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Profiling biomarkers in gingival crevicular fluid using multiplex bead immunoassay

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ABSTRACT

Objective: Biomarkers in gingival crevicular fluid (GCF) have been investigated; however, measurements were limited by the small sample volume available. The aim of this study was to determine the levels of 40 different cytokines and chemokines in GCF samples.
Design: Eleven patients with generalised chronic periodontitis participating in a supportive periodontal therapy programme with remaining probing pocket depths (PDs) of >5 mm were enrolled. One healthy and two diseased sites were sampled in each subject. Forty biomarkers in GCF were examined using a multiplex bead immunoassay. *Porphyromonas gingivalis* from the diseased sites was quantified by real-time polymerase chain reaction.
Results: Twenty-six biomarkers were detected in the GCF samples using the multiplex bead immunoassay. The levels of nine biomarkers were significantly different between the diseased and healthy sites after adjustment with Bonferroni's correction. The level of 26 biomarkers in diseased sites was compared between bleeding on probing (BOP)-positive and BOP-negative sites. Interleukin (IL)-1β and interferon-inducible protein (IP)-10 levels were significantly higher in BOP-positive diseased sites than BOP-negative diseased sites after adjustment for multiple comparisons (IL-1β, $p = 0.0007$, IP-10; $p = 0.0009$). In addition, the levels of IL-1β in GCF were found to be strongly correlated with the *P. gingivalis* ratio ($r = 0.646$, $p = 0.0012$).
Conclusion: IL-1β levels in GCF correlate with the PDs, BOP and the presence of *P. gingivalis* in subgingival plaque. Multiplex bead assays can be useful in GCF studies. These findings can help in identifying new diagnostic methods in the diagnosis of periodontal disease.

Gingival crevicular fluid (GCF) can be collected from the gingival sulcus surrounding the teeth, and it exists either as a serum transudate or more commonly as an inflammatory exudate. GCF contains substances not only from the host but also from microorganisms in the subgingival and supragingival plaque.[1–3] Identifying biomarkers in GCF has become increasingly important in the diagnosis and treatment of periodontal disease.[4,5] The diagnostic potential of GCF has been extensively investigated because of the possibility of non-invasive collection and a rich array of cellular and biochemical molecules associated with disease activity. GCF is the transudate of gingival tissue interstitial fluid, but in periodontal diseases, it is transformed into an inflammatory exudate, which reflects the composition of serum and includes substances derived from the structural tissues of the periodontium and oral bacteria colonising the gingival pocket.[6] The relationships between the subgingival microbiota and host mediators can be examined by analysing subgingival biofilm and GCF samples collected from the same periodontal site. Although Teles et al.[7] and a few other investigators[8–11]

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attempted to correlate the levels of GCF biomarkers with the microbiological data obtained from the same site, most studies were limited by the number of biomarkers investigated.

In the present study, periodontitis was diagnosed almost entirely on the basis of an array of clinical measurements, including the probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP), plaque index (PI) recordings and radiographic findings. However, PD and CAL measurements using periodontal probes and radiographic bone levels provide information about past periodontal tissue destruction but do not elucidate the current state of disease activity or prognosis. Therefore, it is necessary to identify a method for predicting the progression of periodontal diseases or at least to declare the current state of disease activity.Numerous previous studies evaluated the association of periodontal disease with GCF mediators.[12] For example, our group reported the associations of soluble tumour necrosis factor receptor (sTNFR)-1 and sTNFR-2 with periodontitis.[13,14] However, because of the small sample volumes and limitations of the assays used, only a few select cytokines have been evaluated. Therefore, it is difficult to establish an overall perspective of the relationships between various types of cytokines and chemokines. Multiplex immunoassays are highly efficient tools for measuring the levels of multiple proteins in a sample. Several recent reports have quantified cytokines in GCF and saliva using this method.[15–17]

The aim of this study was to detect and quantify the levels of 40 different biomarkers in GCF samples from sites with periodontitis and healthy sites using multiplex immunoassays. In addition, we examined the relationship of *Porphyromonas gingivalis* in the subgingival plaque with the expression of GCF biomarkers in the same diseased sites.

