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Differential gene expression of human β-defensins (hBD-1, -2, -3) in inflammatory gingival diseases

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Antimicrobial peptides, like human β-defensins, play an important role in the epithelial innate defense response. The aim of the present study was to investigate the quantitative expression of human β-defensin-1, -2, and -3 in inflammatory gingival diseases. Gingival biopsies were obtained from patients with healthy gingiva ($n = 10$), patients with gingivitis ($n = 10$), and patients with periodontitis ($n = 10$). The clinical diagnosis was verified by histology. Gingival tissues were used for RNA extraction followed by reverse transcription. Gene expression was quantified by real-time polymerase chain reaction (normalization with GAP-DH). Comparing the tissues with different clinical stages of health and disease, no significant differences in mRNA expression were found for any of the β-defensins studied. Similar levels of expression were found in healthy gingiva, whereas in gingivitis samples there was a significantly higher expression of hBD-2 compared to hBD-1 ($P = 0.004$) and hBD-3 ($P = 0.016$). Likewise, in periodontitis samples, hBD-2 expression was significantly higher than hBD-1 ($P = 0.016$); however, hBD-2 expression was comparable to hBD-3. In conclusion, the results of the present study showed a differential expression of human β-defensins (hBD-1, -2, -3) in tissues with inflammatory gingival disease.

Key words: antimicrobial peptides; gingivitis; human β-defensins; periodontitis; real-time polymerase chain reaction

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The oral epithelium with its moist surface is extremely prone to microbial colonization. Thus, epithelial cells are in close contact with a wide variety of microorganisms and their metabolic products. Nevertheless, no infections occur under normal conditions. The epithelial compartment not only provides a physical barrier to microorganisms but also plays an important role in host defense (3, 4, 21). Bacteria and yeasts surmount the epithelial barrier by disrupting the cell-layer. Host targets being attacked by pathogens are mainly cell–cell junctions, e.g. desmosomes, adherens, and tight junctions (3). Desmoglein 1, a desmosomal protein mediating cell–cell adhesion, is the specific substrate for the proteolytic exfoliative toxin A (ETA) produced by *Staphylococcus aureus*, thus causing loss of cell adhesion (1, 3).

In addition to the physical barrier, the epithelium has established a chemical defense mechanism by expressing antimicrobial peptides. These peptides exhibit a broad range in specificity against gram-positive and gram-negative bacteria, as well as against yeasts and enveloped viruses (3, 21).

Defensins are positively charged antimicrobial peptides with molecular weights ranging from 3.5 to 6.5 kDa. A high number of basic amino acid residues are responsible for their cationic feature. The antimicrobial activity is very likely based on their ability to form aggregates within the pathogen's membrane, thus serving as pores and leading to an osmotic shock (21). Members of the defensin family are highly similar in their primary structure. All defensins share a β-sheet motif stabilized by intramolecular disulfide-bridges. The α- and β-defensins differ in the position and connection of the conserved cysteine residues (4, 21).

To date, 40 different sequences of human defensins are known. Their genes are located on human chromosomes 6, 8, and 20 (18, 21, 22).

β-Defensins have been detected in epithelia of the intestine, respiratory tract,

urinary tract, vagina, and oral cavity (4–9, 13, 15, 16, 23).

Human β-defensin-1 (hBD-1) was isolated from dialysate hemofiltrate of patients with advanced kidney diseases (2). It is constitutively expressed not only in the urogenital tract, but also in salivary glands and other epithelial tissues (9, 13, 16). hBD-1 shows a high activity against gram-negative bacteria (9, 13, 20). Human β-defensin-2 (hBD-2) was isolated from psoriatic skin (10). It possesses a strong bactericidal effect on gram-negative bacteria and a high antimycotic potency, but only a weak bacteriostatic activity against gram-positive *S. aureus* (10). In keratinocyte cell culture, hBD-2 was up-regulated by exposition with TNF-α and bacteria (12). This observation could also be confirmed for the expression of human β-defensin-3 (hBD-3). Human β-defensin-3 was cloned from keratinocytes (11). It is strong potent against gram-negative bacteria, i.e. *Pseudomonas aeruginosa* and *Escherichia coli*, as well as against gram-positive pathogens, i.e. *S. aureus* and *Streptococcus pyogenes* (11). hBD-3 is expressed in keratinocytes, lung epithelium, and various oral tissues (6, 11).

