต่อเนื่อง เพปไทด์ต้านจุลชีพ 2 ครอบครัวใหญ่ที่พบในช่องปาก ได้แก่ ดีเฟนซินและแคทีลิไซดิน โดยสมาชิกในครอบครัวดีเฟนซินประกอบด้วยเพปไทด์ที่มีกรดอะมิโนซิสเทอีนเป็นจำนวนมากซึ่งถูกสร้างโดย พืช แมลง และสัตว์เลี้ยงลูกด้วยนม สำหรับในช่องปาก แอลฟา-ดีเฟนซิน 4 ชนิดถูกสร้างขึ้นและเก็บอยู่ในแกรนูลของนิวโทรฟิล ซึ่งต่อมาจะถูกเปลี่ยนเป็นเพปไทด์ที่ออกฤทธิ์ด้วยกระบวนการแปรรูปโดยการย่อยสลายโปรตีน ในขณะที่บีตา-ดีเฟนซินของมนุษย์พบได้ 3 ชนิด ได้แก่ hBD-1, hBD-2 และ hBD-3 ซึ่งถูกสร้างจากเซลล์เยื่อบุผิวช่องปากเป็นส่วนใหญ่ สมาชิกเพียงตัวเดียวของครอบครัวแคทีลิไซดินที่พบในมนุษย์ได้แก่ LL-37 ซึ่งประกอบด้วยกรดอะมิโน 37 ตัวและมีกรดอะมิโนลิวซีน 2 ตัวอยู่ทางปลายด้านหมู่อะมิโน ในทางคลินิก มีรายงานการแสดงออกที่แตกต่างกันของเพปไทด์ต้านจุลชีพในโรคปริทันต์ชนิดต่าง ๆ และสามารถตรวจพบเพปไทด์ต้านจุลชีพได้ในน้ำลายและน้ำเหลืองเหงือก ในตอนแรกของบทความปริทัศน์ฉบับนี้ ได้อธิบายถึงความรู้พื้นฐานของเพปไทด์ต้านจุลชีพอย่างละเอียด

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Introduction

The warm and moist environment in the oral cavity is a unique niche suitable for a number of microorganisms to colonize, proliferate, and live in harmony as a community, so-called biofilm. Oral epithelium plays a main role as a physical barrier between the microbial biofilm in the external environment and underlying connective tissue and blood vessels. Naturally, this barrier can be disrupted, since the oral epithelium is the only site in the body normally penetrated by a hard tissue, namely, a tooth. The junction between oral epithelium and the tooth is, therefore, considered a site that is readily susceptible to infection from various microorganisms living in dental plaque. Previously, the role of oral epithelium was viewed as that of an innocent bystander. However, it is now apparent that oral epithelial cells can respond to continuous microbial challenges from dental plaque by production of cytokines, chemokines, and antimicrobial peptides, which enhance inflammation and immune response in periodontal tissues. Uncontrolled inflammation from excessive production of these pro-inflammatory molecules is considered one of the etiological factors in the pathogenesis of periodontal disease.

Two well-characterized families of antimicrobial peptides, including defensin and cathelicidin, are present in saliva and GCF, and localized in the oral mucosa.[1] These peptides include β-defensins that are expressed in the oral epithelial cells, α-defensins that are secreted from neutrophil granules, and LL-37, the only human antimicrobial peptide in the cathelicidin family, which mainly derives from neutrophil granules and to a lesser extent from oral epithelial cells.[2] The synthesis of some of these antimicrobial peptides can be considerably up-regulated upon exposure to oral microorganisms; thus, these peptides are regarded as essential effector molecules in innate immunity. In the first part, basic knowledge regarding expression and regulation of defensins and LL-37, including their differential expression in healthy and diseased periodontal tissues and in gingival crevicular fluid (GCF), and their regulation in human primary gingival epithelial cells, will be extensively reviewed. However, a review of other antimicrobial peptides present in the oral cavity, such as calprotectin, adrenomedullin, histatins, etc., is beyond the scope of this article and will not be discussed.

