Characterization of lactoferrin in gingival crevicular fluid of chronic periodontitis patient

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ABSTRACT

Background: Human periodontal diseases are inflammatory disorders as the result of complex interactions between periodontopathogens and the host’s immune response. Periodontitis results in tooth loss and can even lead to systemic diseases if not treated. Gingival crevicular fluid (GCF) reflects the condition of the gingiva and contains proteins transuded from serum or cells at inflamed sites. Polymorphonuclear leukocyte (PMNs) infiltration can be seen in each stage of periodontitis. Lactoferrin is one of the PMN specific granules and could be a useful marker of PMN activity. Purpose: The aim of this study was to determine the band intensity of lactoferrin used as periodontitis biomarker. Methods: Gingival crevicular fluid (GCF) samples were collected using paper point no.30 from 40 subjects, 30 periodontitis patients that devide according to the severity (10 mild periodontitis, 10 moderate periodontitis, and 10 severe periodontitis) and 10 healthy controls, ranging in ages from 20 to 35 years. GCF lactoferrin was analyzed by Western blot and measured the band intensity by quantity one software (bio-rad). Results: The periodontitis sites exhibited significantly greater band intensity of lactoferrin than healthy sites. The band intensity of lactoferrin was positively correlated with the severity of periodontitis (α = 0.05). Conclusion: The study showed that the intensity of the lactoferrin protein bands could be used as biomarkers of periodontitis.

Key words: Gingival crevicular fluid, lactoferrin, periodontitis, band intensity

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INTRODUCTION

Periodontitis is a periodontal disease due to the development of the inflammatory process in the gingival tissue that continues to the other support tissues.\(^1\) Bacterial biofilms are regarded to be the primary aetiologic factor in the initiation of gingival inflammation and subsequent destruction of periodontal tissues and three major specific pathogens have been repeatedly identified as etiologic agents, namely Aggregatibacter (Actinobacillus) actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg) and Tannerella forsythia (Tf). Although chronic exposure to bacteria and their products is a prerequisite for gingival inflammation and periodontal tissue destruction to occur, the major causative factor of soft- and hard- tissue breakdown associated with periodontitis is currently attributed to the host’s immune-inflammatory response to bacterial challenge. Furthermore, the nature of the inflammatory response might determine the destructive character of the disease.\(^2\)

*Porphyromonas gingivalis* is one of the major pathogens in chronic periodontitis. *P. gingivalis* has a number of virulence factors such as capsule, fimbriae, lipopolysaccharide (LPS) and potent proteolytic enzymes such as gingipains. These factors can induce an inflammatory cascade involving pro-inflammatory cytokines, reactive oxygen species and matrix metalloproteinases (MMPs), thus leading to the destruction of supportive soft and hard tissues around the teeth.\(^3\)

The movement of leukocytes from the blood to the local tissue is the key in process of inflammation. Trans-endothelial migration is a selective interaction between leukocytes and the endothelium resulting in leukocytes out of the bloodstream into the tissues. Local damage trigger a variety of inflammatory signals (IL-1\(\beta\), TNF-\(\alpha\), mainly derived from resident leukocytes such as mast cells. Mast cells are an important part which triggered the mobilization of PMN against bacteria and response to anaphylatoxins such as C3a and C5a.\(^4\) PMN acts as a barrier between the mass of plaque and crevicular epithelium.\(^4\)

A diagnosis of periodontitis is established by traditionally used indices, e.g., bleeding on probing and probing depth, which indicate the loss of periodontal tissue attachment to the teeth. Additionally, radiographyvisualizes the loss of periodontal tissue, which supports the diagnosis by determining the amount of bone loss around the teeth. However, these methods are only useful when attachment loss has occurred to some degree. For chair-side and single visits, more reliable biomarkers for periodontitis are needed to provide constant classification and customized treatment and for monitoring periodontal diseases.\(^5\)

Gingival crevicular fluid (GCF) is bodily fluid that reflects the condition of the periodontium, contains various components that originate by transudation of the serum or inflammatory factors and are derived from the interaction between the bacterial biofilm and the cells of periodontal tissues. Additionally, despite a large variation, the amount of GCF tends to increase with the severity of gingival inflammation. In addition to these characterizations, the simple and noninvasive collection of GCF is required for the discovery of periodontitis biomarkers.\(^5,6\)

Lactoferrin is a glycoprotein with a molecular weight of about 80 kDa, which shows high affinity for iron, and classified as a member of the transferrin family, due to its 60% sequence identity with serum transferrin. The ability to keep iron bound even at low pH is important, especially at sites of infection and inflammation where, due to the metabolic activity of bacteria, the pH may fall under 4.5. In such a situation lactoferrin also binds iron released from transferrin, which prevents its further usage for bacterial proliferation.\(^7\)

