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Salivary Cytokine Levels in Chronic Periodontitis and Periodontally Healthy Subjects. A cross-sectional Study

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Abstract

Background and Objective—Saliva has been proposed as a non-invasive diagnostic fluid that could be used in the diagnosis of oral and systemic diseases. The levels of salivary biomarkers such as cytokines could potentially be used as a surrogate to distinguish periodontally healthy from periodontitis subjects. Therefore, the goal of the present investigation was to determine if the levels of 10 cytokines in saliva would differ between a group of periodontally healthy and periodontitis subjects. Correlations between the concentration of these 10 cytokines and clinical parameters of periodontal disease were also examined.

Material and Methods—In this cross-sectional study, 74 chronic periodontitis and 44 periodontally healthy individuals were periodontally examined and had the levels of GM-CSF, IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN- γ and TNF- α measured in whole saliva using a multiplexed bead immunoassay (Luminex). Significance of statistical differences in the levels of salivary cytokines between groups was determined using non-parametric ANCOVA adjusting for age and smoking status. The Spearman rank correlation coefficient was used to explore associations between mean levels of salivary cytokines and mean clinical parameters.

Results—There were no statistically significant differences between groups for any of the cytokines. There were weak statistically significant positive associations between salivary IL-8 and PD ($r_s=0.2$, $p<0.05$) and BOP ($r_s=0.2$, $p<0.05$) and weak negative correlations between salivary IL-10 and AL ($r_s=-0.2$, $p<0.05$) and BOP ($r_s=-0.3$, $p<0.001$).

Conclusion—Mean salivary levels of GM-CSF, IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN- γ and TNF- α could not discriminate between periodontal health and disease.

Keywords

saliva; cytokines; diagnosis; chronic periodontitis; periodontal health

Introduction

Several publications have recently addressed the potential diagnostic properties of saliva. It has been proposed that saliva could not only be used to help diagnose oral diseases, but as “the body’s mirror”, it would also have application in the diagnosis of systemic conditions (1,2). Currently, the clinical diagnosis of periodontal diseases involves primarily the assessment of clinical measures of tissue destruction and signs of tissue inflammation. Although clinicians and researchers have successfully relied on these parameters to determine the periodontal status

of patients and research subjects, the time and expertise required for a full periodontal examination pose severe limitations to epidemiological surveys. Investigations designed to uncover risk factors and risk indicators of periodontal infections would also benefit from a faster screening of subjects, which could facilitate a larger sample size. The same issues are valid for studies of associations between systemic diseases and periodontal infections. Saliva could be used as a non-invasive diagnostic fluid to measure biomarkers released during disease initiation and progression (3–5). Characterization of specific salivary biomarkers associated with periodontal disease presence and severity could have a major impact in the diagnosis and monitoring of periodontal diseases.

Due to their role in the immunopathology of periodontal infections, different cytokines have been measured in gingival crevicular fluid (GCF) and their levels related to the disease status of sites and subjects. For the most part, these studies have found an increase in levels of several GCF cytokines associated with a worsening in clinical parameters of periodontal disease (6–9). In addition, treatment and improvement of the periodontal condition has been associated with decreases in the GCF levels of certain cytokines (10–12). These findings suggest that cytokines could be putative biomarkers of periodontal disease initiation and progression.

Several lines of evidence suggest that the primary source of cytokines in whole saliva is GCF. Ruhl *et al.* (13) measured the levels of Interleukin (IL)-1 α , IL-6, IL-8, epidermal growth factor (EGF), nerve growth factor (NGF) and albumin in parotid, sub-mandibular/sub-lingual and whole saliva. They found that IL-1 α , IL-6 and IL-8 were present in whole saliva at concentrations significantly higher than in major salivary gland secretions. The authors concluded that the inflammatory cytokines detected in whole saliva did not come from the secretions of major salivary glands, and proposed that GCF was the likely source of these cytokines. In another study, transforming growth factor (TGF)- β , IL-1 α and tumor necrosis factor (TNF)- α were statistically significantly higher in whole saliva compared to parotid saliva (14). Although not statistically significant, IL-8 and IL-6 also displayed a trend towards higher levels in whole saliva (14). Therefore, it is possible that whole saliva contains GCF from all periodontal sites providing an assessment of periodontal disease status. For instance, GCF biomarkers that were positively associated with increased attachment loss such as beta-glucuronidase (15) were further examined by the same group of investigators in saliva. They found that the salivary levels of beta-glucuronidase also had a strong positive correlation with clinical parameters of periodontal disease (16).

