LL-37 as an indicator for periodontal disease

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Abstract
LL-37 is part of innate immunity and has activity against oral bacteria. This antimicrobial peptide may play an important role in the defense mechanism within the periodontium. An absence of LL-37 can result in overgrowth of periodontal bacteria, resulting in periodontitis. The aim of this study was to investigate the assumption that LL-37 concentration in gingival crevicular fluid (GCF) is suppressed in patients with periodontitis. The study population consisted of a control group with gingivitis alone (n=12) and a group of patients having periodontitis (n=13). GCF was collected from inflamed sites in subjects with gingivitis (GG), inflamed sites with shallow pockets in subjects with periodontitis (GP) and sites with deep pockets in patients having periodontitis (PP). LL-37 concentrations were determined with ELISA using a quantitative solid-phase sandwich enzyme immunoassay technique. The results showed that there were no statistically significant differences in the concentration of LL-37 in GCF between the three groups, GG, GP and PP. However, a tendency for lower LL-37 value was observed in the GP group in comparison with the PP and GG groups. Thus this study shows an interesting finding that in gingivitis sites in periodontitis patients the LL-37 concentration in GCF was reduced despite the deeper pockets when compared to the gingivitis sites in gingivitis patients. In conclusion, the findings in this study could be interpreted as if patients with periodontitis have a decreased ability to produce LL-37.

Introduction
Gingivitis and periodontitis is characterized by an accumulation of microbes in gingival crevice, followed by inflammation and immune reaction. The inflammation of the periodontal tissue, the tissue supporting the teeth, can be localized to the marginal gingival or and involve degradation of the connective tissue. This can be followed by junctional epithelial migration from the enamelocemental junction and bone resorption (1). The inflammation and tissue destruction are thought to be caused by bacterial antigens, metabolites and enzymes which diffuse into the crevicular epithelium where they lead to immune and inflammatory response. Bacteria such as Porphyromonas gingivalis, Treponema dentocola, Prevotella intermedia, Tannerella forsythis and Actinobacillus actinomycemcomitans produce toxic factors (2,3) that are capable of destroying the crevicular epithelium through stimulating the host immunoinflammatory process (4).
PMNs (polymorphonuclear leucocytes), are found in the first line of defense against periodontal pathogens (1) and can rapidly move through the gingival connective tissue and junctional epithelium into the gingival pocket (2). These inflammatory cells are phagocytic cells present in GCF (gingival crevicular fluid) and belong to innate immunity that also includes cytotoxic cells (T and NK-cells) and their affector and signalling molecules. Innate immunity is gene-encoded and exists both in plants and animals and is characterized by fast expression and delivery and killing of bacteria faster than its growth rate (5,6).
Antimicrobial peptides are the main components of the innate immunity. Earlier studies have shown that mammalian cells produce antimicrobial peptides, such as cathelicidins, alpha-defensin and beta-defensin. Cathelicidins function as antimicrobial peptides against a wide range of bacteria, fungi and leptospirae (7). LL-37 (hCAP18) is the cathelicidin produced in human and is produced in neutrophils and epithelial cells (8, 9). This cathelicidin have activity against oral bacteria, both Gram-positive and Gram-negative bacteria (10) and is present in saliva where it functions as a defence molecule (11).

hCAP18 is the precursor protein of LL-37 and is found in the specific granules of neutrophils (12), lymphocytes, macrophages, and epithelial cells (13,14). The target of LL-37 is the lipopolysaccharide (LPS) in the bacterial membrane and act as a LPS neutralizing factor (15,16). The release of specific LL-37 occurs during the immune response against invading microorganisms. Thus high concentration of LL-37 is released during infection and inflammation due to neutrophil accumulation and has been demonstrated to occur in gingival tissues and in the saliva (17,11).

Actinobacillus actinomycetem comitans is a periodontal pathogen, particularly associated with aggressive periodontitis. Absence of LL-37 promotes the growth of A.a. which could lead to periodontitis (13). This was presented in a study by Katrin Pusep et al. (11) who also found that there were a correlation between LL-37 concentrations and oral health. LL-37 was not found in the saliva or neutrophils in patients with Morbus Kostmann. These patients suffer from a severe congenital neutropenia and are treated by recombinant granulocyte-colony stimulating factor. Kostmann patients who receive this treatment have restored levels of neutrophil, but because the lack of LL-37 in their neutrophils and saliva they have recurrent infections and periodontal disease (11).

