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Bone remodeling-associated salivary biomarker MIP-1α distinguishes periodontal disease from health

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Background and Objective: The field of salivary diagnostics lacks an accepted and validated biomarker of alveolar bone remodeling. To address this, we examined levels of salivary biomolecules specifically associated with biological aspects of bone remodeling in subjects with chronic periodontitis in a case–control study.

Material and Methods: Levels of macrophage inflammatory protein-1α (MIP-1α), osteoprotegerin, C-telopeptide pyridinoline cross-links of type I collagen and β-C-terminal type I collagen telopeptide in unstimulated whole saliva of 80 subjects (40 subjects with moderate to severe chronic periodontitis and 40 sex- and age-matched healthy control subjects) were measured using enzyme immunosorbent assays. Saliva was collected before clinical examination, which included probing depth, clinical attachment loss and bleeding on probing.

Results: The mean level of MIP-1α in subjects with periodontitis was 18-fold higher than in healthy subjects ($p < 0.0001$). Clinical periodontal indices correlated significantly with MIP-1α levels ($p < 0.0001$). Of the biomolecules examined, MIP-1α demonstrated the greatest ability to discriminate between periodontal disease and health as determined by the area under the curve (0.94) and classification and regression tree analysis (sensitivity 94% and specificity 92.7%). Osteoprotegerin levels were elevated 1.6-fold ($p = 0.055$), whereas C-telopeptide pyridinoline cross-links of type I collagen and β-C-terminal type I collagen telopeptide levels were below the level of detection in the majority of subjects.

Conclusion: These findings suggest that the chemokine MIP-1α may aid in identifying periodontitis. Future longitudinal studies are warranted to determine whether this biomarker can help in ascertaining the progression of bone loss in subjects with periodontal disease.

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Periodontal disease results from interaction between oral bacteria and the host inflammatory response. This interaction triggers a cascade of inflammatory events, which in turn promote connective tissue destruction and alveolar bone remodeling (1–7). These unique biological events contain signatures of the microbial ecology, as well as downstream events involving inflammation, attachment loss and bone destruction. It is likely that identification of the dominant signatures for each of these biological phases could provide insight into biomarkers of periodontal disease in oral fluids. To this end, several investigators have identified salivary

biomarkers of biological events associated with aspects of periodontal disease (8–12), and reviews on this topic are available (13–15). However, lacking to date is a focused panel of biomarkers that encompass the early and later biological phases that would yield, at least theoretically, the specificity for the development of a real-time, accurate, biologically based, periodontal disease diagnostic device.

Progress continues to be made towards the achievement of a salivary diagnostic device based on the knowledge that saliva is a rich pool of proteins and molecules that reflects aspects of oral health. In one recent example, we found that biomarkers of inflammation, connective tissue destruction and bone remodeling were present at elevated concentrations in the saliva of patients with periodontal disease (11). In that study, we observed that salivary levels of interleukin-1β and MMP-8 were significantly associated with clinical parameters of periodontal disease, and levels of interleukin-1β and MMP-9 elevated above a threshold (i.e. 2 SD above the mean of healthy controls), together demonstrated an odds ratio of 45 for periodontal disease. While these results demonstrated proof of principle that biomarkers from two distinct biological phases could aid in distinguishing periodontal disease from health, the identification of a biomarker associated with aspects of bone remodeling – a late biological event that could improve the accuracy of salivary diagnostics – remains elusive.

Bone resorption is mediated by osteoclasts that exhibit specific abilities to degrade organic and inorganic components of bone. Different mediators, such as interleukin-1β, prostaglandin E_2, tumor necrosis factor-α, macrophage inflammatory protein-1α (MIP-1α), interleukin-6, interleukin-11 and interleukin-17, act upstream as activators of osteoclastogenesis (16–22). Within the resorption lacunae, RANKL and osteoprotegerin (OPG) are important cytokines belonging to the tumor necrosis factor family, which regulate differentiation of osteoclast progenitor cells into active osteoclasts or inhibit the differentiation process, respectively (23,24). As a result, type I collagen is degraded during bone destruction by proteolytic enzymes such as MMPs and cathepsin K, which lead to release of cross-linked telopeptides into the circulation (serum, saliva and urine) as stable fragments, such as pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP; 25–27) and C-terminal type I collagen telopeptide (β-CTX; 28–31). We and others have investigated salivary levels of several of these important molecules associated with cytokine signaling and alveolar bone resorption (32–39); however, no conclusive information has yet been reported on the best biomarker associated with alveolar bone remodeling in adults. Also, there is a lack of knowledge about whether upstream pathways, midstream osteoclastogenic factors or downstream degradation products are better salivary biomarkers of periodontal disease.

