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Expression of human β-defensin-3 in gingival epithelia

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Objective: This study aimed to investigate the expression patterns of the newly discovered human β-defensin-3 (hBD-3) in human gingiva.

Background: Human β-defensins (hBDs) are a group of small, broad-spectrum, cationic antimicrobial peptides. Our recent study showed that the expression levels of hBD-1 and 2 peptides were associated with periodontal conditions.

Methods: A total of 49 gingival biopsies were collected, including 33 samples from 21 patients with chronic periodontitis and 16 samples from 16 periodontally healthy subjects. The expression of hBD-3 was detected by immunohistochemistry and *in situ* hybridization. Double staining was undertaken to identify hBD-3 peptide-positive cells, using CD-1a and cytokeratin 20 as markers for Langerhans cells and Merkel cells, respectively.

Results: hBD-3 peptide was detected in 88% of the samples, which was confined to the gingival epithelia. In healthy control subjects, hBD-3 peptide was more frequently detected in the basal layer as compared to the patients (53% vs. 18%, $p < 0.05$). In patients, hBD-3 expression extended from the basal layer to the spinous layers (82%), in which hBD-3 was confined to the basal and deep spinous layers in clinically healthy tissues from patients, whereas it extended to the superficial spinous layers in pocket tissues from patients (0% vs. 50%, $p < 0.05$). In both groups, hBD-3 peptide was expressed not only in gingival keratinocytes, but also in Langerhans cells and Merkel cells. hBD-3 transcripts were detected in 90% of the samples and they were confined to the basal and/or suprabasal layers of gingival epithelia.

Conclusions: This study shows that hBD-3 is frequently expressed in gingival epithelia. The appropriate expression of hBD-3 peptide may contribute to the maintenance of periodontal homeostasis, possibly through its antimicrobial effect and promotion of adaptive immune responses.

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Human gingival epithelium is constantly exposed to multiple assaults by microbes that live harmoniously in the oral niche. This harmony depends on a dynamic equilibrium of bacteria–host interactions. Gingival epithelium is the first line of host defense, represented by not only its barrier function that physically prevents microbial invasion, but also its antimicrobial properties that biologically suppress the propagation of putative pathogens (1, 2). Periodontal diseases are initiated by a breakdown of the bacteria–host equilibrium that results in the inflammatory destruction of tooth-supporting tissues (3, 4).

Recently, a group of small cationic cysteine-rich peptides, termed defensins, have been investigated widely. Human defensins have a broad-spectrum antimicrobial activity against gram-positive and gram-negative bacteria, fungi, and viruses (2). On the basis of the cysteine spacing and

connecting patterns of three disulfide bonds, human defensins are classified into two subfamilies, the α-defensins and β-defensins (5). Six α-defensins (6–9) and four β-defensins (2, 10–12) have been identified.

Human β-defensins (hBDs) have been detected in gingival epithelia (13), salivary glands, saliva (14), and gingival crevicular fluid (1). The most investigated hBDs thus far are hBD-1 and hBD-2. Both are active against gram-negative bacteria and yeast, with restricted activity against gram-positive bacteria (2). Most workers have found that hBD-1 is constitutively expressed in various epithelia (14, 15), whereas hBD-2 is significantly induced in inflamed tissues (14, 16). However, others have shown the up-regulation of hBD-1 (13) and decreased expression of hBD-2 in inflamed tissues (17), revealing the complexity of inflammatory diseases and the insufficiency of our knowledge on defensins.

hBD-3 was first isolated from human lesional psoriatic scales and later found to be widely expressed in healthy or diseased tissues (18, 19). Compared with hBD-1 and hBD-2, hBD-3 has potent antimicrobial activities against both gram-positive and gram-negative bacteria, even at low concentrations (10). Moreover, its antimicrobial activity is salt-insensitive at physiologic salt concentrations, which makes hBD-3 of particular value in diseases with elevated salt concentrations (10). The expression of hBD-3 in keratinocytes was found to be regulated by several inflammatory stimuli. Tumor necrosis factor-α and interferon-γ stimulation induced hBD-3 gene expression, whereas IL-13 and IL-4 inhibited the induced hBD-3 expression (20). In addition to its antimicrobial activity, hBD-3 also contributes to the innate immune response by chemoattracting monocytes (19), which may lead to an increase of macrophage-secreted inflammatory factors, which in turn, regulate the production of hBD-3.

