

Changes of Four Proinflammatory Proteins in Whole Saliva during Experimental Gingivitis

Min ZHOU^{1,2}, Huan Xin MENG¹, Yi Bing ZHAO¹, Zhi Bin CHEN¹

Objective: To examine changes of four proinflammatory proteins in whole saliva in the early stage of plaque-induced experimental gingivitis.

Methods: Eleven young male volunteers were recruited following the cessation of all oral hygiene measures for a period of 21 days. The levels of Interleukin-6 (IL-6), Interleukin-1 β (IL-1 β), calprotectin in saliva were determined with enzyme linked immunosorbent assay. The activity of elastase in saliva was examined.

Results: IL-1 β , IL-6 and calprotectin in saliva increased gradually as plaque accumulated and peaked on the 14th and 21st day respectively. Moreover, the three proinflammatory proteins showed good correlations with clinical parameters, with IL-1 β correlating with clinical parameters more closely in particular. The activity of elastase in saliva elevated rapidly and peaked on the second day ($P < 0.01$). However, after the seventh day, elastase activity declined slowly continuously. The change of IL-6 and IL-1 β in saliva showed a similar tendency throughout the experiment, the correlation coefficient was $r = 0.687$ ($P < 0.01$), but there was no obvious correlation between calprotectin and elastase, even though both mainly come from neutrophils.

Conclusion: Our study demonstrated that IL-6, IL-1 β and calprotectin concentrations in saliva could reflect the degree of gingival inflammation. The longitudinal change of elastase activity in saliva during the experimental gingivitis period was quite different from that of other pro-inflammatory proteins; reasons for the decrease of elastase activity in the late gingivitis period need further study.

Key words: saliva, interleukin-6, interleukin-1 β , calprotectin, elastase, experimental gingivitis

A series of studies have demonstrated that substances from periodontopathic bacteria initiate and drive the inflammatory response. Nevertheless, endogenous molecules mediate the inflammatory process and play major roles in their amplification, perpetuation, and the ensuing tissue destruction^{1,2}. In 1965, L \ddot{o} e et al³ con-

ducted experimental gingivitis model for the first time, which exhibited gingival inflammation progress following the dental plaque accumulation dynamically. Since then many researchers have used this model to study longitudinal changes of components in gingival crevicular fluid (GCF), such as tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), elastase (EA), interleukin-8 (IL-8), transforming growth factor beta (TGF- β) and calprotectin, to learn more about early gingival inflammation⁴⁻⁹. Most studies used GCF to investigate host response, since GCF is in the vicinity of the infectious site and could reflect the periodontal disease process sensitively¹⁰. In recent years, detections of substances in whole saliva have gradually increased. Saliva can be easily collected and represents a pooled sample from all

1 Department of Peridontology, Peking University School and Hospital of Stomatology, Beijing, P.R. China.

2 Tongji University School of Stomatology, Shanghai, P.R. China.

Corresponding author: Dr Huan Xin MENG, Department of Peridontology, Peking University School and Hospital of Stomatology, #22 Zhong-guancun Nandajie, Haidian District, Beijing 100081, P.R. China. Tel: 86-10-82195522; Fax: 86-10-62173402; E-mail: kqhxmeng@126.com

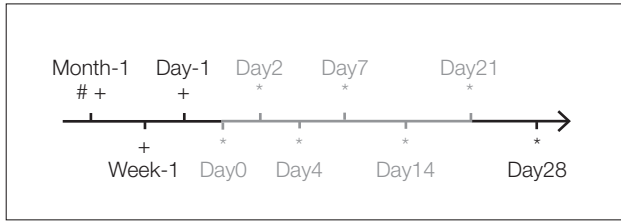
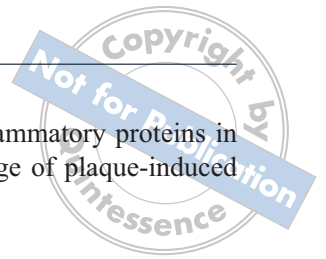


Fig 1 Process chart: # Prophylaxis; + Polishing; * saliva collection

periodontal sites, thereby giving an overall assessment of periodontal disease status^{11–13}.