The purpose of this study, therefore, was to test the hypothesis that a specific salivary biomarker associated with bone remodeling could be identified that would distinguish healthy subjects from those with periodontal disease. Proteins associated with the upstream, midstream and downstream processes of osteoclastogenesis (i.e. MIP-1α, OPG, β-CTX and ICTP) were selected for evaluation.

Material and methods

Participants

Eighty subjects were enrolled in this case–control, cross-sectional study. Participants were recruited from the general clinic population of the College of Dentistry, as well as the surrounding counties, by advertisement. Inclusion criteria included age over 18 years, good general health (excluding the case definition) and a minimum of 20 teeth. Participation in the control group ($n = 40$) required bleeding on probing in $< 10\%$ of sites, probing depth ≥ 5 mm in $< 2\%$ of sites, and clinical attachment loss > 2 mm in $< 1\%$ of sites. Subjects of the test group ($n = 40$) had the diagnosis of generalized moderate to severe chronic periodontitis, based on the criteria defined by the American Academy of Periodontology (40,41). Inclusion criteria of the test group were five sites in two quadrants with a minimum of two affected teeth in each quadrant. Each site had ≥ 5 mm probing depth, clinical attachment loss ≥ 3 mm, and bleeding on probing with score ≥ 2 (0 = one, 1 = pinpoint, 2 = interdental bleeding and 3 = spontaneous/heavy bleeding). Exclusion criteria were as follows: a history of alcoholism; liver, kidney or salivary gland dysfunction; infectious diseases; inflammatory bowel disease; rheumatoid arthritis; granulomatous diseases; diabetes; or undergoing or having undergone organ transplant or cancer therapy. In addition, pregnancy or lactation, use of glucocorticoids, cyclo-oxygenase inhibitors, bisphosphonates, antibiotics or immunosuppressant medication within the last 6 mo, need for antibiotics for infective endocarditis prophylaxis during dental procedures, symptoms of acute illness (i.e. fever, sore throat, body aches or diarrhea), orthodontic appliances or presence of an oral mucosal inflammatory condition (e.g. aphthous, lichen planus, leukoplakia or oral cancer) were also exclusion criteria. The study was performed at the University of Kentucky between August 2005 and August 2009 and was approved by the University Institutional Review Board. All subjects understood the study, provided written informed consent and received incentives (i.e. monetary compensation and a clinical examination) as part of the study protocol.

Clinical evaluation

Complete medical and dental histories were obtained from the patient's records and confirmed by interview. All subjects received comprehensive oral and periodontal examinations that included bleeding on probing, probing depth and clinical attachment loss, assessed as previously described (42). Briefly, probing depths were measured at six locations per tooth (mesial buccal, mid-buccal, distal buccal, mesial lingual, mid-lingual and distal lingual) using a 15 UNC probe. After the

measurement of probing depths, all sites were observed for bleeding on probing. The degree of bleeding was estimated and recorded (0 = no bleeding, 1 = light bleeding, 2 = moderate bleeding and 3 = heavy bleeding) for each probed site. Clinical attachment levels were measured at all six locations per tooth. Three periodontists, blinded to the participant group assignments, performed the clinical evaluations.

Saliva collection

Unstimulated whole expectorated saliva (5 mL) was collected from each subject between 09.00 and 11.00 h, according to a modification of the method described by Navazesh (43). Subjects were asked to avoid oral hygiene measures (i.e. flossing, brushing and mouth rinses), eating, drinking or gum chewing for 1 h before collection. Subjects rinsed their mouth with tap water, then expectorated whole saliva into sterile tubes containing a protease inhibitor solution (SIGMA-FAST; Sigma, St Louis, MO, USA) while seated in an upright position. Collected samples were placed immediately on ice and aliquoted prior to freezing at −80°C. Samples were thawed and analysed within 6 mo of collection. Samples from all participants were collected prior to the periodontal evaluation.

Biomarker analysis

Concentrations of β-CTP, ICTP, OPG and MIP-1α in saliva were determined in duplicate for each subject using enzyme immunosorbent assays (EIA) kits, according to the manufacturer's directions, by technologists in the Clinical Laboratory Improvements Amendments (CLIA)-certified General Clinical Research Core laboratory at the University of Kentucky Medical Center. The β-CTX Urine Crosslaps ELISA and ICTP serum Crosslaps ELISA (Nordic Bioscience Diagnostics A/S, Herlev, Denmark), OPG (Osteoprotegerin EIA kit; ALPCO Diagnostics, Salem, NH, USA) and MIP-1α kit (Millipore/Milliplex® map kit, Billerica, MA, USA) were used to evaluate the analytes. Standards were included on all runs, and all results were reported within the linearity of the assays.