General Information on Human Cathelicidin and Defensin

Cathelicidin is a family of antimicrobial peptides that contain a cathelin domain at their N-terminus and a mature peptide at their C-terminus (Figure 1).[3] Whereas the amino acid sequence of the cathelin domain is conserved throughout animal species tested to date, the sequence of the mature peptide exhibits considerable variations, accounting for various molecular structures, such as α-helix, β-sheet, etc., possibly reflecting the nature of microbial diversity (Figure 1). The cathelin domain primarily functions as a cathepsin L inhibitor, from which the name of this domain is derived.[4] However, it was later demonstrated that this domain also possesses an antimicrobial function against *Escherichia coli* and methicillin-resistant *Staphylococcus aureus*, yet its antimicrobial mechanism is still largely unknown.[5] The first cathelicidin antimicrobial peptide was isolated from bovine neutrophils.[6] Subsequently, several cathelicidin peptides were identified in various mammals, particularly humans. The only cathelicidin in humans, LL-37, an α-helical peptide (Figure 1A), is derived from proteolytic processing of a precursor peptide, human cationic antimicrobial protein-18, and contains two leucines at its N-terminus.[7,8]

Defensin is a family of small cationic antimicrobial peptides. Their molecular structure is an anti-parallel β-pleated sheet (red arrows in Figure 2) with six conserved cysteine amino acids that form three disulfide bonds (blue lines in Figure 2), functioning in stabilization of their β-sheet structure.[9] Moreover, some defensins, especially human β-defensin-2 (hBD-2) and human β-defensin-3 (hBD-3), contain an α-helical domain

at their N-terminus (purple in Figures 2B and 2C). Defensins, comprising several positively charged amino acids that favorably interact with negatively charged microbial membranes, can form a complex structure, such as a dimeric structure.[10] In addition, the defensin peptides contain both hydrophobic and hydrophilic domains in their molecules, a so-called amphipathic structure. All of these properties, thus, make the defensins suitable for membrane integration that eventually leads to a pore formation in the membrane. The pore-forming mechanism of the defensins is then believed to be a crucial process in their antimicrobial