Several reports have demonstrated a wide expression of hBD-3 in human tissues; however, there is little information on its expression pattern in gingival tissues. In this study, we have, for the first time, investigated the co-expression of hBD-3 peptide and mRNA in gingival health and unresolved chronic periodontitis in order to explore the possible role of hBD-3 in the host response of human gingiva.

Material and methods

Subjects

Twenty-one 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. Sixteen periodontally healthy subjects (age range 13–32 years) were recruited as controls. They did not show any sites with probing depth > 4 mm or attachment loss > 1 mm in any quadrant or radiographic evidence of bone loss, and exhibited bleeding on probing in < 20% 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

Twenty periodontal pocket tissues were collected during periodontal surgery in unresolved periodontitis sites with probing depth ≥ 6 mm, attachment loss ≥ 5 mm, and significant loss of alveolar bone on radiographs following non-surgical treatment. Thirteen clinically healthy tissues were collected from the clinically healthy sites adjacent to the pocket sites with probing depth ≤ 3 mm, attachment loss ≤ 1 mm, and absence of bleeding on probing (21). Sixteen gingival biopsies were obtained from the non-inflamed sites of periodontally healthy subjects as healthy controls during tooth extraction for orthodontic reasons. All samples were immediately fixed in 4% paraformaldehyde for 24 h, dehydrated, and embedded in paraffin. The embedded samples were routinely sectioned to yield 3-μm thick specimens and mounted onto slides for immunohistochemistry and *in situ* hybridization procedures.

Immunohistochemistry

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. Then the sections were incubated at 4°C overnight with rabbit anti-human hBD-3 polyclonal IgG (Sage BioVentures Inc., Carlsbad, CA, USA) diluted in 3% NaCl, 0.5% Tween 20 in 1 × Tris-buffered saline. Rabbit non-specific IgG was added as the negative control. After incubation with biotinylated secondary antibody, the reaction was detected using an avidin-biotin peroxidase complex kit (DakoCytomation, Glostrup, Denmark) 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. In addition, the expression of hBD-1 and 2 peptides was detected with goat anti-human hBD-1 and 2 polyclonal IgG according to our established methods (13). For double staining, the second staining procedures were done following the first visualization. The protocol was applied as described above, except that mouse anti-human CD-1a monoclonal IgG_1 (Clone 010, DakoCytomation) (22) and mouse anti-human cytokeratin 20 IgG (Clone Ks20.8, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) (23) were used as the primary antibodies, respectively, polyclonal rabbit anti-mouse IgG (DakoCytomation) was used as the secondary antibody, and mouse monoclonal alkaline

phosphatase anti-alkaline phosphatase antibody (DakoCytomation) was used for visualization.

Subcloning of complementary DNA for human β-defensin-3

Total RNA was extracted from homogenized biopsies using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The first-strand complementary DNA (cDNA) was synthesized from 1 μg of RNA in a final volume of 20 μl using the SuperScript™ First-Strand Synthesis System for reverse transcription–polymerase chain reaction (RT–PCR) (Invitrogen Corp., Carlsbad, CA, USA). A total volume of 50 μl PCR reaction contained 1 × PCR buffer, 1.5 mM $MgCl_2$, 160 μM each dNTP, 2 U of *Taq* DNA polymerase (Invitrogen Corp.), 400 μM of each forward and reverse primer, and 2 μl of the cDNA solution. The sequences of the intron-spanning primers for hBD-3 were as follows: forward: 5′-CCCAAGCTTATGAGGATCCATTATCTTCTG-3′, which contained a recognition site of *Hin*dIII, and reverse: 5′-CGGGGTACCTTATTTCTTTCTTCGGCAGCA-3′, which contained a recognition site of *Kpn*I. The PCR process consisted of an initial denaturation at 94°C for 2 min, then 35 cycles of amplification (94°C for 30 s, 58°C for 30 s, 72°C for 1 min) and a final extension at 72°C for 10 min. It was performed in GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) and the products were separated by electrophoresis on 1.5% agarose gels. After detection with ethidium bromide staining, the 204-bp DNA band was cut off and purified with Gel-M Gel Extraction System (Viogene, Sunnyvale, CA, USA), and its identity was confirmed by sequencing analysis using ABI Prism 310 Genetic Analyzer (Applied Biosystems).