Once an inflammatory response is initiated, neutrophils are the first cells to be recruited to sites of infection or injury. Resting neutrophils can become primed by agents, including bacterial products and cytokines, and then primed neutrophils are mobilised to the sites of infection or inflammation for clearing pathogens. During inflammation, there is a dramatic increase in chemokine secretion resulting in selective recruitment of leukocytes in the injured tissue¹⁴. Proinflammatory cytokines may act in concert with chemotactic factors in the activation of polymorphonuclear leucocyte (PMN) inflammatory potential^{5,15}.

IL-1 and IL-6 are known as multifunctional cytokines involved in the regulation of the immune response, haematopoiesis and inflammation. IL-6 and IL-1, which are mainly produced by monocyte/macrophage and lymphocytes, have proinflammatory qualities during infection and they play important roles in the initiation of immune response^{16,17}.

Calprotectin and elastase are both important proteins that reflect neutrophils activity. Calprotectin, which can be found in the cytosol of neutrophils, monocytes, human gingival keratinocytes and oral epithelial cells^{18,19}, is a most abundant Ca²⁺ binding protein complex in neutrophils and constitutes 45% of neutrophil cytosolic protein²⁰. Elastase is one of the major components in the azurophil granules of neutrophils, which is released from dead and dying neutrophils of the periodontium.

This study was based on the experimental gingivitis model, in which 11 young male subjects with healthy gingiva were measured following the cessation of all oral hygiene for a period of 21 days²¹. The levels of IL-6, IL-1 β , calprotectin and elastase in whole saliva were assayed, and their relationships, as well as their correlations with clinical periodontal parameters, were analysed following the development of experimental gingivitis. The aim of this study was to learn

the changes of these four proinflammatory proteins in whole saliva during the early stage of plaque-induced gingival inflammation.

Methods

Subjects

A total of 11 non-smoking male dental students at a mean age of 21.5 years old (range 21–22), with no evidence of periodontal disease and unremarkable medical histories, were recruited in this study. All subjects had more than 24 teeth, normal occlusion, good oral hygiene and healthy gingiva. Exclusion criteria were the intake of antibiotics and periodontal treatment within the preceding 6 months of the study. This study had obtained ethical approvals and each participant was provided written informed consent prior to their enrolment into the study.

Study design

Unstimulated whole saliva samples were collected at the baseline (day 0), then 2, 4, 7, 14 and 21 days after cessation of all oral hygiene, and at the 28th day (7 days after resuming oral hygiene). The figure below exhibits the process of this experimental gingivitis study (Fig 1).

Saliva collection

Approximately 0.5 ml of unstimulated whole saliva samples were collected by passive drooling into a sterile universal container, and then centrifuged at 13,000 rpm (diameter 5.5 cm) for 15 minutes to remove cells and debris, and then the supernatant were frozen at –70 for later assay. The clinical parameters including Silness-Löe Plaque Index (PLI), probing depth (PD), Mazza’s bleeding index (BI)²² and gingival index (GI) of each tooth were recorded at different time points.

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of IL-6, IL-1 β and calprotectin in saliva were determined by ELISA according to the kit’s instructions.

