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Salivary interleukin-1β concentration and the presence of multiple pathogens in periodontitis

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Abstract

Aim: This study aimed to find salivary enzymes and/or cytokines that would reflect periodontitis, alone or in combination with salivary microbial markers.

Material and Methods: The salivary concentrations of elastase, lactate dehydrogenase, interleukin-1β (IL-1β), interleukin-6, and tumour necrosis factor-α, and the presence of five periodontal pathogens, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, and *Treponema denticola*, were analysed from salivary specimens of 165 subjects, a subpopulation of Health 2000 Health Examination Survey in Finland; 84 of the subjects had probing pocket depth (PPD) of ⩾4 mm at 14 or more teeth (the advanced periodontitis group), while 81 subjects had no teeth with PPD of ⩾4 mm (the control group). All subjects had at least 20 teeth and no systemic diseases.

Results: Among the salivary cytokines and enzymes tested, IL-1β was the only biomarker associated with periodontitis. An association was also found with the presence of multiple periodontal pathogens. Salivary IL-1β and the presence of multiple periodontal pathogens were associated with periodontitis at the same magnitude, when they were in the logistic regression model individually or together.

Conclusion: We suggest that salivary IL-1β and the presence of multiple periodontal pathogens in saliva should be studied more thoroughly as markers of periodontitis.

Key words: bacteria; cytokines; enzymes; periodontitis; saliva

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Periodontitis is an inflammatory condition of the tooth-supporting tissues initiated by the pathogenic bacterial biofilm at and below the gingival margin. Subgingival plaque harbours several hundreds of identified bacterial species. Among those, a rather limited number of species, such as *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia* (*forsythensis*), and *Treponema denticola*, have been associated with periodontitis (Paster et al. 2001, Pihlstrom et al. 2005). Periodontal pathogens activate host cells to produce proinflammatory mediators and enzymes, which in turn promote the destruction of periodontal tissues (Offenbacher 1996). Stimulation of epithelial cells, neutrophils, macrophages, and lymphocytes with microbial components increases cellular secretion of inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) (Silva et al. 2008, Dogi et al. 2009). The release of lysosomal and cytoplasmic enzymes, e.g., neutrophil-based elastase, myeloperoxidase, matrix metalloproteinases (MMPs), and lactate dehydrogenase (LDH), into periodontal tissues is higher in the areas with inflammation (Lamster et al. 1987, Uitto et al. 1996, Correa et al. 2008).

Conflict of interest and sources of funding statement

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The potential use of proteins of inflamed host tissue and infecting bacteria as markers of periodontitis has been investigated for many years, as reviewed in detail by Ozmeric (2004). The majority of studies analysing host and bacterial biomarkers as diagnostic tools for periodontal destruction are based on the use of gingival crevicular fluid (GCF) (Lamster & Ahlo 2007) and, to a lesser extent, saliva has been used as study specimens (Taba et al. 2005). Although GCF is considered as the main source of periodontitis-associated cytokines and host-derived enzymes (Ruhl et al. 2004), these agents will eventually enter also into saliva, which, unlike GCF, offers an easily and non-invasively collected specimen for their detection.

Common aims in periodontal research on salivary diagnostics are to find marker(s) that could be used, preferably as chair-side tests, for example, to determine the activity of periodontitis or the results of periodontal treatment, or to a lesser extent to detect periodontitis in field studies. Various enzymes, cytokines, and biomarkers of bone turnover have been found to be elevated in saliva of periodontitis patients in comparison with periodontally healthy controls (Miller et al. 2006, Kinney et al. 2007, Scannapieco et al. 2007, Tobón-Arroyave et al. 2008, Teles et al. 2009). In addition, saliva contains not only locally and systemically deriving biomarkers of host origin but also microbial markers of periodontal diseases (Kaufman & Lamster 2000, Könönen et al. 2007).

Our aim in this study was to find host-derived enzymes or cytokines, alone or together with aetiologic microbial markers, in saliva to detect periodontitis.

Material and Methods

Study population and specimen collection

A nationally representative population survey ''Health 2000 Health Examination Survey'' was conducted in Finland by the National Institute for Health and Welfare (THL) [formerly National Public Health Institute (KTL)] in the years 2000–2001. The study population comprised of 8028 subjects, aged ⩾30 years and steadily living in Finland. General and oral health examinations were performed in the field in five districts. Periodontal status was examined by specially trained dentists, as described in the report of the National Public Health Institute (http://www.terveys2000.fi/indexe.html). Data on health behaviour and smoking habits were collected by interviews. About 89% of the invited subjects participated in interviews and 85% in health examinations. All protocols were approved by the institutional ethics committees.