In previous studies we have demonstrated by qualitative standard polymerase chain reaction (PCR) that human β-defensins are widely expressed in various oral tissues (6, 7).

Experiments with cell cultures have shown a dependency of gene regulation of β-defensins caused by bacterial metabolites from pathogens (12).

The objective of this study was a quantitative analysis of gene expression of human β-defensins in oral tissues under inflamed and noninflamed conditions. For this purpose, comparative quantitative real-time PCR was performed. A different transcriptional level of β-defensins *in vivo* in health and disease could be demonstrated.

Materials and methods

Tissue samples

Thirty biopsies of clinically inflamed ($n = 20$) or noninflamed ($n = 10$) gingival epithelium were isolated from 30 different patients during routine surgical procedures. All patients had been informed about this study and had signed a letter of informed consent. The study was approved by the Institutional Review Board. The distinction between noninflamed and inflamed tissues was made on the basis of clinical evaluation using generally accepted definitions. As opposed to healthy gingiva (no redness, no bleeding on probing, no attachment loss), samples of gingivitis ($n = 10$) were taken from areas which showed redness and/or bleeding on probing, but no clinical attachment loss. In areas with periodontitis, the tissues showed bleeding on probing, had a probing depth > 5 mm, and clinical attachment loss. All samples were taken from adult patients with chronic periodontitis ($n = 10$). The clinical diagnosis was confirmed histologically, based on HE-stained frozen sections. The samples were immediately frozen in liquid nitrogen and stored at − 70°C.

RNA extraction and cDNA synthesis

RNA was extracted from homogenized biopsies according to standard protocols using TRIzol reagent (WAK-Chemie, Bad Homburg, Germany). Complementary DNA (cDNA) was synthesized from 1 μg of RNA using the AMV RT-Kit (Promega, Mannheim, Germany).

Detection of transcripts for human β-defensins by real-time PCR

Expression of transcripts was determined by means of PCR using 2 μl of a 1 : 5 dilution of the cDNA-solution as a template. PCR amplification were performed using the LightCycler® (Roche, Mannheim, Germany). Reactions were carried out in a total volume of 18 μl with LightCycler®-DNA Master SYBR Green-Mix (Roche). Buccal mucosa served as positive control for human β-defensins (dilutions of 1 : 5; 1 : 10; 1 : 50; 1 : 100; 1 : 500), GAP-DH for normalizing the threshold cycle (C_t), while H_2O was used as negative control. All measurements were performed in duplicate. Every set of experiments comparing the expression of the β-defensins (hBD-1, -2, -3) was carried out with cDNA from the same sample.

Primer sequences: hBD-1 (sense: 5′-TTG TCT GAG ATG GCC TCA GGT GGT AAC-3′; antisense: 5′-ATA CTT CAA AAG CAA TTT TCC TTT AT-3′), hBD-2 (sense: 5′-ATC AGC CAT GAG GGT CTT GT-3′; antisense: 5′-GAG ACC ACA GGT GCC AAT TT-3′), hBD-3 (sense: 5′-AGC CTA GCA GCT ATG AGG ATC-3′; antisense: 5′-CTT CGG CAG CAT TTT CGG CCA-3′), GAP-DH (sense: 5′-CCA GCC GAG CCA CAT CGC TC-3′; antisense: 5′-ATG AGC CCC AGC CTT CTC CAT-3′). The results were analyzed by using the comparative Ct method (19). This method is based on the assumption that target and reference template DNA amplifies with the same efficiency.

Only PCR experiments producing a single DNA fragment, analyzed by gel electrophoresis, were used for the statistical analysis.

Statistical analysis

For comparison of the frequencies of hBD-1, -2, and -3 expression in noninflamed and inflamed oral tissue samples, nonparametric median tests were employed (Mann–Whitney and Wilcoxon signed-rank tests). The significance level was set at $P < 0.05$.

