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Expression of human β-defensins-1 and -2 peptides in unresolved chronic periodontitis

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Background: Human β-defensins (hBDs) are antimicrobial peptides which contribute to host innate immunity by disrupting the membrane integrity of a broad spectrum of microorganisms.

Objectives: This study aimed to determine the expression profiles of hBD-1 and -2 peptides in gingiva and to assess the possible relations of these antimicrobial peptides with periodontal health and disease.

Methods: Seven periodontally healthy subjects and 22 patients with unresolved chronic periodontitis were recruited and the gingival biopsies collected consisted of healthy tissues from the healthy subjects (HT-C); periodontal pocket tissues (PoT) and inflamed connective tissues (ICT) from the base of pocket, i.e. granulation tissues, as well as clinically healthy tissues (HT-P) from the adjacent clinically healthy sites from the patients. The expression of hBD-1 and -2 peptides was detected by immunohistochemistry and quantitatively analyzed with a computerized image processing system.

Results: Both hBD-1 and -2 peptides were detected in all periodontally healthy subjects, while hBD-1 was detected in all patients and hBD-2 was found in most of the patients. Their expression was mainly confined to the granular and spinous layers of gingival epithelium, in which hBD-1 was detected in both intercellular spaces and cytoplasm, whereas hBD-2 was mainly observed in the cytoplasm. HT-C expressed significantly higher levels of hBD-2 than HT-P ($p < 0.05$). Within the patients, both defensins were up-regulated significantly in PoT as compared with the adjacent HT-P ($p < 0.05$).

Conclusions: The present study showed that hBD-1 and -2 were frequently expressed in the granular and spinous layers of gingival epithelia and their expression may be associated with periodontal health and disease.

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Human gingival epithelium is a specialized, stratified squamous epithelium that is delineated and compartmentalized as oral epithelium, sulcular epithelium, and junctional epithelium. Environment niches lined by these epithelia are constantly challenged by bacteria both in health and disease. The gingival epithelium therefore plays a crucial role in containing microbial challenge in addition to initiating an inflammatory response in periodontal diseases. Periodontal diseases are characterized by bacteria-induced inflammatory destruction of tooth-supporting tissues. The severity of periodontal diseases is somewhat dependent on a dynamic equilibrium of bacteria–host interactions (1, 2). As it takes at least three to five days for adaptive immunity to be operative and

effective, the more economic and promptly triggered innate arm of the immune system appears to be crucial in preventing the early onset of infection (3). Of the various attributes contributing to the innate immunity, a group of well-evolved and conserved antimicrobial peptides, which are detected in gingival epithelia, are now considered to be of major importance (4). Here, termed human defensins are a group of small cationic cysteine-rich peptides with potent antimicrobial activity against both gram-positive and gram-negative bacteria, fungi, and viruses (5). Human defensins are classified into α and β subforms according to the cysteine spacing and connecting patterns of three disulfide bonds (6). Six human α defensins (7–10) and four β defensins (hBDs) (11–13) have been identified.

hBD-1 was initially isolated from human hemofiltrates (14) and subsequently identified in the epithelia of a wide range of tissues, such as kidney, pancreas, urogenital and respiratory tracts (15, 16), saliva and gingival epithelia (17). Most of these studies have revealed the constitutive expression of hBD-1 in various epithelia, suggesting a 'surveillance-like' role in the absence of infection and a protective role in the presence of infection (15, 17). Some studies showed the up-regulation of hBD-1 in inflamed epithelia (18, 19), whereas others demonstrated its increased expression upon encounter of certain pathogens (20) and inflammatory mediators (21).

hBD-2 in contrast is an inflammation-induced defensin originally isolated from psoriatic scale keratinocytes and it could be detected in skin (5), trachea (22), saliva and gingiva (17). It is active against gram-negative bacteria and *Candida albicans*, but has more restricted activity against gram-positive bacteria (5). Most workers have noted abundant hBD-2 expression in inflamed tissues (17, 21), whereas one study reported its decreased expression in atopic dermatitis (23). Yet a number of *in vitro* studies have shown the inducibility of hBD-2 in human keratinocytes exposed to proinflammatory cytokines and microorganisms, implying its more specialized role in the innate epithelial defense compared with hBD-1, which is less active in diseased state (17, 24).