The assays of IL-6 and IL-1 β were conducted with commercial ELISA kits (R&D Systems). Samples and standards were incubated for 2 hours at room temperature in microplate wells, which were coated with monoclonal anti-IL-6 or anti-IL-1 β antibody (mouse anti-human) respectively. After decanting and washing, biotinylated detection antibodies (goat anti-human IL-6 or anti-IL-1 β) were added and incubated for 2 hours

Table 1 IL-6, IL-1 β and calprotectin concentrations and EA activity in saliva during experimental gingivitis

Days	IL-6 pg/ml (Mean \pm SE)	IL-1 β pg/ml (Median \pm interquartile range)	Calprotectin μ g/ml (Median \pm interquartile range)	EA activity A/ml (Mean \pm SE)
0	6.699 \pm 0.273	18.317 \pm 1.41	2.479 \pm 1.540	1.984 \pm 0.069
2	7.119 \pm 0.258	19.709 \pm 3.34	2.356 \pm 1.081	2.39 \pm 0.062*#+
4	6.880 \pm 0.273	19.973 \pm 5.01	2.285 \pm 1.706	2.29 \pm 0.065*#+
7	7.269 \pm 0.290	20.964 \pm 4.42	2.450 \pm 3.248	2.36 \pm 0.069*#+
14	7.225 \pm 0.273	23.990 \pm 10.65*#	3.159 \pm 2.478	2.137 \pm 0.065#
21	7.858 \pm 0.258*	23.402 \pm 10.18*	5.006 \pm 3.551#	1.914 \pm 0.062
28	7.053 \pm 0.258	19.845 \pm 4.28	1.757 \pm 0.389	1.714 \pm 0.062

Note: the comparison of IL-1 β and calprotectin concentrations on different time points based on their logarithm values after logit transformations.

*: vs day 0, $P < 0.05$; #: vs day 21, $P < 0.05$; #: vs day 28, $P < 0.05$.

again at room temperature. Streptavidin-HRP (streptavidin conjugated to horseradish-peroxidase) were added to each well and incubated for 20 min afterward. The enzymatic reaction was stopped with 2N sulphuric acid and the plates were read at 450 nm wavelength using a microplate reader (BIO-RAD 680). Concentrations of the cytokines in each sample were determined according to the standard curve for comparison and defined as pg/ml.

Calprotectin was detected and quantified using CALPREST ELISA kit (NovaTec Immundiagnostica) according to the manufacturer's instructions. The optical density (OD) of the samples was read at 405 nm. The concentrations were defined as μ g/ml.

EA activity assay

Forty μ l supernatants of saliva samples were added into each well at first, and 200 μ l substrate solutions (Meosuc-ala-ala-pro-val-NA, Sigma, 1 mmol/L, mixed with 0.1% dimethyl sulphoxide) were added to each well afterward. Phosphate-buffered saline (PBS) was used as blank control. Ninety-six-well microtiter plates were placed in the incubator at 37 for 2 hours away from light. The results were read using a microplate reader at 405 nm wavelength. The enzyme activity is expressed as optical density per milliliter (A/ml).

Statistical analysis

Data management and statistical analysis were performed using Statistical Package for Social Science

(SPSS 12.0) software. Each variable was tested for normality, and non-normally distributed variables were converted to normal distribution by logit transformations. Analysis of variance was used to compare changes among values on days 0, 2, 4, 7, 14, 21 and 28. Multiple comparisons were carried out subsequently by the Bonferroni method for variables with equal variances and Games-Howell for variables with unequal variances. The relationships between levels of IL-6 and IL-1 β on different time points, as well as with clinical parameters were analysed by correlation analysis (Pearson's correlation coefficient for measurement data, while Spearman's rank correlation coefficient for ranked data).

P -values < 0.05 were accepted as statistically significant.

Results

Changes of IL-6, IL-1 β , calprotectin and EA in saliva

During the period of 21-day oral hygiene cessation, the concentrations of IL-1 β , IL-6 and calprotectin in saliva increased gradually as plaque accumulated and peaked at the 14th day and the 21st day respectively (Table 1 and Fig 2a). The highest concentrations of IL-6 and IL-1 β showed significant differences compared with the baseline values ($P = 0.040$, $P = 0.0004$ respectively). After oral hygiene resumed, the concentrations of IL-6 and IL-1 β fell back closely to the baseline values. Calprotectin concentration on the 21st day was higher than that on the 28th day with a significant difference ($P =$

