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Human β defensin-1 and -2 expression in the gingiva of patients with specific periodontal diseases

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Background and Objective: β defensin antimicrobial peptides are important in epithelial innate immunity, and their differential expression is associated with periodontal diseases. The aims of this study were to determine the mRNA expression of human β defensin-1 and -2 in the gingival tissue of patients with gingivitis, aggressive periodontitis and chronic periodontitis, and to evaluate the relationship between defensin expression and type and/or severity of periodontal destruction.

Material and Methods: Fifteen patients in each group with gingivitis, aggressive periodontitis and chronic periodontitis, and 10 healthy subjects, were included in the study ($n = 55$). The periodontal status of the subjects was determined by periodontal clinical measurements and radiographical evaluations. Transcriptional levels of human β defensin-1 and -2 genes in gingival samples were assessed by using the quantitative real-time polymerase chain reaction technique, and the data were evaluated statistically by the RELATIVE EXPRESSION Software Tool 2 for groupwise comparisons.

Results: Expression of the human β defensin-1 gene was lower in gingivitis and aggressive periodontitis groups, and significantly higher in the chronic periodontitis group, than in the control group ($p < 0.001$). Human β defensin-2 mRNA expression in the gingivitis group was lower than in the control group; however, the difference was statistically significant only in half of the gingivitis patients ($p < 0.001$). Human β defensin-2 mRNA levels were higher in some chronic periodontitis patients, but lower in the others when compared with the control group ($p < 0.001$). Expression of the human β defensin-2 gene increased in the aggressive periodontitis group relative to the control group.

Conclusion: This study suggests that human β defensin-1 and -2 genes in the gingival epithelium show differential expression in patients with specific periodontal diseases, and aggressive and chronic periodontitis types demonstrate different gingival β defensin-1 and -2 expression patterns.

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The oral epithelium, which is constantly exposed to microbial factors, functions as a physical barrier (1,2). Recent findings showed that the epithelium is not only a physical barrier, but also has chemical defense mechanisms containing antimicrobial peptides, such as defensins (3). Antimicrobial peptides belong to the first line of host defense mechanisms against infections in epithelial tissue (4). As part of innate immunity,

defensins playing an important role in maintaining tissue integrity in oral health are small cationic cysteine-rich antimicrobial peptides (2,5) and have broad-spectrum antimicrobial activity against gram-positive and gram-negative bacteria, fungi and viruses (6).

Human defensins are classified into two subgroups: α defensins and β defensins. Six α defensins (hND-1 to -6) and four β defensins (human β defensin-1 to -4) have been defined in humans. Polymorphonuclear leucocytes express α defensins as part of their nonoxidative antimicrobial mechanisms, and β defensins are expressed by mucosal epithelial cells (5).

Human β defensin-1 is constitutively expressed in various types of epithelia, whereas human β defensin-2 is induced under conditions of inflammation (7). Nevertheless, there are some conflicting data showing that human β defensin-1 is also expressed in inflamed gingival tissue, and human β defensin-2 is expressed in healthy conditions (8). mRNA and peptide expressions of human β defensin-1, -2 and -3 has been shown *in vitro* in oral epithelial cell cultures and *in vivo* in gingival tissue samples (7,9,10). It has been reported that α and β defensins are localized in different parts of the gingival epithelium. β defensins are expressed in the oral and sulcular stratified epithelium of gingiva, but α defensins are expressed in the junctional epithelium (11). Dale *et al.* showed immunohistochemically that human β defensin-1 and -2 was dense in marginal epithelium and inflamed sulcular epithelium, whereas these are the most exposed sites of gingiva against microbial dental plaque (11). However, the exact role of β defensins in oral health and disease remains unclear, and there are very limited data related to gingival β defensin expression in different types and/or severity of periodontal destruction.

Therefore, the aim of the present study was to determine the expression of gingival human β defensin-1 and -2 mRNA in patients with specific periodontal diseases, such as gingivitis, aggressive periodontitis, and chronic periodontitis, using quantitative real-time polymerase chain reaction (PCR), and to investigate the possible role of β defensins in the host response of different periodontal disease categories.

Material and methods

Patient selection and collection of gingival tissue samples

This study was approved by the Ethics Committee of Ege University, Izmir, and written informed consent, in line with the Helsinki declaration, was obtained from each subject before his or her enrollment in the study. A total of 55 subjects (22 men, 33 women) were recruited in this study, including 15 patients with gingivitis (mean age 29 ± 8.6 years), 15 patients with aggressive periodontitis (mean age 30 ± 7.2 years), 15 patients with chronic periodontitis (mean age 47 ± 5.9 years), and 10 periodontally healthy subjects (mean age 27.7 ± 11.9 years). Patients with a history of systemic disease or taking medications likely to influence periodontal health, who had received periodontal treatment within the last 6 months or used antibiotics and/or anti-inflammatory drugs within the last 3 months, were excluded from the study. The patients were selected according to the clinical and radiographic criteria proposed by the 1999 International World Workshop (12). The clinical periodontal parameters, including probing depth, clinical attachment level, plaque index (13), and papilla bleeding index (14), were recorded at the sampling sites. The Williams probe was used for clinical periodontal measurements, and all measurements were performed by the same investigator (SV-S).