Although the study of antimicrobial peptides has recently been pursued in many fields, there is little information on the relationship between human β-defensins and periodontal diseases. To our knowledge, none relates their expression levels to periodontal health and disease. Hence, we examined the expression of hBD-1 and hBD-2 in gingiva of periodontally healthy subjects as well as in the inflamed periodontal pockets and the clinically healthy gingiva from patients with chronic periodontitis.

Materials and methods

Subjects

Twenty-two Chinese adults, age range 35–55 years, were recruited for the study. They had untreated advanced chronic periodontitis, with ≥ 5.0 mm of probing depth, ≥ 3.0 mm of clinical attachment loss and radiographic evidence of alveolar bone loss on at least two teeth per quadrant. Following basic periodontal treatment, all subjects exhibited unresolved periodontitis in need of periodontal surgery. Seven periodontally healthy subjects (mean age 21.6 ± 5.4 years) were recruited as controls. They did not show any sites with probing depth > 4 mm or clinical attachment loss > 1 mm in any quadrant or radiographic evidence of bone loss, and exhibited bleeding on probing in < 15% of sites. The general health of all subjects recruited was good and none received antibiotics within the preceding 6 months. None reported receiving any prior immunosuppressive therapy. Written and oral informed consent was obtained from all recruits and the study protocol was approved by the Ethics Committee, Faculty of Dentistry, the University of Hong Kong.

Collection of samples

Gingival biopsies were collected during periodontal surgery in unresolved periodontitis sites with probing depth ≥ 6 mm, clinical attachment loss ≥ 5 mm and significant loss of alveolar bone on radiographs following non-surgical treatment, consisting of periodontal pocket tissues (PoT) and inflamed connective tissues (ICT) from the base of pocket, i.e. granulation tissues. Clinically healthy tissues (HT-P) were collected from the clinically healthy sites adjacent to the pocket sites with probing depth ≤ 3 mm, clinical attachment loss ≤ 1 mm and absence of bleeding on probing (25). When collectable, one biopsy of each category of tissues (PoT, ICT and HT-P) was obtained from each patient. Seven gingival biopsies were obtained from seven periodontally healthy subjects as healthy controls (HT-C) during tooth extraction for orthodontic reasons. These sites sampled met the following criteria: (i) probing depth not exceeding 3 mm; (ii) absence of bleeding on probing; (iii) clinical attachment loss not exceeding 1 mm; and (iv) no radiographic evidence of alveolar bone loss.

Immunohistochemistry

The gingival biopsies were immediately fixed in 4% paraformaldehyde for 24 h, dehydrated, and embedded in paraffin. The embedded samples were routinely sectioned to yield 4-μm thick specimens and mounted onto slides for immunohistochemistry procedure. Serial paraffin sections were deparaffinized, rehydrated, and then soaked in deionized water containing 3% hydrogen peroxide for 10 min to block endogenous peroxides. Non-specific binding was blocked for 30 min with 3% bovine serum albumin (Sigma, St. Louis, MO, USA) in Tris-buffered saline. The sections were incubated at 4°C overnight with goat polyclonal IgG antibodies to hBD-1 and hBD-2 (Santa Cruz Biotechnology, CA, USA) at 1 : 200 and 1 : 100 dilution, respectively. After sections were rinsed in Tris-buffered saline, the reaction was detected using an avidin–biotin peroxidase complex kit (Santa Cruz Biotechnology) according to the manufacturer's instructions. Visualization was performed using 3,3-diaminobenzidine (Sigma), followed by a rinse in running tap water. Sections

were counterstained with Mayer's hematoxylin, dehydrated, and permanent mounted. Negative control experiments were performed by incubation with Tris-buffered saline instead of the primary antibodies, and with hBD antibodies preincubated in an excess of recombinant hBDs (Santa Cruz Biotechnology).

Image analysis

A computer-assisted image analysis system was used to quantify the proportion of positive-stained areas within the epithelial tissues of the sample section (26), as all the positive staining was observed in the epithelial region. The image analysis system comprised a true-color red–green–blue video camera (Leica DC 300 V 2.0, Leica, Wetzlar, Germany, 2088 × 1550 pixel) connected to a microscope (Leica DMLB, Leica) via a special B-mount adapter, and a computer equipped with Leica Qwin software (Qwin V 2.4, Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). The high definition images of sections were acquired and evaluated at a magnification of ×20. Discrimination between the positive-stained tissues and the negative-counterstained tissues was semi-automatically recognized by the image analysis system. The hue-intensity-saturation analysis system was used, which performed in a way quite close to the behavior of human eyes (26). Prior to detection of the positive-staining areas, two thresholds were set by defining the weakest and strongest positive areas and the range between them represented the chromaticity of the positive staining that was recognized by the system. For each gingival specimen, the proportion of total positive area to total epithelial area was calculated and presented as area‰.

