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Relationship between periodontal disease status and combination of biochemical assays of gingival crevicular fluid

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Background: Currently, no biochemical assay involving gingival crevicular fluid is utilized routinely as a screening test for periodontal disease.

Objective: The objective of the present study was to evaluate the potential of gingival crevicular fluid assay as a screening methodology.

Methods: The subject population was comprised of 27 volunteers. Nine participants were classified as 'subject with periodontal destruction' (SPD) exhibiting at least one site with pocket depth and attachment loss > 3.5 mm, whereas the remaining individuals were categorized as 'subject with minimal periodontal destruction' (SMD). Gingival crevicular fluid was collected from fixed sites via a standardized method. Biochemical assays of 12 substances (hemoglobin, albumin, transferrin, α_1-antitrypsin, fibronectin, IgA, IgG, IgM, lactoferrin, myeloperoxidase and neutrophil elastase) were conducted at a commercial laboratory. Power transformation of total quantities in gingival crevicular fluid was performed for statistical analysis.

Results: Relationships between total quantity of each substance and periodontal disease status were unclear. Logistic regression analysis yielded six predictive models, which consisted of substance pairs: neutrophil elastase/IgA, neutrophil elastase/hemoglobin, neutrophil elastase/α_1-antitrypsin and neutrophil elastase/IgG, and IgA/albumin and IgA/transferrin ($p < 0.05$). Regression lines for SPD and SMD on a scattergram of IgA and neutrophil elastase were nearly parallel within the range of amounts in gingival crevicular fluid. The predictive model derived from both substances afforded sensitivity and specificity of 88% and 94%, respectively.

Conclusions: These results indicated that the combination of IgA and neutrophil elastase in gingival crevicular fluid may be crucial for prediction of periodontal disease status. Furthermore, these data suggested that biochemical assays employing both substances in gingival crevicular fluid may provide a satisfactory screening test for periodontal disease.

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Periodontal disease is characterized by chronic inflammatory lesions and destruction of supportive periodontal tissue. Subjective symptoms are typically mild during progression of the disease. In the early phase of periodontal disease, subjects tend to ignore the condition until more severe symptoms appear, for example, increased tooth mobility. Periodontal disease may be a major cause of tooth loss. In Japan, periodontal disease and dental caries display similar significance in terms of reason for tooth extraction (1). Accumulation of tooth loss leads to impairment of essential functions in quality of later life, i.e. conversation and eating. Thus, early detection of periodontal disease may be important with respect to quality of later life.

Gingival crevicular fluid and saliva contain substances associated with the host response, which could reflect periodontal disease status; therefore, determination of biochemical substances in oral fluid may be appropriate for early detection of periodontal disease (2, 3). Saliva is secreted from salivary glands; consequently, basic components may reflect a general condition rather than oral disease status. Salivary cotinine, a metabolite of nicotine, is utilized routinely as an indicator of current smoking (4). In lieu of crude saliva, whole saliva may include substances derived from various sites in the oral cavity. A few substances in whole saliva were correlated with periodontal disease: beta-glucuronidase activity (5), IgA1 (6) and elastase (7). Hemoglobin in whole saliva was associated with gingival inflammation (8, 9).

Several studies suggested the availability of assays employing saliva for early detection of periodontal disease; however, none of these tests is, at present, applied routinely (3). Gingival crevicular fluid contains informative substances close to periodontal tissue; as a result, gingival crevicular fluid may be a more suitable screening test sample in comparison with saliva. However, several barriers to routine utilization of gingival crevicular fluid screening methodology exist. Samples, which are collected from a single site in the mouth, may not reflect the whole mouth status of the subject. Cost-effectiveness of bulk biochemical assays could also be an important consideration in terms of widespread application of this screening approach. In general, reagents essential for the assay of special substances may be expensive. A model capable of predicting periodontal disease activity using gingival crevicular fluid was developed (10). However, this model, which requires assessment of six substances, necessitates special assays.

Inexpensive assays of peripheral blood and urine specimens are performed routinely in bulk by commercial laboratories designed for general-health examination of large populations. The examination evaluates individual health status via a combination of several assays. Therefore, combination of those biochemical assays commonly applied to specimens of peripheral blood and urine could also be applied to gingival crevicular fluid samples. The objectives of the present investigation were to examine relationships between periodontal disease status and combination of biochemical assays with respect to gingival crevicular fluid samples and to evaluate a predictive model pertaining to subjects with periodontal disease.

