Influence of Smoking on Interleukin-34 Levels in Gingival Crevicular Fluid and Plasma in Periodontal Health and Disease: A Clinico-biochemical Study

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Abstract

Interleukin-34 (IL-34), an alternative ligand for macrophage colony-stimulating factor receptor, plays an important role in osteoclastogenesis. The aim of this study was to analyze the effect of smoking on IL-34 levels in gingival crevicular fluid (GCF) and plasma in individuals with healthy periodontium and chronic generalized periodontitis (CP). A total of 60 individuals ranging in age from 25 to 55 years were enrolled in the study. The participants were divided into 4 groups: Group A, 30 samples (15 GCF and 15 plasma) obtained from 15 non-smokers with healthy periodontium; Group B, 30 samples (15 GCF and 15 plasma) from 15 smokers with healthy periodontium; Group C, 30 samples (15 GCF and 15 plasma) from 15 non-smokers with CP; and Group D, 30 samples (15 GCF and 15 plasma) from 15 smokers with CP. The Gingival Index and probing depth scores, together with the Clinical Attachment Level, were assessed in each group as clinical periodontal parameters. Levels of IL-34 in GCF and plasma were quantified using enzyme linked immunosorbent assay. The results showed that the mean IL-34 concentrations in GCF and plasma were highest in Group D, followed by Group C, Group B, and Group A, and the difference among them was statistically significant (p<0.05). The relatively elevated IL-34 levels observed here in smokers with CP suggest that this cytokine offers a potential inflammatory marker of periodontal disease in smokers.

Key words: Smoking — Inflammation — Periodontitis — Gingival crevicular fluid — Plasma

Introduction

Periodontitis is a dysbiotic inflammatory disease with an adverse influence on systemic health. It occurs as a result of the interaction of environmental, genetic, host, and microbial factors, and leads to the destruction of tooth-supporting tissues in susceptible subjects due to a shift in the balance of preventive and destructive immune mechanisms against microbial pathogens. Human periodontal ligament cells are exposed to various
periopathogenic factors, including inflammatory cytokines\textsuperscript{4,30,31}, which play a role in osteoclastogenesis through the expression of receptor activator of nuclear factor kappa B ligand (RANKL) on their cell surface\textsuperscript{15,20}.

In a model of bone cell formation, differentiation of osteoclasts was demonstrated to require interaction between receptor activator of nuclear factor kappa B (NF-$\kappa$B) which is expressed on the surface of osteoclasts, and RANKL in the presence of macrophage colony-stimulating factor (M-CSF/CSF-1)\textsuperscript{35}. The role of M-CSF has been demonstrated in osteopetrotic mutant mice, which suffer from congenital osteopetrosis due to deficiency of osteoclasts associated with an absence of M-CSF\textsuperscript{37}. This indicates that M-CSF is required for osteoclastogenesis, stimulating both adhesion and proliferation of osteoclast precursors\textsuperscript{3}.

A novel cytokine, designated interleukin-34 (IL-34), was identified by functional screening of a library of secreted proteins. It stimulates the viability of monocytes and macrophage colony formation from bone marrow cells. The receptor of IL-34 was discovered by screening extracellular domains of transmembrane proteins, and was found to be the already established M-CSF receptor (also called CSF-1 receptor, or c-fms). Interleukin-34 mRNA is expressed in various tissues, including heart, brain, lung, liver, kidney, spleen, thymus, testes, ovary, small intestine, prostate, and colon\textsuperscript{25}. Interleukin-34 plays a vital role in RANKL-induced osteoclastogenesis, as it can substitute for M-CSF, supporting osteoclast differentiation in the same way\textsuperscript{12}.

The effect of cigarette smoking on the pathogenesis of periodontal disease is well understood\textsuperscript{4,22,35}. Smoking can exert a negative impact on periodontal health by interfering with the immune system and altering host response in the presence of bacterial plaque\textsuperscript{2}. Earlier studies reported significantly increased levels of tumor necrosis factor $\alpha$ (TNF-$\alpha$), but not of IL-6 or IL-1$\beta$, in the gingival crevicular fluid (GCF) of current and former smokers with periodontal disease in comparison to in non-smokers\textsuperscript{7,9,10}. This indicates that smoking interferes with the inflammatory process by affecting the release of proinflammatory cytokines\textsuperscript{22}.