Statistical analysis

The difference in hBD expression levels between the paired samples (PoT and HT-P) within the same subject was assessed by the paired *t*-tests. The significance of the difference in hBD expression between healthy controls and patients under different conditions was evaluated by ANOVA. Statistical analyses were performed using SPSS 10.0 software. A p-value of < 0.05 was considered statistically significant.

Results

Expression patterns of hBD-1 and hBD-2

The expression of hBD-1 and hBD-2 peptides in gingival epithelia was mainly confined to the granular and spinous layers in both healthy controls and patients, despite some variations in their expression patterns among subjects (Figs 1 and 2). In a few samples, hBD-1 and hBD-2 were also spottily and sparsely distributed in the basal layers (Figs 1 and 2b, 2c, 2e–h). hBD-1 was detected in both cytoplasm and intercellular spaces (Figs 2g, h and k), whereas hBD-2 was mainly confined to cytoplasm of the cells concerned (Figs 2i, j and l). In no cases were the hBD-1 and -2 detected in connective tissues. Of the 17 ICTs, three ICTs with abundant epithelial components expressed both hBD-1 and hBD-2.

With regard to the expression levels of both antimicrobial peptides in gingival epithelia, hBD-1 expression level was relatively higher than hBD-2 in 26 out of 32 samples (19 PoTs and 13 HT-Ps) from patients and in contrast, hBD-2 expression was relatively stronger than hBD-1 in five out of seven healthy controls.

Expression levels of hBD-1 and hBD-2 in periodontal health and disease

Both hBD-1 and -2 were expressed in all seven HT-Cs. hBD-1 was detected in all HT-Ps (13/13) and PoTs (19/19), whereas hBD-2 was found in 10 HT-Ps (10/13) and 14 PoTs (14/19). The expression levels of hBD-1 and -2 varied greatly in PoTs (Fig. 3). The mean expression levels of hBD-1 in PoTs (18.12 ± 16.71‰) were significantly higher than in HT-Ps (8.79 ± 7.04‰) ($p < 0.05$) and in HT-Cs (6.67 ± 4.86‰) ($p < 0.05$). No significant difference was found between HT-Cs and HT-Ps. For hBD-2, the mean expression levels in HT-Cs