Saliva was collected from 1294 subjects living in southern Finland. The paraffin-stimulated whole saliva samples, collected by expectoration, were frozen at −70°C until further used. For the present study, saliva samples of a subpopulation of 165 subjects were selected on the basis of their periodontal status; 84 of the subjects had probing pocket depth (PPD) ⩾4 mm at 14 or more teeth (the advanced periodontitis group), while 81 subjects had no teeth with PPD ⩾4 mm (the control group). All subjects had at least 20 teeth and no systemic diseases. The procedures of saliva collection as well as oral examinations have been described in detail previously (Könönen et al. 2007).

Cytokine and enzyme analysis

Frozen saliva samples were thawed and centrifuged at 10,000 r.p.m. for 3 min. and the supernatants were aliquoted for assays of salivary IL-1β, IL-6, and TNF-α determined using commercial ELISA kits (GE Healthcare, Buckinghamshire, UK). The LDH activity was measured with a colorimetric cytotoxicity detection kit (Roche, Mannheim, Germany). The tests were performed according to the manufacturers' instructions. According to the information provided by the manufacturers, the minimum detectable doses of cytokines by the ELISA kits were as follows: IL-1β and IL-6 < 1 pg/ml, and TNF-α < 5 pg/ml.

The elastase activity was measured using the chromogenic substrate succinyl–alanyl–valine–*p*-nitroanilide as previously described (Uitto et al. 1996).

Microbiological analysis

The presence and level of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. denticola*, and *T. forsythia* in the saliva samples were obtained by quantitative real-time polymerase chain reaction (qPCR) (Hyvärinen et al. 2009). Briefly, total bacterial DNA was extracted from saliva samples using the ZR Fungal/Bacterial DNA KitTM (Zymo Research, Orange, CA, USA). Species-specific primer and TaqMan probe sets were based on conserved regions from single copy genes. The qPCR assays were performed using Mx3005P real-time QPCR System (Stratagene, La Jolla, CA, USA). The fluorescence increase was monitored during the PCR amplification and all data were analysed using the Mx3005P Real-Time QPCR System software (Stratagene). The exact genome sizes for the target pathogens were obtained from Oralgen Databases (http://www.oralgen.lanl.gov/), and the bacterial DNA levels were quantified by real-time PCR and calculated from the standard curve equations created by serial dilutions. The detection limit for *A. actinomycetemcomitans* is two genome equivalents (GE), for *P. gingivalis* 23 GE, for *P. intermedia* 27 GE, for *T. denticola* 28 GE, and for *T. forsythia* 34 GE, calculated from the standard curves used.

Statistical analysis

The SPSS (version 11.0; SPSS Inc., Chicago, IL, USA) statistical programme was used to analyse the data. The Mann–Whitney *U*- and Kruskal–Wallis *H*-tests were used for comparisons of cytokine concentrations and enzyme activities between the groups. A logistic regression analysis was used to study the association of IL-1β used in analyses as quartiles and as a continuous variable with periodontitis by controlling for gender and smoking. Separate models were constructed using the presence of periodontal pathogens alone and together with IL-1β as the main explanatory variables.

Results

Characterization of the study population by age, gender, periodontal status, and smoking habits is presented in Table 1a. In addition to the differences in the periodontal status between the two study groups, the mean age and the proportions of men and smokers were higher in the periodontitis group than in the control group.

IL-1β was detected in all samples, whereas the number of samples below the detection limit was 16 (19.7%) and 8 (9.6%) for IL-6 and 1 (1.2%) and 3 (3.6%) for TNF-α in the healthy and periodontitis group, respectively. Of the three cytokines tested (IL-1β, IL-6, and

Table 1. Study population by age, gender, smoking habits, periodontal status (PPD) (a), and by salivary biomarkers, including the examined pathogens (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, and/or *Treponema denticola*), enzyme activities, and cytokine concentrations (b)