Statistical analysis

Demographic variables and smoking status were compared between groups using Fisher's exact test. Comparison of analytes between test and control groups was performed by box plots. Mean periodontal indices, age and concentration of salivary biomarkers were compared between test and control groups using two analyses of covariance to adjust for differences in patient demographics. Relationships between analytes and periodontal indices were determined using Spearman's correlation coefficient. Analyte levels associated with the clinical parameters of periodontitis, as well as those that discriminated periodontitis from health, were determined using logistic regression and classification and regression tree (CART) analysis. In the latter analysis, measurements below the detection limit were set at one-half the detection limit. All analyses were performed using the PC SAS 9.1 (SAS Institute Inc., Cary, NC, USA), with statistical significance determined at the 0.05 level.

Results

Eighty adults (40 with chronic periodontitis and 40 healthy control subjects), ranging in age from 21 to 60 years old, were evaluated (Table 1). Participants were age and sex matched. Test subjects were predominantly non-Caucasian, whereas the control subjects were predominantly Caucasian ($p < 0.001$). Smokers were only in the test group ($p < 0.001$). Each group had a similar mean number of teeth. As expected, all periodontal indices were significantly higher in the test group than in the control group ($p < 0.0001$).

Salivary analyte levels

Comparisons of salivary levels of OPG and MIP-1α are shown in Fig. 1. Levels of β-CTX were at or below the level of detection (i.e. 0.80 μg/L) in all samples. Levels of ICTP were detectable (i.e. > 0.020 ng/mL) in only six control subjects and one subject with periodontal disease. These data sets were too small to detect differences between the groups. Osteoprotegerin was detectable in all samples. The mean level of OPG was 1.6-fold higher in the periodontal disease group ($p = 0.0553$). In contrast, levels of MIP-1α were detected in all test subjects but only two control subjects. The mean level of MIP-1α was 18-fold higher in the periodontal disease group than the control group ($p < 0.0001$).

Relationships between salivary analyte levels and parameters of periodontal disease

We next analysed for relationships between levels of each analyte and clinical parameters of periodontitis. Here, MIP-1α demonstrated a strong positive correlation, with Spearman correlation coefficients ranging from 0.75 to 0.8 for percentage bleeding on probing, percentage probing depth ≥ 4 mm, percentage probing depth ≥ 5 mm and percentage clinical attachment loss ($p < 0.0001$). Osteoprotegerin also demonstrated a significant correlation with all four clinical parameters of periodontitis, with coefficients ranging from 0.22 to 0.36 ($p \leq 0.05$), whereas ICTP demonstrated a weak and negative correlation ($p > 0.05$). Correlations with β-CTX were indeterminate owing to too few samples having detectable levels.

To determine whether any of the four analytes discriminated periodontal health from disease, logistic regression models were performed and analysed. In the receiver operator characteristic analyses (Fig. 2), only MIP-1α showed a significant association with periodontitis, with an area under curve of 0.94 ($p < 0.0002$). The CART analysis also agreed with the logistic model, in that only MIP-1α was a significant discriminator of periodontitis. Here, the CART determined that the threshold for periodontitis was 1.12 pg/mL. This threshold displayed a sensitivity of 94.9%, specificity of 92.7%, positive

Table 1. Comparison of demographics and clinical characteristics between study groups

Parameter	Healthy subjects (n = 40)	Subjects with periodontal disease (n = 40)	p-Value
Age (years; mean ± SD)	35.8 ± 9.0	36.8 ± 9.0	—
Female (%)	50.0	50.0	—
White (%)	90.0	35.0	< 0.0001*
Hispanic (%)	2.5	17.5	< 0.0001*
African American (%)	0.0	10	< 0.0001*
Asian (%)	2.5	10	< 0.0001*
Other	5.0	27.5	< 0.0001*
Current tobacco use (%)	0.0	30.0	< 0.0001*
Number of teeth	27.6 ± 1.5	27.4 ± 1.8	—
Periodontal indices (percentage of sites; means ± SD)			
Bleeding on probing sites	3.2 ± 6.2	64.5 ± 24.6	< 0.0001
Probing depth sites ≥ 4 mm	1.6 ± 2.6	25.8 ± 13.6	< 0.0001
Probing depth sites ≥ 5 mm	0.3 ± 0.5	13.3 ± 9.4	< 0.0001
Clinical attachment loss ≥ 2 mm	0.1 ± 0.2	19.3 ± 13.3	< 0.0001

*Demographic data were determined by Fisher's exact test.
Periodontal indices were analysed by ANCOVA.