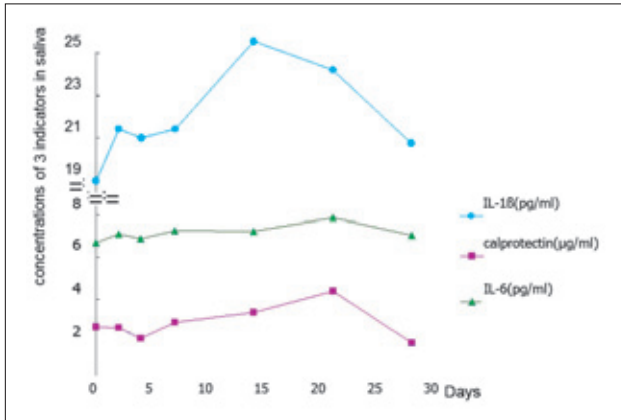


Fig 2a Linear graph of IL-6, IL-1β and calprotectin concentrations in saliva during experimental gingivitis (concentrations of IL-1β and calprotectin based on geometric means).

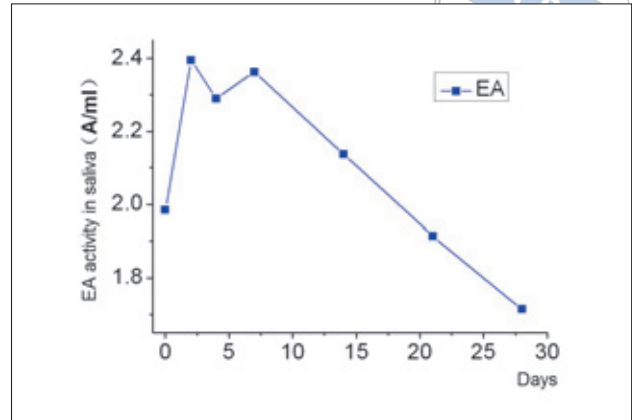


Fig 2b The longitudinal change of EA activity in saliva during experimental gingivitis.

0.01). Salivary elastase activity ranged between 0.98 to 3.8A/ml with a small standard deviation of about 0.02-0.03A/ml. The EA activity in saliva elevated rapidly and peaked on the second day, which was significantly higher than the baseline value with a statistical significance ($P = 0.001$). Moreover, it continued to increase till the seventh day. After the seventh day, EA activity declined slowly to 1.714A/ml on the 28th day (Fig 2b).

The relationships among detection measurements and clinical parameters

The concentrations of IL-6, IL-1β and calprotectin were positively correlated with clinical parameters PLI, GI and BI, except PD. Among these detection measurements, IL-1β concentration showed a little stronger relationship with GI and BI ($r = 0.459$, $r = 0.463$ respectively, detailed in Table 2). There were no significant correlations between EA activity and clinical parameters.

The change of IL-6 and IL-1β in saliva showed a similar tendency throughout the experiment, the cor-

relation coefficient between IL-6 and IL-1β levels in saliva was $r = 0.687$ ($P < 0.01$). Calprotectin and EA exhibited no significant relationships with other pro-inflammatory proteins. Even though both calprotectin and elastase mainly come from neutrophils, there was no obvious correlation between each other ($r = -0.030$) (detailed in Table 3).

Discussion

In recent years, many researches have focused on the changes of components in saliva during experimental gingivitis, such as elastase, cystatins and immunoglobulin A8,²³⁻²⁶. In Lie and van Gils's studies^{23,25}, smokers showed a decrease in cystatin activity and cystatin C, as well as lower outputs of cystatin activity and cystatin C at the end of the 14-day experimental gingivitis period. While for non-smokers, no significant changes of these inflammatory proteins have been found over time. However, in this study, IL-6, IL-1β and calprotectin exhibited a significant increase following the plaque

