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Levels of specific immunoglobulin G to the forsythia detaching factor of *Tannerella forsythia* in gingival crevicular fluid are related to the periodontal status

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Background and Objective: Forsythia detaching factor (FDF) is a putative virulence factor of *Tannerella forsythia* that induces detachment of adherent cells and interleukin-8 production in human fibroblasts. The objective of the present study was to clarify the relationship between anti-FDF IgG levels in gingival crevicular fluid and the clinical status in patients with periodontitis and in healthy subjects.

Material and Methods: Gingival crevicular fluid and subgingival plaque samples were obtained from both the diseased and healthy sites of 37 patients with periodontitis and from 30 healthy subjects. Anti-FDF IgG levels were evaluated, and both the *fdf* gene and *T. forsythia* 16S ribosomal RNA (rRNA) were detected using the PCR.

Results: Anti-FDF IgG levels (of both diseased and healthy sites) of patients with periodontitis were significantly higher than those of healthy subjects. Among the patients with periodontitis, anti-FDF IgG levels of healthy sites were significantly higher than those of diseased sites and the levels showed negative correlations with probing pocket depth and clinical attachment level. Among the patients with periodontitis, *T. forsythia* 16S rRNA was detected in 18 of 37 diseased sites and in 5 of 29 healthy sites, and the *fdf* gene was detected in 19 of 37 diseased sites and in 7 of 29 healthy sites. By contrast, no healthy subjects were positive for *T. forsythia* 16S rRNA or the *fdf* gene.

Conclusion: These data suggest that anti-FDF IgG levels in gingival crevicular fluid are related to the periodontal status.

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It has been established that the etiology of human periodontal disease is dental plaque, a biofilm consisting of more than 500 different bacterial species and their products (1,2). Epidemiological data, from both longitudinal and cross-sectional studies, strongly suggest that a population shift towards gram-negative anaerobic species in

dental plaque is responsible for the initiation and progression of periodontal disease. Therefore, understanding the microbial pathogenesis of periodontal disease requires a detailed analysis of the complicated interactions between the host and gram-negative anaerobic bacterial species in the periodontal environment. *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* comprise a 'red complex' of species that is highly correlated with periodontal disease sites (3). One of these species of bacteria, *T. forsythia,* is a gram-negative, anaerobic, fusiform bacterium. The association of *T. forsythia* with periodontitis, including chronic and aggressive periodontitis, has been observed in a number of studies from populations around the world (4). The roles of *P. gingivalis* and *T. denticola* are well established in periodontitis development and progression. However, only a few putative virulence factors of *T. forsythia* have been identified, such as a trypsin-like protease (5), a sialidase (6), a cell-surface-associated and secreted BspA protein (5), a hemagglutinin (7), components of the bacterial S-layer (8), methylglyoxal production (9), an apoptosis-inducing activity (10) and forsythia detaching factor (FDF) (11). These factors may initiate disease by providing bacterial growth in the periodontal pockets by eliminating host immune cells through the induction of apoptosis or necrosis. Recently, we reported that FDF induced the production of a pro-inflammatory cytokine, interleukin-8, in normal fibroblasts (12). There are some reports that patients with periodontitis have high levels of antibody against periodontopathic bacteria (13–16). However, few reports have examined the immune response against purified antigens of *T. forsythia* in patients with periodontitis and none has examined the correlation between periodontitis and FDF. The objective of this study was to determine the relationship between anti-FDF immunoglobulin G (IgG) levels in gingival crevicular fluid and clinical parameters in patients with periodontitis and periodontally healthy subjects, and to confirm the existence of the *fdf* gene in subgingival plaque using the conventional PCR.

Material and methods

Study population

Thirty-seven patients with chronic or aggressive periodontitis (14 men and 23 women; mean age 48.6 ± 13.0 years) were recruited from the Section of Periodontology, Dental Hospital, Tokyo Medical and Dental University. Each patient had more than 20 teeth present and exhibited at least two teeth with > 4 mm probing depth in half of the jaw. Thirty periodontally healthy subjects (9 men and 21 women; mean age 27.3 ± 7.3 years) constituted the control group. In neither group did subjects have severe systemic disease that might affect the immunological response, had not previously received periodontal treatment, and had received no antibiotics for the past 3 mo.

A summary of the clinical characteristics of the study population and sampling sites are listed in Table 1A,B, respectively. All subjects gave informed consent for the collection of gingival crevicular fluid and subgingival plaque samples and for periodontal status assessment. This study was approved by the Ethical Committee of our university.

Collection of gingival crevicular fluid

In patients with periodontitis, gingival crevicular fluid samples were collected from two selected sites according to probing pocket depth: healthy sites ≤ 3 mm (n = 29) and diseased sites ≥ 4 mm (n = 37). In periodontally healthy subjects, a site with a periodontal index score of 0 and no bleeding on probing was selected as the collection site. Generally, the samples were taken from the mesio-buccal site of the maxillary left first premolar; for subjects missing this tooth, samples were taken from the maxillary right first premolar. Areas of collection were isolated with cotton rolls to avoid contamination with saliva, gently air dried and supragingival plaque was carefully removed using sterile cotton. Gingival crevicular fluid was collected on sterile paper strips (Periopaper®; Ora*flow* Inc., Smithtown, NY, USA). The paper strip was inserted at the bottom of the pocket or sulcus until slight resistance was felt, held in place at the site for 30 s and transferred to a microtube following measurement of the volume of gingival crevicular fluid. The volume of gingival crevicular fluid in the paper strip was measured using a Periotron-6000® reader (Ora*flow* Inc). Sufficient amounts of gingival crevicular fluid for ELISA were obtained from the same sites. Gingival crevicular fluid was extracted from the paper strips into 200 μL of phosphate-buffered saline (PBS: 50 mM, pH 7.2, containing 0.05% Tween 20) by gentle shaking for 15 min. Paper strips suspected of being contaminated with blood and saliva were excluded from the study. The samples were stored at −80°C until required for assay. Following collection of the gingival crevicular fluid samples, clinical measurements were carried out to characterize the sampled sites.