1. Material and methods

1.1. Study population

Clinical examinations were performed, and GCF samples were obtained from 11 subjects with generalised chronic periodontitis from Niigata University Medical and Dental Hospital, Japan. Patient recruitment occurred between September and December 2010. All patients were previously treated for chronic periodontitis and placed on a maintenance regimen for at least 6 months. The patients met the following inclusion criteria: (1) good general health and non-smoking; (2) presence of at least 20 teeth; (3) history of chronic periodontitis; (4) presence of at least three teeth with PDs $\geq$5 mm with concomitant BOP; and (5) signed informed consent. The following were the exclusion criteria: (1) use of systemic or local antibacterial agents in the past 3 months and (2) scaling and root planning treatment under local anaesthesia in the past 3 months. The experimental protocol was approved by the Institutional Review Board of Niigata University Medical and Dental Hospital. Informed consent was obtained from all participants.

1.2. Clinical assessments

All patients were clinically evaluated by a periodontist (YS), licensed by the Japanese Society of Periodontology, for the following indicators of their periodontal condition: (1) number of missing teeth; (2) PD, which was expressed as the mean distance from the free gingival margin to the bottom of the pocket; (3) CAL, which was expressed as the mean distance from the cemento–enamel junction to the bottom of the pocket; and (4) BOP. PD and CAL were assessed using a probe at six sites around each tooth: mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual and distolingual locations. The clinical characteristics of the patients are presented in Table 1.

Table 1 – Clinical parameters of healthy sites and disease site.

	Healthy site	Disease site	p-value
n	11	22	–
Mean PD (±SD)(mm)	2.8 ± 0.4	5.5 ± 0.8	<0.0001
Mean CAL (±SD)(mm)	2.9 ± 0.3	5.8 ± 1.1	<0.0001
Plaque ± (sites)	9/2	5/17	0.002
BOP ± (sites)	11/0	13/9	<0.0001
GCF volume (μl)	1.1 ± 0.2	4.7 ± 0.3	<0.0001
P.g -/+ (sites)	–	9/13	–

1.3. Plaque and GCF collection

One healthy (PD < 3 mm) and two diseased sites (PD = 5–6 mm) were sampled in each subject. Before sampling, the individual tooth site was isolated with cotton rolls, supragingival plaque was removed carefully and the site was gently air-dried with an air syringe. Subgingival plaque samples were taken from the diseased site before GCF sampling. The subgingival plaque samples were collected using paper points (10 s × two times), and the samples were placed in separate microcentrifuge tubes. Then, paper strips were placed into the pocket until mild resistance was sensed and left in place for 30 s to obtain GCF samples. Samples visually contaminated with blood or diluted with saliva during sampling were discarded. The procedure was repeated four times at the same site using new strips each time. The GCF volume was determined on the basis of measurements made using a Periotron 8000 (Oraflow Inc., Plainview, NY, USA). After recording the measurements, they were converted into actual GCF volumes using a calibration graph. The GCF samples were placed in tubes with a transport medium containing 200 μl of phosphate-buffered saline (PBS) without calcium chloride or magnesium chloride, supplemented with 0.5% bovine serum albumin. After shaking for 15 min, the eluates were centrifuged for 10 min at 12,000 × *g* to remove plaque and cellular elements and the strips were removed. The samples were frozen at −80 °C until further analysis.

1.4. Cytokine and chemokine analysis

The following cytokines and chemokines were analysed in this study – cytokines: interleukin (IL)-1α, IL-1β, IL-1 receptor antagonist (IL-1RA), IL-4, IL-6, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17A, IL-21, IL-23 and tumour necrosis factor (TNF)-α; chemokines: macrophage chemotactic protein (MCP)-1, MCP-3, regulated on activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and interferon-inducible protein (IP)-10; matrix