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ORIGINAL ARTICLE

Antimicrobial peptide hCAP-18/LL-37 protein and mRNA expressions in different periodontal diseases

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OBJECTIVE: To investigate the levels of antimicrobial peptide hCAP-18/LL-37 protein and mRNA expression in gingival tissues with different periodontal disease.

MATERIALS AND METHODS: Ten patients with generalized aggressive periodontitis, 10 patients with chronic periodontitis, and 10 healthy controls were included in this study. Periodontal parameters including probing depth, clinical attachment level, plaque index, and papilla bleeding index were assessed in study subjects. hCAP-18/LL-37 mRNA analysis by RT-PCR and immunohistochemistry were performed in 19 samples provided enough RNA in terms of concentration and integrity.

RESULTS: This study demonstrated that hCAP-18/LL-37 was a product of neutrophils. Tissue samples of chronic periodontitis patients had significantly higher immunostaining of hCAP-18/LL-37 on neutrophils infiltrating in both epithelium and connective tissue compared with controls. hCAP-18/LL-37 mRNA expression levels in tissue samples of chronic periodontitis patients seemed to be upregulated compared with controls. While two generalized aggressive periodontitis patients showed downregulated hCAP-18/LL-37 mRNA expression levels, one generalized aggressive periodontitis patient showed slightly increased hCAP-18/LL-37 mRNA level compared with controls.

CONCLUSIONS: hCAP-18/LL-37 has an important role in innate response during periodontal inflammation. Local deficiency in hCAP-18/LL-37 might be a confounding effect in the pathogenesis of generalized aggressive periodontitis.

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Keywords: periodontitis; cathelicidin antimicrobial peptide; gene expression; immunohistochemistry

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Introduction

Antimicrobial peptides are important defense molecules in oral cavity because oral epithelium is constantly exposed to variety of microbial pathogens (Dale *et al*, 2001). Antimicrobial peptides have a broad-spectrum antimicrobial activity against gram-positive and Gram-negative bacterial, fungal and viral pathogens (Boman, 2000; Zaiou *et al*, 2003). Human cationic antimicrobial protein of 18 kDa (hCAP18) consists of a conserved prosequence called the cathelin-like domain and a C-terminal peptide called LL-37 (Bals, 2000). hCAP18 is stored in the secondary granules of neutrophils as an inactive precursor (Bals, 2000; Puklo *et al*, 2008). After the neutrophils stimulation, hCAP18 are cleaved to release the mature peptide LL-37, which is the peptide of 37 amino acid residues starting with two leucine residues (Sorensen *et al*, 2001; Pütsep *et al*, 2002). LL-37 is expressed in epithelium especially following inflammatory stimuli (Frohm *et al*, 1997, 1999; Dale *et al*, 2001; Bals and Wilson, 2003). In addition, both LL-37 protein and mRNA have been detected in human tongue and buccal mucosa and saliva (Frohm *et al*, 1999; Murakami *et al*, 2002). The lack of the salivary LL-37 production in patients with morbus Kostmann syndrome who have increased susceptibility to periodontal disease might suggest the protective role of this molecule in host defense (Pütsep *et al*, 2002).

LL-37 regulates the inflammatory and immune responses, accelerates the angiogenesis, promotes wound healing and re-epithelization, and neutralizes the lipopolysaccharides (Niyonsaba *et al*, 2002; Ciornei *et al*, 2005; Zheng *et al*, 2007). Recently, it has been also shown that LL-37 chemoattracts mast cells, activates these cells to release pro-inflammatory mediators (Niyonsaba *et al*, 2001, 2002). Recruited neutrophils, crucial members of innate immunity, release antimicrobial peptides such as LL-37 and amplify innate immune responses against invading pathogens (Zheng *et al*, 2007). It has been proposed that the association between a specific microbial environment and a modified host response could take place during the development of aggressive periodontitis (Meng *et al* 2007; Zheng *et al*, 2007).

Researchers have stated that LL-37 has killing effect on *Aggregatibacter actinomycetemcomitans* (Pütsep *et al*, 2002, de Haar *et al*, 2006, Tanaka *et al*, 2000) which is an important periodontopathogen for aggressive periodontitis (Gronert *et al*, 2004; Sosroseno *et al*, 2008). It has also been stated that functional defects in polymorphonuclear leukocytes and monocytes may increase host susceptibility to tissue destruction in aggressive periodontitis (Liu *et al*, 2001; Gronert *et al*, 2004).