The purified PCR product and vector pT7/T3 18 (Ambion, Inc., Austin, TX, USA) were cut out with restriction enzymes *Hin*dIII and *Kpn*I (Invitrogen Corp.) and ligated to each other. The ligation product was transformed into DH5α competent cells (Invitrogen Corp.) for amplification, and purified using Minipreps DNA Purification System (Promega Corp., Madison, WI, USA). The purified vector was digested with *Hin*dIII and *Kpn*I, and electrophoresed on a 1.5% agarose gel to determine whether a DNA fragment had been inserted. The identity of the inserted fragment was confirmed by sequencing analysis and compared with the full cDNA sequence of hBD-3 in the GenBank database (Accession no. AJ237673). After linearization of the inserted vector with *Hin*dIII and *Kpn*I, respectively, digoxigenin-labeled sense and antisense RNA probes were transcribed using digoxigenin RNA labeling mix (Roche, Penzberg, Germany) and T7 or T3 polymerase according to the manufacturer's manual (Roche).

In situ hybridization

The deparaffinized sections were digested with 5 μg/ml proteinase K (Sigma) at 37°C for 25 min and post-fixed in 4% paraformaldehyde in phosphate-buffered saline. The sections were then acetylated with 0.25% acetic anhydride (Sigma) in 0.1 M triethanolamine for 10 min and air dried for at least 30 min. After prehybridization with hybridization buffer at 46°C for 2 h, the sections were incubated with 10 μg/ml digoxigenin labeled RNA sense and antisense probes for hBD-3 at 46°C for 18 h. The hybridization buffer was composed of 50% deionized formamide, 1 × Denhardt's (Sigma), 0.3 M NaCl, 0.005 M EDTA, 0.02 M Tris pH 8.0, 10% dextran sulfate, 500 g/ml yeast tRNA (Roche), and 500 μg/ml denatured salmon sperm DNA (Sigma). Stringent washes were conducted for 30 min at 46°C in 50% formamide and 2 × saline sodium citrate buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), then 30 min at 46°C in 2 × saline sodium citrate buffer, and 10 min at 37°C in TNE buffer (0.5 M NaCl, 0.001 M EDTA, 0.05 M Tris, pH 8.0). Single-stranded RNA was removed by treating the sections with 20 μg/ml RNase A (Roche) at 37°C for 30 min. After incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) at 1:500 for 2 h, visualization was performed by detection of the alkaline phosphatase with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche) for 4 h at room temperature.

Statistical analysis

The differences in hBD-3 peptide expression pattern between healthy control subjects and periodontitis patients as well as between the pocket tissues and clinically healthy tissues were assessed by Fisher's exact test. Statistical analyses were performed using SPSS 11.5 software. A p-value of < 0.05 was considered statistically significant.

Results

Expression of human β-defensin-3 peptide in gingival tissues

The expression of hBD-3 peptide was detected in 88% (43/49) of the samples, with 94% in healthy tissue from controls (15/16), 92% in healthy tissue from patients (12/13), and 80% in pocket tissues from patients (16/20) (Table 1). It was observed only in the cytoplasm of gingival epithelia but not in the underlying connective tissues. Two types of expression patterns of hBD-3 peptide were observed: the expression confined to the basal layer (Fig. 1a, Figs 2a and c) or the expression extended from basal layer to the spinous layers

Table 1. Expression frequencies of human β-defensin-3 (hBD-3) peptide and mRNA in healthy tissues from healthy control subjects (HT-C), clinically healthy tissues (HT-P), and periodontal pocket tissues (PoT) from patients

hBD-3	HT-C	HT-P	PoT
Peptide	15/16 (94%)	12/13 (92%)	16/20 (80%)
mRNA	15/16 (94%)	10/13 (77%)	19/20 (95%)