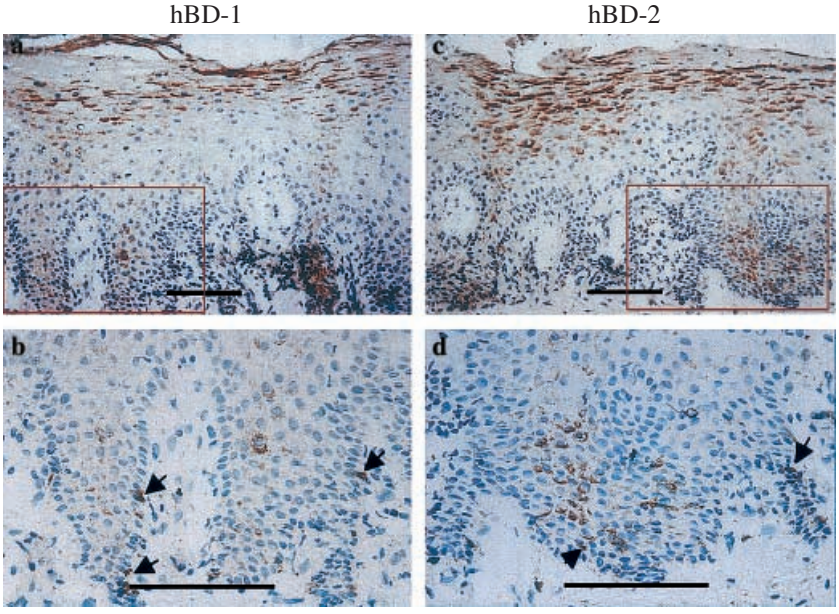

Fig. 1. Immunohistochemical demonstration of human β-defensins-1 (hBD-1) and hBD-2 in gingival epithelia of periodontally healthy subjects. Gingival samples of periodontally healthy subjects were processed with goat polyclonal IgG antibodies to hBD-1 (a, b) and hBD-2 (c, d) using a standard immunohistochemical protocol. Periodontally healthy tissues show intense expression of hBD-2 (c, d) and less intense expression of hBD-1 (a, b) in the granular, spinous and basal cell layers of gingival epithelia. The encircled areas in (a) and (c) are amplified in (b) and (d), respectively. Arrows: positive expression in basal layers. Scale bar: 100 μm.

(11.25 ± 5.49‰) were markedly higher than in HT-Ps (1.98 ± 2.30‰) ($p < 0.05$) and higher than in PoTs (5.85 ± 7.91‰). No significant difference was found between HT-Ps and PoTs.

Expression levels of hBD-1 and hBD-2 in subjects with unresolved chronic periodontitis

Of the samples obtained from the 22 patients, 12 pairs of PoTs and HT-Ps were collected from 12 patients (1 pair per subject). As shown in Table 1, eight subjects exhibited higher levels of hBD-1 in PoTs than in the corresponding HT-Ps, with a ratio of PoT to HT-P ranging from 1.36 to 19.57, and four subjects presented a reverse situation with a ratio of HT-P to PoT from 1.20 to 1.79. For hBD-2, seven subjects showed higher levels in PoTs than in HT-Ps with a ratio of PoT to HT-P from 2.15 to 217.0, and four subjects exhibited higher levels in HT-Ps with a ratio of HT-P to PoT from 1.92 to 20.0. hBD-2 was not detected in both HT-P and PoT in one subject. The overall data showed higher expression levels of both defensins in PoTs than in HT-Ps ($p < 0.05$).

Discussion

Human periodontitis is a common oral disease initiated and perpetuated by a group of predominantly gram-negative anaerobic bacteria, such as *Porphyromonas gingivalis*, *Bacteroides forsythus* and *Actinobacillus actinomycetemcomitans* (27). The ability of gingival sulcular and junctional epithelia to control bacterial challenge and prevent tissue destruction is crucial for maintenance of periodontal health. A recent study has shown the expression of human β-defensins in oral and sulcular epithelia as well as α-defensins and LL-37 in junctional epithelia, suggesting defensins serve different roles in various regions of the periodontium (4). In the present study, we observed both hBD-1 and hBD-2 peptides expression in gingival epithelia from periodontally healthy subjects as well as clinically healthy and pocket sites of patients with unresolved chronic periodontitis. As it has been shown previously that junctional epithelium contains no hBDs, their expression in pocket epithelium as shown here seems to imply not only the inducibility of both hBD-1 and hBD-2 upon periodontal infection, but also the different activities of keratinocytes at junctional epithelium and pocket epithelium.

In contrast to previous studies in skin (28), kidney (21) and gastric antrum (20) which reported undetectable