Interleukin-34 is also likely to play a role in inflammation, as it increases IL-6 and chemokine levels in human whole blood\textsuperscript{19}. It is also expressed in rheumatoid arthritis (RA) synovium, where it has shown a correlation with severity of synovitis, and is more highly expressed in the serum and synovial fluid of RA patients\textsuperscript{11}. It is secreted by synovial and gingival fibroblasts in response to TNF-$\alpha$ and IL-1$\beta$ through the NF-$\kappa$B and c-Jun N-terminal kinase pathways\textsuperscript{6,11}. Expression of IL-34 is also up-regulated in intestine in patients with inflammatory bowel disease (IBD)\textsuperscript{40} and in inflamed salivary glands in patients with Sjögren’s syndrome\textsuperscript{13}.

Understanding the association between IL-34 levels and susceptibility to and severity of periodontitis in smokers might reveal a hitherto unknown pathway of bone destruction in diseases such as periodontitis. To our knowledge, no studies to date have compared IL-34 levels in GCF and plasma between healthy and diseased periodontium to investigate their association with smoking. The purpose of the present study, therefore, was to clarify the role of IL-34 in the pathogenesis of periodontal disease and to study the effect of smoking on the IL-34 levels in periodontally diseased individuals.

### Materials and Methods

#### 1. Study population

The study population comprised 60 individuals aged between 25 and 55 years recruited from the outpatient section of the Department of Periodontology and Implantology at the Government Dental College and Research Institute, Bengaluru, India. The research protocol was submitted to Institutional Ethical Committee and Review Board of that institution and approval obtained (approval no: GDCRI/ACM(2)/PG/PhD/5/2016-2017). The study was conducted between April 2017 and September 2017 in accordance with the
Declaration of Helsinki 1975, as revised in 2013. The protocol of the study was explained and written informed consent obtained from all participants.

2. Selection criteria

Only individuals with a minimum of 20 natural teeth and the clinical signs required for each projected study group were enrolled in the study. The exclusion criteria were as follows: chronic inflammatory disease, such as RA and IBD; respiratory disease, such as chronic obstructive pulmonary disease, asthma, and bronchitis; immunodeficiency due to causes such as HIV; diabetes mellitus; pregnancy; giant cell tumors of the bone; coronary heart disease; hypertension; aggressive periodontitis; betel/areca nut chewing; alcoholism; steroid use; contraceptive use; anti-inflammatory drug or antibiotic use; and periodontal treatment within the preceding 6 months. Eligible participants were selected randomly and first categorized as having chronic generalized periodontitis (CP) or healthy periodontium based on their bleeding on probing, gingival index (GI), probing depth (PD), and Clinical Attachment Level (CAL) scores together with radiographic evidence of bone loss. They were then categorized into the following 4 groups: Group A, 30 samples (15 GCF and 15 plasma) from 15 non-smokers with healthy periodontium; Group B, 30 samples (15 GCF and 15 plasma) from 15 smokers with healthy periodontium; Group C, 30 samples (15 GCF and 15 plasma) from 15 non-smokers with CP; and Group D, 30 samples (15 GCF and 15 plasma) from 15 smokers with CP. Individuals with healthy periodontium had a GI score of 0, PD ≤3 mm, a CAL score of 0, and no radiographic evidence of bone loss. Chronic generalized periodontitis was defined as having a GI score of ≥1, a PD score of ≥5 mm, and CAL score of ≥3 mm together with radiographic evidence of bone loss at more than 30% of sites. The CAL was the distance in millimetres from the cemento-enamel junction to the bottom of the periodontal pocket. A participant was classified as a smoker if he or she smoked ≥100 cigarettes over their lifetime, or a non-smoker if the number was ≤100 cigarettes over their lifetime in accordance with the Centre of Disease Control and Prevention criteria.