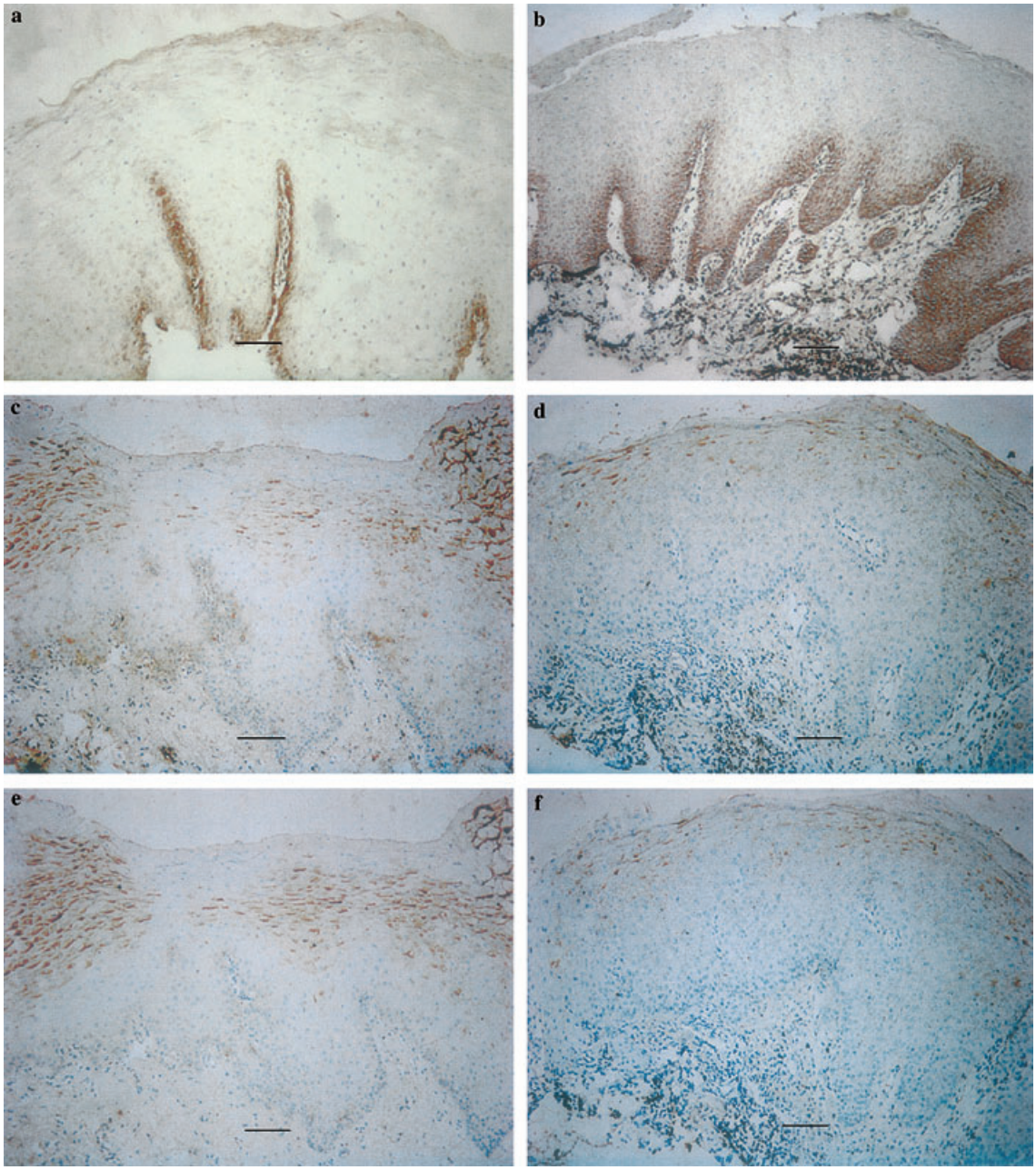

Fig. 1. Expression patterns of human *β*-defensin (hBD)-1, 2, and 3 peptides in human gingival epithelia. Gingival samples were processed with goat polyclonal IgG antibodies to hBD-1 and 2 and rabbit polyclonal IgG antibody to hBD-3, using a standard immunohistochemical protocol. Two types of expression patterns of hBD-3 peptide were shown: expression confined to the basal layer (a) or extended from basal layer to the spinous layers (b). The expression of hBD-1 and 2 peptides was also shown: expression of hBD-1 (c) and hBD-2 (e) in the granular and superficial spinous layers; expression of hBD-1 (d) and hBD-2 (f) in the granular layers. (a), (c), and (e) were derived from a healthy gingival biopsy in a healthy control subject, and (b), (d), and (f) were derived from a clinically healthy gingival biopsy in a subject with chronic periodontitis. Scale bar: 100 μm.

(Fig. 1b, Figs 2e and g). The former was frequently observed in healthy tissue from controls (8/15, 53%), whereas the latter was predominantly found in healthy tissue (10/12, 83%) and pocket tissue from patients (13/16, 81%) (Fig. 3). On the basis of subject, the expression of hBD-3 peptide was more frequently located in the basal layer in the healthy subjects (53%), whereas it was more frequently observed in both the basal and spinous layers in the periodontitis patients (82%) ($p < 0.05$). Within the patient group, the expression of hBD-3 was frequently extended to the superficial spinous layers in pocket tissues (8/16, 50%), whereas no expression of hBD-3 extended to these layers in healthy tissues ($p < 0.05$). No significant correlation