	Periodontitis ($n = 84$)	Control ($n = 81$)
(a)		
Age in years (mean ± SD)	49.6 ± 5.2*	47.9 ± 5.7
% of men	59.5*	35.8
Smoking status		
% of non-smokers	47.6*	81.5
1–19 cigarettes/day	23.8*	13.6
>19 cigarettes/day	28.6*	3.7
No. of teeth (mean ± SD)	27.0 ± 2.3	27.1 ± 2.4
No. of teeth with PPD ⩾ 4 mm (mean ± SD)	19.3 ± 3.6*	0.0
No. of teeth with PPD ⩾ 6 mm (mean ± SD)	4.2 ± 5.1*	0.0
(b)		
Carriage of pathogens		
% of subjects without pathogens	0	9.9
with 1–2 pathogens	27.4*	55.6
with 3–5 pathogens	72.6*	34.6
Relative LDH activity (mean ± SD)	2.7 ± 1.1	2.9 ± 0.8
Relative elastase activity (mean ± SD)	13.4 ± 15.7	12.4 ± 17.5
IL-1β concentration (pg/ml) (mean ± SD)	665.7 ± 267.5*	467.8 ± 279.8
IL-6 concentration (pg/ml) (mean ± SD)	3.6 ± 5.9	3.1 ± 3.6
TNF-α concentration (pg/ml) (mean ± SD)	2.9 ± 4.0	2.7 ± 2.8

*p-value <0.05.
IL-1β, interleukin-1β, IL-6, interleukin-6; LDH, lactate dehydrogenase; PPD, probing pocket depth; TNF-α, tumour necrosis factor-α.

Table 2. IL-1β concentrations in saliva of subjects with and without periodontitis, stratified by the presence of periodontal pathogens (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, and/or *Treponema denticola*) or by smoking. The asterisk indicates the p-value <0.05

		IL-1β concentration (pg/ml)	
		periodontitis ($n = 84$)	control ($n = 81$)
Presence of pathogens in saliva	No pathogen	–	392 ± 205
	1–2 pathogens	639 ± 254	463 ± 289
	3–5 pathogens	676 ± 274	497 ± 287
Smoking status	Non-smokers	612 ± 230	464 ± 272
	Current smokers	706 ± 288	479 ± 311

IL-1β, interleukin-1β.

TNF-α) and enzymes (LDH and elastase), IL-1β was the only salivary biomarker with higher concentrations in the advanced periodontitis group than in the control group (Table 1b). The level of salivary LDH was higher ($p = 0.006$) in subjects with periodontitis (3.36 ± 0.69) when compared with control subjects (3.05 ± 0.70), but only when non-smoking subjects were included. When subjects were grouped as smokers and non-smokers, regardless of their periodontal status, the relative LDH activity ($p < 0.001$) and TNF-α concentration ($p = 0.03$) were significantly lower and the IL-1β ($p = 0.026$) concentration significantly higher in smokers than in non-smokers.

Table 2 presents the salivary IL-1β concentration in both study groups stratified by the presence of multiple periodontal pathogens in saliva and by smoking behaviour. In the categories of 1–2 and three to five pathogens in saliva, higher IL-1β concentrations were found in the periodontitis group when compared with the control group. Also, the difference of IL-1β concentration between the study groups was significant both in non-smokers ($p = 0.005$) and smokers ($p = 0.004$).

To study the gender- and smoking-adjusted associations between periodontitis and IL-1β as well as the presence of microbes, logistic regression models included first the interaction term between IL-1β and smoking. Because it was shown to be insignificant (data not shown), the final models were constructed without the interaction term. As shown in Table 3, the IL-1β concentration associated in a dose-dependent manner with advanced periodontitis (Model 1). This was found using IL-1β concentration either as a continuous variable or as a categorized variable (quartiles). Also, the presence of three to five pathogens was associated with advanced periodontitis (Model 2). When both of these biomarkers were in the same model adjusted for gender and smoking (Model 3), the estimates of IL-1β changed only slightly when compared with those seen in Models 1. The same concerned the microbial variable (Model 2).

Discussion

IL-1β is produced during periodontal inflammation and tissue destruction, while the presence of periodontal pathogens represents the aetiologic burden of periodontitis. The main finding of this study was that the salivary IL-1β concentration was associated with advanced periodontitis. Furthermore, inclusion of both marker candidates (IL-1β and the presence of periodontal pathogens) in the logistic regression model resulted in a similarly strong association with periodontitis to the one achieved by the use of each marker individually.

The observation of the present study is preliminary. The results are based on the comparison between the two selected groups, i.e., 84 subjects with advanced periodontitis, determined as having at least 14 teeth with PPD ⩾ 4 mm, and 81 subjects without any teeth with PPD ⩾ 4 mm as their controls. This limits the generalization of the results, which should be verified in a larger population with different stages of periodontal diseases. It is also important to point out that the estimates of the odds ratio in this case do not describe the actual risk. Another important point is that gingival inflammation itself can affect IL-1β secretion and consequently, its concentration in saliva, regardless of the individual subject's pocket depth. In the present study, in order to minimize the effect of gingival inflammation, we carried our stratified analyses in distinct groups (i.e., periodontally healthy subjects *versus* patients with advanced periodontitis).