Although it is known that antimicrobial peptides are important contributors to the maintenance of the balance between health and disease in oral cavity, there are very limited numbers of studies investigating the role of hCAP-18/LL-37 in different periodontal disease (Hosokawa *et al*, 2006; Puklo *et al*, 2008; Türkoğlu *et al*, 2009). Puklo *et al* (2008) investigated GCF LL-37 levels in aggressive and chronic periodontitis patients using Western blot technique. Researchers stated that local deficiency in LL-37 could be considered as a supporting factor in the pathogenesis of severe periodontitis (Puklo *et al*, 2008). In other study, Hosokawa *et al* (2006) searched the expression pattern of antimicrobial peptides in gingival tissues. They demonstrated that neutrophils expressed LL-37, and that expression was more prominent in the inflammatory lesions when compared with healthy gingival (Hosokawa *et al*, 2006). Researchers also suggested that gingival epithelial cells expressed LL-37 antimicrobial peptides, irrespective of the presence or absence of inflammation (Hosokawa *et al*, 2006). Dale *et al* (2001) showed that antimicrobial peptides in the gingiva are localized in specific sites, and have different roles in various regions of the periodontium. Researchers stated that LL-37 was expressed from neutrophils in gingival tissues and particularly in junctional epithelium which serves as a route for neutrophil migration from the connective tissue into the gingival crevice (Dale *et al*, 2001).

It is well known that both chronic and aggressive periodontitis are multifactorial inflammatory diseases, and tissue destruction depends on the balance between both host protective and destructive mechanisms (Kinane and Hart, 2003). Although Dale *et al* (2001) and Hosokawa *et al* (2006) showed LL-37 expression pattern in inflamed as well as healthy gingival tissues, there is no study investigating LL-37 protein and mRNA expressions in gingival tissues with different periodontal diseases by immunohistochemistry and real time-PCR. We hypothesized that lack of hCAP-18/LL-37 mRNA and/or protein expression in gingival tissues with periodontitis can contribute to multifactorial etiology of periodontitis, by modifying host responses. Therefore, we aimed to investigate the levels of hCAP-18/LL-37 protein and mRNA expression in periodontally diseased gingival tissues by immunohistochemistry and RT-PCR techniques.

Materials and methods

Study population

A total of 30 subjects were included in the present study. All subjects were recruited from the Ege University, School of Dentistry, and Department of Periodontology over a period of 1 year between 2006 and 2007. At the prescreening stage, 55 subjects were examined. Fifteen subjects did not fulfill the inclusion criteria and 10 subjects did not accept to attend the study. Finally, a total of 30 subjects who fulfilled all inclusion criteria entered the study. Prior to participation, the purpose and procedures were fully explained to all patients and all participants gave written informed consent in accordance with Helsinki Declaration. The study protocol was approved by Ethics Committee of the Ege University School of Medicine.

All the patients were systemically healthy in this study. None of the subjects had received antibiotics within the past 3 months or treatment for periodontal disease within the past 6 months. Women who were pregnant, breastfeeding, or using oral contraceptives were excluded from the study. Smokers were also not included in this study.

Determination of periodontal status

The clinical periodontal parameters were assessed at six sites around each tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual) for the whole mouth excluding third molars and included probing depth (PD), clinical attachment level (CAL), plaque index (PI) (Quigley and Hein, 1962), and bleeding on probing (BOP). Additionally, papilla bleeding index (PBI) (Saxer and Mühlemann, 1975) was determined. Measurements of PD and CAL were performed with calibrated Williams periodontal probe (Hu-Friedy, Chicago, IL, USA). Clinical measurements were carried out by the same periodontist (O.T) to minimize variability. The intra-examiner reliability was high as was revealed by intraclass correlation coefficient 0.87 for PD measurements and 0.85 for CAL measurements, respectively.

The diagnosis of subjects was established on the basis of clinical and radiographic criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Diseases and Conditions (Armitage, 1999). In this case control study, the subjects was categorized into three groups as follows:

Chronic periodontitis group. Ten generalized severe chronic periodontitis patients (Armitage, 1999) were included in this group. The patients who had at least four non-adjacent teeth with sites CAL $\geq$5 and PD $\geq$6 mm were defined as chronic periodontitis patients. Additionally, they had BOP at $>$ 50% for whole mouth. Diagnosis of chronic periodontitis was made if the CAL was commensurate with the amount of plaque accumulation of the patients. All patients were older than 35 years of age, and had at least 16 teeth in their mouth.

Generalized aggressive periodontitis group . Ten patients were included in this group. These patients demonstrated a generalized pattern of severe destruction and CAL $\geq$5 and PD $\geq$6 mm on eight or more teeth, at least three of these were other than central incisors of first molars. Additionally, the level of attachment loss was

not consistent with the amount of plaque accumulation or local contributing factors. All patients were younger than 35 years of age, and had at least 16 teeth in their mouth.