3. Selection of site and collection of GCF fluid

One examiner was responsible for allocating participants to each group and selecting sites for sample collection (ARP). The clinical parameters (GI, PD, and CAL) were evaluated by a calibrated examiner (CNG) using a periodontal probe (UNC PCP-15, Hu-Friedy, Chicago, IL, USA). The same examiner also performed radiographic assessment and collected the GCF samples. Intra-examiner calibration was determined before the start of the study by examination of 30 sites twice at an interval of 24 hr. Calibration was accepted at the 95% level, if measurements at baseline and at 24 hr later were within 1 mm of each other. Gingival crevicular fluid was collected from the site with the maximum CAL in Group C and D, but from multiple sites in the periodontally healthy groups to ensure an adequate amount. The GCF was collected at the same time of the day (in the forenoon) to circumvent potential problems with the circadian variation usually seen in GCF volume. On the following day, the site for sample collection was well isolated and supragingival plaque removed while taking care not to make contact with the marginal gingiva. A standardized volume of 3 μl GCF was collected by placing a microcapillary pipette (Sigma-Aldrich, St. Louis, MO, USA) at the entrance of the gingival sulcus (extrasulcular method). Micro-pipettes contaminated with blood or saliva were excluded from the study. The collected GCF samples were transferred to airtight plastic vials and stored at −70°C until assayed.

4. Blood collection and plasma extraction

Two millilitres of blood was collected from the antecubital fossa by venepuncture using a 2-ml syringe and 20-gauge needle, and transferred immediately to EDTA-containing vials.
Table 1 Descriptive statistics of study population (mean ± SD)

| Study Group | Group-A (n = 15) | Group-B (n = 15) | Group-C (n = 15) | Group-D (n = 15) | t value | p-value |
|-------------|------------------|------------------|------------------|------------------|--------|--------|
| Age (in years) | 35.3 ± 7.825 | 35.3 ± 7.287 | 39.7 ± 7.995 | 39.4 ± 6.468 | 1.634 | 0.192 |
| PD (in mm) | 2.33 ± 0.724 | 2.33 ± 0.724 | 6.00 ± 1.134 | 6.20 ± 1.014 | 84.522 | <0.001* |
| GCF IL-34 (pg/ml) | 366.71 ± 36.13 | 392.25 ± 48.26 | 710.80 ± 162.08 | 826.44 ± 161.28 | 56.670 | <0.001* |
| Plasma IL-34 (pg/ml) | 76.79 ± 5.45 | 120.69 ± 29.75 | 397.52 ± 95.59 | 573.28 ± 107.57 | 153.463 | <0.001* |

*p<0.001 is significant

Table 2 Independent student t-test to compare mean values between Group C and Group D

| Study Group | Group C (n = 15) | Group D (n = 15) | t value | p-value |
|-------------|------------------|------------------|--------|--------|
| GI score | 2.167 ± 0.339 | 1.980 ± 0.332 | 2.318 | 0.139 |
| CAL in mm | 4.53 ± 0.915 | 5.13 ± 1.060 | 2.752 | 0.108 |

p<0.001 is significant

Plasma was separated from blood by centrifuging at 3,000 rpm for 5 min. The plasma was immediately transferred to a plastic vial and stored at −70°C until the time of assay.

5. IL-34 analysis

The GCF and plasma samples were assayed for IL-34 levels using a highly sensitive enzyme linked immunosorbent assay (ELISA) kit (human IL-34 Catalog Number: DY5265, R & D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. All samples were run in duplicate and the mean value used for the analysis.

6. Statistical analysis

The SPSS statistical software package (SPSS version 18.0, Chicago, IL, USA) was used for the statistical analysis. Based on the results of a pilot study including 5 participants in each group, it was estimated that a 15 participants would be needed in each group to achieve 90% power and detect a difference of 0.5 ± 0.687 between the hypotheses with a significance level (alpha) of 0.05 using a two-sided, two-sample t-test. The mean values of the demographic and clinical parameters were compared by using a one-way ANOVA. The GI and CAL values were compared between Group C and Group D by using an independent student t-test. A pair-wise comparison of IL-34 concentration in GCF and plasma between groups was performed using the Tukey test. The mean intra-examiner standard deviation of differences in repeated PD and CAL measurements was obtained using single passes of measurements (correlation coefficients between duplicate measurements; r = 0.95).