Material and methods

The current study deals with a screening test; as a result, subjects were selected from the general population. Participants consisted of 27 volunteers (15 males and 12 females, 41.8 ± 11.0 years of age, average ± SD). Subjects were office personnel employed by a business in Kyoto prefecture, Japan. The protocol, which was designed within the guidelines of the Declaration of Helsinki of the World Medical Association, was approved by the company's occupational safety and health committee. Informed consent was obtained from all participants prior to the study.

Collection of gingival crevicular fluid and periodontal assessments were performed on a portable bed under sufficient light provided by a light apparatus (Cold Light Supply, Nagashima Inc., Tokyo, Japan) at the workplace. Gingival crevicular fluid samples were collected with a paper strip at three fixed sites per subject: mesio-palatal line angles of the upper maxillary first molars on both sides and at the deepest pocket within the mouth. The deepest pocket was identified in advance during a pilot examination 3 months prior to this investigation. Prior to collection of gingival crevicular fluid, supragingival plaque was removed with a cotton ball. A paper strip (Periopaper, Proflow Inc., Amityville, NY, USA) was inserted into the gingival crevice until resistance was encountered; it remained in place for 30 s. The paper strip was removed and stored in a sampling tube containing 1 ml of 0.05 M Tris-HCl buffer (pH 7.5) with 0.1% bovine serum albumin (Serological Proteins Inc., Kankakee, IL, USA) and 0.1% sodium azide (Nacalai Tesque, Kyoto, Japan) as preservative. The samples were then delivered to a commercial laboratory for the assays.

Following collection of gingival crevicular fluid, clinical assessments were conducted: pocket depth and attachment loss were measured at the mesial surfaces of the labial/buccal and lingual/palatal aspects of all existing teeth, with the exception of the third molars, with a pressure-sensitive probe (Vivacare TPS Probe, Vivadent, Schaan, Lichtenstein). Supragingival plaque and gingival inflammation were assessed with the plaque index (11) and the modified gingival index (12), respectively. Generally, screening tests are utilized for early detection of non-apparent disease, whereas dichotomous classification such as 'negative' and 'positive' functions in the tests to distinguish corresponding disease status. Therefore, in the present study, two categories, 'subject with periodontal destruction (SPD)' and 'subject with minimal periodontal destruction (SMD)' were employed, despite the general utility of three categories, namely, healthy, gingivitis and periodontitis, for periodontal diseases in the clinical setting. The periodontal disease category was defined on the basis of pocket depth and attachment loss dichotomy. Individuals demonstrating at least one site at which both pocket

depth and attachment loss exceeded 3.5 mm were defined as SPD. Remaining subjects, including gingivitis subjects, were defined as SMD.

At the commercial laboratory, elution of constituents from the paper strips was performed with 1 ml of samples by gentle agitation for 30 min. Gingival crevicular fluid samples were diluted for the multiple assays of 12 substances. Hemoglobin, albumin, transferrin, α_1-antitrypsin, fibronectin, IgA, IgG, IgM, lactoferrin, myeloperoxidase and neutrophil elastase were assessed by sandwich enzyme-linked immunosorbent assays (13, 14). Anti-human polyclonal antibodies of hemoglobin, albumin transferrin, α_1-antitrypsin IgA, IgG, IgM, myeloperoxidase (preceding seven acquired from Dakopatts, Glostrup, Denmark), fibronectin, neutrophil elastase and lysozyme (preceding three acquired from Serotec, Oxford, England) were introduced to the wells of 96-well microplates and absorbance was measured spectroscopically (Multiscan MS UV, Labsystems, Helsinki, Finland). The analyzer, which displayed sufficient sensitivity so as to permit detection of substances, required a 0.05-ml aliquot of sample in each assay for total amount of proteins in gingival crevicular fluid. Assay of lactoferrin was completed with a commercially available kit (Shionogi & Co., Ltd, Osaka, Japan). These assays were performed according to the manufacturer's recommended protocols.

Screening tests were evaluated with respect to sensitivity and specificity (15). Screening tests, which accurately identify individuals presenting with disease as well as disease-free subjects, are usually required. Sensitivity is the proportion of persons with active disease identified as positive by the screening test. Specificity is the proportion of persons without active disease correctly identified by the screening test. The probability of sensitivity or specificity ranges from 0 to 1. Probabilities approaching 1 result in categorization of a test as either highly sensitive or highly specific.