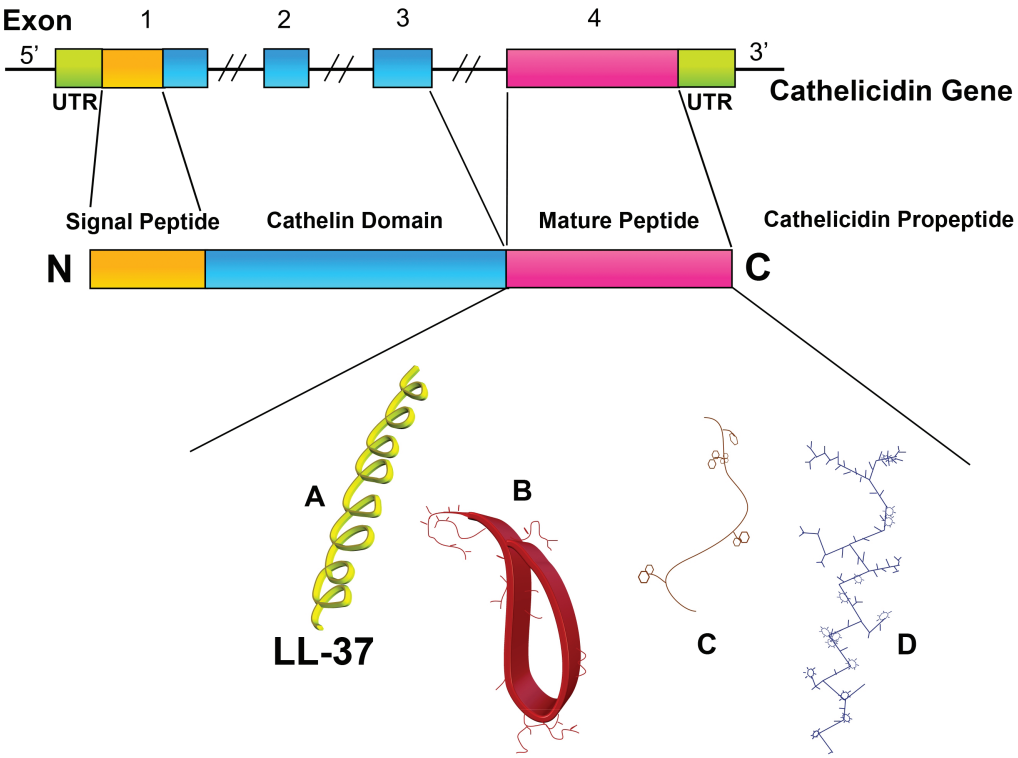

Figure 1 *The cathelicidin gene and propeptide. The different molecular structures of mature peptides comprise an α-helix (A), such as LL-37, a cysteine-rich sheet (B), a tryptophan-rich linear peptide (C), and a proline-rich linear peptide (D). UTR = an untranslated region, N = N-terminus, C = C-terminus.*

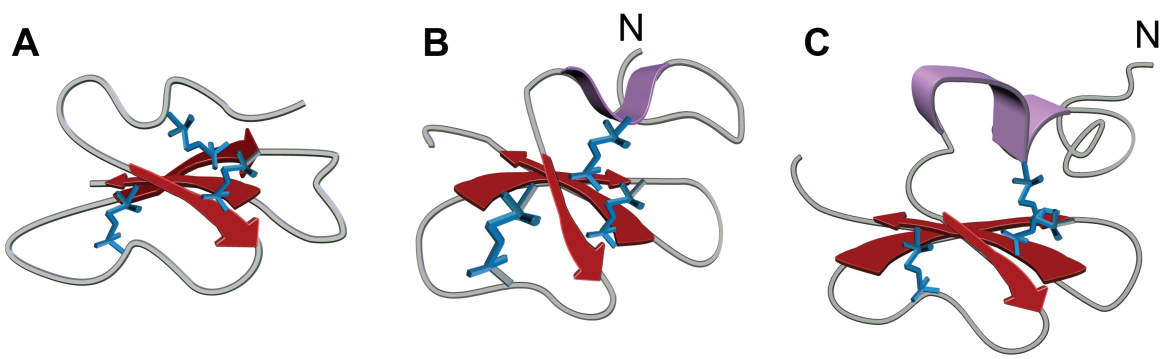

Figure 2 *The molecular structure of hBD-1 (A), hBD-2 (B), and hBD-3 (C). Each is an anti-parallel β-pleated sheet (red arrows) that contains three disulfide bonds (blue lines). Furthermore, an additional α-helical domain (purple) near the N-terminus is present in hBD-2 (B) and hBD-3 (C).*

function. Therefore, it has been shown by a number of studies that the defensins exert their broad spectrum of antimicrobial activities against gram-negative and gram-positive bacteria, fungi, and some enveloped viruses.[9]

The human defensin family can be further divided into two subfamilies, including α-defensin and β-defensin subfamilies. In the α-defensin subfamily, four of the six α-defensins, human neutrophil peptide-1, -2, -3, and -4 (HNP-1, -2, -3, and -4), are synthesized and stored in neutrophil granules,[11,12] while the other two α-defensins, human defensin-5 and -6 (HD-5 and -6), are synthesized and stored in the granules of Paneth cells, specialized epithelial cells located at the crypts of Lieberkühn of the small intestine.[13,14] Being encoded by the same gene, the pro-peptide of HNP-1, -2, and -3 comprises 94 amino acids, which is successively cleaved by putative proteolytic enzymes, yielding different sizes of the mature peptides that are stored in azurophilic granules.[15] The number of amino acids in the mature peptides of HNP-1, HNP-2, and HNP-3 varies from 29 to 30 amino acids. On the other hand, HD-5 and HD-6 are stored in Paneth cell granules as pro-peptides, and are subsequently activated by trypsin digestion upon release into the intestinal lumen.[16] HNP-4 is encoded by another gene, and its amino acid sequence completely differs from that of HNP-1, HNP-2, and HNP-3, leaving only the identical characteristic cysteines and some arginines.[12]

In the β-defensin subfamily, four human β-defensins, human β-defensin-1, -2, -3, and -4 (hBD-1, -2, -3, and -4), are principally expressed in epithelial cells that cover several tissues and organs, particularly skin and the mucosal surfaces of gastrointestinal, respiratory, and urogenital tracts. However, only hBD-1, -2, and -3 are expressed in the oral cavity.[17] HBD-1 and hBD-2 peptides are localized in differentiated epithelial cells within the suprabasal layers of normal gingival epithelium,[2] whereas hBD-3 peptide is expressed in undifferentiated epithelial cells within the basal layer,[18] suggesting a potential role for hBD-3 as a mediator to signal the underlying connective tissue cells. HBD-1 is constitutively expressed in several epithelial cell types, especially gingival epithelial cells,[19] whereas expression of hBD-2, hBD-3, and hBD-4 is inducible upon stimulation with pro-inflammatory cytokines or contact with microorganisms.