Tissue samples from gingivitis patients were taken from interproximal sites showing redness and/or bleeding on probing, but no clinical attachment loss. In periodontitis patients, the samples were obtained from sites with probing depth ≥ 6 mm, clinical attachment level ≥ 4 mm, bleeding on probing, and ≥ 50% alveolar bone loss on radiographs. In the healthy control group, gingival tissue specimens were taken during tooth extractions for orthodontic reasons or crown-lengthening procedures. All tissue samples were placed in RNAse- and DNAse-free stabilizing solution and stored at −80°C until the day of laboratory analysis.

Total RNA extraction

Total RNA was extracted from all tissue samples by using NucleoSpin RNA II (Catalog No: 740955.50; Macherey-Nagel GmbH, Düren, Germany) according to the manufacturer's instructions. All RNA samples were treated with DNAse I to remove genomic DNA contamination. RNA concentration and purity were measured photometrically using an Eppendorf Biophotometer (Catalog no: 6131,000.012; Eppendorf GmbH, Hamburg, Germany). RNA integrity was determined by 1.2% formaldehyde agarose-gel electrophoresis. The formaldehyde agarose gel was stained by SYBR® Green II staining solution (Catalog No: S-9305; Sigma GmbH, Taufkirchen, Germany). RNA bands were visualized under an ultraviolet transilluminator.

Fifteen tissue samples from each disease group, and 10 samples from the healthy control group, were obtained at baseline. However, only eight samples from patients with gingivitis, nine samples from patients with chronic periodontitis, nine samples from patients with aggressive periodontitis, and six samples from the control group provided enough total RNA (in terms of concentration and integrity) for further experiments.

cDNA synthesis and real-time PCR

Total RNA (1000 ng) was reverse transcribed into cDNA in a 20 µl volume by using the iScript™ cDNA synthesis Kit (Catalog No: 170-8891; Bio-Rad GmbH, Munchen, Germany). cDNA synthesis was performed at 42°C for 30 min, according to the manufacturer's instructions. One microlitre of cDNA was used in a 25 µl volume of real-time PCR reaction. Real-time PCR was carried out using iQ™ SYBR Green Supermix (Catalog No: 170–8880; Bio-Rad GmbH) (15).

Real-time PCR was performed on The iCycler iQ Real-Time PCR

Detection System (Catalog No: 170–8740; Bio-Rad GmbH). PCR conditions were an initial denaturation step of 95°C for 15 min, followed by 42 repeats of 94°C for 15 s, 60°C for 30 s, 72°C for 30 s, and 80°C for 15 s. A melt-curve protocol immediately followed amplification with 95°C for 1 min and 55°C for 1 min, followed by 80 repeats of heating for 10 s, starting at 55°C with 0.5°C increments (16).

A negative control without the cDNA template was run with every assay to evaluate the overall specificity. Reverse transcriptase minus the control was used to detect DNA contamination in the RNA samples. To confirm the accuracy and reproducibility of real-time PCR, the intra-assay precision was determined in three repeats within the iCycler. Inter-assay variation was investigated in two different experiment runs, performed on two separate days, in the iCycler (16).

Standard curves

Standard curves for each gene were generated by a 1 : 5 dilution of cDNA to determine the efficiency of target and reference reactions. Standard curves were obtained by using serial dilutions of five different concentrations of standard and by plotting the C_t vs. Log_{10} [cDNA]. The slope (S) obtained from the standard curve was used to calculate the PCR efficiency using the following equation:

$$\text{PCR efficiency} = [10^{(1/-S)}].$$

The mRNA expression level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for normalization of RNA quantity and quality differences in all samples (16).

Primer design

The cDNA sequence of human (*Homo sapiens*) glyceraldehyde-3-phosphate dehydrogenase was obtained from the GenBank database. Primers for selected genes were designed via the Vector NTI (version 5.0 demo; Invitrogen Ltd., Karlsruhe, Germany) that were synthesized in Sigma Genosis Company (Taufkirchen, Germany) (Table 1). Primers for human β defensin-1 and human β defensin-2 were synthesized according to the previously used primer sequences (17).