Table 2 The relationship between laboratory measurements and clinical indicators

	PLI	GI	BI	PD
IL-6 (pg/ml)	0.237*	0.368**	0.419**	0.061
IL-1β(pg/ml)	0.387**	0.459**	0.463**	0.159
Calprotectin (μg/ml)	0.354**	0.391**	0.336*	-0.057
EA activity (A/ml)	-0.102	-0.187	-0.17	-0.216

Note: * $P < 0.05$, ** $P < 0.01$.

accumulation in whole saliva based on non-smokers. The increase may be explained by three reasons: Firstly, the increasing content of inflammatory mediators was continuously washed into whole saliva from GCF during experimental gingivitis; Secondly, the inflammation will not only confine to the vicinity of the local gingival crevicular and gingival tissue, but also affect salivary glands²⁷⁻²⁹. Additionally, oral antigens that persist in the plaque would provide continuous stimulation to the local glands. The indirect evidences of a local immune system associated with salivary glands were derived from topical immunisation studies. These studies indicated that intimate contacts of plaque covered molar surfaces can enable an antigen uptake through the mucosal surface to the parotid immune tissue^{29,30}. Thus Seemann's finding of slightly raised IgA secretions in the parotid but not in the submandibular/sublingual glands might be explained simply by the anatomical situation that plaque-covered buccal surfaces have intimate contact with parotid tissues²⁶.

However, among the four laboratory measurements, elastase was the only one that showed a reverse longitudinal change during the experimental gingivitis period and exhibited no significant relationships with other proinflammatory proteins. In Uitto's study, there was a good correlation between the salivary elastase activity and the number of deep periodontal pockets (≥ 6 mm) and the average community periodontal index of the subjects. Moreover, it also found that elastase activity was not a good indicator of gingivitis⁸. As we know, in the early stage of inflammation, neutrophils are the major cells migrating from the circulation to inflammatory sites. And elastase is an abundant proteinase released from the azurophilic granules of neutrophils, which is an indicator of neutrophil activity³¹. In addition, GCF flows from the gingival crevice, carrying elastase into the oral cavity. The inflamed gingiva was probably the source of nearly all the elastase detected in the oral cavity³². Elastase activity in saliva should increase following the development of the gingival inflammation. However, the results we obtained were inconsistent with the theory above. The EA activity in saliva elevated rapidly and peaked on the second day, and then dropped after the seventh day continuously to the 28th day.

GCF and whole saliva also contain serum-derived alpha-1 proteinase inhibitor ($\alpha 1$ PI) and Secretory leukocyte protease inhibitor (SLPI), the predominant physiological inhibitors of neutrophil elastase (NE)^{33,34}. The primary function of $\alpha 1$ PI is to control the activity of neutrophil elastase through the formation of $\alpha 1$ PI-elastase complex³⁵. In Cox's study, $\alpha 1$ PI, originating

Table 3 Correlations among laboratory measurements

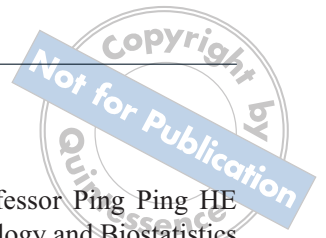
	IL-6 (pg/ml)	IL-1 β (pg/ml)	Calprotectin (μ g/ml)	EA (A/ml)
IL-6		0.687**	0.017	-0.060
IL-1 β	0.687**		0.098	-0.064
Calprotectin	0.017	0.098		-0.030

Note: ** $P < 0.01$

from GCF, was evidently diluted in whole mixed saliva (its concentration in whole mixed saliva was about 1/30 compared to that in GCF), whereas SLPI in whole mixed saliva was supplemented by contributions from glandular saliva³². In mixed saliva, SLPI accounted for more than 70% of the molar concentration of the granulocyte elastase inhibitors studied³⁴. During the inflammation process, elastase was released into oral cavity with GCF, it could do harm to gingival tissue and oral mucosa, but inhibitory effect of SLPI and $\alpha 1$ PI presumably to limit self-damaging excessive inflammation and keep balance.