Despite the recognized diagnostic potential of saliva, only a few reports have attempted to correlate the levels of cytokines in saliva with the periodontal condition of the subjects. Miller *et al.* (17) conducted a study to determine if salivary biomarkers specific for three aspects of periodontitis: inflammation, collagen degradation and bone turnover, correlated with clinical features of periodontal disease. They examined the relationship between clinical parameters of periodontal disease and the levels of IL-1 β , matrix metalloproteinase (MMP)-8, and osteoprotegerin (OPG) in whole saliva. They reported that the mean levels of IL-1 β and MMP-8 in saliva were significantly higher in periodontitis subjects than in periodontally healthy controls. MMP-8 and IL-1 β correlated with periodontal indices, whereas, after adjustment for confounders, OPG did not. Combined elevated salivary levels of MMP-8 and IL-1 β increased the risk of experiencing periodontal disease 45-fold.

Ng *et al.* (18) in a cross-sectional study evaluated the association between radiographic evidence of alveolar bone loss and the concentration of host-derived bone resorptive factors [IL-1 β , TNF- α , IL-6 and prostaglandin E₂ (PGE₂)], as well as markers of bone turnover [pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP), osteocalcin and osteonectin] in stimulated whole saliva collected from 110 untreated dental patients. Variables positively associated with increased bone loss score were: age, current smoking, use

of bisphosphonate drugs, and salivary IL-1 β levels above the median. Increased levels of salivary osteonectin were associated with less bone loss.

Therefore, the goal of the present investigation was to perform a cross-sectional study to compare the levels of granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, interferon (IFN)- γ and TNF- α in samples of whole saliva from periodontally healthy and chronic periodontitis subjects. In addition, we examined correlations between the concentration of these 10 cytokines and clinical parameters of periodontal disease.

Material and Methods

Subject population and study design

Seventy four chronic periodontitis and 44 periodontally healthy subjects were recruited for this cross-sectional study at The Forsyth Institute. The periodontally healthy subjects were 18 to 65 years of age, with at least 20 natural teeth and no pocket depth or attachment level measurements > 3 mm. The periodontal disease subjects were 18 to 65 years of age with at least 20 natural teeth, and $\geq$ 8 sites with pocket depths > 4 mm and attachment level > 3 mm. Exclusion criteria included: presence of orthodontic appliances; abnormal salivary function; use of prescription drugs; use of antibiotics in the month prior to the study; use of any over the counter medications other than analgesics; diseases of the soft or hard oral tissues and systemic conditions which might place subjects in a high-risk category or could influence the course of periodontal diseases and periodontal therapy within 6 months prior to the baseline examination. Only subjects who were ambulatory and volunteered to come to Forsyth were included. The Institutional Review Board at The Forsyth Institute approved the study protocols including the recording of clinical measurements and collection of saliva samples. All subjects signed informed consent prior to entry into the study.

Clinical exam

Clinical measurements were taken at 6 sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual) at all teeth excluding third molars (a maximum of 168 sites per subject) as previously described (19). The clinical parameters measured and the order of measurement were as follows: 1) Presence of plaque (0 or 1); 2) Pocket Depth (PD [mm]); 3) Attachment level (AL [mm]); 4) Bleeding on probing (BOP [0 or 1]) and 5) Suppuration (0 or 1). Probing pocket depth and attachment level measurements were made to the nearest mm using a North Carolina periodontal probe. Pocket depth and attachment levels were measured twice by the same examiner and the average of the pair of measurements was used for analysis. Saliva samples for cytokine assessment were taken prior to the clinical measurements. All clinical data were recorded on data sheets and then entered into a computer using a prompted data entry program.

Saliva samples

Subjects refrained from brushing for 12 h and from drinking, eating or chewing gum for 1½ h prior to sample collection. Subjects expectorated 1–2 ml of accumulated whole saliva into a container. Saliva samples were cleared by centrifugation at 10,000 RPM for 10 min to pellet bacteria and the supernatant was kept at –80°C until assay.