Preparation of the recombinant FDF

The recombinant FDF used in this study has been reported previously (11). Briefly, the expression plasmid, pQE-FDF, which contains the entire *fdf* structural gene, was constructed. The transgenic *Escherichia coli* strain BL21 (DE3) carrying this plasmid was cultured in Luria–Bertani broth and induced with 400 μM isopropyl-β-D-thiogalactopyranoside. The bacterial cells were harvested, sonicated with phosphate buffer (10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 400 mM KCl) and centrifuged to obtain the cleared lysate. The lysate was loaded onto a Nickel–Sepharose column and the recombinant FDF was eluted using imidazole concentrations of between 300 and 500 mM without any contaminants. The purified protein was desalted with PBS, concentrated and stored at −80°C.

Biotinylation of recombinant FDF

Recombinant FDF (rFDF) was biotinylated using the EZ-Link® Sulfo-NHS-LC-Biotinylation kit (Pierce,

Table 1. Clinical characteristics of (A) the study population and (B) sampling sites

(A)

	Periodontally healthy subjects	Periodontitis patients
	$n = 30$	$n = 37$
Age (years)	27.3 ± 7.3	48.6 ± 13.0*
Gender (male : female)	9 : 21	14 : 23
Number of teeth	27.4 ± 1.2	26.9 ± 3.2
Bleeding on probing (% of sites)	2.3 ± 6.8	48.0 ± 30.0*
Mean probing pocket depth (mm)	1.7 ± 0.2	3.4 ± 1.0*
Mean clinical attachment level (mm)	1.7 ± 0.3	3.8 ± 1.4*

(B)

		Periodontitis patients	
	Periodontally healthy subjects	Diseased sites PPD ≥ 4 mm	Healthy sites PPD ≤ 3 mm
	$n = 30$	$n = 37$	$n = 29$
Probing pocket depth (mm) (sampling site)	2.3 ± 0.6	5.7 ± 1.6**	2.1 ± 0.7
Clinical attachment level (mm) (sampling site)	2.4 ± 0.6	6.3 ± 1.7**	2.4 ± 1.0
Gingival crevicular fluid volume (μL)	0.79 ± 0.25	0.86 ± 0.23***	0.72 ± 0.15

Data are expressed as mean ± standard deviation.
PPD, probing pocket depth.
*Statistically significantly higher than periodontally healthy subjects (Mann–Whitney *U*-test, $p < 0.05$).
**Statistically significantly higher than healthy sites (Mann–Whitney *U*-test, $p < 0.05$).
***Statistically significantly higher than healthy sites in periodontitis patients (Mann–Whitney *U*-test, $p < 0.05$).

Rockford, IL, USA) according to the instructions given in the manufacturer's manual (PIERCE 21435). In brief, 1 mg (approximately 17 nmoles) of rFDF was dissolved in 1 mL of PBS and reacted with 30 μL of 10 mM EZ-Link® Sulfo-NHS-LC-Biotinylation reagent (0.3 μmoles) for 1 h on ice. The reaction mixture was then applied to the Sephadex G-50 column (5-mL bed volume) equilibrated with PBS and the protein fraction and the salt-fraction containing the unreacted reagent were separated.

ELISA for anti-FDF IgG

Measurement of anti-FDF IgG concentrations in the gingival crevicular fluid was carried out using an ELISA. In brief, avidin-conjugated ELISA plates (NUNC IMMOBILIZER STREPTAVIDIN CLEAR F96; Nunc A/S, Roskilde, Denmark) were coated with 100 μL of biotinylated FDF diluted to 0.01 mg/mL in PBS buffer (pH 7.5) and incubated at room temperature for 2 h. After washing, 20 μL of gingival crevicular fluid in 80 μL of 0.05% fetal calf serum/PBS was loaded into duplicate coated wells and incubated at room temperature for 1 h. Then, 100 μL of rabbit anti-human horseradish peroxidase-conjugated IgG, diluted 1 : 20,000 in PBS-Tween, was added and incubated for 1 h with shaking at room temperature. After extensive washing of the plates, 100 μL of tetramethyl-benzidine (Pierce) substrate solution was added and incubated at room temperature with shaking for 15 min. Finally, 100 μL of 2 M H_2SO_4 was added to stop the reaction and initiate the final color change. Plates were read at A_{450} and background A_{640} on an automated microplate spectrophotometer (VERSA max™; Tunable Microplate Reader; Molecular Devices Corporation, Sunnyvale, CA, USA). The amount of anti-FDF IgG in the samples was calculated using a standard curve obtained with polyclonal anti-rabbit-FDF (11). To transform the Periotron digital readings for each paper strip into volumes, known volumes of distilled water were delivered to Periopaper® in a range of volumes (0.1–1.0 μL). Each measurement was performed three times, and the mean value for each volume was used in a linear regression analysis from which the slope and the intercept were used to determine the volumes of gingival crevicular fluid collected. The concentration of anti-FDF IgG (ng/μL) was calculated by using the following formula: total amount of anti-FDF IgG (ng)/gingival crevicular fluid volume (μL). The concentration of IgG was designated as the IgG level in this report.