Total amount of each substance in the sample was enumerated. Distribution of gingival crevicular fluid sample quantities was usually skewed to lower values; therefore, data were transformed according to the established formula; $X = ((x - a)^p - 1)/p$. If $p = 0$, $X = \log(x - a)$. Differences between SPD and SMD in terms of total amount in gingival crevicular fluid were examined with the Mann–Whitney test. Multiple logistic regression analysis was employed to develop a predictive model of periodontal disease status (SPD or SMD). Quantities of the substances in the sample served as independent variables in the backward stepwise elimination method. Statistical analyses including the power transformation were conducted with computer software (STATFLEX, Artech, Osaka, Japan). The level of significance was set at 5%.

Results

Following clinical assessment, nine (six males and three females) and 18 subjects were identified as SPD and SMD, respectively (Table 1). Mean values of the plaque index and the modified gingival index were similar between groups: 1.1 for plaque index and 0.9 for modified gingival index ($p > 0.05$). Pocket depth and attachment loss in SPD were significantly higher than corresponding parameters in SMD ($p = 0.0152$ and 0.0024, respectively). Mean values of pocket depth and attachment loss were calculated at two fixed sites where gingival crevicular fluid samples were collected. Pocket depth was 2.5 ± 0.6 mm for SMD and 3.2 ± 1.0 mm for SPD, whereas attachment loss values were 0.5 ± 0.9 mm and 2.4 ± 1.7 mm, respectively. The values at fixed sites were higher than the mean values of the whole mouth. Differences between SMD and SPD were significant in pocket depth and attachment loss ($p = 0.0367$ and $p = 0.0096$, respectively).

Descriptive statistics of total quantity of 12 substances in gingival crevicular fluid are presented in Table 2. Mean value of total amount exceeded median value for all substances. For hemoglobin, albumin, transferrin and fibronectin, mean value of total amount exceeded the 75th percentile. Distributions of total amount were apparently skewed to lower levels for all substances (data not shown).

Table 1. Comparisons of clinical indices between subject with minimal periodontal destruction (SMD) and subject with periodontal destruction (SPD) groups

Clinical indices	All ($N = 27$)	SMD ($N = 18$)	SPD ($N = 9$)	p
Plaque index	1.1 ± 0.7	1.1 ± 0.8	1.1 ± 0.7	0.9211
Modified gingival index	0.9 ± 0.6	0.9 ± 0.7	0.9 ± 0.6	0.9208
Pocket depth (mm)	2.2 ± 0.4	2.1 ± 0.4	2.5 ± 0.5	0.0152
Attachment level (mm)	0.4 ± 0.5	0.2 ± 0.4	0.8 ± 0.5	0.0024

Table 2. Total amounts of biological markers in gingival crevicular fluid samples from 81 sites in 27 subjects

Biological marker	Mean ± SD	Median	IQR[a]
Hemoglobin (μg)	7.0 ± 26.4	0.4	0–3.1
Albumin (μg)	13.9 ± 26.7	3.1	2.1–10.7
Transferrin (μg)	0.55 ± 0.63	0.34	0.22–0.53
α_1-antitrypsin (μg)	0.72 ± 0.62	0.57	0.26–1.01
IgA (μg)	0.39 ± 0.31	0.28	0.17–0.52
IgG (μg)	2.5 ± 2.4	1.5	1.1–3.1
IgM (μg)	0.14 ± 0 .21	0.06	0.02–0.14
Myeloperoxidase (ng)	86.0 ± 64.0	71.6	39.2–114.3
Fibronectin (ng)	115.9 ± 183.3	47.4	23.9–104.3
Neutrophil elastase (ng)	40.0 ± 40.0	24.8	15.7–48.4
Lysozyme (ng)	53.1 ± 49.1	36.5	20.7–73.9
Lactoferrin (ng)	150.9 ± 133.7	113.2	50.5–186.7

[a]IQR, inter-quartile range (25th percentile–75th percentile).