Healthy control group . Ten systemically and periodontally healthy subjects exhibiting PD ≤3 and CAL ≤2 mm at more than or equal to 90% of the measured tooth sites were included in healthy control group. They had BOP score < 15% at examination and no alveolar bone loss present in radiography (i.e., distance between the cemento–enamel junction and bone crest ≤3 mm at > 95% of the proximal tooth sites). All the subjects had at least 20 teeth and were older than 35 years.

Sample collection

Gingival tissue samples were obtained from 30 subjects for immunohistochemistry and hCAP-18/LL-37 mRNA analysis. Gingival tissue biopsies including oral epithelium, gingival crevicular or pocket epithelium, junctional epithelium, and connective gingival tissue were taken from interproximal sites under local anesthesia. The tissue samples from periodontitis patients were collected from sites with CAL ≥5 and PD ≥6 mm at baseline before treatment. Gingival tissue samples of the healthy control group were obtained during crown-lengthening operation for prosthodontic purposes. The selected sites for healthy control had PD ≤3 mm, and they had no clinical attachment loss and radiographic bone loss. PBI scores of healthy sites tissue samples were equal to 0. The gingival tissue biopsies were divided into two portions: a two-third portion of tissue sample was immediately fixed in 4% paraformaldehyde (Sigma Chemical Co., St Louis, MO, USA) for immunohistochemistry, one-third portion of tissue sample was immediately put in 1.0 ml RNA stabilization reagent (RNA Later, Ambion Co., Foster City, CA, USA) and frozen −40°C for real RT-PCR). Both portions of sampled gingival tissues for protein and mRNA analysis of hCAP-18/LL-37 had same tissue layers (oral epithelium, gingival crevicular or pocket epithelium, junctional epithelium, and connective gingival tissue).

RNA extraction

Gingival tissue samples were homogenized using homogenizator (DI 18 Dispersor IKA Yellow Line, Rio de Janeiro, Brazil) for 3 min at 4°C at 18 000 g. Homogenate was centrifuged at 2000 g for 5 min at 4°C and homogenate supernatant was obtained. Total RNA was extracted from homogenate supernatant using a commercially available tissue RNase Mini isolation kit (Qiagen GmbH, Hilden, Germany), according to manufacturer's instructions. The concentration and purity of the RNA were estimated by the A260/A280 ratio on a spectrophotometer Nanodrop (Thermo Fischer Scientific, Wilmington, DE, USA) The A_{260}/A_{280} ratio of the RNA samples was between 1.8 and 2.1.

Ten tissue samples from each diseased and healthy group for RT-PCR were obtained at baseline. However, only six samples from chronic periodontitis patients, eight samples from generalized aggressive periodontitis patients, and five samples from the control group provided enough total RNA (in terms of concentration and integrity) for further experiments. Therefore, only these samples were subjected to further procedures for RT-PCR.

cDNA synthesis

One microgram of RNA was converted in 20 μl of cDNA by two steps using QuantiTect Reverse Transcription kit (Qiagen GmbH, Hilden, Germany). At first step, genomic DNA elimination reaction were performed. The 14 μl genomic DNA elimination reaction mixture, composed of gDNA wipeout buffer, 7 × (2 μl), template RNA and RNase free water, were prepared and incubated for 2 min at 42°C in a ABI 9700 Thermal Cycler (Applied Biosystems, Foster City, CA, USA), then immediately placed on ice. At second step, reverse transcription master mix including Quantiscript Reverse Transcriptase (1 μl), Quantiscript RT Buffer, 5 × (4 μl), RT Primer Mix (1 μl) were prepared. After that, 14 μl template RNA from first step were added to tube containing the 6 μl of reverse transcription master mix (20 μl of total volume), and mixture were mixed. Prepared 20 μl of reaction mixtures including RNA were incubated for 30 min at 42°C, and 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase. cDNA samples were kept in −20°C.

Quantitative RT-PCR

hCAP-18/LL-37 mRNA levels were measured quantitatively by the RT-PCR. RT-PCR was performed using QuantiTect Prob RT-PCR kit (Qiagen GmbH) and primers for hCAP-18/LL-37 and GAPDH labeled with FAM and TAMRA dye (Invitrogen, Paisley, UK). About 5 μl cDNA was added into reaction mixture which consisted of 2 × QuantiTect Probe RT-PCR Master Mix, reverse and forward primers, probe, and RNase-free water. The 50-μl reaction mixtures including cDNA were subjected to 95°C for 15 min (one cycle), 94°C for 15 s (40 cycles), 56°C for 30 s (40 cycle). Amplifications were performed in ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). hCAP-18/LL-37 mRNA levels were standardized by GAPDH housekeeping gene. The comparative threshold cycle or C_t (the number of PCR cycles necessary to get threshold signal of fluorescence) method was used to quantify the amplified transcripts. Relative quantification was presented as the change in expression of the target gene in the patient sample relative to that of a control transcript by using the $2^{-\Delta\Delta Ct}$ calculation (Arocho *et al*, 2006).