Uitto⁸ conducted a period of 15 days experimental gingivitis study with 12 adults, in which elastase average levels in rinse saliva decreased from 0.30 ± 0.41 units to 0.09 ± 0.13 units (the difference was not statistically significant). And he additionally pointed out that elastase activity was not a good indicator of gingivitis. This may be also related to the elastase inhibitor present in saliva and GCF mentioned above. In Nieminen's study³⁶, elastase activity in whole saliva was significantly higher in the advanced periodontitis group than in the control group with healthy periodontium, and the activity of salivary elastase correlated significantly with the number of deep gingival pockets (PD ≥ 6 mm) and with either gingival index (GI) or the percentage of bleeding sites (BOP%). We surmise that in the stage of advanced periodontitis, the inhibitors are not enough to inhibit elastase activity, thus perhaps leading to increased EA activity.

IL-6, IL-1 β and calprotectin concentrations in saliva showed good correlations with clinical parameters such as PLI, GI and BI. A number of studies have demonstrated that the levels of some proinflammatory



proteins in GCF could reflect the degree of gingival inflammation. Moreover, our study showed that IL-6, IL-1 β and calprotectin concentrations in saliva also mirror gingival inflammation conditions. Of the three measurements, IL-1 β showed a better correlation with clinical parameters. Zhang's study indicated that IL-1 β release rates increased in the GCF as early as 3 days later of plaque accumulation³⁷. It is generally agreed that IL-1 β is a good indicator of reflecting the severity of periodontal disease and an important mediator in the pathogenesis of periodontal disease³⁸.

The change of IL-6 and IL-1 β in saliva showed a similar tendency throughout the experiment period, the correlation coefficient between IL-6 and IL-1 β levels in saliva was $r = 0.687$ ($P < 0.01$). During the early stage of inflammation, periodontal bacteria and their virulence products could stimulate immune cells, mainly monocytes to produce IL-1 β . At the same time, IL-6 is not spontaneously produced by intact normal cells – in general its secretion requires a stimulus, for example following cellular activation by bacterial LPS or cytokines such as IL-1 β and tumour necrosis factor- α (TNF- α)³⁹. The expression of interleukin-1 receptor 1 (IL-1R1) on the membrane increases and combines with IL-1. The transcription factor nuclear factor κ B (NF- κ B) is activated through Interleukin-1 receptor-associated kinase (IRAK) complex, which could induce IL-6 transcription and release.

There was no obvious relationship between calprotectin and elastase in this study, although both mainly come from neutrophils. The reason may be EA activity was degraded or inhibited by some enzymes in saliva, so its activity decreased unexpectedly following the gingival inflammation progress. Whereas, calprotectin is remarkably resistant to proteolytic degradation and shows good thermal stability^{40,41}. Therefore, during experimental gingivitis, calprotectin concentrations gradually increased and can be easily detected compared with elastase.

This study selected the subjects, who were all non-smoking males aged between 21 and 22 years old and every one had meals in the canteen of the hospital, to ensure consistency and to avoid individual differences as much as possible. It has detected IL-6, IL-1 β , calprotectin and elastase in experimental gingivitis at the same time, which is the first time that we know of. It helped us to learn more about the occurrence and development of gingival inflammation and the host immunity. What is more, our study demonstrated that IL-6, IL-1 β and calprotectin concentrations in saliva could reflect the degree of gingival inflammation. Whether elastase activity was affected by inhibitors in saliva needs further study.

Acknowledgements

The authors thank Assistant Professor Ping Ping HE from the Department of Epidemiology and Biostatistics of Peking University Health Science Centre for her help in the statistical analysis. And the authors also thank Professor Yue Qin SHA and Dr Zhen HUANG for their assistance in the laboratory work.