Table 1. Gene names, GenBank, and EMBL access numbers of the human nucleic acid sequences used, the primer sequence from 5′ to 3′, and the length of the polymerase chain reaction product

Genes	Access no.	Primer sequence		Length (bp)
GAPDH	NM_002046	Forward	5′-ACCACAGTCCATGCCATCAC-3′	452
		Reverse	5′-TCCACCACCCTGTTGCTGTA-3′	
Human β defensin-1	NM_005218	Forward	5′-ATGAGAACTTCCTACCTTCTGCT-3′	207
		Reverse	5′-TCACTTGCAGCACTT-3′	
Human β defensin-2	AF040153	Forward	5′-CCAGCCATCAGCCATGAGGGT-3′	255
		Reverse	5′-GGAGCCCTTTCTGAATCCGCA-3′	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Data and statistical analysis

The real-time PCR results were evaluated statistically by the RELATIVE EXPRESSION Software Tool 2 (REST) for groupwise comparisons (18), and the α significance level was 0.001. The significance of each gene's expression level was calculated by a pairwise fixed reallocation test. The software computes a relative expression ratio (R) considering the control group. Target gene expression (human β defensin-1 and human β defensin-2) was normalized via the reference gene (glyceraldehyde-3-phosphate dehydrogenase), according to the following equations (18, 19):

$$\text{Relative quantity} = \text{Efficiency}^{(\text{control}C_t - \text{unknown}C_t)} \quad \text{(eqn 1)}$$

$$\text{Normalization factor} = (\text{quant.1}^* \ldots ^* \text{quant.N})^{(1/N)} \quad \text{(eqn 2)}$$

$$\text{Expression} = \text{Relative quantity} \div \text{Normalization factor} \quad \text{(eqn 3)}$$

GENE EXPRESSION MACRO Version 1.1 (Bio-Rad GmbH) software was used to draw graphs of expression levels and evaluate the gene expression levels. In addition, REST software has a different calculation algorithm to give relative expression levels. REST is the most common software used to calculate the relative gene expression levels. Both software programs (REST and Gene Expression Macro) were used in this study to obtain more reliable values. For up-regulation, the value of the expression level is equal to the given value which we obtain from equation 3. In the case of down-regulation, the regulation factor is illustrated as a reciprocal value (1/expression ratio). For example, if the expression value is 1.3, which is > 1, this means that there is 1.3-fold up-regulation. However, if the expression value is 0.5, which is < 1, this means that there is 1/0.5 = twofold down-regulation. To determine the gene expression level, the relative quantity and normalization factor is calculated in equations 1 and 2. The expression level is calculated according to equation 3.

Each experiment for each tissue sample was performed three times. The statistical analyses were carried out in six healthy control samples. Three mean expression values were obtained from six controls in three different assays. The mean expression levels were used in statistical analysis. Three control bars in the figures show the mean ± standard deviation of three assays of six control samples. For the other study groups, data were shown for each patient instead of the mean of the group. Standard deviation bars show the standard deviation of three different assays of the same specimen.

Inter- and intragroup comparisons of relative gene expressions of human β defensin-1 and -2, and intergroup comparisons of periodontal clinical measurements were analyzed by the nonparametric Wilcoxon Signed Rank test and Mann–Whitney *U*-test. A *p*-value of < 0.05 was considered statistically significant.

Table 2. Clinical periodontal measurements of the study groups (mean ± standard deviation)

	PD (mm)	CAL (mm)	PBI	PI
Control ($n = 10$)	1.70 ± 0.67	0	0	0.30 ± 0.48
Gingivitis ($n = 15$)	2.40 ± 0.5	0	2.53 ± 0.52[a]	2.33 ± 0.49[a]
AgP ($n = 15$)	7.11 ± 0.98[a,b]	7.55 ± 1.23[a,b]	2.59 ± 0.5[a]	1.59 ± 0.85[a]
CP ($n = 15$)	6.99 ± 1.26[a,b]	7.82 ± 1.44[a,b]	2.58 ± 0.51[a]	2.79 ± 0.54[a,c]

AgP, aggressive periodontitis; CP, chronic periodontitis; CAL, clinical attachment level; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBI, papilla bleeding index; PD, probing depth; PI, plaque index.
[a]Significantly higher than the healthy control group ($p < 0.05$).
[b]Significantly higher than the gingivitis group ($p < 0.05$).
[c]Significantly higher than the aggressive periodontitis group ($p < 0.05$).

Results

Clinical findings

There were no statistically significant differences between study groups in terms of smoking and gender distribution ($p < 0.05$). Clinical measurements, including probing depth, clinical attachment level, papilla bleeding index and plaque index of individuals from all study groups, are shown in Table 2.

Integrity and content of total RNA

Extracted total RNA from tissue cells represented no variation in RNA integrity and content. All total RNA samples were run in formaldehyde agarose gel electrophoresis for checking integrity and content. 18s rRNA and 28s rRNA were abundant in all samples.

Real time PCR sensitivity

The sensitivity of iCycler real-time PCR was evaluated by using different amounts of RNA from human lung total RNA (Catalog No: 7968; Ambion Inc., Cambridgeshire, UK). Human lung total RNA was chosen because expression of human β defensin-1 and human β defensin-2 mRNA in lung has been reported previously (20,21). According to the standard graphs for all set primers, test linearity (r^2) was between 0.900 and 0.997 for glyceraldehyde-3-phosphate dehydrogenase, between 0.947 and 0.987 for human β defensin-1, and between 0.980 and 0.984 for human β defensin-2 (Table 2). In all amplification graphs of all genes, no increase in fluorescence was observed for the nontemplate control or the nonreverse transcription mRNA control samples, indicating the absence of contaminating DNA in the system and the samples (data not shown).