The aim of the present study was to investigate if LL-37 is suppressed in the presence of periodontitis. To test this we compare LL-37 concentrations in GCF in healthy and periodontitis subjects.

**Materials and methods**

**Subjects and site selection**

The study was approved by the ethics committee at Huddinge University Hospital, Sweden.

Totally 25 subjects were included in the study (14 men, 11 women; mean age 36, 5, range 24-72 years) who were patients and staff at Institution of Odontology, Karoliska Institutet, Huddinge. Written info prior to participation in the study was gives to all the patients. Thirteen patients with chronic periodontitis were allocated as a test group. The group of periodontitis patients consisted of 8 men and 4 women. The healthy control group consisted of 12 patients, 6 men and 7 women who did not show any signs of periodontitis. Details about the study population are presented in table 1.

In this study we excluded patients who have been undergoing periodontal treatment for the last 4 months, patients suffering from diabetes, bleeding disorders. Intake of antibiotics or an extensive intake of anti-inflammatory drugs in the previous 6 months was also an exclusion criteria.
Table 1: Study population; age, gender and smoking habits.

<table>
<thead>
<tr>
<th>Category</th>
<th>Age (years) Mean</th>
<th>Gender M/F</th>
<th>Smoking S/NS</th>
</tr>
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<tbody>
<tr>
<td>Patients n=12</td>
<td>52.2</td>
<td>8/4</td>
<td>6/6</td>
</tr>
<tr>
<td>Controls n=13</td>
<td>47.1</td>
<td>6/7</td>
<td>2/11</td>
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Collection of Gingival Crevicular Fluid (GCF)

In the test-group, samples were taken from sites with deep pockets (PP-periodontitis sites in periodontitis patients) and from shallow pockets (GP-gingivitis sites in periodontitis patients). In the control group samples were taken from inflamed shallow sites (GG-gingivitis sites in gingivitis patients).

Sampling of GCF preceded all other clinical assessment, in order to avoid tissue irritation and blood contamination of the samples. Supragingival plaque was carefully removed and the sites were isolated with cotton rolls and gently air-dried with an air syringe before sampling.

The clinical measurement included probing pocket depth and registration of gingival inflammation (18) after sampling.

GCF was collected using a modification of the intracervicular washing device described by Salonen & Paunio (19). Each pocket was washed with Phosphate-Buffer-Saline (PBS), pH 7, 4, up to approximately 500 microliters. The GCF samples were centrifuged at 10 000 rpm for 5 minutes and then stored in a freezer (-80 ºC) until used for LL-37 estimation.

LL-37 assay

LL-37 levels were determined with a commercial enzyme-linked Immunosorbant assay (ELISA). LL-37 levels were determined using a Human LL-37-ELISA KIT (Cell Sciences, Canton, MA, USA). Standard series were prepared according to the manufacturers instructions and standard range from 0.14 to 100 ng/ml was achieved.

GCF samples were diluted 20 times in coating buffer. The ELISA procedure employed a quantitative solid-phase sandwich enzyme immunoassay technique wherein a monoclonal antibody specific for LL-37 had been precoated to micro-titer plate provided in the kit. 100 microliters of the diluted samples and the standards were coated on to the 96 wells plate. The wells were washed with dilution buffer after one hour incubation at room temperature. After washings a biotin conjugated polyclonal antibody was added to the wells. Streptavidin-peroxidase conjugate (100 microliter) was added to each well and then incubated for one hour at room temperature. The plate was washed again and incubated for one hour at room temperatur. 100 microliter of TMB substrate solution was added to each wellplate and the plate was incubated for 30 minutes at roomtemparature. The reaction was stopped by adding 100 microliters of stop solution. Plates were read in a spectrophotometer (Multiscan MS, Labsystems, Finland) and the absorbance was measured at 450 nm. Amounts of LL-37 were expressed in ng/ml.

Polyclonal antibody against human LL-37 conjugated to horseradish peroxidase was used as a secondary antibody.