Primers and probes

Primer/probes were designed from the published hCAP-18 cDNA sequence (NM_004345). hCAP-18 cDNA sequence starts with ATG start codon at nucleotide 141 position and stops by TAG stop codon at nucleotide 651 position. This region codes amino acid sequence of hCAP-18 peptide. Forward primer, reverse primer and probe specific to the hCAP-18 cDNA were designed as

follows: 5′F-ACACAGCAGTCACCAGAGGATTGT-3′ (nucleotide 408–431), 5′-R-AAATCACCCAGCAG-GGCAAATCTC-3′ (nucleotide 530–553) and 5′FAM-ACTTCAAGAAGGACGGGCTGGTGAA-3′ TAMR-A (nucleotide 433–457). Primers and probes for hCAP-18 and for GAPDH (5′F-TCGACAGTCAGCCGCATC-TTCTTT-3′, 5′RACCAAA-TCCGTTGACTCCGAC-CTT-3′, 5′-FAM-AGCCACATCGCTCAGACACCAT GGG3′-TAMRA) were obtained from Invitrogen (Invitrogen, Paisley, UK).

Immunohistochemical analysis

Gingival tissue pieces were fixed in 4% paraformaldehyde for 24 h and processed for embedding in paraffin wax using routine protocols. About 4-μm-thick coronal sections were taken from paraffin blocks using a microtome (Leica Microsystems Launches Branding Campaign, Wetzlar, Hesse, Germany); they were then dewaxed and rehydrated through a graded ethanol series using routine protocols and applied to charged slides (X-ra TM Surgipath Medical Industries, Richmond, IL, USA).

Immunohistochemical staining was performed on an automated immunostainer (BenchMark® XT, Ventana Medical Systems S.A, Parc d'Innovation, Kaysersberg, France) with streptavidin biotin-peroxidase complex technique using the 3,3 diaminobenzidine tetrahydrochloride as the chromogen. Counter-staining was performed using hematoxylin (Mayers Hematoxylin Zymed Laboratories Inc., South San Francisco, CA, USA). The primary antibody (LL37/CAP18, Human: Mab, Clone 3D11, Hycult, Canton, MA, USA) against to hCAP-18/LL-37 was used at a dilution of 1:50.

An investigator blind (G.K.) to the study groups examined the samples preparing for immunohistochemistry. Cell counting was performed at ×400 magnification. Two sections were determined in each individual subject for immunohistochemical evaluation. Immunoreactive cells were counted in three or five high power field areas in epithelium and connective tissue. Total cell count per unit area was calculated by taking average of the total immunoreactive cells in interested three or five high power field areas for each sample. In brief, total cell count in three or five high power field areas was divided to the number of power field areas examined.

Nineteen samples, which provided enough RNA for RT-PCR, were also evaluated by immunohistochemically.

Statistical analysis

Considering a difference of 50% in mean number of hCAP-18/LL-37 expressing total cell counts and assuming standard deviations to be maximum 80% of the mean values and accepting a power of 80%, *P*-value of 5% in healthy and diseased groups, minimum sample size was calculated. All data analysis was performed using a statistical package (SPSS Inc., ver. 14.0, Chicago, IL, USA). Statistical analysis was performed using both parametric and non-parametrical techniques. Comparison of clinical periodontal parameters among the study groups was made by Kruskal–Wallis test and *post hoc* analysis by Mann–Whitney *U*-tests. Similarly, comparison of immunostaining for hCAP-18/LL-37 in epithelium and connective tissues among the study groups was made by Kruskal–Wallis test and *post hoc* analysis by Mann–Whitney *U*-tests.

Results

Demographic characteristics and clinical periodontal parameters

Power calculation analysis revealed that the minimum required sample size was five subjects for each group. Table 1 shows demographic and clinical characteristics of the subjects whom gingival tissue samples were subjected to mRNA and immunohistochemical analysis. With respect to gender of patients no significant difference was observed among the study groups ($P > 0.05$). Generalized aggressive periodontitis patients were younger than the other subjects, and the difference was statistically significant ($P < 0.05$). Whole mouth clinical periodontal parameters (PD, CAL, PI, PBI, and BOP) of the healthy subjects were significantly lower than those of the other patients ($P < 0.05$) (Table 1). Similarly, PD, CAL, PI and PBI scores of sampling sites were significantly lower in healthy subjects than those of the others ($P < 0.05$) (Table 1). There was no significant difference in clinical periodontal parameters between generalized aggressive and chronic periodontitis patients ($P > 0.05$).