Table 3. Comparisons of total amount of biological markers in gingival crevicular fluid on a site basis between subject with minimal periodontal destruction (SMD) and subject with periodontal destruction (SPD)

	SMD ($N = 54$)		SPD ($N = 27$)	
Biological marker	Median	IQR[a]	Median	IQR[a]
Hemoglobin (μg)	0.3	0–2.5	0.4	0–4.7
Albumin (μg)	3.2	2.4–10.6	2.7	1.8–14
Transferrin (μg)	0.33	0.25–0.48	0.37	0.13–0.86
α_1-antitrypsin (μg)	0.49	0.27–0.89	0.74	0.23–1.21
IgA (μg)	0.24	0.16–0.45	0.43	0.21–0.77
IgG (μg)	1.4	1.1–3.1	1.8	0.9–3.1
IgM (μg)	0.07	0.02–0.15	0.05	0.02–0.14
Myeloperoxidase (ng)	71.6	33.6–120.8	79.5	42.5–113.3
Fibronectin (ng)	49.5	19.2–78.7	47.1	25.2–159.4
Neutrophil elastase (ng)	28.8	16.3–50.3	20.1	8.7–43.8
Lysozyme (ng)	40.1	19.6–75.2	34.4	23.3–57.3
Lactoferrin (ng)	118.6	48.4–211.5	104.0	52.7–161.4

[a]IQR, inter-quartile range.
Significant differences were not detected for any substance.

Total amount of each substance was compared between the SPD and SMD groups on a site basis (Table 3). SPD was greater than SMD with respect to median values of hemoglobin, transferrin, α_1-antitrypsin, IgA, IgG and myeloperoxidase. However, no significant difference was observed between SMD and SPD for any substance.

Periodontal disease status was predicted by multiple logistic regression analysis. Transformed amounts served as independent variables. Analyzed samples were utilized to determine whether any combinations of biochemical substances in gingival crevicular fluid were predictive of periodontal disease status. The deepest site was identified in advance following periodontal examination; therefore, data obtained from the deepest site was excluded from this model. The periodontal examination is itself definitive for periodontal disease status. Regression analyses were performed exclusively with data derived from maxillary first molars. These analyses yielded six predictive models (Table 4). All models consisted of two significant variables ($p < 0.05$). Neutrophil elastase was included in four models, whereas IgA was a component of three models. Albumin, transferrin, hemoglobin, α_1-antitrypsin and IgG were included in each model.

Total amounts of two substances, which were significant for that specific model, were plotted on a scattergram; subsequently, regression lines for SPD and SMD were generated separately (Fig. 1). Regression lines for SPD and SMD crossed within the range of quantities detected in five models (Figs 1A, B, D, E and F). Regression lines were nearly parallel on the scattergram of neutrophil elastase and IgA (Fig. 1C).

On the scattergram of neutrophil elastase and IgA (Fig. 1C), two points, which represent gingival crevicular fluid samples from the same subject, were connected with a line (Fig. 2). Most of the lines were generated in a direction similar to that of the regression lines depicted in Fig. 1(C). Lines, which were directed perpendicular to regression lines, tended to be short, i.e. similar quantities were obtained from both sites in the same subject.

Quantities of neutrophil elastase and IgA were represented by higher values in the samples for each participant in order to develop a practical predictive model of periodontal disease status employing two samples of gingival crevicular fluid from fixed sites. The representative values of both substances in SPD and SMD were then plotted on a scattergram (Fig. 3). Regression lines were, again, parallel. Plots of either SPD or SMD were located close to each line. Most plots for SPD were located beneath those for SMD. This trend was consistent in the range of approximately 100-fold of the amount in gingival crevicular fluid: 0.02–2.0 μg for IgA and 1–100 ng for neutrophil elastase.

Predicted probabilities of SPD and SMD were enumerated in the multiple logistic regression analysis using the pair of representative values. Total amounts of IgA and neutrophil elastase were compared between SPD and SMD (Figs 4A and B, respectively). Predicted values were also compared between SPD and SMD (Fig. 4C). The plots in both groups overlapped with respect to IgA and neutrophil elastase; predicted values provided clearer separation of both groups. When an approximate cut-off point was defined, sensitivity and specificity of the test were 88% and 94%, respectively.