Intra- and interassay coefficients of variation were calculated for each gene to determine the accuracy of the method. To calculate the intra-assay coefficient of variation, a pooled cDNA sample was analyzed three times in the same iCycler reverse transcription-polymerase chain reaction (RT-PCR) run, and to calculate the interassay coefficient of variation, the same-pooled cDNA sample was analyzed in two different iCycler RT-PCR runs. Intra- and interassay coefficient of variations are shown in Table 3.

Confirmation of PCR products

PCR products should have checked by separating the PCR product in agarose-gel electrophoresis because of using the SYBR green I system in real-time PCR. Each PCR product was separated by 2% agarose gel electrophoresis. Each primer set gave a specific band in an expected PCR product length (Fig. 1). By using the melting curve, it was shown that there were no primer dimers in any PCR product. Accurate quantification of the desired PCR product was achieved. All PCR primer sets were designed to have a PCR efficiency of between 1.73 and 2.30 (Table 3).

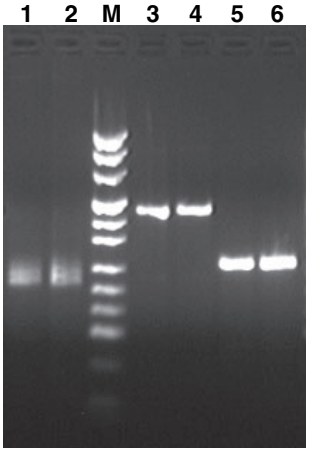

Fig. 1. Two per cent agarose gel electrophoresis of all real-time polymerase chain reaction products derived from the real-time polymerase chain reaction. Lane M, pUC mix marker (Cat No: SM0301; Fermentas GmbH, St Leon-Rot, Germany) (1116, 883, 692, 501, 404, 331, 242, 190, 147, and 110 bp); lanes 1 and 2, human β defensin 1 (207 bp); lanes 3 and 4, glyceraldehyde-3-phosphate dehydrogenase/(452 bp); lanes 5 and 6, human β defensin 2 (255 bp).

Expression level and validation parameters of real-time PCR for human β defensin-1

When periodontal disease groups were compared with the healthy control group, human β defensin-1 relative

Table 3. Characteristics and validation parameters of real-time polymerase chain reaction assays for human β defensin-1 and human β defensin-2

	GAPDH	Human β defensin-1	Human β defensin-2
Test linearity (r) ($n = 3$)	0.900–0.997	0.947–0.987	0.980–0.984
PCR efficiency ($n = 3$)	1.75–2.30	1.73–1.96	1.97–2.04
Interassay CV ($n = 2$)	4.98	4.60	9.40
Intra-assay CV ($n = 3$)	5.49	5.28	7.61

Intra-assay (test precision) coefficients of variation (CV) and interassay (test variability) coefficients of variation of real-time polymerase chain reaction assays are shown together with test linearity, polymerase chain reaction (PCR) efficiency.
GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

gene expression was down-regulated in the group of patients with gingivitis, although expression was statistically significant in seven out of eight patients ($p < 0.001$). Human β defensin-1 expression was significantly up-regulated in eight out of nine patients with chronic periodontitis ($p < 0.001$). On the other hand, the human β defensin-1 gene was significantly down-regulated in seven of eight patients with aggressive periodontitis and up-regulated in the remaining patient ($p < 0.001$) (Fig. 2).

When periodontal disease groups were compared with each other in terms of human β defensin-1 expression, the mRNA human β defensin-1 expression level of the chronic periodontitis group was significantly higher than that of the gingivitis group and the aggressive periodontitis group ($p = 0.001$). There was no statistical difference between aggressive periodontitis and gingivitis groups ($p = 0.156$) (Fig. 3).