Results

The present study investigated the expression of human β-defensins in gingival tissues in different inflammatory stages. Gene expression of hBD-1, -2, and -3 was monitored by real-time PCR after reverse transcription of the corresponding mRNA. The quality of real-time PCR experiments was determined by monitoring reactions with different template concentrations of control buccal mucosa cDNA. There was a linear logarithmic dependency on template concentration (Fig. 1). Figure 2 shows relative expression levels of hBD-1, -2, and -3 in the examined gingival epithelia. No significant differences were found for any of three different β-defensins being analyzed, either in inflamed or in healthy gingiva (Fig. 2). Comparative expression analysis of hBD-1, -2, and -3 showed similar levels in healthy gingiva (Fig. 3A). In samples with gingivitis, however, there was a significantly higher expression of hBD-2 compared with levels of hBD-1 ($P = 0.004$) and hBD-3 ($P = 0.016$) (Fig. 3B). In periodontitis samples, hBD-1 showed a significantly lower gene activation than hBD-2 ($P = 0.016$) (Fig. 3C). The expression level of hBD-3 was comparable to hBD-2 (Fig. 3C).

Discussion

The present study shows the simultaneous expression of human β-defensin-1, -2, and -3 in inflamed and noninflamed gingival tissue samples detected by real-time PCR.

First, the relative transcriptional level of hBD-1, -2, and -3 was examined in healthy gingiva compared to the inflammatory stages of gingivitis and periodontitis. No significant differences could be detected. Previous studies demonstrated the constitutive expression of hBD-1 in