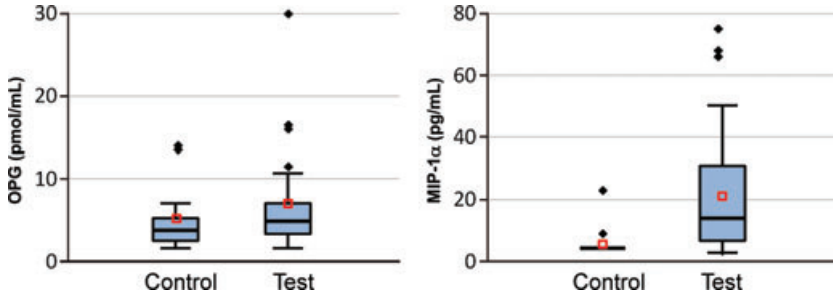

Fig. 1. Box plots for salivary levels of osteoprotegerin (OPG) and macrophage inflammatory protein-1α (MIP-1α). Mean values of MIP-1α were significantly different between the two groups (p < 0.0001); comparisons between groups of the other analytes were not. Outliers are shown as diamonds and means as red squares.

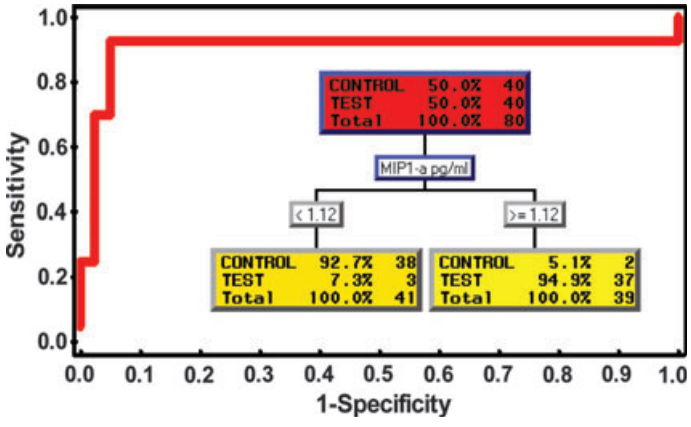

Fig. 2. Receiver operator characteristics of MIP-1α for identification of periodontal disease. Inset shows classification and regression tree analysis, demonstrating that MIP-1α is associated with periodontitis at the concentration ≥ 1.12 pg/mL.

predictive value of 94.9% and negative predictive value of 92.7% for periodontal disease. Additional pairing and combinations of the four biomarkers did not associate better with periodontitis.

Discussion

This cross-sectional, case–control study evaluated select bone remodeling biomarkers in whole unstimulated saliva from 40 adults who had chronic periodontitis and 40 healthy control subjects. The proteins evaluated are associated with important biological phases of bone remodeling. An upstream signaling protein (MIP-1α), a midstream anti-osteoclastic factor (OPG) and two downstream collagen type I degradation products (ICTP and β-CTX) were selected in an effort to address key aspects of alveolar bone remodeling that might be reflected in saliva. Our findings showed that MIP-1α performed well on several measures. Salivary MIP-1α levels were significantly elevated in subjects who had periodontal disease and demonstrated the strongest correlation with clinical parameters of periodontal disease. In the regression models, MIP-1α was the biomarker that best discriminated periodontal disease from health compared with OPG, ICTP and β-CTX.

Several studies have evaluated bone remodeling biomarkers in saliva in relation to periodontal disease, yet only a limited number have detected collagen degradation products in saliva. Both ICTP and β-CTX are well-recognized end-products of the collagenolytic pathway. Of these, β-CTX is generated first, from the organic matrix of type I collagen, by lysosomal cathepsin K attack. Later in the resorption process, MMPs break down cross-link peptides from the carboxy-terminal telopeptide areas of type I collagen into ICTP (44). Prior to the present study, neither β-CTX nor ICTP in saliva had been shown head-to-head to be a better discriminator of periodontal disease. Ng *et al.* (35) reported in a cross-sectional study the inability to detect ICTP in whole, stimulated saliva in 110 untreated dental patients. In contrast, Kinney *et al.* (32) reported the detection of ICTP in saliva and rising levels in patients with gingivitis and mild periodontitis over a 12 mo observation period, and Gurlek *et al.* (38) detected ICTP in the saliva of 67 otherwise healthy adults who had inflammatory