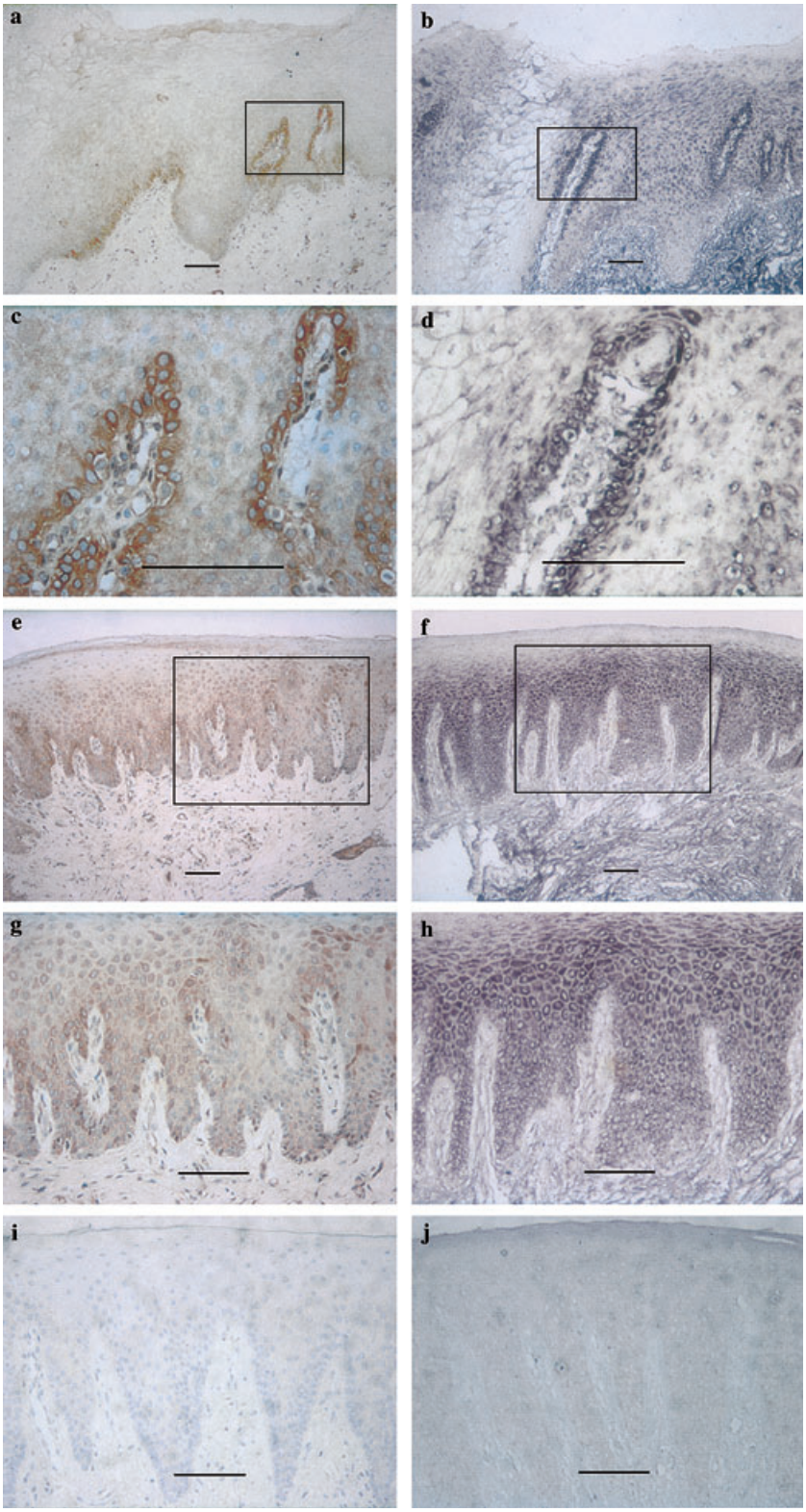

Fig. 2. Expression of human β-defensin-3 (hBD-3) peptide and *in situ* mRNA in human gingival epithelia. Peptide expression was detected by immunohistochemistry with rabbit polyclonal IgG antibody to hBD-3 (a, c, e, and g). Negative control was processed with rabbit non-specific IgG (i). mRNA expression was detected by *in situ* hybridization with digoxigenin labeled RNA antisense probe for hBD-3 (b, d, f, and h). Negative control was processed with sense probe (j). The mRNA expression was mainly confined to the basal layer (b, d) or extended to the spinous layers (f, h). Greater range of mRNA expression was observed in 49% of the samples compared with their peptide expression (e, f, g, h), whereas 37% showed consistent mRNA and peptide expression (a, b, c, d). The encircled areas in (a), (b), (e), and (f) were amplified in (c), (d), (g), and (h), respectively. (a), (b), (c), and (d) were derived from a healthy gingival biopsy in a healthy control subject, and (e), (f), (g), and (h) were derived from a periodontal pocket biopsy in a subject with chronic periodontitis. Scale bar: 100 μm.