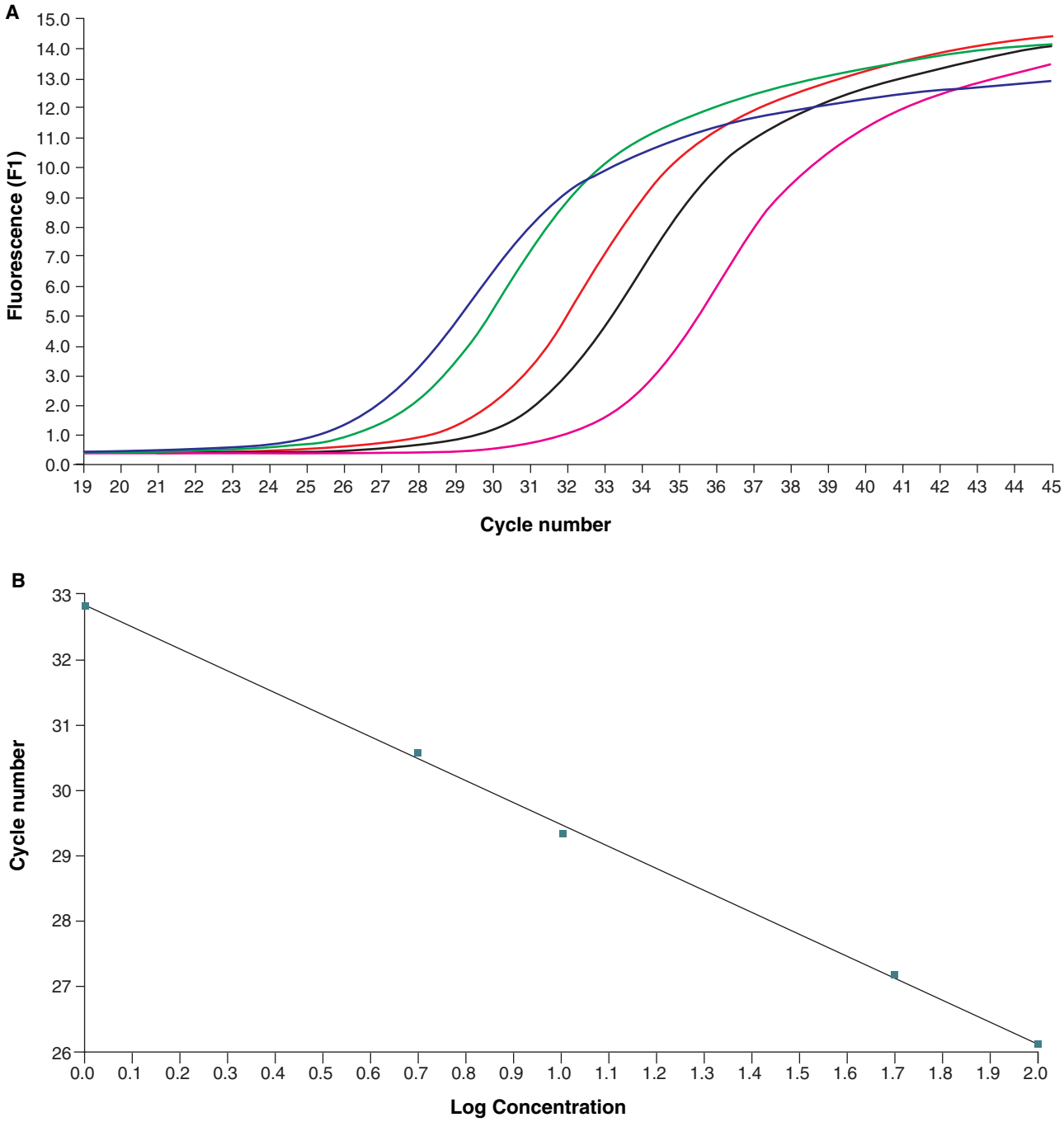

Fig. 1. Graph of real-time PCR for human β-defensin-1. A) The curves represent dilution of template concentrations (1 : 5; 1 : 10; 1 : 50; 1 : 100; 1 : 500). B) Logarithmic graph of standard curve.

oral tissues (3, 6, 16). Considerably high differences of the transcriptional level within a set of experiments were observed. Thirty different individuals were examined, expressing their own pattern of defensins. This might explain the variation within each set of data. Real-time PCR experiments of minor quality could be excluded (Fig. 1). Any deviation of measuring points from the standard curve is minimal (Fig. 1B). Thus, the method of PCR is not the reason for any of the observed variations.

It has been shown in various epithelial tissues that expression of hBD-2 depends on the stage of inflammation (3, 19). In contrast, oral epithelial cells express hBD-2 even under noninflamed conditions (3, 15). This has been suggested to be the result of exposure of the tissue to commensal nonpathogenic bacteria (3, 15). *In vitro* analysis of gene expression demonstrated a transcriptional up-regulation of β-defensin-2 in cultured epithelial cells after treatment with bacteria or bacterial supernatant (14). In addition, interleukin-1 and interleukin-1 receptor antagonist (IL-1 and IL-1RA) play important roles as interfering factors in transcriptional regulation of β-defensins (17). IL-1 alone activates

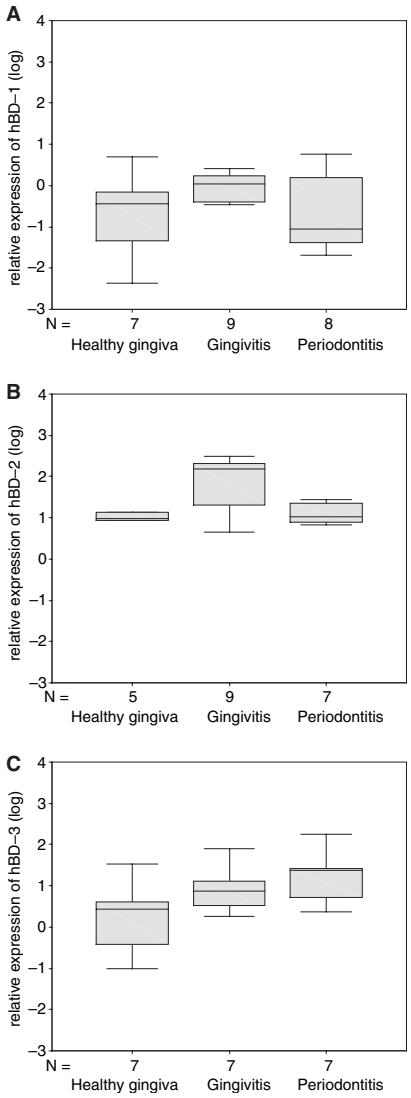

Fig. 2. Relative expression of human β-defensins hBD-1 (A), hBD-2 (B), and hBD-3 (C) in healthy and inflamed gingival tissue. No significant differences were found.