Microbiological study

Subgingival plaque was obtained from the same site from which gingival crevicular fluid was collected in both groups. A subgingival plaque sample was taken using a sterile paper point, as described previously by Takeuchi *et al.* (17), with some minor modifications. Briefly, the tooth was cleaned with sterile cotton for removing supragingival plaque and then an absorbent sterile paper point was pushed down to the bottom of the

Table 2. PCR primers used in this study

Gene target	Primer sequence (5′ → 3′)	Product size (bp)
Tannerella forsythia 16S rRNA	Forward: GCG TAT GTA ACC TGC CCG CA Reverse : TGC TTC AGT GTC AGT TAT ACC T	641
fdf	Forward: ATG AGT TAC AAA AAA T Reverse : TTA CAA ATC TAC TCT C	1611

fdf, forsythia detaching factor gene; rRNA.

selected pocket, where it was left in place for 30 s. The paper point was immediately transferred to a microtube containing sterile distilled water and stored at −80°C until use.

Bacterium-specific PCR

The bacterial cells in the microtube were dispersed by vortexing for 60 s. After washing, genomic DNA was isolated by heating at 100°C for 10 min as described in a previous report (18). The presence of bacterial DNA for *T. forsythia* and *fdf* genes in the plaque samples from each patient was examined using conventional PCR. The PCR primers used in this study are listed in Table 2. The PCR assay was carried out as previously described (18). Briefly, the PCR mixture for *T. forsythia* contained 1.0 μM of the primer pair, 1.5 mM $MgCl_2$, 0.2 mM of deoxyribonucleotide triphosphate (Promega, Madison, WI, USA), 1 × reaction buffer (Promega), 1.25 units of *Taq* DNA polymerase and 5 μL of template DNA in a 50-μL reaction volume. Amplification by PCR was performed in a DNA thermal cycler (Veriti 200; Applied Biosystems Inc., Foster City, CA, USA). The PCR temperature profile for *T. forsythia* included an initial denaturation at 95°C for 2 min, followed by 36 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min and a final extension at 72°C for 2 min. PCR procedures for *fdf* were carried out as follows. The PCR mixture for *fdf* contained 1.0 μM of the primer pair, 1 × PrimeSTAR™ Max Premix (Takara Bio Inc., Shiga, Japan) and 5 μL of template DNA in a 50-μL reaction volume. The PCR temperature profile for *fdf* included an initial step at 95°C for 1 min, followed by 25 cycles of 95°C for 10 s, 55°C for 5 s, 72°C for 5 s and a final extension at 72°C for 7 min. Following amplification, 10-μL aliquots of PCR products were analyzed by electrophoresis on a 1.0% agarose gel. The gel was stained with 0.01 μg/mL of ethidium bromide and photographed under ultraviolet light at 300 nm. The size of the PCR products was estimated using a 1-kb DNA ladder as a molecular weight marker.

Statistical analysis

Data were expressed as means ± standard deviations. The Mann–Whitney *U*-test was applied for evaluating differences in clinical and biochemical parameters between patients with periodontitis (both the diseased and healthy sites) and periodontally healthy subjects and between healthy sites and diseased sites among patients with periodontitis. The correlations of anti-FDF IgG levels to clinical parameters were evaluated using Pearson's correlation coefficient.

Results

Patient characteristics

Table 1A lists the characteristics of the patients with periodontitis ($n = 37$) and those of periodontally healthy subjects ($n = 30$). The mean probing pocket depth, the mean clinical attachment level and bleeding on probing in patients with periodontitis were significantly higher than those in periodontally healthy subjects ($p < 0.05$). In addition, there were statistically significant differences in age, which was higher in the periodontitis group. Table 1B lists the characteristics of the sampling sites in the patients with periodontitis and in the periodontally healthy subjects. Probing pocket depth (at the sampling site) and clinical attachment level (at the sampling site) in diseased sites of patients with periodontitis were significantly higher than those in healthy sites of patients with periodontitis and periodontally healthy subjects ($p < 0.05$). The gingival crevicular fluid volume in diseased sites of patients with periodontitis was significantly higher than that in healthy sites of patients with periodontitis ($p < 0.05$).

Anti-FDF IgG levels in patients with periodontitis (both the diseased and healthy sites) and healthy subjects

Gingival crevicular fluid samples were collected from 37 diseased and 29 healthy sites of 37 patients with periodontitis and from 30 sites of 30 periodontally healthy subjects. The mean values of anti-FDF IgG levels in the gingival crevicular fluid of patients with periodontitis (diseased sites: 2.60 ± 2.58 ng/μL; healthy sites: 15.14 ± 16.90 ng/μL) were significantly higher than those of periodontally healthy subjects (0.79 ± 0.25 ng/μL) ($p < 0.05$) (Fig. 1A,B). Among the patients with periodontitis, mean antibody levels from healthy sites were 15.14 ± 16.90 ng/μL, significantly higher than those from diseased sites, 2.60 ± 2.58 ng/μL ($p < 0.05$) (Fig. 1C).