was found between the expression pattern of hBD-3 peptide and the age of subjects in both healthy subjects and periodontitis patients (pocket tissues and healthy tissues).

Expression of human β-defensin-1 and 2 peptides in gingival tissues

The expression of hBD-1 and 2 peptides was mainly confined to the granular and spinous layers of gingival epithelia (Figs 1c–f) but it was not observed in the underlying connective tissues. In a few samples, hBD-1 and 2 were spottily and sparsely observed in the basal layers. hBD-1 was detected in both cytoplasm and intercellular spaces, whereas hBD-2 was mainly confined to cytoplasm of the cells concerned.

Double labeling

Some of the hBD-3 positive cells in gingival epithelia were not keratinocytes, which was testified by the colocalization of hBD-3 and markers for Langerhans cells and Merkel cells, CD-1a and cytokeratin 20, respectively (Fig. 4). Langerhans and Merkel cells were detected in 33% and 30% of the samples, respectively, which did not show a significant difference between healthy controls and periodontitis subjects. The presence of CD-1a positive Langerhans cells was noted in the spinous and basal layers of gingival epithelium, some of which were also positive for hBD-3 (Figs 4a and c). The basally located cytokeratin 20-positive Merkel cells were also found to express hBD-3 peptide (Figs 4b and d).

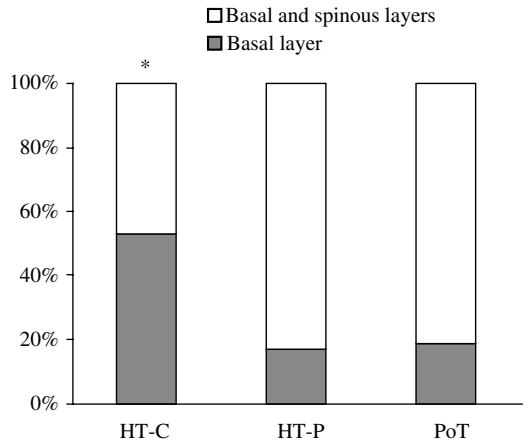

Fig. 3. Expression patterns of human β-defensin-3 (hBD-3) peptide in healthy tissues from healthy control subjects (HT-C), clinically healthy tissues (HT-P), and periodontal pocket tissues (PoT) from patients with chronic periodontitis. The expression of hBD-3 peptide confined to the basal layer was dominant in HT-Cs (8/15, 53%), whereas it extended to the spinous layers in HT-Ps (10/12, 83%) and PoTs (13/16, 81%). On the basis of subjects, significant difference existed between the healthy control subjects (HT-Cs) and periodontitis patients (HT-Ps and PoTs) (*$p < 0.05$).

In situ mRNA expression of human β-defensin-3 in gingival tissues

hBD-3 *in situ* mRNA expression was detected in 90% of the samples, with 15 out of 16 healthy tissues from controls (94%), 10 out of 13 healthy tissues from patients (77%), and 19 out of 20 pocket tissues from patients (95%) (Table 1). It was observed only in gingival epithelia but not the connective tissues, mainly confined to the spinous and basal layers (Figs 2b, d, f and h). Most samples (49%) showed greater range of mRNA expression than their peptide (Figs 2f and h), whereas 37% showed consistent mRNA and peptide expression (Figs 2b and d), and 14% showed either hBD-3 peptide or mRNA expression. No significant difference was observed in the mRNA expression pattern between healthy subjects and periodontitis patients.