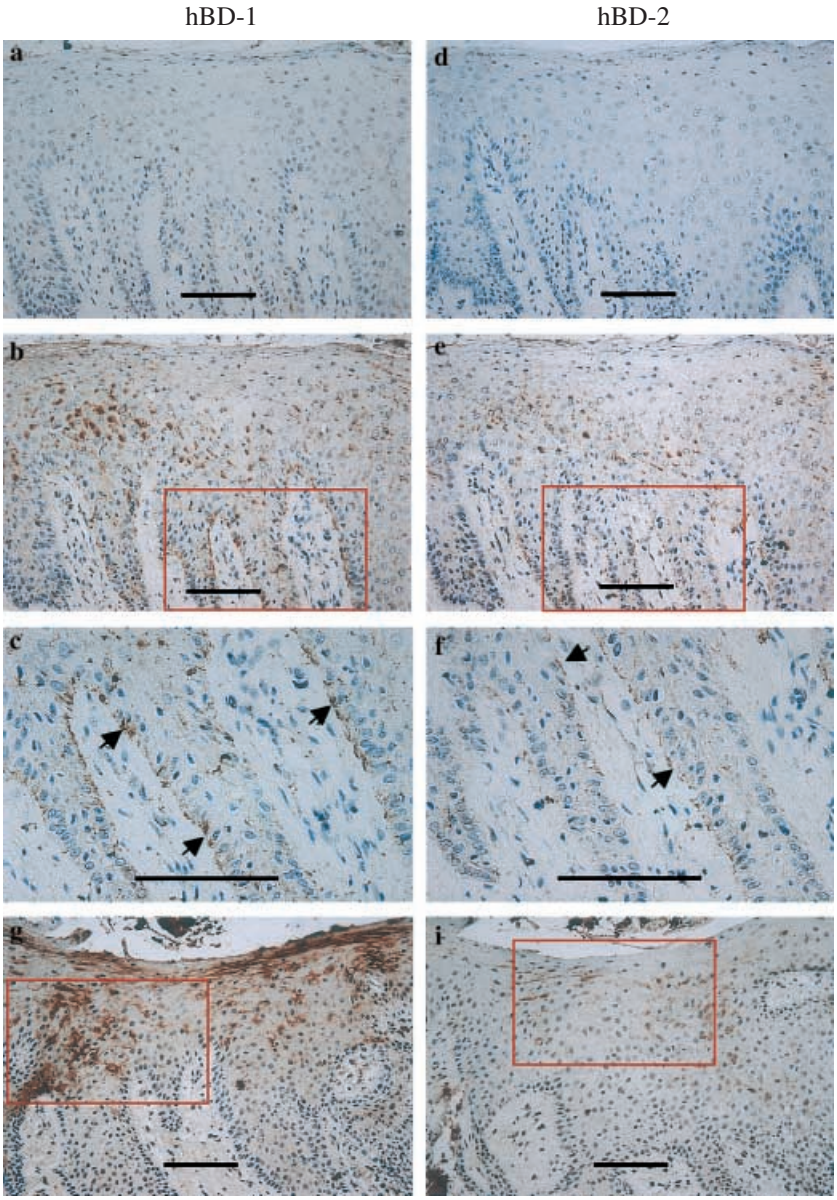

Fig. 2. Immunohistochemical demonstration of human β-defensins-1 (hBD-1) and hBD-2 in the pocket epithelia of patients with chronic periodontitis. Negative controls (a, d) were processed with hBDs antibodies preincubated in an excess of recombinant hBDs. The pocket epithelia of patients were processed with goat polyclonal IgG antibodies to hBD-1 (b, c, g, h, k, m, o) and hBD-2 (e, f, i, j, l, n, p) using a standard immunohistochemical protocol. The expression of hBD-1 and hBD-2 was mainly confined to the granular and spinous layers of gingival epithelia, although they were detected in all layers of pocket epithelia (b, e) including basal layers (c, f). hBD-1 was intensely stained in the intercellular and cytoplasm (g, h, k, m), whereas hBD-2 was sparsely stained in the cytoplasm (i, j, l, n). hBD-1 (o) and hBD-2 (p) may exhibit a similar expression pattern as well. The encircled areas in (b), (e), (g) and (i) are amplified in (c), (f), (h) and (j), respectively. Arrows: positive expression in basal cells. Scale bar: 100 μm.