periodontal disease. Our findings herein and in our previous study are similar to those of Ng *et al*. and in contrast to those of Kinney *et al*. and Gurlek *et al*. Previously, we detected levels of ICTP in only 4.8% of 74 subjects (33) and here detected ICTP levels in only 8.8% of 80 dental patients, and only one patient in the test group. We believe that the use of different assay methods likely yielded higher sensitivity by Kinney *et al*. and Gurlek *et al*., in as much as our detection limit was within the range reported by those authors (38). Of note, β-CTX in the present study was even more difficult to detect, with all our samples having levels at or below the level of detection, and no previous salivary studies found in the published literature for comparison. Together, these findings are consistent with the premise that ICTP is a more sensitive marker of lytic alveolar bone resorption in saliva than β-CTX (45) and suggest that MMP-associated degradation products are predominant in saliva compared with cathepsin K-associated degradation products. Also, the overall context of the findings seems to indicate that downstream collagen degradation products are difficult to detect in the saliva of periodontitis patients when evaluation is performed in a cross-sectional manner, whereas their detection in longitudinal studies, where periods of active bone resorption are more likely to be demonstrated, could prove to be more useful in this area of diagnostic assessment.

Osteoprotegerin, an osteoblast-secreted decoy receptor that inhibits osteoclastogenesis, has been shown to be present at elevated concentrations in the saliva of patients with untreated chronic periodontitis (32,46), to correlate positively with probing depth and bleeding on probing (11) and to correlate with clinical attachment level (47). Osteoprotegerin has also been reported to be present at lower levels in smokers than nonsmokers who have chronic periodontitis (47) and serves as a salivary biomarker when paired with MMP-8 and red-complex anaerobic periodontal pathogens (48). In patients with periodontal disease, salivary OPG levels have diminished after scaling and root planing (32,42). However, as a single biomarker OPG has shown a weaker correlation with clinical parameters of periodontal disease than interleukin-1β and MMP-8 (11). Consistent with that finding, we observed that OPG was less of a discriminator for periodontal disease than MIP-1α, but better than ICTP and β-CTX. Together, these findings suggest that OPG, a factor midstream in the bone remodeling process, is a more sensitive salivary biomarker of periodontal disease than factors further downstream in the process. Consistent with this, others have found salivary RANKL levels to decrease after periodontal therapy (49). However, we are cognizant that it is possible that different saliva management protocols could contribute to alternative interpretations of these data.

Macrophage inflammatory protein-1α/CCL3 is a member of the cysteine-cysteine chemokine family, which is secreted by macrophages, neutrophils, basophils, dendritic cells, lymphocytes and epithelial cells and mediates granulocyte migration and adhesion (50–52). It stimulates monocytes and/or osteoclast progenitor cells to become active osteoclasts in a RANK/RANKL and dose-dependent manner (53). Macrophage inflammatory protein-1α has been detected at higher salivary levels (50-fold) in a longitudinal study of seven adolescents who had aggressive periodontitis compared with control subjects (34) and is secreted by gingival fibroblasts and epithelial cells (51,54). In our study, salivary concentrations of MIP-1α were 18-fold higher in subjects with periodontitis than in healthy subjects ($p < 0.0001$), and periodontal indices demonstrated highly significant correlations with MIP-1α levels ($p < 0.0001$). In regression analyses, MIP-1α offered high specificity (94%) and sensitivity (92.5%) for distinguishing periodontal disease from health, with the CART analysis providing an optimal cut-point of MIP-1α at 1.12 pg/mL and high sensitivity and specificity. These findings suggest that the salivary level of MIP-1α could have clinical utility as a screening tool for moderate to severe periodontal disease. However, its utility for discriminating between intermediate levels of disease (gingivitis and mild periodontitis) and health is not yet known. Also, because our study is limited by several factors (i.e. only 40 subjects per group were studied, reproducibility of each subject was not analysed longitudinally, and we excluded patients with oral mucosal diseases and systemic inflammatory conditions), the overall specificity of these biomarkers for periodontal disease in the general population needs to be further investigated.

Conclusions

This study, which evaluated four biomolecules associated with alveolar bone remodeling, found that salivary levels of MIP-1α can identify persons who have periodontal disease. Our data, along with the findings from previous studies (11,32,48,55), suggest that the combined presence of elevated levels of a panel of salivary biomarkers representing the three biological phases (inflammatory, connective tissue destruction and bone remodeling) of periodontal disease may offer the sensitivity and specificity for screening for periodontal disease in nondental settings, as well as potentially providing an understanding of the dynamics of the periodontitis lesion. Validation of this premise could lead to the use of biofluid panels as adjuncts in the diagnostic assessment of periodontal disease in the near future.

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