References

1. van der Zee E, Everts V, Beertsen W. Cytokines modulate routes of collagen breakdown. Review with special emphasis on mechanisms of collagen degradation in the periodontium and the burst hypothesis of periodontal disease progression. *J Clin Periodontol* 1997;24:297–305.
2. Kantarci A, Hasturk H, Van Dyke TE. Host-mediated resolution of inflammation in periodontal diseases. *Periodontol* 2000 2006;40:144–163.
3. Loe H, Theilade E, Jensen SB. Experimental gingivitis in man. *J Periodontol* 1965;36:177–187.
4. Heasman PA, Collins JG, Offenbacher S. Changes in crevicular fluid levels of interleukin-1 beta, leukotriene B4, prostaglandin E2, thromboxane B2 and tumour necrosis factor alpha in experimental gingivitis in humans. *J Periodontol Res* 1993;28:241–247.
5. Wright HJ, Chapple IL, Matthews JB. Levels of TGFbeta1 in gingival crevicular fluid during a 21-day experimental model of gingivitis. *Oral Dis* 2003;9:88–94.
6. Que ML, Andersen E, Mombelli A. Myeloid-related protein (MRP)8/14 (calprotectin) and its subunits MRP8 and MRP14 in plaque-induced early gingival inflammation. *J Clin Periodontol* 2004;31:978–984.
7. Kinane DF, Winstanley FP, Adonogianaki E et al. Bioassay of interleukin 1 (IL-1) in human gingival crevicular fluid during experimental gingivitis. *Arch Oral Biol* 1992;37:153–156.
8. Uitto VJ, Nieminen A, Coil J et al. Oral fluid elastase as an indicator of periodontal health. *J Clin Periodontol* 1996;23:30–37.
9. Zhao Y, Meng H, Chen Z. Calprotectin and total protein in gingival crevicular fluid during experimental gingivitis (in Chinese). *Beijing Da Xue Xue Bao* 2003;35:668–670.
10. Uitto VJ. Gingival crevice fluid--an introduction. *Periodontol* 2000 2003;31:9–11.
11. Kaufman E, Lamster IB. Analysis of saliva for periodontal diagnosis – a review. *J Clin Periodontol* 2000;27:453–465.
12. Ng PY, Donley M, Hausmann E et al. Candidate salivary biomarkers associated with alveolar bone loss: cross-sectional and in vitro studies. *FEMS Immunol Med Microbiol* 2007;49:252–260.
13. Miller CS, King CP Jr, Langub MC et al. Salivary biomarkers of existing periodontal disease: a cross-sectional study. *J Am Dent Assoc* 2006;137:322–329.
14. Van Dyke TE, Kornman KS. Inflammation and factors that may regulate inflammatory response. *J Periodontol* 2008;79(8 Suppl):1503–1507.
15. Terui T, Kato T, Suzuki R et al. Effect of interleukin 1 on chemotaxis and chemiluminescence of human neutrophils. *Tohoku J Exp Med* 1986;149:317–322.
16. Okusawa, S, Yancey KB, van der Meer JW et al. C5a stimulates secretion of tumor necrosis factor from human mononuclear cells in vitro. Comparison with secretion of interleukin 1 beta and interleukin 1 alpha. *J Exp Med* 1988;168:443–448.
17. Akira S, Hirano T, Taga T et al. Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). *FASEB J* 1990;4:2860–2867.