Quantification of Cytokines Using Luminex

Cytokine levels were determined using a multiplexed bead immunoassay. Prior to assay, further processing of the saliva samples involved adsorption onto cellulose (Whatman filter paper) followed by elution by centrifugation at 10,000 RPM for 10 min, through a 0.22 μ m filter using

Spin-X Centrifuge Tube Filters (Costar®). This was required to reduce mucin content and avoid clogging of the Luminex 100™ machine. Ten cytokines: GM-CSF, IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN- γ and TNF- α were measured using the human ultrasensitive cytokine ten-plex antibody bead kit (BioSource International, Camarillo, CA). The assays were performed in 96-well filter plates as previously described (20). Briefly, the filter plate was pre-wetted with washing buffer and the solution was aspirated from the wells using a vacuum manifold (Millipore Corporation, Billerica, MA). Microsphere beads coated with monoclonal antibodies against the 10 different target analytes were added to the wells. Samples and standards were pipetted into the wells and incubated for 2 h with the beads. The wells were washed using a vacuum manifold (Millipore Corporation, Billerica, MA) and biotinylated secondary antibodies were added. After incubation for 1 h, beads were washed followed by an incubation of 30 min with streptavidin conjugated to the fluorescent protein, R-phycoerythrin (streptavidin-RPE). After washing to remove the unbound streptavidin-RPE, the beads (minimum of 100 per analyte) were analyzed in the Luminex 100™ instrument (MiraiBio, Alameda, CA). The Luminex 100™ monitors the spectral properties of the beads to distinguish the different analytes, while simultaneously measuring the amount of fluorescence associated with R-phycoerythrin, reported as median fluorescence intensity. The concentrations of the unknown samples (antigens in GCF samples) were estimated from the standard curve using a third order polynomial equation and expressed as pg/ml after adjusting for the dilution factor. Samples below the detection limit of the assay were recorded as zero, while samples above the upper limit of quantification (ULQ) of the standard curves were assigned the highest value of the curve.

Data Analysis

The outcome variables evaluated in this study were the mean clinical parameters and mean levels of 10 salivary cytokines for each subject. Values for each clinical parameter were averaged within a subject and then averaged across individuals in the periodontitis and periodontally healthy groups separately. Values for the cytokines were averaged across subjects in each clinical group. The differences in mean clinical parameters between periodontally healthy and periodontitis subjects were determined using the unpaired student t-test, while differences in the distribution of males and smokers were examined using the Chi-square test. Significance of statistical differences in the levels of salivary cytokines between groups was initially examined using the Mann-Whitney test but ultimately determined using non-parametric analysis of covariance (ANCOVA) adjusting for age and smoking status. The Spearman rank correlation coefficient was used to explore associations between levels of salivary cytokines and clinical parameters of disease such as mean pocket depth, mean attachment level and mean prevalence of bleeding sites. Correlation coefficients were calculated using the entire data set for periodontally healthy and periodontitis subjects.

Results

Table 1 summarizes the mean demographic and clinical parameters for the two clinical groups. All clinical parameters of periodontal disease were statistically significantly higher in the chronic periodontitis subjects. In addition, there were statistically significant differences in age, % of males and % of smokers, which were all higher in the periodontitis group. Further characterization of the level of disease in the periodontitis population revealed that 25% of the subjects presented a minimum of 18% of the sites with PD > 4 mm and CAL > 3 mm. Since age is a risk indicator and smoking is a risk factor for periodontal disease, analysis of covariance (ANCOVA) adjusting for these variables was performed. The results (data not shown) remained statistically significant for mean PD, % of sites with BOP and % of sites with plaque, confirming the difference in level of periodontal disease between the groups.

The results from the multiplexed bead immunoassays revealed that the levels of most cytokines were within the range of quantification of the assay. For GM-CSF, IL-1 β , IL-4, IL-6 and TNF- α , over 90% of the samples were within the dynamic range of the standard curves. In the case of IFN- γ , 83% of the samples were quantifiable, while for IL-2, IL-5, IL-8 and IL-10 approximately 50% of the samples were in the dynamic range of the assay. IL-10 had the highest percentage of negative samples: 33%, while IL-8 had the highest percentage of values above the ULQ: 34%

The mean levels of the 10 biomarkers were initially compared between periodontitis and periodontally healthy subjects using the Mann-Whitney test. The results showed a statistically significant higher level of mean salivary IL-10 ($p < 0.01$) and IL-5 in the healthy group ($p < 0.05$). However, the non-parametric ANCOVA adjusting for age and smoking revealed no statistically significant differences between groups for any of the cytokines (Fig. 1 and Fig 2). IL-8 was the most abundant cytokine in saliva with mean levels in pg/ml ($\pm$ SEM) of 1945 ± 181 and 2268 ± 111 , followed by IL-1 β : 633 ± 91 and 673 ± 69 for periodontally healthy and periodontitis subjects, respectively (Fig. 1). All other cytokines were present at levels ranging from 39 to 327 pg/ml (Fig. 2). The Spearman correlation coefficient revealed weak but statistically significant positive associations between the mean salivary levels of IL-8 and the mean PD ($r_s = 0.2$, $p < 0.05$) and the mean % of sites with BOP ($r_s = 0.2$, $p < 0.05$). Weak negative correlations were found between the mean salivary levels of IL-10 and the mean AL ($r_s = -0.2$, $p < 0.05$) and mean % of sites with BOP ($r_s = -0.3$, $p < 0.001$).