The expression of hCAP-18/LL-37 mRNA in gingival tissue samples

GAPDH mRNA expressions were detected in all samples studied. Positive hCAP-18/LL-37 mRNA expressions were detected in four of six gingival tissue samples with chronic periodontitis (66.7%), three of eight samples with generalized aggressive periodontitis (37.5%), and one of five samples with healthy periodontium (20%) ($P > 0.05$).

Figure 1 shows GAPDH normalized gene transcript levels of hCAP-18/LL-37 in study groups. In all chronic periodontitis patients who were positive for hCAP-18/LL-37 mRNA, gene expression levels seemed to be upregulated when compared with healthy controls (Figure 1). While two generalized aggressive periodontitis patients showed downregulated hCAP-18/LL-37 mRNA expression levels compared with healthy controls, hCAP-18/LL-37 mRNA level slightly increased in one generalized aggressive periodontitis patient compared with healthy controls (Figure 1).

Immunohistochemical evaluation for hCAP-18/LL-37 in gingival tissue samples

Figure 2 demonstrates the number of hCAP-18/LL-37 immunostaining neutrophils in epithelium and connective tissue of gingival tissue samples in the study groups. There were no immunostaining neutrophils for hCAP-18/LL-37 in three of five healthy gingival tissue

Table 1 Demographic and clinical periodontal features of whole mouth and sampling sites in the study groups

	CP (n = 6)	*G-AgP* (n = 8)	*Healthy* (n = 5)
Demographics			
Age	46 (38–62)	29 (26–34)*	38 (35–45)
Gender			
Female/Male	3/3	5/3	3/2
Whole mouth (median (min-max))			
PD (mm)	3.5 (3–5.3)	3.4 (2.7–5.8)	1.48 (1.3–1.6)*
CAL (mm)	3.9 (3.7–8.1)	4.1 (3.4–6.4)	0.08 (0.02–0.11)*
PI	3.3 (2.4–4.1)	3.02 (1.3–4.2)	1.9 (1.3–2.1)*
PBI	2.5 (1.6–2.9)	2.1 (1.2–2.8)	0.12 (0.06–0.17)*
BOP	89.9 (72.4–100)	77.6 (64.1–95.8)	7.6 (6.8–9.6)*
Sampling sites(median (min–max))			
PD (mm)	7 (6–8.5)	6 (6–10)	2 (2–2)*
CAL (mm)	6.5 (6–9.5)	6.2 (5–10)	0 (0–0)*
PI	4 (3–5)	3 (3–5)	2 (1–3)*
PBI	3 (3–4)	3 (2–4)	0 (0–0)*

*Significant difference from the other groups ($P < 0.05$). CP, chronic periodontitis; G-AgP, generalized aggressive periodontitis; PD, probing depth; CAL, clinical attachment level; PI, plaque index; PBI, papilla bleeding index; BOP, bleeding on probing.

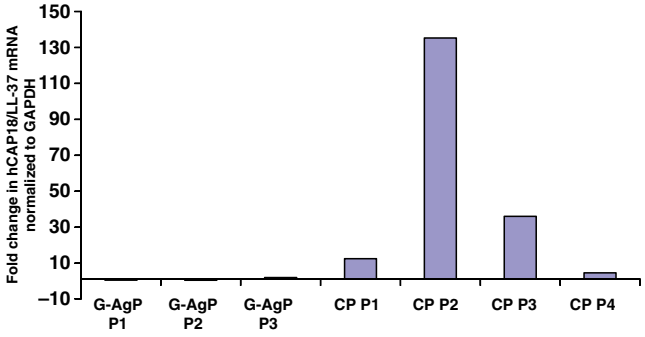

Figure 1 RT-PCR quantification of hCAP-18/LL-37 mRNA expression in each subject. hCAP-18/LL-37 mRNA expression was determined in gingival tissue with chronic and generalized aggressive periodontitis, and expression levels were standardized by GAPDH housekeeping gene. Relative quantification was expressed as fold induction in expression of the target gene in the gingival sample of patient relative to that of a control transcript. G-AgP P = Generalized aggressive periodontitis patient. CP P = Chronic periodontitis patient

