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Lysine-specific proteolytic activity responsible for forsythia detaching factor modification

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ABSTRACT

Objectives: The objective of the present study was to clarify the lysine-specific proteolytic activity derived from periodontal pathogens responsible for Forsythia detaching factor (FDF) modification.
Design: The activity responsible for FDF modification in *Tannerella forsythia* and *Porphyromonas gingivalis* were evaluated by colorimetric assay using Ac-Arg-Ala-Lys-p-nitroaniline as a substrate. FDF modification in *T. forsythia* and *P. gingivalis* were evaluated by Western blotting using recombinant FDF (rFDF) as a substrate. Furthermore, the activity in GCF of 20 patients with periodontitis and 10 healthy subjects was also evaluated by colorimetric assay. Bacteria in subgingival plaque were detected using polymerase chain reaction.
Results: The activity of both bacteria in colorimetric assay were 21.35 unit (*P. gingivalis*) and 3.61 unit (*T. forsythia*), respectively. Western blot analysis revealed that *P. gingivalis* was found to efficiently degrade rFDF and *T. forsythia* partially cleaved rFDF. The activity in GCF from patients with periodontitis (clinically healthy sites: CH, deep bleeding sites: DB and deep non-bleeding sites: DNB) was significantly higher than those from healthy subjects (healthy sites: H). Among the patients with periodontitis, the activity from CH was significantly lower than those from DB and DNB. *T. forsythia* was detected in 68.4% of DNB, in 78.4% of DB and in none of CH. *P. gingivalis* was detected in 63.2% of DNB, in 84.0% of DB and in 10.5% of CH. No bacterium was detected in healthy subjects.
Conclusion: The lysine-specific proteolytic activity responsible for FDF modification correlates with the presence of major periodontal pathogens.

1. Introduction

Cross-sectional and longitudinal studies of predominant cultivable microbiota reveal that human gingival plaques are associated with periodontal disease (Christersson, Zambon, & Genco, 1991). Among those microbiota, *Tannerella forsythia* is a Gram-negative, anaerobic, fusiform bacterium that is found in association with *Porphyromonas gingivalis* and *Treponema denticola* in human subgingival plaque (Haffajee et al., 1998). Together as a consortium they are strongly implicated as periodontal pathogens (Socransky, Haffajee, Cugini, Smith, & Kent, 1998; Tanner and Izard, 2006). The virulence factors of *T. forsythia* are as yet poorly understood, due in part to the difficulty in culturing this fastidious organism, and the difficulty in creating mutants. Therefore, only a few putative virulence factors of *T. forsythia* have been identified, such as a trypsin-like protease (Grenier, 1994), glycosidases (Braham and Moncla, 1992; Ishikura, Arakawa, Nakajima, Tsuchida, & Ishikawa, 2003; Thompson, Homer, Rao, Booth, & Hosie, 2009), a surface-associated as well as secreted *Bacteroides* surface protein A (BspA) (Sharma et al., 1998), a hemagglutinin (Murakami, Higuchi, Nakamura, Yoshimura, & Oppenheim, 2002), components of the bacterial S-layer (Sabet, Lee, Nauman, Sims, & Um, 2003; Sakakibara et al., 2007), methylglyoxal (Maiden, Pham, & Kashket, 2004), apoptosis-inducing activity (Arakawa et al., 2000), karilysin (Karim et al., 2010), PrtH (Nakajima et al., 2006; Saito, Ishihara, Kato, & Okuda, 1997) and forsythia detaching factor (FDF) (Nakajima et al., 2006).

Abbreviations: FDF, forsythia detaching factor; FDFc, forsythia detaching factor C-terminal fragment; rFDF, recombinant forsythia detaching factor; rFDFc, recombinant forsythia detaching factor C-terminal fragment; GCF, gingival crevicular fluid; IL-8, interleukin-8; CH, clinically healthy sites; DB, deep bleeding sites; DNB, deep non-bleeding sites; H, healthy sites; pNA, p-nitroaniline; PD, probing depth; CAL, clinical attachment level.

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Nakajima et al. reported that FDF/PrtH is a bacterial protease composed of 536 amino acids and that part of the *fdf* gene was consistent with putative *prtH* gene (Nakajima et al., 2006). In addition to the cell detaching activity, it impairs the reduction of nicotinamide adenine dinucleotide (NAD) in mitochondria and induces accumulation of reactive oxygen species. A consequence of these effects results in delayed production of proinflammatory cytokine interleukin-8 (IL-8) (Tomi, Fukuyo, Arakawa, & Nakajima, 2008) and FDF has been implicated in the initiation of chronic inflammation and deterioration of periodontitis. Supporting those observations, the level of anti-FDF antibody is significantly higher in patients with periodontitis than healthy subjects (Onishi, Arakawa, Nakajima, & Izumi, 2010). Furthermore, several research group reported that *T. forsythia* prtH genotype is associated with high levels of *T. forsythia* (Hamlet et al., 2008; Tan, Song, & Ong, 2001; van der Reijden, Bosch-Tijhof, Strooker, & van Winkelhoff, 2006). We have previously demonstrated that native FDF purified from sonicated extracts or the culture supernatant of *T. forsythia* contained the C-terminal fragment (FDFc) as a major component of the active fraction (Nakajima et al., 2006), and further investigation revealed that FDFc is more active in cell detaching activity than full-length FDF (personal communication by T. Nakajima). Recombinant FDF (rFDF) expressed in *E. coli* also contained significant amount of the C-terminalfragment correspond to FDFc derived from sonicated extracts of *T. forsythia*. In our recent report, as a first step toward understanding the mechanisms responsible for the interaction between FDF and the local enzymatic environment which affect the initiation and progression of periodontitis associated with *T. forsythia*, we suggested that proteolytic activity, especially lysine-specific proteolytic activity, in GCF to degrade FDF and to generate FDFc from FDF by C-terminal FDF-cleavage at residue Lys268 correlates with severity of periodontitis (Onishi et al., 2013).

