

Comparative Evaluation of Interleukin-35 Levels in Gingival Crevicular Fluid in Patients with Chronic Gingivitis and Chronic Periodontitis

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ABSTRACT

Background: Interleukin (IL)-35, a new member of the IL-12 superfamily, is known for its suppressive action. Due to this, it is classified as an anti-inflammatory cytokine. This study was carried out to evaluate and compare the levels of IL-35 in gingival crevicular fluid (GCF) in patients with chronic gingivitis and chronic periodontitis.

Materials and methods: Gingival crevicular fluid samples were obtained from chronic gingivitis patients (n = 15) and patients with chronic periodontitis (n = 15). Clinical measurements like probing pocket depth, bleeding on probing, papillary bleeding index, and modified plaque index were recorded. Enzyme-linked immunosorbent assay was used for the determination of GCF IL-35 levels in samples.

Results: Clinical parameters were significantly higher in the chronic periodontitis group than the chronic gingivitis group. The GCF IL-35 levels were significantly higher in the chronic gingivitis group than the chronic periodontitis group.

Conclusion: The IL-35 levels were higher in chronic gingivitis group as compared with chronic periodontitis group, indicating that the levels of IL-35 decrease with increase in the inflammatory status, so it might play a role in suppressing gingival inflammation and maintaining periodontal health.

Keywords: Enzyme-linked immunosorbent assay, Gingival crevicular fluid, Gingivitis, Interleukin-35, Periodontitis.

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INTRODUCTION

Chronic periodontitis is an inflammatory disease playing a role in the breakdown of alveolar bone, periodontal

ligament, and cementum, several changes in the immune response, like infiltration of the tissues by neutrophils, macrophages, B cells, and T cells, and the generation of high concentrations in several cytokines and other biochemical mediators. Cytokines are basically soluble proteins that bind to specific receptors on target cells and thus lead to either trigger or inhibition of the intracellular signaling cascades. Inflammatory response is regulated by proinflammatory and anti-inflammatory cytokines which are released by regulatory T cells.¹ Interleukin (IL)-12 superfamily comprises several members which include IL-12, IL-23, IL-27, and IL-35.² Out of these ILs, IL-35 has suppressive effect and it acts as inhibitory cytokine generated by Treg cell populations.³ The IL-35 inhibits proliferation of T cells. Inhibitory action of IL-35 is because of arresting G1 phase of mitosis.⁴ The IL-35 has a potential to activate nTreg cells especially at high inflammation sites.⁵ Nakijima et al⁶ have stated that patients with chronic periodontitis exhibited increased frequency of T lymphocytes and CD4 CD25 T cells in the inflammatory infiltrate of gingival tissues and also exhibited the phenotypic markers of Tregs, such as Foxp3. The search for potential markers associated with the severity, as well as the susceptibility of periodontal disease, has recently been receiving considerable attention.

Few studies have been performed to evaluate the exact role of IL-35 in the etiology of periodontal disease. As of now only one study has compared the levels of IL-35 in plasma, saliva, and gingival crevicular fluid (GCF) in periodontal health and disease.⁷ In our previous study, we have compared the levels of IL-35 in periodontally healthy subjects and patients with chronic periodontitis.⁸ In the present study, the basic aim was to evaluate and compare IL-35 levels in patients with chronic gingivitis and chronic periodontitis.

MATERIALS AND METHODS

Study Design

A total of 30 systemically healthy individuals who are nonsmokers were selected for the present study.

Study Population

A total of 30 patients who reported to the outpatient department of Sharad Pawar Dental College and

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Hospital, Sawangi (M), Wardha, India, were divided into groups I and II. Group I – chronic gingivitis group: No attachment loss, probing pocket depth (PPD) <3 mm, and bleeding on probing (BOP) scores >20% teeth. Group II – chronic periodontitis group: At least four teeth in each jaw, with PPD >5 mm, clinical attachment level (CAL) >4 mm, more than or equal to 50% alveolar bone loss in at least two quadrants, and BOP >50%. Exclusion criteria for the study were (1) patients with systemic diseases that could affect the periodontium, (2) smokers, (3) pregnant patients, (4) patients with history of periodontal treatment received for the past year, (5) individuals who have taken any anti-inflammatory or antibiotic drugs in last 6 months. An informed consent was signed by the patients and the study protocol was approved by the ethical committee of Datta Meghe Institute of Medical Science, Sawangi, Meghe, Wardha, India. All the information related to clinical data, personal data, and others was recorded in specially designed case history pro forma.