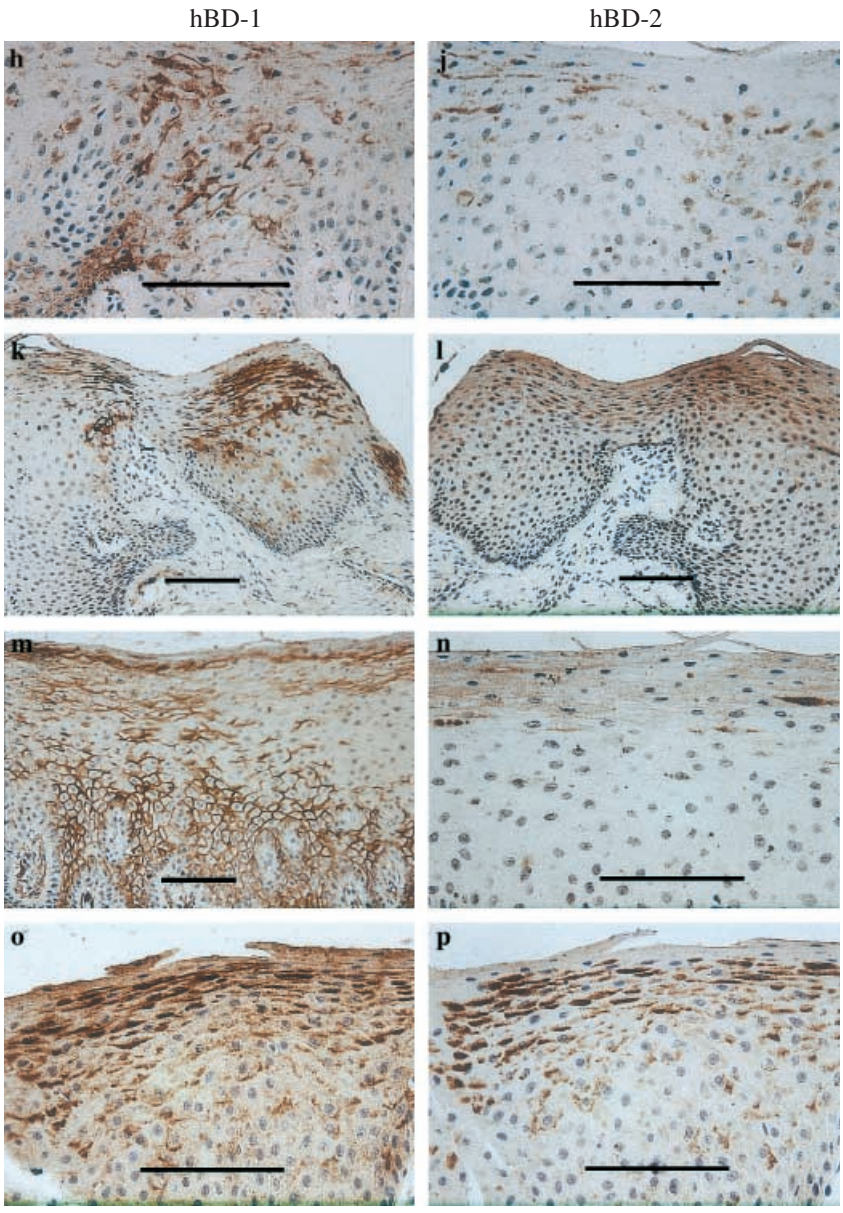

Fig. 2. Continued.

levels of hBD-2 in non-inflamed sites, our present study showed its consistent expression in healthy gingival epithelia as in other epithelial compartments of the oral cavity (29). It was surprising to note that the gingival epithelium from periodontally healthy subjects exhibited a significantly higher hBD-2 expression than the clinically healthy tissues from periodontitis patients. The reason for this observation is unclear. Based upon the evidence of an *in vitro* study that *Fusobacterium nucleatum* significantly stimulated hBD-2 mRNA expression in gingival epithelial cells from healthy tissues (24), it could be speculated that the high level of hBD-2 might be due to the potent and constant background activity of the predominant commensal bacteria and their interactions with the healthy oral tissues. This was also evidenced in an analogous situation in frogs, whose skin is capable of secreting 30–40 antimicrobial peptides when exposed to a variety of natural flora in mud and soils (30). The decreased levels of hBD-2 in the clinically healthy sites of patients may be due to the expense in the battle against periodontal pathogens, which demonstrates the susceptibility of the subjects to periodontitis.

Although hBD-1 and hBD-2 were reported to be expressed in differentiated cells (4), we found a moderate expression of them in the basal layer of gingival epithelia in addition to the granular and spinous layers. The expression of both defensins by basal cells was recently observed in distal outer root sheath of the hair follicle, a region highly exposed to physiologic and pathogenic skin microflora (19). These findings seem to imply that cell differentiation may not be the sole molecular basis for defensins expression. Lipopolysaccharide, interleukin-1β (IL-1β), and tumor necrosis factor-α, known as highly specific β-defensin inducers (24), may putatively bolster the peptide expression in less differentiated cells (31, 32).