Table 3. Unadjusted and adjusted associations of periodontitis with salivary IL-1β concentration (pg/ml) expressed in quartiles and as a continuous variable, and the multiple presence of periodontal pathogens (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis, Prevotella intermedia*, *Tannerella forsythia*, and/or *Treponema denticola*) in saliva

			Odds ratios (95% confidence limits)			
		median (range) (pg/ml)	unadjusted	model 1*	model 2*	model 3*
IL-1β						
	I quartile	1068 (960–12,909)	8.7 (2.5–30.1)	5.5 (1.0–28.7)		5.0 (0.8–29.9)
	II quartile	778 (653–947)	5.6 (2.1–14.9)	4.0 (1.1–14.5)		3.9 (0.9–15.8)
	III quartile	497 (345–640)	3.8 (1.6–9.2)	3.6 (1.2–10.8)		4.1 (1.2–13.9)
	IV quartile	238 (35–341)	1.0	1.0		1.0
	Goodness-of-fit (χ^2/df/p)		0.0/0/	17.8/17/0.403		46.9/41/0.244
	Pseudo R^2 (Nagelkerke)		0.153	0.373		0.476
	p		0.000	0.000		0.000
IL-1β	As continuous variable (pg/ml)		1.003 (1.001–1.004)	1.002 (1.001–1.004)		1.002 (1.001–1.003)
	Goodness-of-fit (χ^2/df/p)		154.8/149/0.356	161.1/112/0.02		168.7/112/0.000
	Pseudo R^2 (Nagelkerke)		0.141	0.359		0.455
	p		0.000	0.000		0.000
Presence of pathogens	3–5 pathogens		4.5 (2.3–8.9)		5.8 (2.1–15.6)	6.0 (2.1–17.1)
	1–2 pathogens		1.0		1.0	1.0
	Goodness-of-fit (χ^2/df/p)		0.0/0/		8.6/7/0.281	46.9/41/0.244
	Pseudo R^2 (Nagelkerke)		0.163		0.423	0.476
	p		0.000		0.000	0.000

*Adjusted for smoking (no. of cigarettes/day 0, 1–19, >19) and gender.

The logistic regression models 1–3 are adjusted for gender and smoking status. In each quartile, the median (range) concentrations of IL-1β are given as pg/ml.

IL-1β, interleukin-1β.

The advantage of using saliva as a study specimen is that it can be easily and non-invasively collected and the collection does not require specially trained dental nurses or assistants. However, one important disadvantage of using saliva for periodontal disease diagnosis is that it fails to detect the exact site of active disease. Furthermore, variations in salivary flow rate, use of antimicrobial medications, and smoking habits can have an impact on salivary analysis. Indeed, one prerequisite for a good periodontal disease marker is its function in the population level, for example independently of smoking behaviours or of other modifiers. Therefore, we presented the IL-1β concentrations stratified by smoking, verified the interaction between IL-1β and smoking, and used smoking variable categorized according to the amount of daily smoking. The adjustment for smoking and gender attenuated the estimates of IL-1β but not of the presence of three to five pathogens. However, these adjustments did not make the use of salivary IL-1β, a non-significant marker of periodontitis.

In a longitudinal study by Kibayashi et al. (2007), where the periodontal status and salivary compositions were analysed, salivary levels of aspartate aminotransferase, LDH, alkaline phosphatase, albumin, and prostaglandin E_2 were found to be significantly lower in smokers than in non-smokers. On the other hand, the mean salivary IL-1β concentration in smokers (163.3 pg/ml) compared with non-smokers (205.7 pg/ml) was not statistically significant, supporting no marked effect of smoking on salivary IL-1β level. In a cross-sectional study by Nishida et al. (2006), the mean salivary IL-1β concentration was significantly higher in passive smokers (199.0 pg/ml) than in non-smokers (164.3 pg/ml) (Nishida et al. 2006). In the present study, all cytokine concentrations and enzyme activities tested were lower in smokers than in non-smokers, with the exception of the IL-1β concentration, which was higher in smokers than in non-smokers in the periodontitis group. It is well known that smoking increases the destruction of periodontal tissues and, consequently, increases the expression of IL-1β. On the other hand, the effect of smoking on salivary cytokine levels and enzyme activities can be either by diminishing the host response and impairing the cellular inflammatory secretions or by directly interacting with the enzyme and inhibiting their activity (Zappacosta et al. 2002, Kibayashi et al. 2007, Torres de Heens et al. 2009).