Gingival Crevicular Fluid Sampling

Gingival crevicular fluid samples were taken from two sites with papillary bleeding index (PBI) >2, PPD >3, and positive BOP in the chronic gingivitis group; from two sites (deepest pocket PD >5) with PBI >2 and positive BOP according to the baseline clinical measurements in the chronic periodontitis group. Maxillary sites were chosen to prevent saliva contamination. Gingival crevicular fluid samples were collected using calibrated capillary tube. Sampling areas were isolated properly. The GCF samples from sites of each individual were placed into one polypropylene tube and pooled before freezing at 70°C. Standard curve was taken as a reference to convert reading to actual volume. All GCF samples were stored at 80°C until laboratory analyses.

Clinical Measurements

The clinical evaluation of the patients was determined by PBI by Muhlemann,⁹ modified plaque index by Turskey et al,¹⁰ BOP, PPD, and CAL.

Measurement of IL-35 in GCF

The IL-35 levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit. Initially, GCF samples were diluted by 300 mL phosphate-buffered saline (PBS). After adding 100 µL of each, dilution plate was incubated for 2 hours at 37°C. Next, 100 mL each of detection reagent A and reagent B was added and incubated for 1 hour and 30 minutes at 37°C respectively, along with 90 mL substrate solution, and the plate was incubated for 15 to 25 minutes at 37°C. Plate was read in ELISA reader. The diluted absorbance values of IL-35 (pg/µL) in GCF were

multiplied by the dilution agent volume (300 mL PBS). The IL-35 concentrations in a unit volume (pg/µL) were then determined by multiplying the GCF IL-35 diluted absorbance values by 300 mL (to account for the dilution) and dividing by the GCF volume (µL).

Statistical Analysis

For all clinical parameters and IL-35 levels, mean and standard deviation were calculated. The following methods of statistical analysis have been used in this study: (1) To test the difference between the groups ANOVA (one-way analysis of variance) was used. F value is calculated and if F value is significant then it means that differences between the groups are significant. To find out which of the two groups mean is significantly different *post hoc*, Scheffe's test is used. In case F value is not significant, it indicates that there is no significant difference between the groups and stops the analysis at this stage and does not use least significant difference test. The formula used was:

$$F = \frac{\text{MS between groups}}{\text{MS within groups}}$$

where MS = Mean Sum of Square (2) Pearson's correlation coefficient test is widely used in the sciences as a measure of the strength of linear dependence between two variables; p-value less than 0.05 was taken as significant in all the above tests. Statistical Package for the Social Sciences was used for the analysis of data. In all the above tests, p-value less than 0.05 was taken to be statistically significant.

RESULTS

Clinical parameters in the study are shown in Table 1. Chronic periodontitis group showed significantly higher parameters statistically as compared with chronic gingivitis group (p < 0.001). Higher volume of GCF was obtained in chronic periodontitis group as compared with chronic gingivitis group. Gingival crevicular fluid levels of IL-35 are shown in Table 2. The GCF concentration of IL-35

Table 1: Mean Clinical Parameters of the study groups

Clinical Parameters	Chronic gingivitis	Chronic periodontitis
PPD	Full mouth	1.5 mm
	Sampled site	1.8 mm
CAL	Full mouth	0.0 mm
	Sampled site	0.0 mm
PBI	Full mouth	1.57
	Sampled site	1.79
PI	Full mouth	1.58
	Sampled site	1.72
BOP	50.3%	70.9%

Table 2: Mean Levels of IL-35 in study groups

Mean IL-35 levels	Chronic gingivitis group	Chronic periodontitis group
	36.28 pg/micro liter	19.93 pg/micro liter

was significantly higher in the chronic gingivitis group as compared with chronic periodontitis group ($p < 0.05$) (chronic periodontitis group = 19.93 pg/ μ L, chronic gingivitis group = 36.28 pg/ μ L).