Correlation coefficients of anti-FDF IgG levels in gingival crevicular fluid and clinical parameters in patients with periodontitis

Correlations between anti-FDF IgG levels in gingival crevicular fluid and clinical parameters of the sampling sites were analyzed using Pearson's correlation coefficient. Table 3 presents the correlation between anti-FDF IgG levels and probing pocket depth or clinical attachment level in 29 healthy sites and 37 diseased sites of the patients with periodontitis. The correlation coefficient between anti-FDF IgG levels in gingival crevicular fluid and probing pocket depth (at the sampling site) was −0.444 and that between anti-FDF IgG levels in gingival crevicular fluid and clinical attachment level (at the sam-

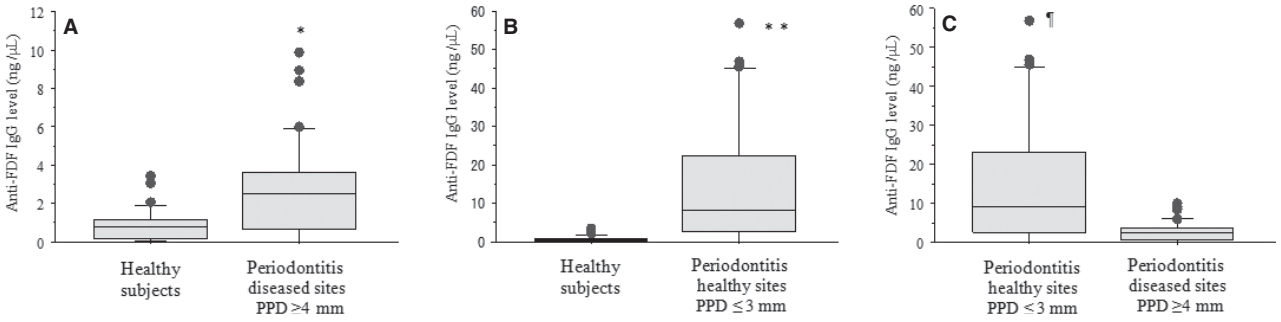

Fig. 1. Comparison of anti-forsythia detaching factor (FDF) immunoglobulin G (IgG) levels between patients with periodontitis (both the diseased and healthy sites) and healthy subjects. (A) *The mean value of anti-FDF IgG levels in the gingival crevicular fluid of patients with periodontitis at the diseased sites (2.60 ± 2.58 ng/μL) was significantly higher than that of periodontally healthy subjects (0.79 ± 0.25 ng/μL) ($p < 0.05$). (B) **The mean value of anti-FDF IgG levels in the gingival crevicular fluid of patients with periodontitis at the healthy sites (15.14 ± 16.90 ng/μL) was significantly higher than that of periodontally healthy subjects (0.79 ± 0.25 ng/μL) ($p < 0.05$). (C) ¶The mean value of anti-FDF IgG levels in the gingival crevicular fluid of patients with periodontitis at the healthy sites (15.14 ± 16.90 ng/μL) was significantly higher than that at the diseased sites (2.60 ± 2.58 ng/μL) ($p < 0.05$).

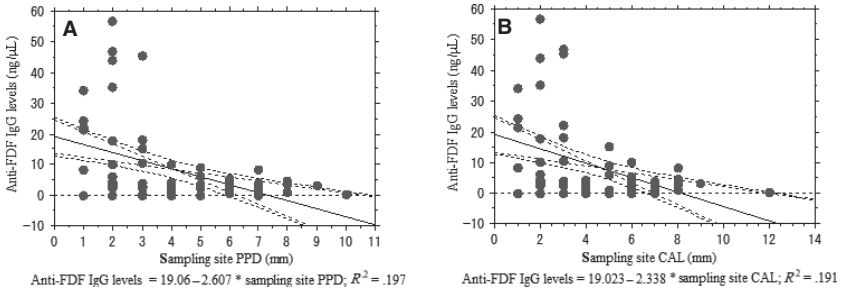

Fig. 2. Correlations between anti-forsythia detaching factor (FDF) IgG levels and probing pocket depth (A) or clinical attachment level (B) in patients with periodontitis (29 healthy sites and 37 diseased sites). The correlation coefficient with probing pocket depth was −0.444 ($p = 0.0002$) and that with clinical attachment level was −0.437 ($p = 0.0002$).

Table 3. Correlation analyses between anti-forsythia detaching factor (FDF) IgG levels and various clinical parameters in periodontitis patients

Clinical parameters	Correlation coefficient	*p*-value
Probing pocket depth (sampling sites)	−0.444	0.0002*
Clinical attachment level (sampling sites)	−0.437	0.0002*

*Statistically significant correlation (Pearson's correlation coefficient, $p < 0.05$).

Table 4. Occurrence of *Tannerella forsythia* 16S rRNA and the forsythia detaching factor (*fdf*) gene in periodontally healthy subjects and periodontitis patients

	No. of positive sites		
		Periodontitis patients	
T. forsythia genes	Periodontally healthy subjects ($n = 30$)	Healthy sites PPD ≤ 3 mm ($n = 29$)	Diseased sites PPD ≥ 4 mm ($n = 37$)
T. forsythia 16S rRNA	0	5	18
fdf	0	7	19

PPD, probing pocket depth.

pling site) was −0.437, suggesting that these correlations were significantly negative ($p < 0.05$) (Fig. 2A,B).