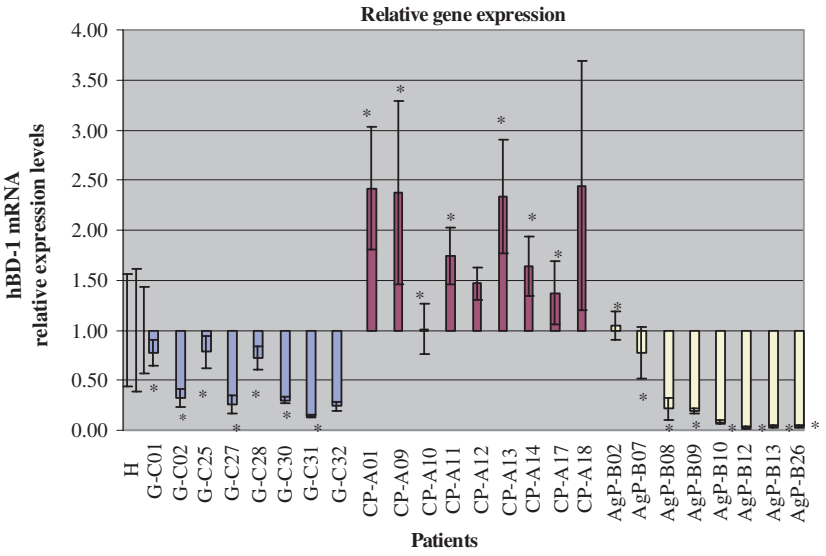

Fig. 2. Relative gene expression levels for human β defensin-1 in each individual. Human β defensin-1 gene expression levels were determined in gingival tissue samples obtained from patients with gingivitis, aggressive periodontitis, and chronic periodontitis. Expression changes were shown as n-fold up-regulation or 1/n-fold down-regulation (expression ratio ± standard deviation). Statistical significance was indicated compared with the healthy control group (*$p < 0.001$). hBD-1, human β defensin-1.

Expression level and validation parameters of real-time PCR for human β defensin-2

Expression of the human β defensin-2 gene was down-regulated in the gingivitis group, but up-regulated in the aggressive periodontitis group relative to the healthy control group. Human β defensin-2 mRNA was up-regulated in half of the chronic periodontitis patients, but down-regulated in the other half (Fig. 4).

When periodontal disease groups were compared with each other in terms of human β defensin-2 expression, the mRNA expression level of human β defensin-2 in patients of the chronic periodontitis group was higher than that of patients in the gingivitis group, although the difference was not statistically significant ($p = 0.083$). Expression of human β defensin-2 in patients of the aggressive periodontitis group was significantly higher than those in the gingivitis ($p = 0.006$) and chronic periodontitis ($p = 0.031$) groups (Fig. 5).

Considering intragroup comparisons of human β defensin-1 and -2 expression, human β defensin-2 expression

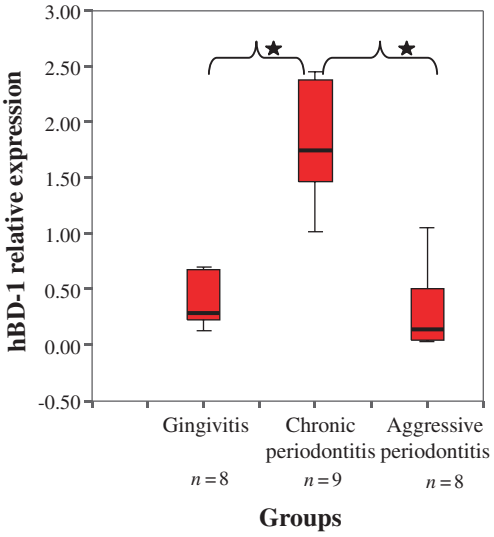

Fig. 3. The comparison of human β defensin-1 expression levels between gingivitis, aggressive periodontitis and chronic periodontitis groups. Statistically significant difference between the study groups (*$p < 0.05$). hBD-1, human β defensin-1.

was slightly higher than human β defensin-1 in the gingivitis group, but the difference was not statistically significant ($p > 0.05$). In the chronic periodontitis group, human β defensin-2 expression was significantly lower than human β defensin-1 expression ($p = 0.011$). In patients of the aggressive periodontitis group, human β defensin-2 mRNA expression was higher than that of human β defensin-1, although the difference was not statistically significant ($p > 0.05$) (Fig. 6).

Discussion

In the present study, the expression of human β defensin-1 and -2 mRNA in

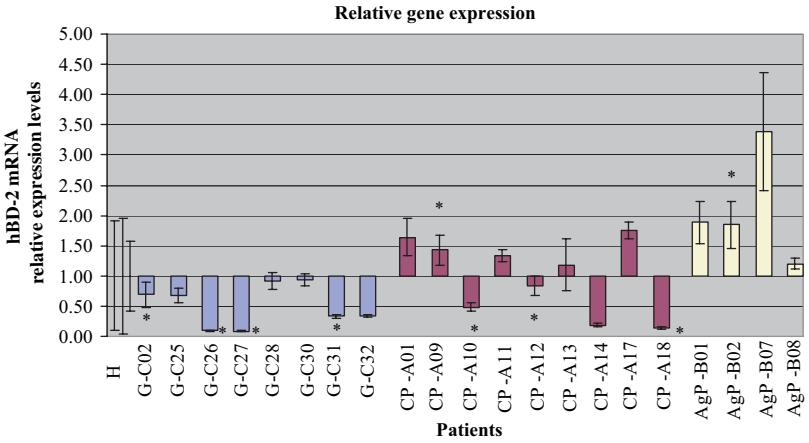

Fig. 4. Relative gene expression levels for human β defensin-2 in each individual. Human β defensin-2 gene expression levels in gingival tissue samples were obtained from patients with gingivitis, aggressive periodontitis, and chronic periodontitis. Expression changes were shown as n-fold up-regulation or 1/n-fold down-regulation (expression ratio ± standard deviation). Statistical significance was indicated compared with the healthy control group (*$p < 0.001$). hBD-2, human β defensin-2.