DISCUSSION

The IL-35 is an anti-inflammatory cytokine and is a member of IL-12 superfamily. It arrests mitosis in G1 phase and inhibits proliferation of T cells; IL-35 also induces the development of iTreg35 cells, a subset of Treg cells.⁴ There are three other studies in the literature evaluating the relationship of IL-35 and chronic periodontitis. Kalburgi et al¹¹ evaluated the levels of IL-35 messenger ribonucleic acid (mRNA) in gingival tissues of healthy individuals and individuals with chronic periodontitis and aggressive periodontitis patients, showing higher levels of IL-35 mRNA in periodontitis group.

Mitani et al¹² measured the concentration of IL-35 in GCF and gingival tissue expression of EB13 and IL-12A (heterodimers of IL-35) in healthy individuals and individuals with CP. The gingival tissue expression of EB13 and IL-12A was significantly higher in the CP group than the healthy group. The findings of the present study are consistent with these results. Gingival crevicular fluid was used in this study because GCF has been the focus of most research in recent years. Gingival crevicular fluid is an exudate that flows into oral cavity from periodontal pockets. As a result of gingival inflammation, GCF is formed when fluid leaks from dilated blood vessels within the gingival connective tissue. As this fluid flows through the inflamed connective tissue, it picks up enzymes and other mediators involved in immune response. In this regard, it is an "inflammatory soup" containing subgingival bacteria and host cells. So, it offers a great potential as a source of factors that may be associated with disease activity. Koseglu evaluated the levels of IL-35 in plasma, saliva, and GCF in periodontally healthy individuals, gingivitis patients, and chronic periodontitis patients. In this study, mean GCF IL-35 level in healthy individuals was 63.19 pg/ μ L, 33.02 pg/ μ L in gingivitis patients, and 20.57 pg/ μ L in chronic periodontitis patients. The results in our study are comparable to the study by Koseglu.⁷ In this study, the mean GCF IL-35 levels in chronic gingivitis patients were 36.28 and 19.93 pg/ μ L in chronic periodontitis patients. Authors have published a similar kind of the study evaluating the IL-35 levels in periodontally healthy subjects and subjects with chronic gingivitis⁸ and the results are comparable with this study as well as the study by Koseglu, with level of IL-35 in periodontally healthy subjects (68.90 pg/ μ L) and chronic gingivitis patients (35.02 pg/ μ L). These findings lead to a consideration that the increased level

of IL-35 may be necessary to resolve the inflammation in periodontitis. Inflammatory bowel disease and rheumatoid arthritis are chronic inflammatory diseases, as is gingivitis. In a study conducted by Wirtz et al, mice with experimental inflammatory bowel disease were treated with recombinant IL-35. They found that it significantly reduced the development of experimental bowel disease. Their results show that IL-35 has an important role in the control of intestinal immune responses by regulating overwhelming T-cell activation. Moreover, recombinant IL-35 injections preserved mice from rheumatoid arthritis. The IL-35 could be an alternative treatment method for inflammatory diseases in the future.¹³ These correlations support the hypothesis that the level of IL-35 increases in inflammation sites. Higher IL-35 levels characterize healthy periodontal and gingival conditions, which demonstrate its anti-inflammatory action. However, IL-35 is not the only anti-inflammatory cytokine and there are other anti-inflammatory cytokines, such as IL-6, IL-4, and IL-16, although IL-35 is the new anti-inflammatory cytokine to be used as a predictor of future disease site. However, the results might alter due to changes in the concentration of other anti-inflammatory cytokines, which cannot be denied. This is one of the important limitations of this study.

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