Expression and Regulation of Human Cathelicidin and Defensins

Human cathelicidin is mainly isolated from neutrophil granules distinct from those that store proteolytic enzymes, such as neutrophil elastase, proteinase-3, etc., to prevent premature activation of the cathelicidin peptide inside the neutrophils. Upon being released into neutrophil phagosomes after bacterial phagocytosis, the neutrophil cathelicidin is proteolytically cleaved into a mature LL-37 peptide by proteinase-3.[20] Moreover, cathelicidin expression in other cell types can be controlled by exposure to microorganisms, growth factors, and differentiating agents. For instance, LL-37 expression in skin keratinocytes and gastric epithelial cells is induced by *Staphylococcus aureus* and *Helicobacter pyroli*, respectively.[21,22] Furthermore, LL-37 expression is up-regulated by insulin-like growth factor-I and vitamin D, known to promote wound healing and differentiation, respectively.[23,24]

In the oral cavity, LL-37 is expressed in buccal and tongue mucosa,[25] and its expression is up-regulated in the inflamed gingival tissues.[26] Correspondingly, the concentrations of LL-37 in the gingival tissue, whether derived from neutrophils or from gingival epithelium, correlate positively with the depth of the gingival crevice, suggesting that LL-37 levels may be used as one diagnostic tool in inflammatory periodontal disorders.[26] In addition, LL-37 peptide is detected in saliva[27] and GCF[28], and the LL-37 levels in GCF are significantly elevated in patients with chronic periodontitis compared to those in patients with gingivitis

or to those in healthy volunteers.[29]

The neutrophil α-defensin gene (*DEFA1*) is located on chromosome 8 (8p23).[9] HNP-1, HNP-2, and HNP-3 mRNAs are mainly expressed in neutrophils, and their respective proteins were first characterized from azurophilic granules.[11] Moreover, expression of HNP-1, HNP-2, and HNP-3 can be detected in Langerhans cells in the vicinity of epithelial dysplasia adjacent to precancerous lesions and oral squamous cell carcinoma, but their expression is not found in normal oral mucosa.[30] They are also present in ductal cells of submandibular salivary glands from patients with oral cancer.[31]

With respect to periodontal tissue, the detectable amounts of HNP-1, HNP-2, and HNP-3 in GCF can vary from 270 to 2000 nanogram per site (or approximately equivalent to mg/ml), which is sufficient for their antimicrobial function in periodontium.[32] By virtue of matrix assisted laser desorption ionization mass spectrometry, it has been demonstrated that HNP-1 is most abundant in GCF, whereas HNP-3 is least abundant.[33] Moreover, the concentrations of HNP-1, HNP-2, and HNP-3 have been quantified in saliva. These concentrations (up to twelve μg/ml) are variable in the human population, and the median levels of HNP-1,HNP-2, and HNP-3 in saliva are significantly higher in children without dental caries than in those with dental caries experience, suggesting the protective role of neutrophil α-defensins against dental caries.[34]

The sizes of β-defensins are somewhat larger than those of α-defensins. The first human β-defensin is hBD-1, isolated from hemofiltrate passing through the kidney at the nanomolar levels.[35] The gene encoding hBD-1, *DEFB1*, is on chromosome 8, in close proximity to *DEFA1*, around 100-150 kilobases apart.[36] *DEFB1* contains two exons and one large 6962 base pair (bp) intron, and the two exons encode a 362 bp complementary DNA (cDNA) that is translated into an hBD-1 pro-peptide.[36] The hBD-1 pro-peptide is subsequently cleaved into several hBD-1 mature peptides, ranging from 36 to 47 amino acids long. Widespread and low expression of hBD-1 has been detected in various epithelia lining several organs, such as trachea, bronchus, skin, small intestine, salivary glands, etc.[9]