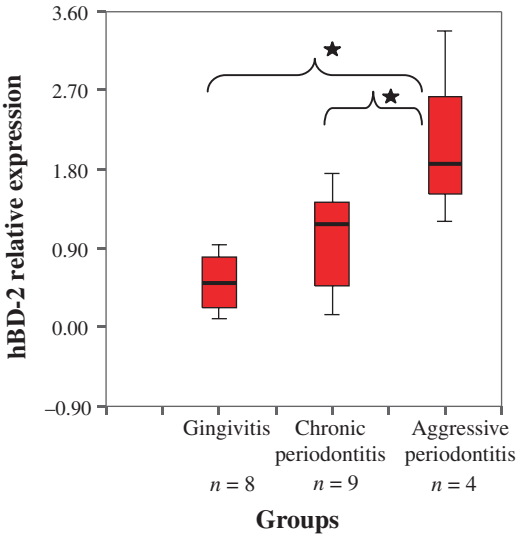

Fig. 5. Comparison of human β defensin-2 expression levels in gingivitis, aggressive periodontitis, and chronic periodontitis groups. A statistically significant difference was observed between the study groups (*$p < 0.05$). hBD-1, human β defensin-1; hBD-2, human β defensin-2.

the gingiva of patients with various types of periodontal diseases was investigated by quantitative real-time PCR. Human β defensin-1 and -2 genes demonstrated differential expression in patients with gingivitis, aggressive periodontitis, and chronic periodontitis. In addition, aggressive and chronic periodontitis followed different patterns in gingival defensin expression. To the best of our knowledge, the present study is the first study to determine the expression of human β defensin-1 and -2 mRNA in all specific periodontal diseases, such as gingivitis, aggressive periodontitis, and chronic periodontitis.

As real-time PCR gives more sensitive and confident data for comparing different disease categories (22), the quantitative real-time PCR technique was used in the present study instead of the semiquantitative RT-PCR method. Defensin gene expression changes were statistically analyzed relative to the reference gene and the healthy control group by the REST Software Tool 2 (18), and the α significance level was 0.001. Therefore, it was considered that the present study confidently determined the β-defensin levels of gingiva in specific periodontal diseases.

Gingival human β defensin-1 and -2 mRNA decreased in the gingivitis group compared with the control group in the present study. The decrease in β defensin levels in the gingival epithelium of gingivitis patients may increase the susceptibility of individuals to periodontal infections. In addition, the decrease in production of β defensins in gingival and sulcus epithelium may facilitate the invasion of dental plaque microorganisms and their products to the connective tissue and initiate gingival infection. It is well known that the clinical symptoms of gingivitis appear 10–20 d after microbial dental plaque accumulation starts. However, some subjects are resistant to gingivitis, but others can get gingivitis easily (23). In this respect, it might be speculated that the decrease in gingival β defensin expression may facilitate gingivitis in some individuals.

Healthy gingival epithelium cells constitutively synthesize human β defensin-1 as part of their physiological structure without any exogenous induction (10). By contrast, human β defensin-2 is expressed only in inflamed tissue in some organs (24,25), although gingival epithelium cells, which are constantly exposed to commensal bacteria, express human β defensin-2, even in the healthy state (9). Therefore, human β defensin-2 expression in healthy gingiva is considered as one of the defense mechanisms of gingival epithelium (11). Likewise, human β defensin-2 mRNA expression was determined in both inflamed and healthy gingival tissue samples in the present study. It is still not clear what breaks down β defensin synthesis in the gingival epithelium of healthy individuals, which may cause gingival inflammation. Genetic polymorphisms of β defensin genes may decrease their expression in gingiva, and this can