Lactoferrin can be found in saliva and crevicular fluid and acts bacteriostatic and bacteriocid. In the crevicular fluid significant higher levels of lactoferrin have been found at periodontitis sites in comparison to healthy sites.\(^6\) Lactoferrin is one of the PMN specific granules and could be a useful marker of PMN activity. Increased lactoferrin levels have been reported in severe infections, for example, meningitis, in burns patients, rheumatoid arthritis, and chronic salivary gland diseases. There are few reports about the quantification of lactoferrin in GCF. Lactoferrin of gingival crevicular fluid levels in gingivitis, periodontitis, and localized aggressive periodontitis patients were two-fold higher than in periodontally healthy individuals. Gingival crevicular fluid production is a result of the increase in permeability of microvasculatures of the periodontal tissue due to inflammatory reactions. Lactoferrin released from PMNs into the GCF is a good indicator of periodontal inflammation.\(^8\) The objective of this study was to determine the band intensity of lactoferrin used as periodontitis biomarker.

MATERIALS AND METHODS

GCF samples were collected from periodontitis patients and healthy individuals for the identification of periodontitis biomarkers at the Department of Periodontology, Faculty of Dental Medicine Universitas Airlangga. The standard protocol was approved by the KKEPK (Komisi Kelaikan Etik Penelitian Kesehatan) of Faculty of Dental Medicine Universitas Airlangga (KKEPK NO. 151/KKEPK.FKG/X/2013). Informed consent was obtained from all donors.

Forty male patients in range of age 20 to 45 years old participated in the study. All participants were free of systemic disease and had not taken medication (such as anti-inflammatory agents, antibiotics, immunosuppressants, or contraceptives) that could affect their periodontal status for at least six months prior to the study. Radiographic examination and clinical periodontal assessment were performed. To stratify chronic periodontitis patients, the clinical periodontal parameters from average 6 sites per individual were assessed at the initial examination for mean probing depth (PD), clinical attachment loss
Characterization of lactoferrin in gingival crevicular fluid (CAL), and bleeding on probing (BOP). According to the criteria, we divided the samples into 4 groups, healthy, mild periodontitis, moderate periodontitis, and severe periodontitis based on clinical attachment loss (CAL), healthy subjects with no CAL, mild periodontitis with 1-2 mm of CAL, moderate periodontitis with 3-4 mm of CAL, and severe periodontitis with more than 5 mm of CAL.

GCF was taken from the tooth at posterior region of maxilla with the deepest pocket. Each tooth was isolated with a cotton roll and the supragingival plaque was carefully removed without touching the marginal gingiva. The gingiva was then gently dried with an air syringe. Gingival crevicular fluid was collected using paper point No. 30 at the orifice of the sulcus 1-2 mm subgingivally for 30 seconds. The same procedure was repeated after 1 minute. The paper points were then stored in 100 μL PBS (pH 7.4) and then stored at -40° C until further analysis.

The entire sample were calibrated for their total protein concentration using a nano drop before SDS-PAGE analysis. SDS-PAGE analysis performed to separate proteins based on molecular weight. After SDS-PAGE analysis, proteins in the gel were then transferred onto nitrocellulose (NC) membranes. NC membranes were incubated in primary antibody (anti-lactoferrin antibody) at a ratio of 1: 1000 in 1% skim milk solution overnight, then washed with PBST. After the membranes were incubated in secondary antibody labeled with alkaline phosphatase which has a ratio of 1: 2500 for 1 hour, then washed with PBST. The membranes were incubated with Western blue substrate solution in the dark until visible color bands or overnight, then washed with distilled water. Then the membranes were dried at room temperature.

The membranes were documented, furthermore, each image is analyzed to calculate the intensity of bands with Quantity One software. Data analysis was performed using one-way ANOVA. The correlation among the intensity of lactoferrin band and the severity of chronic periodontitis were assessed using Tukey HSD test.

Results

Figure 1 showed that protein bands were thicker with a higher color intensity than others. The increase of lactoferrin intensity was shown in Figure 2.

![Figure 1](image1.png)

**Figure 1.** Western blot result of lactoferrin; M = Marker; Co = kontrol; Mi = mild; Mo = moderate; Se = severe.

![Figure 2](image2.png)

**Figure 2.** The increase of lactoferrin intensity.

**Table 1.** Lactoferrin bands intensity of gingival crevicular fluid from each severity of chronic periodontitis

<table>
<thead>
<tr>
<th>No</th>
<th>Control (int/mm²)</th>
<th>Mild periodontitis (int/mm²)</th>
<th>Moderate periodontitis (int/mm²)</th>
<th>Severe periodontitis (int/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>12.22*</td>
<td>12.78</td>
<td>13.29</td>
<td>13.87</td>
</tr>
<tr>
<td>SD</td>
<td>0.25</td>
<td>0.17</td>
<td>0.2</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*Data presented as mean ± SD