PCR detection and frequency

The specificities of the *T. forsythia*-specific 16S rRNA and *fdf*-specific primers were evaluated using the chromosomal DNA extracted from the parental strain *T. forsythia* ATCC43037. The primers for amplification of the fragments are listed in Table 2. Amplicons appearing to be of the expected sizes of 641 bp for 16S rRNA and 1611 bp for *fdf* genes were obtained. No PCR bands were obtained when the primers were tested against DNA extracted from the other oral anaerobes (data not shown). These results further confirm the specificity of these primers for *T. forsythia*. From the full restriction enzyme map of the *fdf* gene, digestion of the *fdf* amplicon with *Eco*RV was expected to give two DNA fragments of 1190 bp and 421 bp (data not shown). The *fdf* PCR amplicon from the plaque sample was identical to the *fdf* PCR amplicon from the parental strain.

Detection frequencies in the subgingival plaque sample were presented as the percentage of positive sites of the target gene. The presence of the *T. forsythia* gene was indicated by a 641-bp band and the presence of the *fdf* gene was indicated by a 1611-bp band appearing on an agarose gel following electrophoresis. As shown in Table 4,

T. forsythia 16S rRNA and the *fdf* gene were not detected in periodontally healthy subjects. By contrast, among the patients with periodontitis, *T. forsythia* 16S rRNA was detected in 18 of 37 diseased sites and in 5 of 29 healthy sites. Furthermore, 19 of 37 diseased sites and 7 of 29 healthy sites were positive for the *fdf* gene.

All of the sampling sites in patients with periodontitis were next divided into two groups based on the presence of *T. forsythia* 16S rRNA or the *fdf* gene. As shown in Fig. 3, the mean values of probing pocket depth and clinical attachment level of both the *T. forsythia* 16S rRNA-positive group and the *fdf*-positive group were significantly higher than those of the *T. forsythia* 16S rRNA-negative and the *fdf*-negative groups ($p < 0.05$). Anti-FDF IgG levels of both the *T. forsythia* 16S rRNA-positive and *fdf*-positive groups were significantly lower than those of the *T. forsythia* 16S rRNA-negative and the *fdf*-negative groups ($p < 0.05$).

Discussion

The data reported here indicate that there was a significant difference in the anti-FDF IgG levels in the gingival crevicular fluid between patients with periodontitis (both the diseased and healthy sites) and healthy subjects. Furthermore, we also found an obvious difference in the anti-FDF IgG levels in gingival crevicular fluid between healthy sites and diseased sites among the patients with periodontitis. It was concluded that the anti-FDF IgG levels in gingival crevicular fluid might be related to the periodontal status, which is not detectable by general clinical examinations.

In this study, we used the 60-kDa recombinant protein FDF of *T. forsythia* as a specific antigen (11) because it is considered to be one of the etiological agents of this bacterium. This factor might be a key virulence factor for cell adhesion and the cause of inflammation (12).

In several studies on the humoral immune response to virulence factors of periodontopathogens in gingival crevicular fluid, the results have suggested that gingival crevicular fluid contains a variety of cellular and biochemical mediators that reflect the metabolic status of periodontal tissues. Several materials, including plasma proteins, enzymes with collagenolytic activity, other microbial and host cell enzymes, and inflammatory mediators, have been analyzed in attempts to identify factors to facilitate the diagnosis of periodontal disease (19). According to Armitage, more than 65 gingival crevicular fluid constituents have been evaluated as potential diagnostic markers of periodontal disease progression. These markers can be divided into three groups: host-derived enzymes and their inhibitors; inflammatory mediators and host-response modifiers (such as IgG); and products of tissue breakdown. The amount of gingival crevicular fluid produced at a given site significantly increases with the severity of gingival inflammation, as assessed clinically (20). Although there is extensive variation, severely inflamed sites generally produce more gingival crevicular fluid than less inflamed sites. In this study, we continuously collected gingival crevicular fluid from the same sites until sufficient amounts of gingival crevicular fluid for ELISA were obtained. Furthermore, we expressed anti-FDF IgG levels as concentrations (ng/μL). Using this evaluation, we found that the difference in anti-FDF IgG levels was not caused by the difference in gingival crevicular fluid volumes.