In oral mucosa, hBD-1 expression is found in gingival epithelium, but is not associated with the amount of IL-8 expression in the gingival tissue, suggesting that the amount of hBD-1 expression does not correlate with the degree of tissue inflammation, but varies among different individuals.[19] Confluent cultured gingival epithelial cells constitutively express hBD-1 mRNA at baseline levels; however, its expression is up-regulated in a post-confluent culture, representing the state of cellular differentiation *in vitro*.[2] On the other hand, it has been demonstrated that increased hBD-1 expression can, in turn, induce differentiation in skin keratinocytes.[37]

By using a protein chip array together with surface enhanced laser desorption/ionization and time-of-flight mass spectrometry, hBD-1 peptide at a molecular mass of about 4.7 kilodalton is detected in culture medium of gingival epithelial cells.[38] Highly variable amounts of hBD-1 peptide have been found in saliva and GCF, collected from different normal individuals.[38] It is possible that salivary ductal cells may contribute some hBD-1 peptide, detected in saliva, apart from the hBD-1 peptide synthesis by oral epithelial cells.[39] It is noteworthy that hBD-1 and hBD-2 are neither expressed in cultured gingival fibroblasts [19,40] nor found in the underlying connective tissue of the oral mucosa.[2]

The second human β-defensin (hBD-2) was first isolated in large amounts from psoriatic skin keratinocytes.[41] The gene encoding hBD-2 is *EFB4*, which is located on chromosome 8, region 8p22-p23.1, in close proximity to *DEFA1* and *DEFB1*.[42] *DEFB4* contains one 1639 bp intron and two small exons that encode a signal peptide domain and a mature peptide, whose sizes are 23 and 41 amino acids long, respectively.[42] Expression of both hBD-1 and hBD-2

is localized in the suprabasal layers of normal epidermis,[43] identical with their expression in normal oral mucosa.[2] HBD-2 peptide is stored in lamellar granules in the spinous layer of epidermis, and later released into the extracellular environment with other lipids in the granular layer, suggesting that lipids covering the skin function as a natural barrier against water permeability and microbial invasion due to the presence of antimicrobial peptides.[44]

Similar to the inducible expression of hBD-2 by microorganisms and pro-inflammatory cytokines in other cell types, hBD-2 mRNA is up-regulated in cultured gingival epithelial cells in response to stimulation with IL-α, TNF-β, phorbol ester, a potent epithelial activator, and gram-negative periodontal bacteria, including *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*.[40,45-48] Nevertheless, unlike the critical role of CD14, a lipopolysaccharide (LPS) co-receptor, and nuclear factor-kappa B (NF-κB) in hBD-2 induction in respiratory epithelial cells and mononuclear phagocytes,[49,50] CD14 and NF-κB are neither critical nor essential for hBD-2 up-regulation in gingival epithelial cells.[51] In fact, a purified LPS fraction of *Fusobacterium nucleatum* or *Aggregatibacter actinomycetemcomitans* is a poor hBD-2 activator in gingival epithelial cells.[40,48] Furthermore, p38 MAP kinase and c-Jun N-terminal MAP kinase (JNK) control hBD-2 mRNA up-regulation in response to *Fusobacterium nucleatum* in gingival epithelial cells.[51] Likewise, the MAP kinase pathways, but not the NF-κB transcription factor, are critical for hBD-2 up-regulation by the outer membrane protein 100 of *Aggregatibacter actinomycetemcomitans*.[52] Taken together, these findings suggest different cellular receptors and intracellular signaling mechanisms to control hBD-2 up-regulation by different stimulants in distinct cell types. In addition to the involvement of p38 MAP kinase and JNK in hBD-2 up-regulation by *Fusobacterium nucleatum*, it is shown that an increase in intracellular calcium ion and phosphorylated phospholipase D, two important molecules in regulating epithelial cell differentiation, are involved in hBD-2 up-regulation by *Fusobacterium nucleatum*.[53,54] Accordingly, the regulation of hBD-2 expression can be controlled by both inflammation from bacteria and epithelial differentiation.