Results

Table 1 shows the descriptive statistics (mean ± SD) of the study population. A total of 60 participants were included in the study. The mean age in Group C (39.7 ± 7.995 years)
was higher than in the other groups. A comparison of the mean GI scores and CAL between Group C and Group D revealed no statistically significant difference (Table 2). The mean IL-34 concentrations in GCF (826.44 ± 161.28 pg/ml) and plasma (573.28 ± 107.57 pg/ml) were highest in Group D, and the difference was statistically significant, with a p-value of <0.001 (Table 1). Further multiple comparisons using the Tukey test were performed to determine which pair or pairs differed significantly. A statistically significant difference was observed in IL-34 concentrations in GCF and plasma in Group C and D (p<0.05), but not in Group A and B (Tables 3, 4). Spearman’s rho demonstrated a positive correlation between IL-34 concentration in GCF and plasma, but there was no statistically significant correlation between the clinical parameters and IL-34 concentrations in GCF and plasma (Table 5).

**Discussion**

Periodontitis is a chronic inflammatory oral disease of the adult population characterized by a gingival inflammatory reaction against pathogenic bacterial microflora, resulting in alveolar bone loss and eventually tooth loss. By interfering with the immune system and altering the host response in the presence of bacterial plaque, smoking might have a harmful impact on periodontal health. Increasing evidence points to smoking as a major risk factor for periodontitis, affecting the prevalence, extent, and severity of disease. The link between periodontitis and smoking may have relevant public health implications because both diseases are important risk factors for cardiovascular disease. To the best of our knowledge, this is the first study in any type of population to use ELISA to investigate the effect of smoking on IL-34 levels in GCF and plasma in individuals with healthy periodontium and CP.

Interleukin-34, an alternative ligand for the M-CSF receptor, is considered a novel non-canonical pathway of osteoclast formation, as it can substitute for M-CSF in osteoclast differentiation, and plays an important role in RANKL-induced osteoclastogenesis. Patients with inflammatory periodontal disease often have elevated serum levels of pro-inflammatory cytokines. Inflammation and osteoclastogenesis were triggered by pro-inflammatory cytokines and brought about alveolar bone resorption in periodontitis.

By a mechanism involving NF-κB and mitogen-activated protein kinase, the proinflammatory cytokines TNF-α and IL-1β regulate IL-34 expression in synovial and gingival fibroblasts. One study found that CSF-1 and IL-34 had complementary roles in periodontal disease, with IL-34 active in the steady state and CSF-1 in inflammation. Meanwhile, another study found that IL-34 mRNA expression in periapical lesions was significant.
significantly higher than that in normal periodontal ligament tissue, indicating that IL-34 is closely involved in inflammation in chronic apical periodontitis<sup>28</sup>. Thus, the increase in concentrations of IL-34 in GCF and plasma in both non-smokers and smokers with CP in the present study may be attributable to the proinflammatory properties of this protein. The precise effects of nicotine on cell cytokines and cytokine quantities in smokers have