samples (epithelium and connective tissues) examined (Figures 2a,b and 3a). There were a few positive staining neutrophils for hCAP-18/LL-37 in two epithelium and little staining neutrophils in two connective tissues of five healthy tissue samples (Figure 2a,b). All gingival tissue samples with chronic periodontitis had positive staining neutrophils for hCAP-18/LL-37 in epithelium and connective tissue (Figure 2a,b). There was higher immunostaining of hCAP-18/LL-37 on neutrophils infiltrating epithelium and connective tissue in chronic periodontitis patients than those of healthy subjects, and the difference was statistically significant ($P < 0.05$) (Figures 2a,b and 3c). In three out of eight gingival tissue samples of generalized aggressive periodontitis there were no immunostaining neutrophils for hCAP-18/LL-37 in epithelium, whereas neutrophils had strong immunostaining for hCAP-18/LL-37 in epithelium of one gingival tissue sample with generalized aggressive periodontitis (Figure 2a). Four of eight samples with generalized aggressive periodontitis had positive immunostaining neutrophils in epithelium in varying degrees (Figure 2a). There were no or a few immunostaining neutrophils for hCAP-18/LL-37 in four of eight connective tissue samples of generalized aggressive periodontitis patients, however there were immunostaining neutrophils for hCAP-18/LL-37 in four samples with generalized aggressive periodontitis patients, in varying degrees (Figures 2b and 3b).

Discussion

This study demonstrated that hCAP-18/LL-37 was a product of neutrophils. We also showed that the immunostaining of hCAP-18/LL-37 in epithelium and connective tissue of the gingival tissues is significantly higher in patients with chronic periodontitis than those of healthy controls. Additionally, mRNA expression levels of hCAP-18/LL-37 in tissue samples with chronic periodontitis seemed to be upregulated compared with healthy controls. On the contrary, generalized aggressive periodontitis patients showed downregulated hCAP-18/LL-37 mRNA expression levels compared with healthy controls. Based on the present data we might suggest that hCAP-18/LL-37 plays an important role in host immune response in periodontal disease, and local deficiency of hCAP-18/LL-37 in generalized aggressive periodontitis patients might reflect confounding effect of this antimicrobial peptide on the rapid destruction of periodontal tissues in generalized aggressive periodontitis.

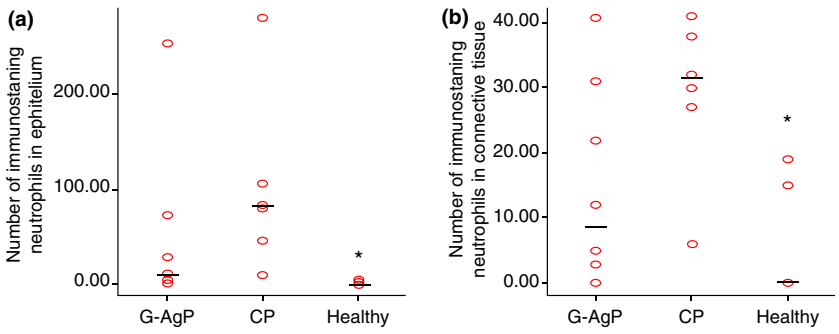

Figure 2 The scatter plot of study groups demonstrates the number of hCAP-18/LL-37 immunostaining neutrophils in epithelium and connective tissue. Chronic periodontitis patients had significantly higher number of hCAP-18/LL-37 immunostaining neutrophils in epithelium (**a**) and connective tissue (**b**) of gingival tissue samples than healthy controls. Median values of total cell count per unit for hCAP-18/LL-37 positively stained neutrophils. *Significant difference from chronic periodontitis group ($P < 0.05$). G-AgP = Generalized aggressive periodontitis. CP = Chronic periodontitis