Discussion

The results of our study did not demonstrate a significant difference between chronic periodontitis and periodontally healthy subjects in the levels of any of the 10 cytokines tested. In addition, there were only weak statistically significant associations among mean clinical parameters of periodontal disease and mean salivary levels of IL-8 and IL-10. These results cast doubt upon the use of salivary cytokines as biomarkers of periodontal disease. The present results are in contrast to reports by other investigators that have described elevated levels of salivary biomarkers such as IL-1 β and MMP-8 in subjects with periodontitis compared to periodontally healthy subjects (17,18). In one study, the levels of IL-1 β and MMP-8, measured using ELISA, were statistically significantly higher in moderate to severe periodontitis subjects ($n = 28$) than in control subjects ($n = 29$) and levels of both analytes correlated with clinical parameters of periodontal disease such as bleeding on probing and percentage of sites with PD ≥ 4 mm (17). The reported mean levels ($\pm$ SD) in pg/ml of IL-1 β were 213 ± 167 and 753 ± 1022 for periodontally healthy and periodontitis subjects, respectively. In the present study, the corresponding values were 633 ± 602 and 673 ± 590 for periodontally healthy and chronic periodontitis subjects, respectively. A possible explanation for the discrepancy between these results might reside in the level of disease of the two study populations. Miller's periodontitis subjects presented with more severe periodontal disease, exemplified by their higher mean % of sites with PD ($\pm$ SD) ≥ 4 mm of 45.2 ± 21.9 compared to 27.6 ± 18.2 in our population. In addition, their mean % of sites with BOP (mean $\pm$ SD) was also higher than ours: 45.9 ± 15.6 vs. 28.4 ± 15.3 .

Another recent report described a positive association between the mean levels of salivary IL-1 β and radiographic signs of bone loss in a population of 110 "untreated dental patients" (18). Since the authors did not record traditional clinical parameters of periodontal disease, it is difficult to comment on potential differences in the level of disease between their study population and the individuals reported here. However, the authors highlighted in their discussion that several subjects having high salivary levels of IL-1 β showed no signs of bone loss. Eight subjects in our periodontally healthy group also displayed very high levels of IL-1 β . Given these observations, it is very unlikely that salivary IL-1 β could be used as a

discriminatory biomarker to distinguish periodontally healthy and diseased subjects. In addition, differences in methods of saliva collection (stimulated or unstimulated), processing (speed and time of centrifugation), storage (time, temperature and addition or not of protease inhibitors) and in the methodology used for the quantification of the biomarkers (ELISA vs. Luminex) might also have impacted the difference in results.

Part of the rationale for investigating cytokines present in saliva is based on the concept that these mediators find their way into whole saliva through GCF. Since GCF levels of cytokines are elevated in chronic periodontitis sites compared to periodontally healthy sites (6–9), it was hypothesized that they would also be elevated in whole saliva of periodontitis individuals. However, cytokines are also known to be produced by human buccal and gingival epithelial cells, which could provide an additional or alternative source of these mediators in whole saliva (21). Further, there is evidence that the secretion of cytokines by oral epithelial cells increases with an increase in periodontal chronic inflammation and infection with periodontal pathogens (21–23), suggesting that secretion of cytokines by oral epithelia might enhance differences in the levels of whole saliva cytokines between chronic periodontitis and periodontally healthy subjects.

The lack of association between levels of salivary biomarkers and periodontal disease status reported here could be explained, in part, by the extensive dilution of the gingival crevicular fluid containing these cytokines in saliva. The amount of gingival crevicular fluid produced per site per hour has been estimated to be 3 μ l/h for healthy sites, 20 μ l/h for intermediate pockets and 44 μ l/h for deep pockets (24). Considering the turnover of the salivary compartment to be 20 ml/h, this would result in dilution factors of 1:6,666, 1:1,000 and 1:455 per site, depending on the periodontal condition of the site. The final dilution provided by saliva is difficult to estimate since it would depend on the distribution of healthy, intermediate and deep sites in a subject. However, the mean percentage of sites greater than 4 mm in our population was 14% (mean of 20 sites per subject), and the percentage of pockets greater than 6 mm was only 2.7% (mean of 3.8 sites per subject), suggesting a limited contribution of GCF from pathologically deepened sites to the composition of whole saliva for most patients.