The highest hBD-2 expression in gingival tissue is found at the gingival margin, adjacent to microbial plaque accumulation, and hBD-2 expression is localized in differentiated epithelial cells within the suprabasal layers of gingival epithelium.[2] Moreover, the localization of hBD-2 peptide is found not only in cultured gingival epithelial cells that express involucrin, another marker for differentiation, but also in stimulated cells with infectious and pro-inflammatory stimulants.[2] In contrast, neither hBD-1 nor hBD-2 is expressed in junctional epithelium, which consists of relatively undifferentiated epithelial cells, implying that the junctional epithelium may be more susceptible to infection than other areas of gingival epithelium because of the lack of some antimicrobial peptides.[2] However, it is probable that other antimicrobial peptides, such as α-defensins, LL-37, etc., released from neutrophils that transmigrate from blood vessels into the junctional epithelium and gingival crevice, may perform this antimicrobial function instead.[1]

Using biochemical and molecular biology techniques, the gene encoding hBD-3 (*DEFB103*) has been cloned from skin keratinocytes and alveolar epithelial cells, and the amino acid composition of hBD-3 has been sequenced and classified as a novel peptide in the β-defensin subfamily.[55] *DEFB103*, containing two small exons, is located 13 kb upstream from *DEFB4* that encodes hBD-2 on chromosome 8.[56] HBD-3 cDNA is translated into an hBD-3 pro-peptide that comprises a signal peptide domain and a mature peptide (22 and 45 amino acids long, respectively). The amino acid sequence of hBD-3 is 43% identical to that of hBD-2.[56]

In the oral cavity, hBD-3 mRNA and peptide

are localized in the basal layer of normal gingival epithelium.[18] Furthermore, hBD-3 mRNA is expressed in both inflamed and non-inflamed epithelium and salivary glands,[57] and its expression is up-regulated in leukoplakia and oral lichen planus.[58] *In vitro*, hBD-3 mRNA expression is induced in cultured epithelial cells that are stimulated with IFN-γ, TNF-α, and IL-1β,[55,56] although IFN-γ does not up-regulate hBD-2 mRNA.[59] Consistently, it is later demonstrated that IFN-γ is a primary inducer for hBD-3 expression, whereas IL-1β and TNF-α are major stimulants for hBD-2 expression.[60]

With respect to up-regulation of hBD-3 by oral microorganisms, hBD-3 mRNA expression is induced by live nonperiodontopathic bacteria, including *Streptococcus sanguinis* and *Streptococcus gordonii*,[61] and some periodontopathic bacteria, including *Aggregatibacter actinomycetemcomitans*,[62] *Prevotella intermedia*, and *Fusobacterium nucleatum*.[61] In contrast, three well known causative pathogens in chronic periodontitis, including *Porphyromonas gingivalis*, *Tanerella forsythia*, and *Treponema denticola*, down-regulate hBD-3 mRNA expression, as well as IL-8 production and secretion in an oral epithelial cell line.[61] This indicates that these so-called red-complex periodontal pathogens may suppress innate immune responses of oral epithelial cells by an immune-evading mechanism, known as chemokine paralysis. Furthermore, the red-complex bacteria can tolerate the host immune response by being more resistant to LL-37 and phagocytosis by neutrophils, indicating their strong implication with chronic periodontal infection.[63]

Conclusions

Substantial variations in expression of small cationic antimicrobial peptides, including LL-37 and defensins, in periodontal tissues, GCF, and saliva, exist and may be correlated with the pathogenesis of periodontal disease, as well as that of other oral inflammatory and infectious diseases. Therefore, the association between altered expression of antimicrobial peptides and some types of periodontitis should be further explored in detail. Moreover, expression of some antimicrobial peptides and their clinical significance in other oral diseases should be further studied. Perhaps, some peptides could be further developed as biomarkers for diagnosis and/or prognosis of oral diseases in the future.

It is also necessary to continue regulation studies of some inducible antimicrobial peptides in order to understand the mechanisms used to enhance expression of these peptides. In quest of new adjunctive treatment modalities for periodontitis, it is probable that enhancement of antimicrobial peptide expression by putative components of commensal bacteria that are not harmful to the human body or by non-toxic agents, similar to vaccination, may be of significant interest in controlling the number of periodontal pathogens.

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