Discussion

It is now well recognized that epithelia serve not merely as physical barriers to microbial challenges but rather as reservoirs of antimicrobial peptides, which enable them to survive under normal as well as harsh environmental conditions. Human β-defensins are one member of the antimicrobial family, whose expression has been widely observed in epithelial tissues. In the present study, for the first time, we demonstrated the localization of hBD-3 peptide and mRNA in gingival tissues. Consistent with our earlier investigation of β-defensins, hBD-1 and 2 (13), hBD-3 peptide was expressed in the cytoplasm of gingival epithelial cells but not the connective tissues. Compared with hBD-1 and 2, whose expression was restricted to differentiated granular cells (13, 24), the expression of hBD-3 peptide was observed in the undifferentiated basal cells and less differentiated spinous cells. Basal cells are the most active cells in the metabolism of gingival epithelia, being responsible for the epithelial renewal and materials exchange with the subepithelial tissues. Recent studies have shown that hBDs are chemotactic for immature dendritic cells, memory T cells, and monocytes (19, 25). Thus, the basal cell expression of hBD-3 appears to be a special 'messenger mechanism' that facilitates the cross-talk between gingival epithelia and connective tissues, serving as a link between the innate and adaptive immune systems. Taken together with the expression patterns of hBD-1, 2, and 3, a new picture of the antimicrobial barriers in gingival epithelia is emerging, which is represented by the functionality of different defensins in specific zones.

In addition to keratinocytes, gingival epithelia contain three less abundant cell types: Langerhans cells, Merkel cells, and melanocytes. Langerhans cells are immature dendritic cells, characterized by their typical dendritic morphology and outstanding capacity to initiate primary immune responses to microbial antigens at the gingival margin. Merkel cells have been identified in gingival epithelia and Malassez epithelia in periodontal ligaments (26), which contain various neuropeptides and function as sensory mechanoreceptors and neuroendocrine cells. Furthermore, these cells recruit more antigen-presenting cells (25) or immune cells (19) to the epithelia and hence enable an amplified immune response and a linkage between innate and adaptive immunity. As these cells comprise about 10% of the total cell population in gingival epithelium and are found mainly in the spinous and basal layers, we hypothesized and then confirmed that Langerhans cells and Merkel cells also expressed hBD-3. Although it remains to be determined whether hBD-3 produced by Langerhans cells and Merkel cells is involved in the specialized function of these cells, it is tempting to speculate that such cells may take an active part in the antimicrobial activity through secretion of hBD-3.

An increasing number of reports have shown that hBD-3 expression is regulated by several inflammatory mediators associated with tissue inflammation (20). Human periodontitis is a multifactorial inflammatory disease with a complex pathogenesis. As the composition of subgingival microflora varies significantly between healthy and periodontitis sites (27), this raises the question whether there is a difference in hBD-3 expression between these sites. Interestingly, our study indicates that the expression of hBD-3 in the basal layer is frequent in the samples from healthy subjects, but it extends to the deep spinous layers in the clinically healthy samples from patients and to the superficial spinous layers in the pocket tissues. It is known that in the normal situation basal layer