Another possible confounder in the interpretation of data based on levels of cytokines in saliva is the presence of putative inhibitors. The inhibitory effects of whole and parotid saliva on the levels of several cytokines were investigated by Wozniak *et al.* (14), who concluded that most cytokines tested were statistically significantly reduced in concentration in whole saliva compared to parotid saliva. Only the concentration of IL-1 α was not affected by the addition of saliva. The authors suggested that sequestration of cytokines by mucin-like proteins or other large molecules and enzymatic degradation could be potential inhibitory mechanisms present in saliva. The higher level of inhibition demonstrated for whole compared to parotid saliva also suggests that whatever mechanisms are operating, they are either present at higher levels or more efficient in whole saliva.

Similar results were reported by Ng *et al.* (18). *In vitro* testing of the inhibitory effects of whole saliva using a similar methodology to Wozniak *et al.* (14), revealed a 75% reduction in salivary cytokine levels detected using Luminex, compared to samples diluted in buffer. They also reported that the addition of protease inhibitors did not reverse the reduction in levels of cytokines resulting from addition of whole saliva. They suggested that the reduction in cytokine detection was a consequence of sequestration by large salivary proteins such as mucins. Also in agreement with Wozniak *et al.* (14), whole saliva had a greater inhibitory effect than parotid saliva on the detectable levels of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α .

In the present study we did not explore the presence of inhibitory mechanisms in saliva; however, it is fair to assume that they were also in place in our samples. Although inhibitory

mechanisms in saliva might have resulted in an underestimation of the salivary levels of the cytokines tested, unless these mechanisms are enhanced by the periodontal disease process, they should have affected both periodontally healthy and periodontitis subjects to the same extent. Therefore, the influence of such inhibitory mechanisms in our results might have been limited.

In summary, we could not find an association between levels of salivary cytokines and clinical parameters of periodontal disease. The dilution of GCF components in saliva seems to mask existing differences in the levels of these biomarkers at the site level. Other biomarkers associated with the onset and development of periodontal diseases not examined here might still be present in saliva at levels that could be used to discriminate between periodontal health and disease. Further, other proteomic approaches could be used to explore as yet unidentifiable biomarkers of periodontal disease in saliva.

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Fig. 1.

Mean levels (pg/ml $\pm$ SEM) of IL-1 β and IL-8 in chronic periodontitis and periodontally healthy subjects. Whiskers indicate SEM. The levels of each cytokine were determined in each subject and then averaged across subjects in the 2 clinical groups separately. The significance of differences between the two groups was determined using non-parametric ANCOVA adjusting for age and prevalence of smokers. None of the differences were statistically significant.

Fig. 2. Mean levels (pg/ml $\pm$ SEM) of GM-CSF, IL-2, IL-4, IL-5, IL-6, IL-10, IFN- γ and TNF- α in chronic periodontitis and periodontally healthy subjects. Whiskers indicate SEM. The levels of each cytokine were determined in each subject and then averaged across subjects in the 2 clinical groups separately. The significance of differences between the two groups was determined using non-parametric ANCOVA adjusting for age and prevalence of smokers. None of the differences were statistically significant.

Table 1

Mean ($\pm$ SD) demographic and clinical parameters for periodontally healthy and periodontitis subjects. Significance of differences between groups was determined using the unpaired t-test unless otherwise indicated.

	Periodontitis	Health	p value
n	74	44	
Age (years)	50 $\pm$ 13	31 $\pm$ 8	p < 0.001
% males	65	57	p < 0.05*
% smokers	43	0	p < 0.001*
<i>% of sites with:</i>			
visible plaque	59 $\pm$ 22	13 $\pm$ 19	p < 0.001
bleeding on probing	28 $\pm$ 15	4 $\pm$ 8	p < 0.001
suppuration	1 $\pm$ 2	0	p < 0.01
PD < 4 mm	72 $\pm$ 15	100	p < 0.001
PD 4 to 6 mm	25 $\pm$ 13	0	p < 0.001
PD > 6 mm	3 $\pm$ 5	0	p < 0.01
mean PD (mm)	3.0 $\pm$ 0.6	2.2 $\pm$ 0.3	p < 0.001
mean AL (mm)	3.4 $\pm$ 1.0	2.1 $\pm$ 0.5	p < 0.001
no. missing teeth	3.5 $\pm$ 3.4	0.4 $\pm$ 1.1	p < 0.001

* Chi-square test