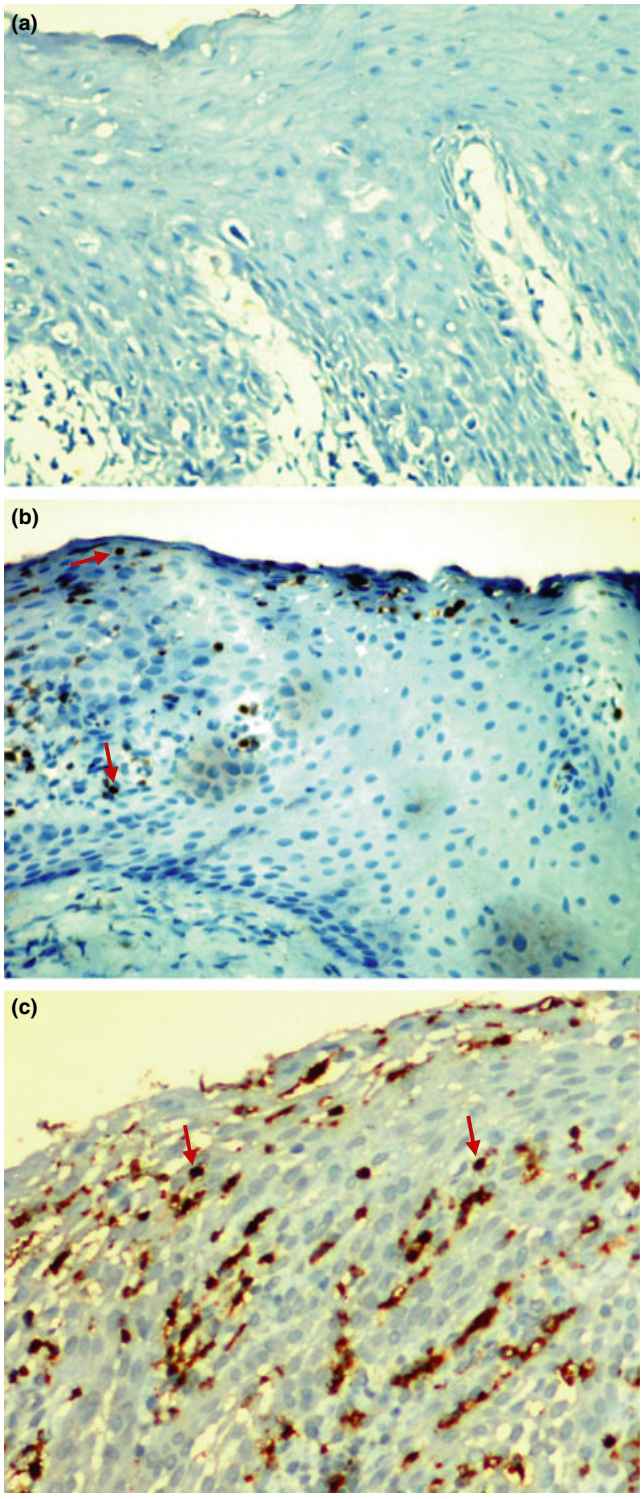

Figure 3 Immunohistochemical analysis of human antimicrobial peptide hCAP-18/LL-37 expressions in the gingiva. Sections were counterstained with hematoxylin. Arrows show positive immunostaining. (**a**) No immunostaining neutrophils for hCAP-18/LL-37 (magnification ×400). (**b**) Immunostaining neutrophils in both epithelium and connective tissue of gingival tissue sample with generalized aggressive periodontitis (magnification ×400). (**c**) Strong immunostaining for hCAP-18/LL-37 in neutrophils in both epithelium and connective tissue of gingival tissue sample with chronic periodontitis (magnification ×400)

In this study, immunostaining of hCAP-18/LL-37 in epithelium and connective tissue of gingival tissue samples with chronic periodontitis was significantly higher compared with healthy controls. Additionally, hCAP-18/LL-37 mRNA expression was detected in only one gingival tissue samples of healthy controls. These findings are consistent with the findings of the study by Hosokawa *et al* (2006) who showed that hCAP-18/LL-37 positive staining neutrophils in epithelium and connective tissues in four of six inflamed gingiva but none in six non-inflamed gingival tissues. Researchers also demonstrated that hCAP-18/LL-37 mRNA was expressed only in inflamed tissues (four of six samples), while none of the four non-inflamed gingival tissues. hCAP18, which is full chain composed of LL-37 and cathelin-like domain, was stored in neutrophil granules. After inflammatory stimulus hCAP18 is cleaved to LL-37 and cathelin-like domain by proteinase 3 (Sorensen *et al*, 2001). In the absence of clinical inflammation, neutrophils do not become activated, and do not express mature LL-37. Therefore, it is not surprising that hCAP-18/LL-37 both protein and mRNA expressions are very low in gingival tissue samples of healthy control subjects.

Epithelial lining, the first line of defense between an organism and the environment, plays an active role in host innate immunity (Weinberg *et al*, 1998). It was demonstrated that the level of hCAP-18/LL-37 mRNA was similar in cultured gingival epithelial cells that were unstimulated as well as in those stimulated by bacterial extracts (Dale *et al*, 2001). The expression of antimicrobial peptides in epithelial cells might be constitutive, inducible, or decreased by different stimuli (Hiemstra, 2001). Although the microbial dental plaque is closely contacted with epithelial surface, epithelial cells do not seem to express hCAP-18/LL-37 in this study. However, this microbial stimulus might cause the expression of other antimicrobial peptides from epithelial cells, such as alpha defensins. Additionally it has been shown that some of the bacteria can suppress the expression of LL-37 from epithelial cells (Islam *et al*, 2001). Therefore, the studies evaluating the bacterial challenge of sites with periodontitis for hCAP-18/LL-37 would be interesting in view of the assessment of effect of specific bacteria to hCAP-18/LL-37 expression in both epithelial cells and neutrophils in periodontitis patients.