18. Ross KF, Herzberg MC. Calprotectin expression by gingival epithelial cells. *Infect Immun* 2001;69:3248–3254.
19. Kido J, Hayashi N, Kataoka M et al. Calprotectin expression in human monocytes: induction by porphyromonas gingivalis lipopolysaccharide, tumor necrosis factor-alpha, and interleukin-1 beta. *J Periodontol* 2005;76:437–442.
20. Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. *J Leukoc Biol* 1993;53:197–204.
21. Zhao YB, Meng HX, Chen ZB. The clinical observations and the measurement of gingival crevicular fluid volume during the experimental gingivitis (in Chinese). *Zhonghua Kou Qiang Yi Xue Za Zhi* 2004;39:42–44.
22. Mazza JE, Newman MG, Sims TN. Clinical and antimicrobial effect of stannous fluoride on periodontitis. *J Clin Periodontol* 1981;8:203–212.
23. Lie MA, Loos BG, Henskens YM et al. Salivary cystatin activity and cystatin C in natural and experimental gingivitis in smokers and non-smokers. *J Clin Periodontol* 2001;28:979–984.
24. Lie MA, Myint MM, Schenck K et al. Parotid salivary S-IgA antibodies during experimental gingivitis in smokers and non-smokers. *J Periodontol Res* 2002;37:86–92.
25. van Gils PC, Brand HS, Timmerman MF et al. Salivary cystatin activity and cystatin C in experimental gingivitis in non-smokers. *J Clin Periodontol* 2003;30:882–886.
26. Seemann R, Hagewald SJ, Sztankay V et al. Levels of parotid and submandibular/sublingual salivary immunoglobulin A in response to experimental gingivitis in humans. *Clin Oral Investig* 2004;8:233–237.
27. Nair PN, Schroeder HE. Retrograde access of antigens to the minor salivary glands in the monkey *Macaca fascicularis*. *Arch Oral Biol* 1983;28:145–152.
28. Marcotte H, Lavoie MC. Oral microbial ecology and the role of salivary immunoglobulin A. *Microbiol Mol Biol Rev* 1998;62:71–109.
29. Emmings FG, Evans RT, Genco RJ. Antibody response in the parotid fluid and serum of Iru monkeys (*Macaca fascicularis*) after local immunization with *Streptococcus mutans*. *Infect Immun* 1975;12:281–292.
30. Nair PN, Schroeder HE. Local immune response to repeated topical antigen application in the simian labial mucosa. *Infect Immun* 1983;41:399–409.
31. Loos BG, Tjoa S. Host-derived diagnostic markers for periodontitis: do they exist in gingival crevice fluid? *Periodontol 2000* 2005;39:53–72.
32. Cox SW, Rodriguez-Gonzalez EM, Booth V et al. Secretory leukocyte protease inhibitor and its potential interactions with elastase and cathepsin B in gingival crevicular fluid and saliva from patients with chronic periodontitis. *J Periodontol Res* 2006;41:477–485.
33. Beatty K, Bieth J, Travis J. Kinetics of association of serine proteinases with native and oxidized alpha-1-proteinase inhibitor and alpha-1-antichymotrypsin. *J Biol Chem* 1980;255:3931–3934.
34. Ohlsson M, Rosengren M, Tegner H et al. Quantification of granulocyte elastase inhibitors in human mixed saliva and in pure parotid secretion. *Hoppe Seylers Z Physiol Chem* 1983;364:1323–1328.
35. Travis J, Salvesen GS. Human plasma proteinase inhibitors. *Annu Rev Biochem* 1983;52:655–709.
36. Nieminen A, Nordlund L, Uitto VJ. The effect of treatment on the activity of salivary proteases and glycosidases in adults with advanced periodontitis. *J Periodontol* 1993;64:297–301.
37. Zhang J, Kashket S, Lingstrom P. Evidence for the early onset of gingival inflammation following short-term plaque accumulation. *J Clin Periodontol* 2002;29:1082–1085.
38. Stashenko P, Jandinski JJ, Fujiyoshi P et al. Tissue levels of bone resorptive cytokines in periodontal disease. *J Periodontol* 1991;62:504–509.
39. Irwin CR, Myrillas TT. The role of IL-6 in the pathogenesis of periodontal disease. *Oral Dis* 1998;4:43–47.
40. Naess-Andresen CF, Egelanddsdal B, Fagerhol MK. Calcium binding and concomitant changes in the structure and heat stability of calprotectin (L1 protein). *Clin Mol Pathol* 1995;48:M278–284.
41. Marshall WJ and Bangert SK. *Clinical biochemistry: metabolic and clinical aspects*, ed 2. New York: Churchill Livingstone, 2008:246.