Table 4. Combinations of significant substances capable of predicting periodontal disease in six regression models

# of Model	Independent variable #1	*p*	Independent variable #2	*p*
1	IgA	0.0131	Albumin	0.0436
2	IgA	0.0098	Transferrin	0.0313
3	IgA	0.0272	Neutrophil elastase	0.0196
4	Hemoglobin	0.0098	Neutrophil elastase	0.0189
5	α_1-antitrypsin	0.0394	Neutrophil elastase	0.0486
6	IgG	0.0421	Neutrophil elastase	0.0275

Discussion

The ultimate goal of the present investigation was to examine evidence regarding the potential utilization of gingival crevicular fluid assays as a screening test for periodontal disease. As expected, relationships between total quantity of substances in gingival crevicular fluid and periodontal disease status were unclear. In logistic regression analysis of periodontal disease status, which functioned as the

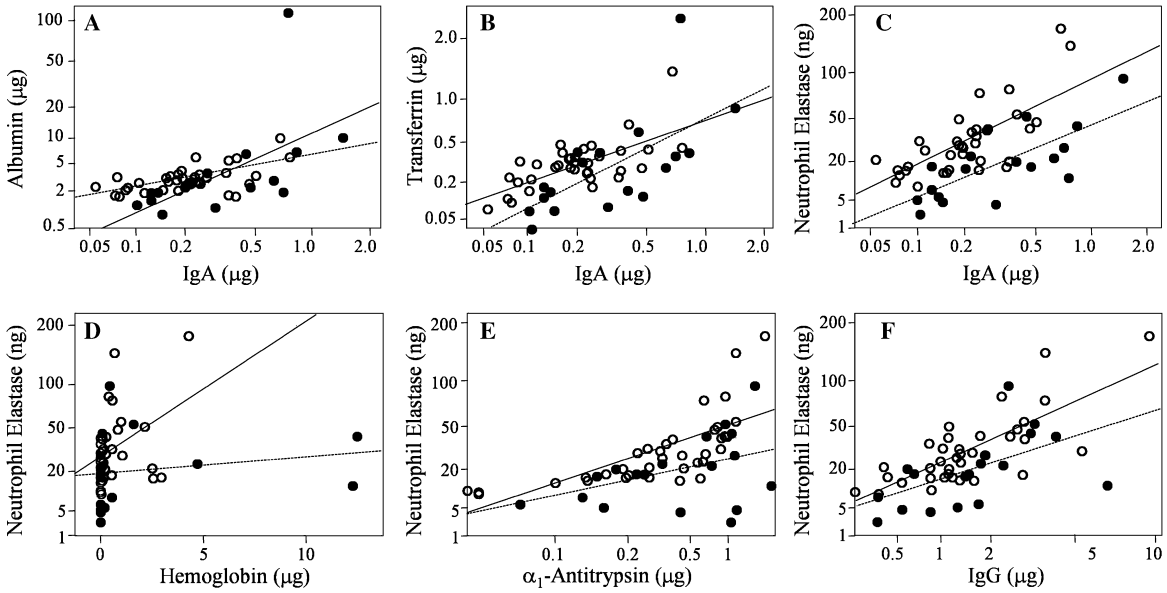

Fig. 1. Scatter plots of total quantities of two substances that were significant in each predictive model. Regression lines were generated for subject with periodontal destruction (SPD: broken line for closed circles) and subject with minimal periodontal destruction (SMD: solid line for open circles) samples.

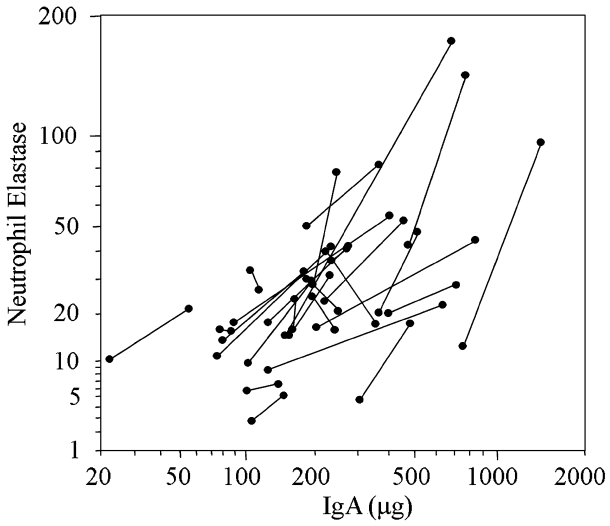

Fig. 2. Scattergram of total amount between neutrophil elastase and IgA: each line is derived from two points, which represent gingival crevicular fluid samples from the same subject.

dependent variable, the output consisted of the combination of total amounts of two substances. Scatter plots constructed from such substances indicated that the combination of IgA and neutrophil elastase was the most satisfactory predictive model of periodontal disease status. Subsequently, IgA and neutrophil elastase were employed in the predictive model of periodontal disease status; additionally, sensitivity and specificity of the test were satisfactory.