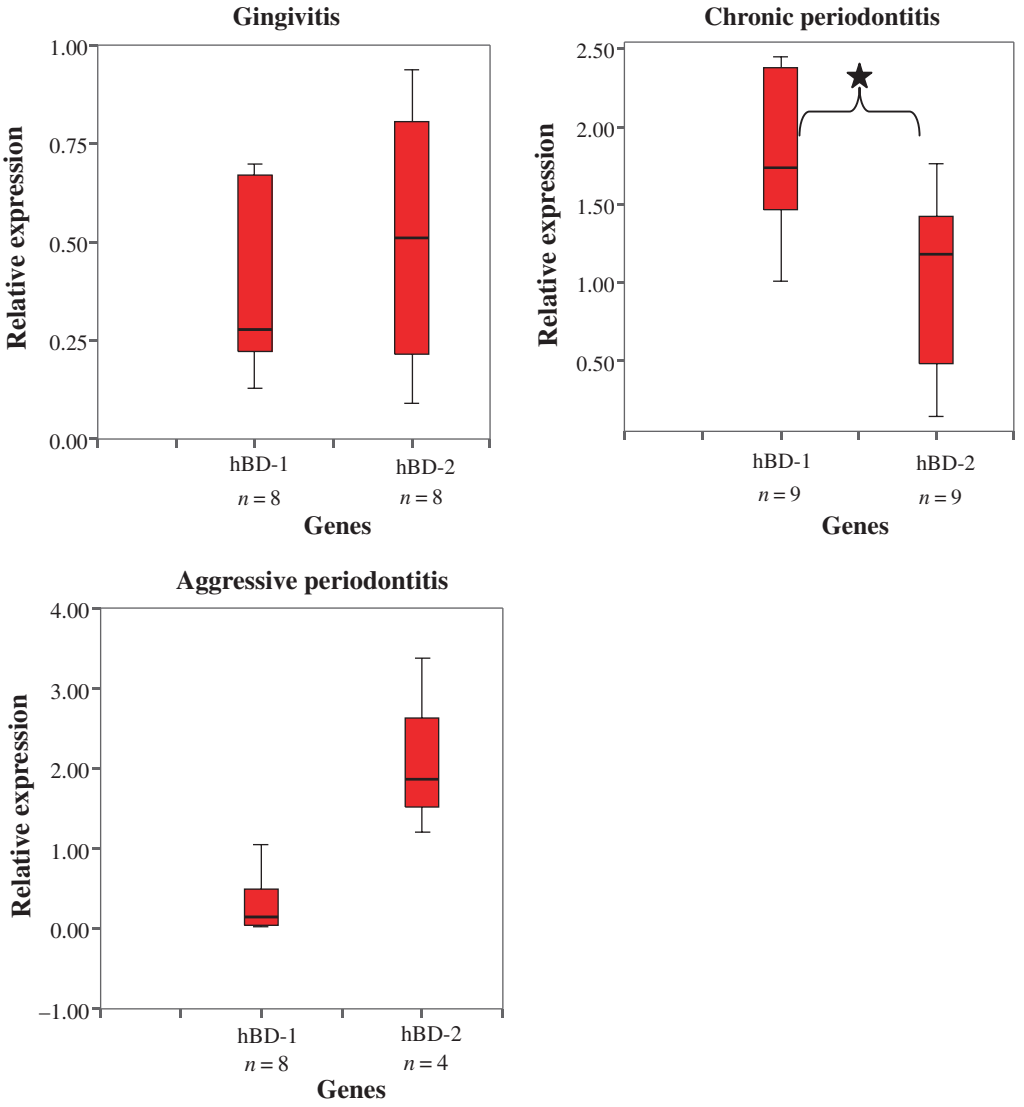

Fig. 6. Human β defensin-1 and human β defensin-2 comparative gene expression analysis in the gingiva of patients with gingivitis, chronic periodontitis, and aggressive periodontitis. Statistical significance was determined by the Wilcoxon Signed Ranks test (*$p < 0.05$).

increase the susceptibility to periodontal diseases because periodontal infection starts in the gingival epithelium. It is known that gingivitis (i.e. reversible inflammation of gingiva) can be readily treated by the elimination of dental plaque from teeth surfaces. Whether the elimination of dental plaque – the primary etiological agent of periodontal disease – changes β defensin expression in gingiva, can explain this better. It is possible to plan experimental animal studies to investigate this in greater detail because it is not ethically appropriate to take gingival samples from humans after periodontal treatment.

It has been shown that stratified gingival epithelium synthesizes human β defensin-2, even in the healthy state (10), which is different from other epithelial tissues (24,25). Krisanaprakornkit *et al.* (9) have reported that commensal *Fusobacterium nucleatum* induces human β defensin-2 expression in human oral keratinocyte cells. *F. nucleatum* is a gram-negative anaerobic bacterium isolated from the dental plaque of periodontally healthy and diseased individuals (26). It has been considered to function as a bridge bacterium between pathogenic bacteria, such as *Porphyromonas gingivalis*, *and* early colonies of dental pellicle, rather than an etiologic agent of periodontal disease. Therefore, *F. nucleatum* may help to keep gingival epithelial cells stimulated to produce β defensins, and thus to provide effective and continuous host defense (9). For these reasons, the healthy gingiva expresses human β defensin-2. *F. nucleatum* has been shown to stimulate human β defensin-2 expression more than *P. gingivalis*, a major pathogen of chronic periodontitis (9). In our study, the decreased expression of human β defensin-2 in the gingivitis group, relative to the control group, provides indirect evidence that periodontopathogens causing gingivitis cannot induce the

expression of human β defensin-2. Human β defensin-2 might not be expressed in the gingivitis group as a result of the changes in gingiva and microorganism composition of dental plaque after gingival inflammation. At this point, it may be difficult to explain the reasons and the results. Gingivitis may occur because the epithelium cannot function properly as a result of the genetic β defensin deficiency. After gingivitis starts, defensin levels can stay low because of environmental changes during inflammation and eventually this can increase periodontal infection.