In this study, in all chronic periodontitis patients who were positive for hCAP-18/LL-37 mRNA, gene expression levels seemed to be upregulated when compared with healthy controls. After stimulation, neutrophils migrate into periodontium from connective tissue, which results in increased numbers of neutrophils in gingival tissues and release of mature hCAP-18/LL-37 from activated neutrophils. We have recently shown that GCF hCAP-18/LL-37 levels were significantly increased in chronic periodontitis patients compared with healthy controls (Türkoğlu *et al*, 2009). Although we stated that the high expression levels of hCAP-18/LL-37 have been found in chronic periodontitis, it has been shown that *P. gingivalis,* an important periodontopatogen for chronic periodontitis (Bragd

et al, 1985), is able to resist the antimicrobial action of a number of antimicrobial peptides, including adrenomedullin, beta defensin and cathelicidin LL-37 (Guthmiller *et al*, 2001; Joly *et al*, 2004; Allaker *et al*, 20007). Therefore, we consider that hCAP-18/LL-37 levels are not effective to being protected from chronic periodontitis.

Various studies have investigated genetic factors, functional defects of polymorphonuclear leukocytes, and inflammatory mediators and cytokines, which are related to host susceptibility to severe tissue destruction in aggressive periodontitis (Liu *et al*, 2001; Garlet *et al*, 2003, 2004; Gronert *et al*, 2004; Meng *et al*, 2007). It can be suggested that possible genetic defects in CAMP gene might affect mRNA expression, or post-transcriptionally defects might affect the conversion from mRNA to mature protein. In other words, the lack of hCAP-18/LL-37 might be an effective factor for generalized aggressive periodontitis to have rapid and severe progressive characteristics. In this study, generalized aggressive periodontitis patients showed less immunostaining neutrophils in tissue samples, besides downregulated mRNA levels for hCAP-18/LL-37. These findings show that the lack of this antimicrobial peptide might be an important contributing factor for pathogenesis of generalized aggressive periodontitis, because of its known effects on *Aggregatibacter actinomycetemcomitans* (Tanaka *et al*, 2000; Pütsep *et al*, 2002; de, Hiemstra, van Steenbergen, Everts, and Beertsen, 2006), which is important periodontopathogen for aggressive periodontitis (Gronert *et al*, 2004; Sosroseno *et al*, 2008). Just as, Puklo *et al* (2008) investigated GCF LL-37 levels using Western blot technique in both chronic and aggressive periodontitis patients. Researchers stated that while patients with chronic periodontitis had significantly elevated amounts of LL-37 in GCF than those of controls, no significant increase could be obtained in GCF LL-37 levels of aggressive periodontitis patients (Puklo *et al*, 2008). Our results are consistent with the opinion of Puklo *et al* (2008) who suggested that a local deficiency in hCAP-18/LL-37 could play a role in the pathogenesis of severe cases of periodontitis.

In conclusion, our results suggest that mature LL-37 release is mainly due to neutrophil priming and is not originated from epithelial cell activation during periodontal inflammation. In addition, we might suggest that hCAP-18/LL-37 plays an important role in host immune response in periodontal disease, and local deficiency of hCAP-18/LL-37 antimicrobial peptide reflect confounding effect of this antimicrobial peptide on the destruction of periodontal tissues in generalized aggressive periodontitis. To the best of our knowledge, this is the first study investigating both hCAP-18/LL-37 protein and mRNA expressions in gingival tissues of patients with chronic and generalized aggressive periodontitis by immunohistochemistry and RT-PCR. The cross-sectional feature of this study prevents the assessment of cause and effect relationship between periodontitis and hCAP-18/LL-37 antimicrobial peptide. Therefore, further studies with large sample size are required to display both protein and mRNA expression levels of hCAP-18/LL-37 in gingival tissues with different periodontal diseases as well as investigating these levels after periodontal treatment in order to enlighten the role of this peptide in the pathogenesis of periodontal diseases. Additionally, studies investigating possible neutrophil functional defects, such as chemotaxis and expression of antimicrobial peptides, would help to explain the pathogenesis of generalized aggressive periodontitis.

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