Among 12 substances in gingival crevicular fluid evaluated in logistic regression models, IgA and neutrophil elastase, individually, displayed meaningful predictive ability with respect to SPD. Furthermore, combination of IgA and neutrophil elastase afforded the most predictive model. Thus, IgA and neutrophil elastase may be key substances for prediction of SPD. Protective roles of IgA in gingival crevicular fluid against periodontal destruction have been demonstrated (16–18). IgA in gingival crevicular fluid was associated with periodontal disease (16). In saliva, secretory IgA constitutes the main specific immune defense mechanism and may play an important role in the homeostasis of oral microorganisms (19). Detection of IgA antibodies specific to *Porphyromonas gingivalis* in gingival crevicular fluid could be utilized as a predictive parameter of periodontal infection (20). Neutrophil elastase, which was derived from the effector system of acute inflammatory response, was also identified in gingival crevicular fluid of patients with periodontal disease (21).

Biological rationale as to the connection between IgA and neutrophil elastase in gingival crevicular fluid is interesting. Predicted value, which was calculated from transformed amounts of IgA and neutrophil elastase, may express degree of relative connection between systems for protection by IgA and for acute inflammatory response. In the present investigation, relative rate of the quantity of IgA transformed to neutrophil elastase in gingival crevicular fluid was higher in SPD than

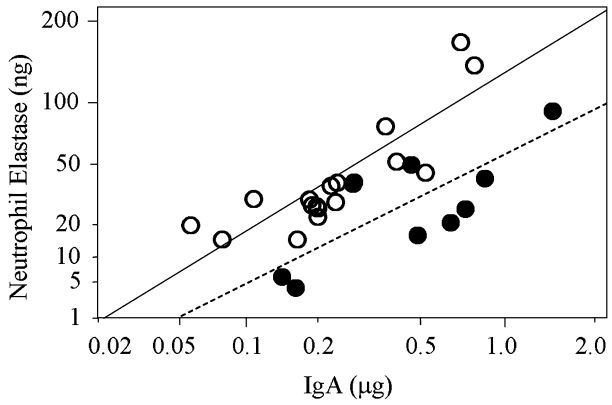

Fig. 3. Scatter plots of total amount with higher values of neutrophil elastase and IgA in two samples from the same subject. Regression lines represent subject with periodontal destruction (SPD: broken line for closed circles) and subject with minimal periodontal destruction (SMD: solid line for open circles).

that in SMD. Thus, an imbalance between both systems might appear in the gingival crevicular fluid of SPD. Recent studies regarding intensive monitoring of periodontal disease status focused on matrix metalloproteinase in gingival crevicular fluid (22–24). In IgA nephropathy, an IgA-specific disease in kidney, matrix metalloproteinases play an important role in mesangial cell activation (25, 26). These findings may underscore the importance of the role of IgA observed in this study.

Procedures capable of predicting periodontal disease status developed in the present investigation may be uncomplicated: two samples of gingival crevicular fluid were obtained via placement of a standardized paper strip at fixed sites for 30 s. Samples, which were stored in sampling tubes, were then delivered to the commercial laboratory for biochemical assay. The assays were conducted routinely in the laboratory; thus, these protocols could be performed in bulk in a satisfactory manner.

On the scatter plots, the lines between two points, which represented data derived from two sites in the same subject, were nearly parallel to regression lines. The other short lines, which were directed perpendicular to the regression line, indicated that similar quantities were obtained from both sites. These findings revealed that, despite the differing quantities of gingival crevicular fluid occurring in two samples from the same subject, the relative relation between IgA and neutrophil elastase is consistent between the two samples. In other words, the amount of gingival crevicular fluid may exert little influence on the relative relation. The difference in the relative relation between IgA and neutrophil elastase in gingival crevicular fluid was consistent throughout the range of approximately 100-fold in terms of the amounts in gingival crevicular fluid. This trend may increase the availability of gingival crevicular fluid assays for screening purposes. The quantity of gingival crevicular fluid may be limited; however, the sample could be utilized for prediction of periodontal disease status. Amount of gingival crevicular fluid did not display normal distribution for all substances. Power transformation of the amounts of gingival crevicular fluid may also contribute to these promising results.