It is known that various periodontal conditions exist among tooth sites within the same periodontitis patient (25, 33, 34). In the present study, we compared the expression levels of hBD-1 and hBD-2 in pocket tissues and the adjacent clinically healthy tissues from the same individual. It was interesting to note that both defensins were expressed at a higher level in pocket epithelia than in the adjacent healthy gingival epithelia. Marked difference in the composition of subgingival microflora between these sites as shown in our previous studies may partly account for this observation (25, 33, 34). There is evidence that different bacteria modulate the production of antimicrobial peptides in distinct ways. *F. nucleatum*, for instance, up-regulated hBD-2 expression, whereas *P. gingivalis* did not (24); interestingly, enteric bacteria such as *Shigellae* down-regulate the genes encoding hBD-1, and *Helicobacter pylori* up-regulates hBD-1 mRNA expression in gastric cells (35). It was postulated that certain pathogens and their products in the unresolved pocket might be responsible for the high expression levels of both defensins. Moreover, inflammatory mediators and cytokines play a crucial role in regulating immuno-inflammatory responses to microbial challenge. Increased expression of IL-4 and IL-13

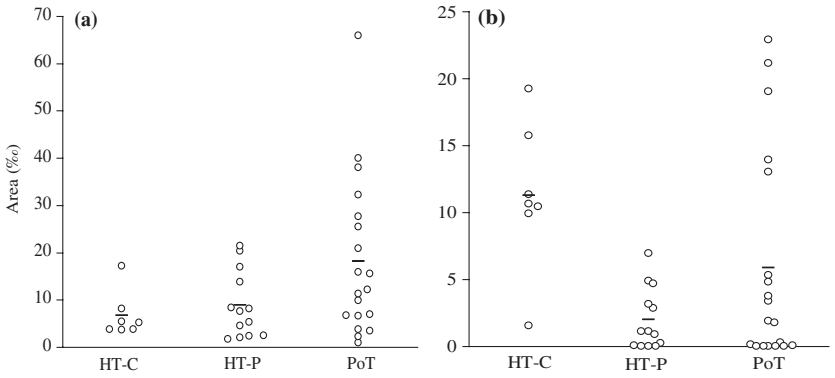

Fig. 3. The individual expression levels of human β-defensins-1 (hBD-1) (a) and hBD-2 (b) in periodontal health and disease. HT-C: the healthy gingival tissues from seven periodontally healthy subjects as controls; HT-P: the clinically healthy tissues from 13 patients with chronic periodontitis; PoT: the periodontal pocket tissues from 19 patients with chronic periodontitis. Horizontal bars indicate means of the value of each category of tissues.

Table 1. The expression levels (area‰) of human β-defensins-1 (hBD-1) and hBD-2 in 12 pairs of periodontal pocket tissues (PoT) and clinically healthy tissues (HT-P) from 12 subjects with unresolved chronic periodontitis

	hBD-1		hBD-2	
Subjects	PoT	HT-P	PoT	HT-P
1	27.64	20.29	0.12	0.23
2	15.48	8.32	22.88	6.93
3	9.78	16.94	13.91	1.11
4	0.91	1.63	0	2.83
5	37.97	1.94	1.89	0.88
6	32.19	2.34	19.01	4.68
7	25.38	4.45	13.02	0.06
8	65.82	13.75	0.05	1.11
9	6.64	8.04	1.75	0
10	11.26	7.56	3.74	0
11	39.9	5.22	0	3.13
12	15.81	21.37	0	0
Mean ± SD	24.07 ± 18.14	9.32 ± 7.08*	6.36 ± 8.43	1.75 ± 2.22*

Significant differences from PoT, *$p < 0.05$.

was highly effective in inhibiting hBD-2 mRNA expression in human keratinocytes and led to a diminished expression of the peptide in atopic dermatitis (23), whereas IL-1 was a potent stimulant of hBD-1 and hBD-2 (20, 24). Thus, it is tempting to speculate that an altered pro- and anti-inflammatory cytokine profiles might up-regulate the production of hBDs in pocket epithelia. However, as human periodontitis is a multifactorial inflammatory disease with a complex pathogenesis, the relationship between bacterial challenge, cytokines and hBDs expression is necessarily complex and should be interpreted with caution. Further study is warranted to confirm our hypothesis. The present study showed that hBD-1 and hBD-2 were frequently expressed in human gingiva and their expression was mainly confined to the granular and spinous layers of gingival epithelium. hBD-2 was detected in the cytoplasm, whereas hBD-1 was found both in the cytoplasm and the intercellular spaces of epithelial cells. hBD-2 expression level was significantly decreased in clinically healthy tissues from periodontitis patients, as compared with those from periodontally healthy subjects. Within the patients, both defensins were up- regulated in pocket tissues. This study suggests that the expression of hBD-1 and hBD-2 in gingival epithelia may be associated with periodontal health and disease.

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