Because gingival crevicular fluid is an inflammatory exudate that reflects

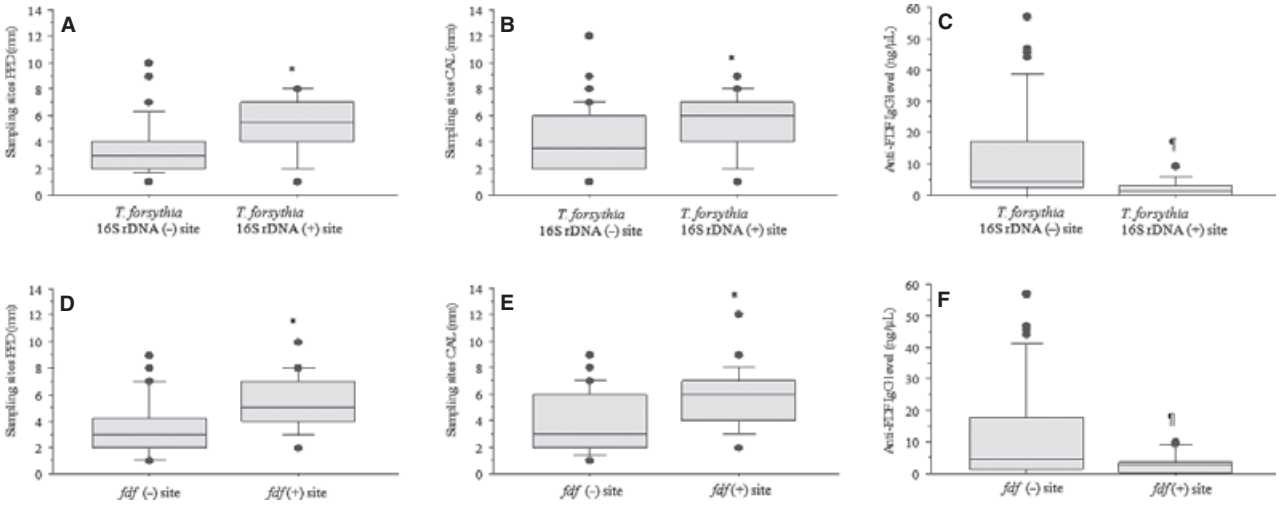

Fig. 3. Comparison of probing pocket depth (mm) and clinical attachment level (mm) between *Tannerella forsythia* 16S rRNA or the forsythia detaching factor (*fdf*) gene positive and negative sites in patients with periodontitis. All sampling sites in patients with periodontitis were divided into two groups based on the presence of *T. forsythia* 16S rRNA or the *fdf* gene. (A, B, D, E) *The mean probing pocket depth and clinical attachment level values of the *T. forsythia* 16S rRNA-positive and *fdf* -positive groups were significantly higher than those of the *T. forsythia* 16S rRNA-negative and *fdf*-negative groups ($p < 0.05$). (C, F) ¶Anti-FDF IgG levels of the *T. forsythia* 16S rRNA-positive and *fdf*-positive groups were significantly lower than those of the *T. forsythia* 16S rRNA-negative and *fdf*-negative groups ($p < 0.05$).

ongoing events in the periodontal tissues, extensive research has been carried out on gingival crevicular fluid components that might serve as potential diagnostic or prognostic markers for periodontitis. Among these, we focused on the host-derived component IgG in the present study. Several studies have examined the antibody response in gingival crevicular fluid to plaque bacteria. Naito *et al*. (14) demonstrated that the antibody levels to *P. gingivalis* in serum and gingival crevicular fluid were significantly higher in a group of patients with periodontitis than in healthy subjects. Plombas *et al*. (21) also reported that the crevicular fluid of patients with periodontitis contained significantly higher levels of IgG to four bacteria (*Aggregatibacter actinomycetemcomitans, P. gingivalis, Prevotella intermedia and Fusobacterium nucleatum*) than that of healthy subjects. Our data showing that anti-FDF IgG levels in gingival crevicular fluid in healthy sites and diseased sites of the patients with periodontitis were significantly higher than those of healthy subjects are consistent with the results of these investigations.

Moreover, we focused on the comparison of anti-FDF IgG levels between healthy sites and diseased sites. It was revealed that anti-FDF IgG levels in healthy sites were significantly higher than those in diseased sites among the patients with periodontitis ($p < 0.05$). These results apparently disagree with the results of comparing the IgG levels between healthy subjects and patients with periodontitis. Various reports have examined the antibody response to some periodontopathic bacteria in gingival crevicular fluid in patients with periodontitis. Suzuki *et al*. (22) found that local production of IgG to *P. gingivalis* was markedly increased in chronic periodontitis. By contrast, Challacombe *et al*. (23) demonstrated that IgG levels to *P. gingivalis* were lower in the gingival crevicular fluid of patients with a high periodontal disease index score. Kinane *et al*. (24) investigated the specific IgG, immunoglobulin A (IgA) and immunoglobulin M (IgM) levels to *P. gingivalis* and *A. actinomycetemcomitans* in serum and gingival crevicular fluid by ELISA and showed that lower gingival crevicular fluid antibody levels to *P. gingivalis* were found in deeper periodontal pockets and in more inflamed sites. This has been confirmed in a cross-sectional study of healthy sites and sites with gingivitis or periodontitis in the same patients with periodontitis (25). These reports supported our findings that anti-FDF IgG levels in healthy sites were significantly higher than anti-FDF IgG levels of diseased sites among patients with periodontitis. However, the IgG levels to *T. forsythia* whole cells were not evaluated in this study. It would therefore be meaningful to examine the correlation between IgG levels to whole cells of this bacterium with those to FDF in gingival crevicular fluid.

Killian *et al*. (26) reported that *P. gingivalis* can degrade human IgG and IgA, suggesting that low IgG levels in the gingival crevicular fluid may be caused by degradation by this bacterium. They also suggested that local antibodies were absorbed by the greater mass of subgingival plaque in the periodontal pockets. Moreover, Jansen *et al*. (27) investigated the proteolytic activity of several subgingival bacteria against IgG and demonstrated that IgG was partially or completely degraded by them. However, Lamster *et al*. investigated the correlation between total IgG in gingival crevicular fluid and specific serum antibody to *P. intermedia*, and found that a local deficiency of IgG to oral pathogens may lead to local disease progression. Serum-derived and locally produced IgG against FDF might be degraded at the diseased sites, but not at the healthy sites. Furthermore, the possibility of the degradation of IgG by proteases derived from host and/ord bacteria in the periodontal pockets has to be considered. In this study, we focused on anti-FDF IgG levels and did not explore the presence of FDF in gingival crevicular fluid. If it is possible to quantify the FDF in gingival crevicular fluid, the mechanism of our findings that anti-FDF IgG levels in healthy sites were significantly higher than those of diseased sites in patients with periodontitis would be elucidated.