**RESULTS**

The mean of the control group was higher than the group with mild periodontitis, moderate and severe, so it can be seen that there is an intensity increase at each level of periodontitis severity (Table 1).
Lactoferrin binds to iron to prevent the progression of fenton reaction, so the increased levels of GCF lactoferrin in periodontitis may act as an antimicrobial agent and antioxidant preventive.12

Gingival crevicular fluid was taken from male patients with chronic periodontitis, do not have a systemic disorder and nonsmokers. The determination of these criteria due to hormonal influences. Sex differences have been reported to affect the body’s response to bacteria.13 Smoking is also associated with the etiology and pathogenesis of periodontal disease, as well as to the results of periodontal treatment.14 Smoking also has a detrimental effect on the survival and function of PMN in GCF in populations with healthy periodontal.15

In this research, lactoferrin in gingival crevicular fluid was characterized using Western blot method. Previous research has shown a positive relationship between the titers of lactoferrin periodontitis in GCF. According to Kivadasannavar et al.,16 a statistically significant difference exists between the LF levels before and after scaling and root planning and periodontal flap surgery. A correlation has been found between the lactoferrin levels in GCF and the treatment modalities carried out. That result is in accordance with the investigation done by Jentsch et al.,6 who conclude that there is a decrease in lactoferrin found in GCF of periodontitis patients during periodontal therapy. Another study conducted by Glimvall et al.,7 concluded that higher levels of lactoferrin were detected in subjects with chronic periodontitis and correlated with probing pocket depth ≥ 6 mm. Although some data have been raised regarding the titers of lactoferrin in GCF of periodontitis patients, but this study is the first to reveal the relationship between the intensity of the band lactoferrin in the GCF of patients with periodontitis severity.

The band is formed because there is a specific reaction between antigen with antibody. Primary antibodies used in this study is a monoclonal antibody lactoferrin, so that antigen-specific binding protein lactoferrin. The specific proteins can be used for serological diagnosis by conducting further tests to obtain high specificity and sensitivity.18

The calculations using Quantity One show that there is an intensity increase of lactoferrin bands in accordance with the increase in periodontitis severity. In the control group appeared lactoferrin band with low intensity. This indicates that the gingival crevicular fluid of healthy periodontal tissues also contained lactoferrin in low concentrations, as in the human oral cavity also contained a constant amount of bacteria in a controlled. Continuous influx of PMN into the gingival pockets surrounding periodontal tissues play a major role in the control,10 whereas in the group with periodontitis, lactoferrin band intensity thicken with
increasing severity of periodontitis. This is in accordance with the opinion of Moosani which states that the PMN was found in the pocket of clinically healthy gingival tissue, and showed an increase in the number of chronic inflammation.9

Anova showed a significant difference in lactoferrin band intensity. According Kusnooto, pure isolates and a good levels of protein homogenates will produce a good and clear protein bands. There is a dominant protein bands with bold colors and there is expressed by the form of a thin bands. This is due to the good level of purity and protein concentration were sufficient. This means that there are differences in the concentration of lactoferrin in any degree of periodontitis severity.20

Tukey test results indicate that there are significant differences between each group of samples. Severity increase of chronic periodontitis followed by an increase in the intensity of lactoferrin bands. The Increased of band intensity indicates an increase in the concentration of lactoferrin that contained in gingival crevicular fluid of each sample. This illustrates that the increase in the chronic periodontitis severity affects the concentration of lactoferrin in gingival crevicular fluid. These results are in accordance with the opinion of Adonogianaki et al.14 and Ozdemir et al.19 that there are different levels of lactoferrin in GCF significantly between patients with gingivitis and periodontitis compared with healthy subjects. Tsai et al.8 also revealed that there is a strong relationship between periodontal clinical parameters with levels of lactoferrin. Increased severity of periodontal inflammation followed by an increase in GCF lactoferrin. Similar results have also been reported that myeloperoxidase derived from PMN associated with GCF volume, in addition to the β-glucorodinase also has a relationship with periodontal pocket depth. It may reflect a decrease in neutrophil chemotactic factor in the pocket due to the elimination of plaque and periodontal inflammation.14

GCF production is the result of an increase in microvascular permeability due to periodontal tissue inflammatory reaction. Lactoferrin that is released from PMN into the GCF could be a useful marker of periodontal inflammation. Therefore lactoferrin levels in GCF is a sensitive and objective method for detecting the severity of periodontitis. The results of this study showed that the intensity of the lactoferrin bands could be used as a biomarker of periodontitis. Further research needs to be directed towards the developing a chairside diagnostic kit to collect and evaluate the concentration of lactoferrin in the clinic.

REFERENCES
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