Mean values of pocket depth and attachment loss at fixed sites were higher than those of the whole mouth. Generally, maxillary molars and mandibular incisors display a potentially greater susceptibility to periodontal destruction relative to other teeth (27). The fixed sites where gingival crevicular fluid samples were collected could reflect periodontal disease status of the subject. Significant differences between SMD and SPD with respect to pocket depth and attachment loss were also apparent at the fixed sites. In contrast to the distinctions in the clinical indices, no significant difference was observed between SMD and SPD in terms of the amount of any substances in gingival crevicular fluid. However, combination of two substances in gingival crevicular fluid at the fixed sites could be crucial for prediction of

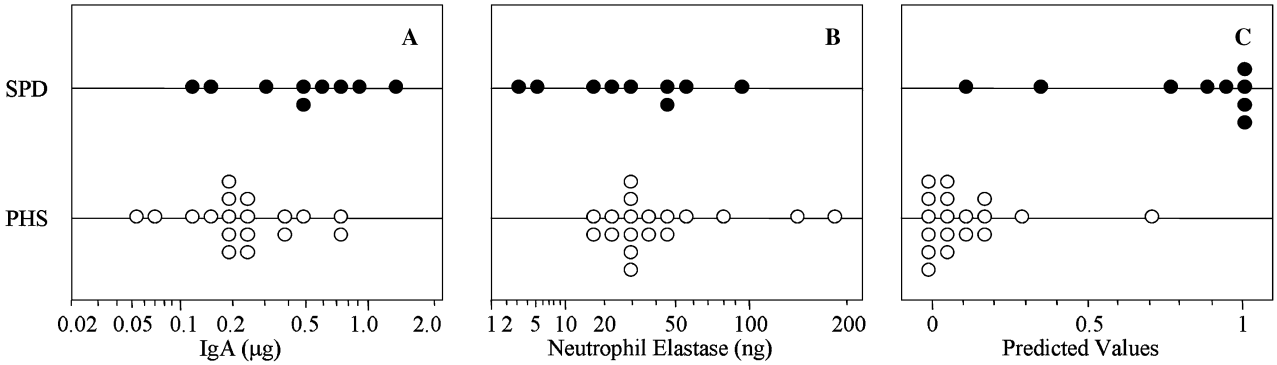

Fig. 4. Comparisons of representative values of IgA (A), neutrophil elastase (B) and predicted values (C) between subject with periodontal destruction (SPD) and subject with minimal periodontal destruction (SMD).

periodontal destruction. Ideally, utilization of a combination of two substances in gingival crevicular fluid collected at any site in the mouth, including healthier sites, may be incorporated for the prediction.

Content of gingival crevicular fluid substances is generally expressed as concentration and/or total amount. In the present study, expression of content in gingival crevicular fluid samples was based on total amount per 30-s sample. In order to express content of gingival crevicular fluid substance as concentration, measurement of gingival crevicular fluid volume is necessary. Gingival crevicular fluid volume in the paper strips that were used for collection of gingival crevicular fluid is determined via electric impedance; thus, the measurement requires a special device as well as an additional procedure. Generally, less expensive, easier screening methods are essential. Several investigators described the superiority of expression as total amount per timed sample (2, 28, 29). Upon comparison of sites characterized by similar amounts of the substance in gingival crevicular fluid, dilution of the substance by fluid may be greater in sites exhibiting larger gingival crevicular fluid volume than in sites with less gingival crevicular fluid volume. In the case of small gingival crevicular fluid volume, which was evident in healthier sites, measurement of fluid volume often resulted in a wide variation of concentration of the substance in gingival crevicular fluid. With respect to the accuracy of measurement, contamination by saliva may exert considerable influence on the concentration of the gingival crevicular fluid substances in sites with less gingival crevicular fluid.

In the present study, combination of IgA and neutrophil elastase in gingival crevicular fluid could afford prediction of periodontal disease status regardless of amount of gingival crevicular fluid. Delivery of samples to the commercial laboratory and biochemical assays were routinely performed in instances involving peripheral blood and urine specimens. In order to establish the gingival crevicular fluid test as a routine method for periodontal disease screening, additional investigation of more convenient sampling procedures and validation may be necessary.

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