In the present study, the relatively decreased human β defensin-1 mRNA levels in the aggressive periodontitis group may increase the susceptibility of patients to periodontitis. Therefore, the decreased human β defensin-1 levels in gingival epithelium might be a putative risk factor for gingivitis and aggressive periodontitis. On the other hand, in the present study, the increased human β defensin-2 expression in aggressive periodontitis can show that the major periodontopathogens of aggressive periodontitis induce human β defensin-2 expression. It has been reported that *Actinobacillus actinomycetemcomitans*, the most potent pathogen of aggressive periodontitis, stimulates human β defensin-2 mRNA expression in human oral keratinocyte cell culture (22). In addition, interleukin-1 and interleukin-1 receptor antagonist play an important role in transcriptional regulation of the human β defensin-2 gene. Interleukin-1 induces human β defensin-2 gene expression by itself, but interleukin-1 receptor antagonist blocks this effect (27). In the present study, the relatively increased human β defensin-2 expression in the aggressive periodontitis group might explain the reason why no severe gingival inflammation, but advanced periodontal destruction, occurs in patients with aggressive periodontitis.

In the present study, the β defensin-1 level of patients in the chronic periodontitis group was found to be higher than that of patients in the control group. Likewise, Lu *et al*. (8) reported that β defensin-1 mRNA levels in the pocket epithelium of patients with severe chronic periodontitis were higher than those in the healthy sulcus epithelium of the same patients. On the contrary, Dommisch *et al*. (28) found decreased human β defensin-1 mRNA levels in the gingiva of patients with chronic periodontitis compared with healthy controls; however, the difference was not statistically significant. Although human β defensin-1 is constitutively expressed by gingival epithelial cells (10), the relatively enhanced human β defensin-1 mRNA levels in chronic periodontitis group in the present study might show that periodontopathogens and/or proinflammatory mediators responsible for chronic periodontitis can stimulate the production of human β defensin-1. Even though human β defensin-1 levels were high in this group, they could not stop periodontal destruction in chronic periodontitis, which may show that the other mechanisms are also important in the first line of host defense.

It may be suggested that aggressive periodontitis differs from chronic periodontitis in terms of gingival β defensin expression, because the present findings showed a statistical difference in human β defensin-1 and -2 expression levels of these two periodontitis groups. In aggressive and chronic periodontitis, the invasion level of pathogenic microorganisms into the gingival connective tissue might depend on β defensin expression of gingival epithelium. Different bacteria can modulate defensin production of epithelial cells in different ways, for example, commensal bacteria induce human β defensin-2 by the mitogen-activated protein kinase (MAPK) pathway: in particular, Jun N-terminal kinase and p38 MAPK, but MAPKs and also nuclear factor-κB, play an important role in human β defensin-2 stimulation by pathogenic bacteria (29). It is important to understand the transcriptional regulation of human β defensin-2, for two reasons: (i) to find out how to increase human β defensin-2 expression in order to control local tissue infection; and (ii) to prevent tissue invasion by periodontopathogens.

The protease type and/or amount from *P. gingivalis*, one of the most potent bacteria in chronic periodontitis, stimulate human β defensin-2 expression of gingival epithelial cells (30). Arginine-specific gingipains induce human β defensin-2 expression more than lysine-specific gingipains, and they activate protease-activated receptor-2 in stimulation (30). In other words, not only the type of microorganism, but also the different properties of the same microorganism, affect human β defensin-2 expression of gingiva to different extents. The abovementioned findings may partly explain the reason why, in the present study, some patients with chronic periodontitis showed relatively higher human β defensin-2 expression than others.

In the present study, the mRNA expression levels of human β defensin-1 and -2 were demonstrated, but the protein levels of these peptides were not shown. Even though real-time PCR data are sufficiently sensitive to compare various periodontal diseases in terms of β defensin expression, in the limitations of the present study, it is difficult to clarify the cells that are producing the defensin message. It would be possible to show immunohistochemically what cells and what compartments of epithelial tissue are predominating β defensin production in various types of periodontal diseases, which is an ongoing study. Dale *et al*. (11) showed, in uninflamed and inflamed gingival tissue samples by *in situ* hybridization, that human β defensin-1 and human β defensin-2 mRNA were localized throughout the gingival epithelial tissue, and also they detected human β defensin-1 and -2 peptide expression within the well-differentiated cells of the upper spinous and granular layers of epithelium by immunostaining. In addition, they showed that localization of the β defensins is associated with the differentiation of cells in stratified epithelia in keratinocyte cell culture.

In a recent study (28), no statistically significant difference was found among the healthy control, gingivitis, and chronic periodontitis groups in terms of human β defensin-1, -2 and -3 expression in gingival tissue samples. However, in the present study, the expression of human β defensin-1 and

-2 mRNA was found to be significantly lower in gingivitis group, but higher in chronic periodontitis group relative to the healthy control group. Although similar defensin detection methods (real-time PCR) were used in both studies, different results have been reported. Taken together, it might be speculated that the factors modulating defensin expression of gingiva may show ethnic differences, which needs to be clarified with new studies including more patients.

In conclusion, in the present study, human β defensin-1 and -2 genes demonstrated differential expression in patients with specific periodontal diseases. In addition, aggressive and chronic periodontitis followed different patterns in gingival defensin expression. Human β defensin expression of gingival epithelium may play an important role in the initiation and progression of periodontal diseases.

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