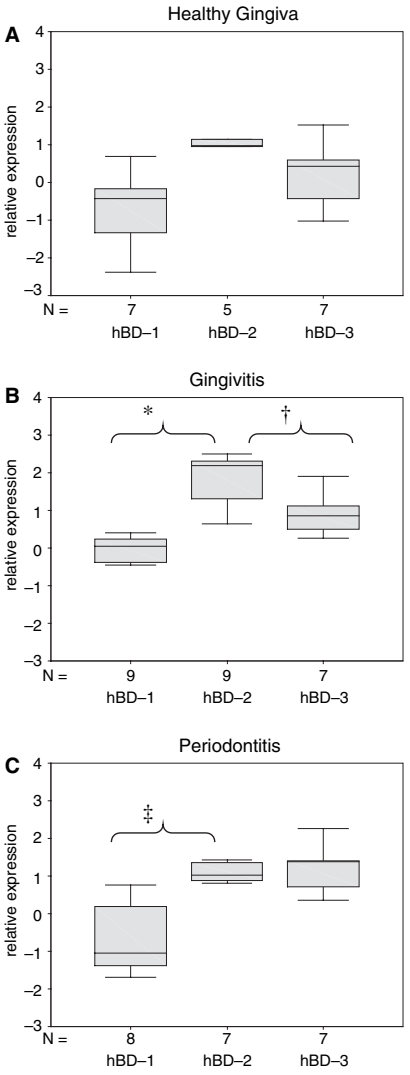

Fig. 3. Comparative analysis of gene expression of human β-defensins hBD-1, hBD-2, and hBD-3 for healthy gingiva (A), and gingiva in the inflammatory stage of gingivitis (B) and periodontitis (C). Significant differences by Wilcoxon-signed-rank-test: (B) $P = 0.004$ for hBD-1 : hBD-2 (*), and $P = 0.016$ for hBD-2 : hBD-3 (†); (C) $P = 0.016$ for hBD-1 : hBD-2 (‡). Values at abscissa indicate the numbers of real-time PCR experiments producing one single PCR fragment. Samples of PCR experiments with more than one band have not been taken into consideration.

gene expression of human β-defensin-2, whereas IL-1RA has been shown to block this effect (17).

Expression of hBD-3 in cultured lung epithelial cells was also induced after stimulation with bacteria or bacterial supernatants (11). Our results are based on the analysis of clinical biopsies. This might explain the differences compared to the published results of *in vitro* studies.

Our comparative analysis of gene expression for hBD-1, -2, and -3 in healthy and inflamed gingiva tissues showed differential transcriptional levels for the three human β-defensins. No significant differences in the expression of hBD-1, -2, and -3 could be detected in healthy gingiva. In samples of gingivitis, hBD-2 expression was at a higher level than hBD-1 and -3 expression. hBD-2 expression is known to be up-regulated after bacterial contact with epithelial cells (15).

In biopsy samples from periodontitis, hBD-2 expression was significantly higher than that of hBD-1, while no statistical significant difference was observed for hBD-2 compared with hBD-3. These data suggest a delayed up-regulation of hBD-3 expression compared with hBD-2.

The first stage of clinical oral inflammation (gingivitis) shows an early up-regulation of hBD-2. Periodontitis leads to gene activation of hBD-3, while hBD-2 is still highly expressed.

It would be interesting to investigate the mutual interacting role of human β-defensins in their transcriptional regulation.

In summary, the expression of human β-defensins was found to be similar in healthy gingiva, while the inducible defensin hBD-2 showed a higher transcriptional level in tissue with inflammatory disease.

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