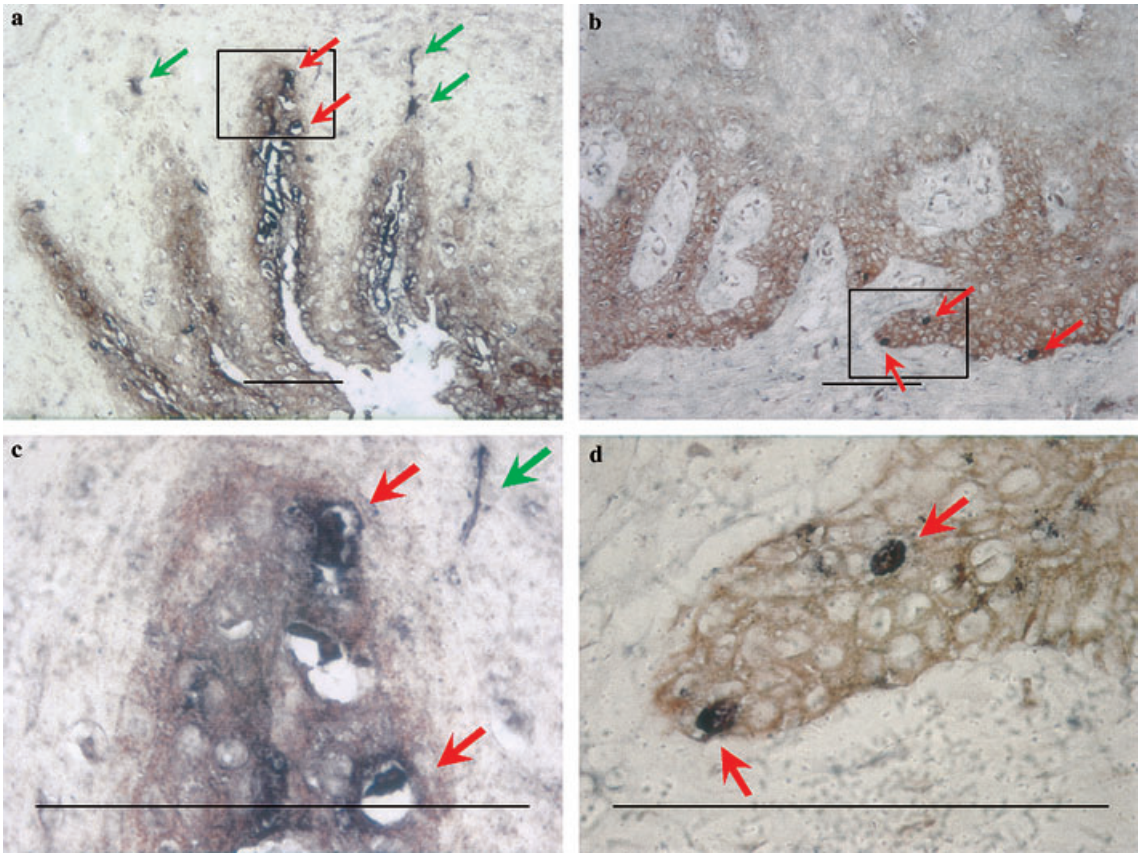

Fig. 4. Co-localization of human β-defensin-3 (hBD-3) and CD-1a in Langerhans cells and CK-20 in Merkel cells in human gingival epithelia. Gingival tissues were stained with hBD-3 streptavidin-biotinylated immunoperoxidase and CD-1a and CK-20 immunoalkaline phosphatase, respectively. Langerhans cells were identified with positive staining of CD-1a (green and red arrows in a and c). hBD-3 was detected not only in keratinocytes, but also in CD-1a positive Langerhan's cells (red arrows shown in a and c) and CK-20 positive Merkel cells (red arrows shown in b and d). The encircled areas in (a) and (b) were amplified in (c) and (d), respectively. (a) and (c) were derived from a healthy gingival biopsy in a healthy control subject, and (b) and (d) were derived from a clinically healthy gingival biopsy in a subject with chronic periodontitis. Scale bar: 100 μm.

contains more functionally active keratinocytes than the upper layers, whereas during inflammation the activity of the keratinocytes increases in the upper layers. The altered localization of hBD-3 peptide observed in the subjects with chronic periodontitis seems to imply an association of hBD-3 peptide expression with the increased activity of keratinocytes in host immune response to inflammation. In contrast, a similar expression pattern of hBD-1 and 2 peptides was observed in healthy and diseased gingival tissues, although our recent study showed that a significant difference existed in the expression levels of hBD-1 and 2 between these two categories of tissues (13). Furthermore, there was no significant difference in the mRNA expression pattern between the healthy subjects and periodontitis patients. hBD-3 transcripts were mainly located in the basal and spinous layers, extending to the granular layers in some of the samples. In a few cases, the exclusive expression of hBD-3 mRNA and peptide in basal layer further confirmed the ability of basal cells in producing hBD-3. Based upon these expression patterns in healthy and inflamed samples, it can be speculated that in health, the level of hBD-3 peptide translated in basal cells appears to be sufficient for the 'surveillance-like' function and maintain the host homeostasis, whereas in the clinically healthy conditions of patients, more peptide was translated in the suprabasal layers, ready to resist the possibly invading pathogens. When finally the tissue destruction occurred, the deeply located hBD-3 peptide could not satisfy the need of the battle, more peptides were produced in the spinous layers to reinforce the antimicrobial activity of hBD-1 and 2 against the invaded pathogens in the granular layers. Based upon the distributions of hBD-1, 2, and 3 peptides in human gingival epithelium, it is likely that the expression of hBDs is associated with periodontal health and disease. Further investigation is warranted to determine the exact role of these antimicrobial peptides in the maintenance of periodontal homeostasis.

In conclusion, hBD-3 was expressed in the basal and spinous layers of gingival epithelia, not only in the

keratinocytes but also in Langerhans cells and Merkel cells. The localization of hBD-3 appears to be different to that of hBD-1 and 2, implying that hBDs compensate each other and act in an orchestrated manner in gingival epithelia to enhance host defense. The expression pattern of hBD-3 peptide seems to be associated with gingival health, implicating the potential contribution of hBD-3 to the maintenance of periodontal homeostasis, possibly through its antimicrobial effect and promotion of adaptive immune responses.

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