In a recent study by Teles et al. (2009), salivary concentrations of IL-1β, −2, −4, −5, −6, −8, and −10 did not differ between 74 subjects with chronic periodontitis and 44 periodontally healthy subjects. The identification of periodontitis in that study was similar with our study; the authors recruited subjects with at least 20 natural teeth and having eight or more sites with PPD > 4 mm and, in addition, attachment level > 3 mm (Teles et al. 2009). The mean levels (± standard deviations) of salivary IL-1β reported were 633 ± 602 pg/ml for periodontally healthy subjects and 673 ± 590 pg/ml for chronic periodontitis patients. The concentration was quite similar with our periodontitis group. Teles et al. (2009) used Luminex technology, which has an advantage to detect multiple cytokines in small sample volumes simultaneously. However, the use of multiplex assay, such as the Luminex system, for analysing several cytokines with highly variable concentrations in the sample fluid may impair the sensitivity for their detection (Leng et al. 2008).

In our study, the salivary concentration of IL-1β also corresponded well with the findings of Tobón-Arroyave et al. (2008), but was clearly higher than those described by others (Miller et al. 2006, Nishida et al. 2006, Ng et al.

2007). Unlike the salivary concentration of IL-1β, those of TNF-α and IL-6 did not differ between the periodontitis group and the control group in any of the comparisons we performed. In most samples, the concentrations of these two latter cytokines were either below the detection limit or, if detected, very low. Similarly, in the study of Ng et al. (2007), some of the concentrations of salivary IL-6 and TNF-α were below the detection limit.

Although elastase in oral fluids has been suggested to be an indicator of periodontal disease (Nieminen et al. 1996, Uitto et al. 1996, Armitage 2003), in the present study, the increased elastase level seen in the periodontitis group was not statistically significant. This is probably due to the precipitation observed in some samples disturbing the analysis. In non-smoking subjects, the relative activity of salivary LDH was higher in those with periodontitis when compared with control subjects. Instead, no difference was found when the comparison was made between the periodontitis and control groups where both smokers and non-smokers were included. It has been suggested that smoking inhibits the activity of LDH in saliva due to its interaction with aldehydes in tobacco smoke (Zappacosta et al. 2002), and therefore the salivary LDH activity cannot be considered a reliable biomarker candidate in population-based studies.

Our microbial data indicate that the presence of multiple periodontal pathogens in saliva could be used as a marker of periodontitis. Recently, the number of pathogenic species in saliva rather than a certain periodontal pathogen was associated with clinical signs of periodontitis (Paju et al. 2009). In contrast to these findings, Ready et al. (2008), who analysed the presence of subgingival *A. actinomycetemcomitans*, *P. gingivalis*, and *T. forsythia* in periodontitis subjects, found that subjects who harboured a single periodontal pathogen had more periodontal pockets than subjects who harboured two or three pathogens. The authors suggested that if the subgingival microbiota is less diverse, the species present may be able to promote a greater inflammatory response. However, the presence of bacteria in saliva provides information about the bacterial challenge that periodontal tissues face, whereas salivary IL-1β, a proinflammatory mediator known to stimulate the production of destructive enzymes, can give information about the initiated and/or increased tissue response (Yan & Boyd 2007). In this regard, microbial markers may provide additional strength to salivary diagnosis of periodontitis when they are used in combination with host-derived markers. Ramseier et al. (2009) showed that combining certain anaerobic periodontal pathogens (such as *P. gingivalis* or *T. denticola*) present in subgingival biofilms with salivary MMP-8 and osteoprotegerin levels predict the status of periodontal diseases.

Saliva is a non-invasive sample material, which enables the detection of various biomarkers, such as microbes, cytokines, and enzymes, in the oral cavity. According to our results, salivary analyses of IL-1β and periodontitis-associated bacteria are a good means of monitoring periodontal status in large population studies. To confirm our promising, though preliminary results, future studies on salivary biomarkers should include a larger population with different stages of periodontal diseases and with different study settings than a cross-sectional one. In conclusion, salivary IL-1β concentrations and the presence of several periodontal pathogens in the saliva may be used as markers of periodontitis in large-scale population studies with no available clinical periodontal data.

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Clinical Relevance

Scientific rationale for the study: Salivary enzymes and/or cytokines alone or in combination with salivary microbial markers may reflect the presence of periodontal infection.
Principal findings: Salivary concentration of IL-1β and presence of multiple periodontal pathogens in saliva were associated with periodontitis when gender, smoking, and the presence of different periodontal pathogens were controlled.
Practical implications: Salivary IL-1β and the presence of multiple periodontal pathogens in saliva could be used as markers of periodontitis in studies where a clinical examination of the periodontal status is not feasible. However, further studies using larger study population with different stages of periodontal diseases are needed.