The results derived from ELISA in this study do not provide any information regarding the functional characteristics of this antibody. However, we have previously reported that FDF induces detachment of adherent cells and interleukin-8 production in human fibroblasts *in vitro* (12). In this study, the IgG levels of diseased sites were significantly lower than those of healthy sites, and the IgG levels of both the diseased and healthy sites of patients with periodontitis were significantly higher than those of healthy subjects in which the *fdf* gene was not detected. Moreover, the correlation coefficient between anti-FDF IgG levels and probing pocket depth and clinical attachment level was negative. These results suggest that FDF would induce the production of neutralizing antibody. However, the neutralizing activity of this antibody should be confirmed.

In addition, we attempted to identify the *fdf* gene in the periodontal pockets. Previously, Saito *et al*. (5) isolated and characterized an *N*-benzoyl-Val-Gly-Arg-*p*-nitroanilide-specific protease gene, designated *prtH*, from *T. forsythia* ATCC 43037. Nakajima *et al*. (11) demonstrated that a portion of the *fdf* gene was consistent with the putative *prtH* gene (GenBank accession no.: AB001892) other than the 5′-terminal 513 bp, and they demonstrated that FDF retained the activity of PrtH.

There are some reports regarding an association of the *prtH* gene and periodontitis. Tan *et al*. reported that *T. forsythia* was detected in 78 (91%) of 86 diseased sites and in 33 (45%) of 74 healthy sites. Among the 86 diseased sites examined, 73 (85%) were colonized by bacteria with the *prtH* genotype. In sites of patients who were periodontally healthy, 7 (10%) of 73 possessed *T. forsythia* with the *prtH* genotype, suggesting a strong association of the *prtH* gene of *T. forsythia* with adult periodontitis (28). Furthermore, Hamlet *et al*. reported that the *prtH* genotype was detected in 13 (23.2%) of 56 subjects harboring

T. forsythia in their subgingival plaque. They also indicated that the odds of periodontal disease (progression and/or current disease) were 1.55 times greater in subjects harboring the *prtH* genotype of *T. forsythia* than in those not harboring it (29). Moreover, they demonstrated that higher levels of the *prtH* genotype were associated significantly with future attachment loss (30). Because the *prtH* gene was part of the *fdf* gene, we conducted a PCR assay using specific primers for the full length of the *fdf* gene. In the results of our study, *T. forsythia* 16S rRNA was detected in 18 of 37 diseased sites and in 5 of 29 healthy sites but not detected in healthy subjects. Furthermore, 19 of 37 diseased sites and 7 of 29 healthy sites and none of the healthy subjects were positive for the *fdf* gene. Our results indicated an association of the *fdf* gene with periodontitis and also confirmed previous reports. However, it would be necessary to isolate *T. forsythia* from the plaque samples and confirm the sequence of *fdf* gene or the existence of FDF.

Next, we analyzed the existence of the *fdf* gene among the patients with periodontitis. Patients with periodontitis were divided into two groups based on the presence of *T. forsythia* 16S rRNA or the *fdf* gene. The mean probing pocket depth and clinical attachment level values of the groups that were positive for *T. forsythia* 16S rRNA and the *fdf* gene were significantly higher than those of the groups that were negative for *T. forsythia* 16S rRNA and the *fdf* gene. However, the mean level of anti-FDF IgG in those positive groups was significantly lower than that of those negative groups. These results suggest that IgG might be degraded by periodontopathogens or that IgG production might be diminished.

The present study demonstrates an association of anti-FDF IgG levels in gingival crevicular fluid with periodontal status of patients with periodontitis and healthy subjects. Further investigation is needed to determine whether anti-FDF IgG would be a useful indicator for the diagnosis and prognosis of periodontal disease.