Statistical analysis

Median and interquartile range were calculated. P values less than 0.05 were considered to be statistically significant. Statistical significances of differences were calculated with
Wilcoxon signed rank test or Mann Whitney u-test. Statistica 7, 1 was the computer program used for statistical analysis.

Results

Clinical data from the population study (n=25) show that the PP have higher GI and PPD values compared to the GP and GG groups (table 2).

There was no statistically significant difference between the three groups, GG, GP and PP in concentration of LL-37 in gingival crevicular fluid. LL-37 mean value in the gingivites sites in periodontitis patients was less than the mean value in periodontitis sites in periodontitis patients and in gingivitis sites in gingivitis patients. A tendency for lower LL-37 value was observed in the GP group in comparison with the PP and GG groups (Fig. 1).

The association between gingival index and the concentration of LL-37 in GCF did not show any correlation, r=0,16 (Fig. 2). In addition there was a high variation in LL-37 concentrations and the pocket depth in the test- and control groups, r=0,19 (Fig. 3). There were no statistical significance in the mean value of LL-37 in GCF between males (10,3 ng/site, SD ±8,5 ng/site) and females (15,7 ng/site, SD ±14,4) or between smokers (10,8 ng/site, SD ±9,0) and non-smokers (13,4 ng/site, SD 12,6 ng/site) when calculated on shallow sites and deep pockets. Age and LL-37 did not correlate (Fig. 4).

Table 2: Clinical data, comparison of mean values (±standard deviation) between GG (gingivitis in gingivitis patients), GP (gingivitis in periodontitis patients) and PP (periodontitis in periodontitis patients). GI (Gingival Index) and PPD. GI (gingival index) and PPD (probing pocket depth).

<table>
<thead>
<tr>
<th>Site</th>
<th>GI Mean (±SD)</th>
<th>PPD (mm) Mean (±SD)</th>
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<tbody>
<tr>
<td>GG</td>
<td>0,9 (±0,6)</td>
<td>2,3 (±0,4)</td>
</tr>
<tr>
<td>GP</td>
<td>1,4 (±0,6)</td>
<td>2,3 (±1,3)</td>
</tr>
<tr>
<td>PP</td>
<td>1,9 (±0,8)</td>
<td>6,9 (±0,7)</td>
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Fig. 1: Distribution of LL-37 in GCF in GG, GP and PP sites. LL-37 is expressed in pg/site.
Fig. 2: The correlation between LL-37 concentration in GCF and gingival index.

Fig. 3: The relationship between LL-37 concentrations in GCF from 32 sites and probing pocket depth.
Discussion

Periodontitis is a multi etiological disease where the bacterial invasion plays an essential role through stimulating the host tissue to express an immunoinflammatory response. This process may lead to resorption of the alveolar bone depending on host-response.

The intracellular space in the junctional epithelium offers an entrance for bacterial invasion of the tissue in periodontal disease. Antimicrobial peptides may play an important role in the barrier function within the periodontium. It was shown in an earlier study by Katrin Pütsep et al. (11) that granulocyte-colony stimulating factor (G-CSF)-treated morbus Kostmann patients suffer from deficiency in salivary LL-37 despite sufficient neutrophil number.

In the present study it was not possible to show significant differences in LL-37 in GCF between patients with periodontitis and healthy subjects. However, there is a trend showing that levels of crevicular LL-37 in the GP group was reduced compared to that in the PP and GG groups. Furthermore, we could not show any correlation between LL-37 concentrations with age, gender or smoking habits.

Neutrophils and epithelial cells producing and releasing LL-37 plays an important role in the innate immunity in the periodontium. During infection and inflammation high concentration of LL-37 is released in gingival tissue (17). Thus this study shows an interesting finding that in gingivitis sites in periodontitis patients, the LL-37 concentration in GCF was reduced despite the deeper pockets when compared to the gingivitis sites in gingivitis patients.

The sample size in the present study was small. An increase of the population study to 30 patients in the control group and 30 patients in the test group could give significance in differences between patients and controls if the differences are persistent. The findings in this study could be interpreted as if patients with periodontitis have a decreased ability to produce LL-37.

Considering the small number of subjects the results must be interpreted with caution but the findings still are intriguing enough to merit an extended study.
Acknowledgment: I would like to express my gratefulness to my adviser Prof. Anders Gustafsson for his help and support throughout the study.

References