| Group     | Correlation   | p-value | Group     | Correlation   | p-value | Group     | Correlation   | p-value |
|-----------|---------------|---------|-----------|---------------|---------|-----------|---------------|---------|
| A         | IL-34 GCF     | 0.263   | —         | 0.216        | —       | IL-34 Plasma | -0.109       | —       |
|           | p-value       | 0.344   | —         | 0.440        | —       | p-value     | 0.700        | —       |
| B         | IL-34 GCF     | 0.757   | —         | -0.174       | —       | IL-34 Plasma | -0.491       | —       |
|           | p-value       | 0.001*  | —         | 0.535        | —       | p-value     | 0.063        | —       |
| C         | IL-34 GCF     | 0.581   | -0.430    | -0.105       | 0.032   | IL-34 Plasma | -0.343       | -0.243  |
|           | p-value       | 0.023   | 0.110     | 0.709        | 0.911   | p-value     | 0.211        | 0.423   |
|           | GI            | Correlation | -0.008      | 0.086   |
|           | p-value       | 0.896   | 0.978     |
| PD        | Correlation   | 0.688   | —         | —         |
|           | p-value       | 0.005   |           |           |
| D         | IL-34 GCF     | 0.664   | 0.080     | 0.248      | 0.035   | IL-34 Plasma | 0.193       | 0.074   |
|           | p-value       | 0.007   | 0.778     | 0.374      | 0.901   | p-value     | 0.491        | 0.793   |
|           | GI            | Correlation | 0.204       | 0.272      |
|           | p-value       | 0.467   | 0.327     |
| PD        | Correlation   | 0.771   | —         | —         |
|           | p-value       | 0.001*  |           |           |

*p<0.001 is significant
yet to be fully clarified. Studies analyzing concentrations of nicotine and other chemicals or noxious stimuli related to smoking can enhance our understanding of the direct effects of nicotine on cytokine levels\(^22\). Gingival crevicular fluid provides one source of evidence in evaluating the multiple effects of smoking on host response in the periodontium. Earlier studies found elevated GCF levels of TNF-α\(^7\) and IL-8 in smokers\(^17\). The results of the present study revealed a significant increase in mean IL-34 levels in GCF (826.44 ± 161.28 pg/ml) and plasma (573.28 ± 107.57 pg/ml) in smokers with CP (Group D) (p<0.001) in comparison with in the other groups. This is in accordance with the results of earlier studies showing higher GCF levels of proinflammatory cytokines IL-1β and IL-6 or TNF-α in smokers\(^9,17,39\). One study, however, found a decrease in several proinflammatory cytokines and chemokines together with certain regulators of T-cells and natural killer cells in GCF in smokers\(^36\).

In the present study, microcapillary pipettes were used to collect GCF to avoid nonspecific attachment of the analyte, something which can occur with filter paper fibers, resulting in a false decrease in detectable IL-34 levels that, in turn, can lead to miscalculation of the correlation of IL-34 levels with severity of disease. The disadvantage of the method used in the present study is the possibility of trauma to the marginal gingiva, but utmost care was taken to avoid this during GCF collection here\(^32\).

The present findings indicate that smoking and periodontitis can, independently or jointly, alter GCF and plasma IL-34 levels. The concentration of IL-34 observed here was higher in GCF than in plasma, which could be explained by local production of IL-34 in diseased periodontal tissues, suggesting that IL-34 levels might serve as a marker of local disease activity. Interleukin-34 levels in GCF and plasma showed an increase in smokers with healthy periodontium and in those with CP compared with in non-smokers with healthy periodontium and those with CP, indicating that smoking upregulates secretion of IL-34 and may play an important role in the progression of periodontal disease.

To our knowledge, this cross-sectional study is the first to evaluate GCF and plasma levels of IL-34 in smokers and non-smokers with or without periodontal disease and to investigate potential correlations between severity of disease and GCF and plasma levels of IL-34 in an Indian population. Further longitudinal, prospective, interventional, multicentre studies are required to clarify the role of IL-34 and other potential markers of inflammation in CP and assess smoking as an important risk factor in the progression of periodontal disease.

**Conclusion**

Within the limitations of this study, the present results suggest that IL-34 offers a potential inflammatory marker of periodontal disease. The levels of IL-34 were highest in smokers with CP, which may indicate that this cytokine is active in the inflammatory process, both systemically and locally, in periodontal tissues. Determining IL-34 levels may therefore be valuable in detecting individuals at high risk of periodontitis, particularly in smokers. This has relevant public health implications, as both smoking and periodontitis are important risk factors for cardiovascular disease. Further multicentre, longitudinal, interventional, prospective studies of IL-34 and other markers are needed to clarify their role in the pathogenesis of periodontal disease in smokers.

**Conflict of Interest**

The authors declare no conflict of interest. They declare no financial support or relationships that may pose a conflict of interest.
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