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References

1. Kroes I, Lepp PW, Relman DA. Bacterial diversity within the human subgingival crevice. *Proc Natl Acad Sci U S A* 1999;**96:**14547–14552.
2. Paster BJ, Boches SK, Galvin JL *et al.* Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001;**183:**3770–3783.
3. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol* 1998;**25:**134–144.
4. Tanner AC, Izard J. *Tannerella forsythia*, a periodontal pathogen entering the genomic era. *Periodontol 2000* 2006;**42:** 88–113.
5. Saito T, Ishihara K, Kato T, Okuda K. Cloning, expression, and sequencing of a protease gene from *Bacteroides forsythus* ATCC 43037 in *Escherichia coli*. *Infect Immun* 1997;**65:**4888–4891.
6. Ishikura H, Arakawa S, Nakajima T, Tsuchida N, Ishikawa I. Cloning of the *Tannerella forsythensis* (*Bacteroides forsythus*) *siaHI* gene and purification of the sialidase enzyme. *J Med Microbiol* 2003;**52:**1101–1107.
7. Murakami Y, Higuchi N, Nakamura H, Yoshimura F, Oppenheim FG. *Bacteroides forsythus* hemagglutinin is inhibited by N-acetylneuraminyllactose. *Oral Microbiol Immunol* 2002;**17:**125–128.
8. Sabet M, Lee SW, Nauman RK, Sims T, Um HS. The surface (S-) layer is a virulence factor of *Bacteroides forsythus*. *Microbiology* 2003;**149:**3617–3627.
9. Maiden MF, Pham C, Kashket S. Glucose toxicity effect and accumulation of methylglyoxal by the periodontal anaerobe *Bacteroides forsythus*. *Anaerobe* 2004;**10:**27–32.
10. Arakawa S, Nakajima T, Ishikura H, Ichinose S, Ishikawa I, Tsuchida N. Novel apoptosis-inducing activity in *Bacteroides forsythus*: a comparative study with three serotypes of *Actinobacillus actinomycetemcomitans*. *Infect Immun* 2000;**68:** 4611–4615.
11. Nakajima T, Tomi N, Fukuyo Y *et al.* Isolation and identification of a cytopathic activity in *Tannerella forsythia*. *Biochem Biophys Res Commun* 2006;**351:**133–139.
12. Tomi N, Fukuyo Y, Arakawa S, Nakajima T. Pro-inflammatory cytokine production from normal human fibroblasts is induced by *Tannerella forsythia* detaching factor. *J Periodontal Res* 2008;**43:**136–142.
13. Gmur R, Hrodek K, Saxer UP, Guggenheim B. Double-blind analysis of the relation between adult periodontitis and systemic host response to suspected periodontal pathogens. *Infect Immun* 1986;**52:**768–776.
14. Naito Y, Okuda K, Takazoe I. Immunoglobulin G response to subgingival gram-negative bacteria in human subjects. *Infect Immun* 1984;**45:**47–51.
15. Naito Y, Okuda K, Takazoe I, Watanabe H, Ishikawa I. The relationship between serum IgG levels to subgingival gram-negative bacteria and degree of periodontal destruction. *J Dent Res* 1985;**64:** 1306–1310.
16. Yoneda M, Hirofuji T, Motooka N *et al.* Humoral immune responses to S-layer-like proteins of *Bacteroides forsythus*. *Clin Diagn Lab Immunol* 2003;**10:**383–387.
17. Takeuchi Y, Umeda M, Ishizuka M, Huang Y, Ishikawa I. Prevalence of periodontopathic bacteria in aggressive periodontitis patients in a Japanese population. *J Periodontol* 2003;**74:**1460–1469.
18. Ashimoto A, Chen C, Bakker I, Slots J. Polymerase chain reaction detection of 8 putative periodontal pathogens in subgingival plaque of gingivitis and advanced periodontitis lesions. *Oral Microbiol Immunol* 1996;**11:**266–273.
19. Armitage GC. Analysis of gingival crevice fluid and risk of progression of periodontitis. *Periodontol 2000* 2004;**34:** 109–119.
20. Armitage GC. Clinical evaluation of periodontal diseases. *Periodontol 2000* 1995;**7:**39–53.
21. Plombas M, Gobert B, De March AK *et al.* Isotypic antibody response to plaque anaerobes in periodontal disease. *J Periodontol* 2002;**73:**1507–1511.
22. Suzuki JB, Martin SA, Vincent JW, Falkler WA Jr. Local and systemic production of immunoglobulins to periodontopathogens in periodontal disease. *J Periodontal Res* 1984;**19:**599–603.
23. Challacombe SJ, Wilton JM. A study of antibodies and opsonic activity in human crevicular fluid in relation to periodontal disease. *J Periodontal Res* 1984;**19:** 604–608.
24. Kinane DF, Mooney J, MacFarlane TW, McDonald M. Local and systemic antibody response to putative periodontopathogens in patients with chronic

periodontitis: correlation with clinical indices. *Oral Microbiol Immunol* 1993;**8:** 65–68.

25. Mooney J, Kinane DF. Levels of specific immunoglobulin G to *Porphyromonas gingivalis* in gingival crevicular fluid are related to site disease status. *Oral Microbiol Immunol* 1997;**12:**112–116.
26. Kilian M. Degradation of immunoglobulins A2, A2, and G by suspected principal periodontal pathogens. *Infect Immun* 1981;**34:**757–765.
27. Jansen HJ, van der Hoeven JS, van den Kieboom CW, Goertz JH, Camp PJ, Bakkeren JA. Degradation of immunoglobulin G by periodontal bacteria. *Oral Microbiol Immunol* 1994;**9:** 345–351.
28. Tan KS, Song KP, Ong G. *Bacteroides forsythus prtH* genotype in periodontitis patients: occurrence and association with periodontal disease. *J Periodontal Res* 2001;**36:**398–403.
29. Hamlet SM, Taiyeb-Ali TB, Cullinan MP, Westerman B, Palmer JE, Seymour GJ. *Tannerella forsythensis prtH* genotype and association with periodontal status. *J Periodontol* 2007;**78:** 344–350.
30. Hamlet SM, Ganashan N, Cullinan MP, Westerman B, Palmer JE, Seymour GJ. A 5-year longitudinal study of *Tannerella forsythia prtH* genotype: association with loss of attachment. *J Periodontol* 2008;**79:**144–149.