In this study, we investigated the lysine-specific proteolytic activity derived from *P. gingivalis*, *T. forsythia* and GCF from healthy subjects and patients with periodontitis.

2. Materials and methods

2.1. Recombinant FDF

rFDF were constructed to imitate native FDF with (His) 8- and HA-tags at the C-terminal ends. rFDF were expressed and partially purified from *Escherichia coli* (*E. coli*). Some extra bands as well as 60kDa-rFDF band were detected by Western blotting using rabbit anti-FDF polyclonal antibodies. We speculated that these extra bands might be derived from *E. coli*, because extra bands were not detected by Western blotting using the same antibodies in native FDF purified from sonicated extracts of *T. forsythia*. rFDF were kindly provided by Dr. Takuma Nakajima. The rFDF used in this study has been reported previously (Nakajima et al., 2006). Briefly, the transgenic *E. coli* strain BL21 (DE3) carrying expression plasmids for full-length FDF was cultured in Luria-Bertani broth and induced with 400 μM isopropyl-β-D-thiogalactopyranoside. Bacterial cells were harvested, sonicated with binding buffer (10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, and 400 mM KCl) and centrifuged in order to obtain clear lysate. The lysate was loaded onto a nickel-bound Chelating-Sepharose FF column and rFDF was eluted with imidazole concentrations between 200 and 500 mM. Eluted fractions were desalted with PBS, concentrated and stored at −80 °C.

2.2. Bacterial culture and cell free extracts

T. forsythia ATCC43037 and *P. gingivalis* ATCC33277 were used in this study. These bacteria were kindly provided by Prof. Makoto Umeda in Osaka dental university. *T. forsythia* ATCC43037 was grown for 7 days on Brucella agar (Kyokuto Pharmaceutical Industrial Co.,Ltd., Tokyo, Japan) supplemented with 5% naked rabbit blood, 2.5 mg/ml of hemin, 5.0 mg/ml of menadione, 0.01% dithiothreitol, and 10 mg/ml of N-acetylmuramic acid and *P. gingivalis* ATCC33277 was grown for 3 days on Brucella agar (Kyokuto Pharmaceutical Industrial Co.,Ltd., Tokyo, Japan) supplemented with 5% naked rabbit blood, 2.5 mg/ml of hemin, 5.0 mg/ml of menadione, 0.01% dithiothreitol at 37 °C under anaerobic conditions 10% (v/v) CO_2, 10% (v/v) H_2, and 80% (v/v) N_2, respectively. These cells were harvested by scraping colonies from Brucella agar plates, washing them 3 times with phosphate-buffered saline (PBS). For preparation of cell free extracts, the bacterial cells were then suspended in 0.05 M Tris-HCl, 0.15 M NaCl pH 7.6 and sonicated on ice for 10 min in 30 s bursts using a Bioruptor (Tosyo Electric Co., Ltd., Kanagawa, Japan). Cell free extracts were then centrifuged at 15,000*g* for 15 min to remove cellular debris and then the supernatant was collected, and the protein concentration of the extracts was determined with a Protein Assay Kit (Pierce-Warriner, Warrington, UK). The extracts was then diluted to a final protein concentration of 0.5 mg/ml and stored at −20 °C.

2.3. Study population and collection of gingival crevicular fluid

Subjects were recruited as previously described (Onishi et al., 2013). Briefly, patients and healthy subjects were selected from among the study population of Meikai University Dental Hospital. All of the subjects recruited for this study read an information sheet and gave their consent in writing. Criteria for inclusion of chronic periodontitis were presence of at least one site with ≤ 3 mm periodontal probing depth and no bleeding on gentle probing (clinically healthy site: CH), one site ≥ 6 mm that bled upon gentle probing (deep bleeding site: DB) and one site ≥ 6 mm with no bleeding on probing (deep non-bleeding site: DNB), and these sites (CH, DB and DNB) were then used as sampling sites. On the other hand, criteria for inclusion of periodontally healthy subjects were no presence of site with ≥ 4 mm periodontal probing depth and no bleeding on gentle probing. A site with ≤ 3 mm periodontal probing depth and no bleeding on gentle probing (healthy site: H) was used as a sampling site in healthy subjects. Criteria for exclusion were (1) relevant medical history; (2) pregnant; (3) using mouth rinses or oral irrigators; and (4) periodontal therapy in the preceding 3 months, including root surface debridement, or adjunctive use of local or systemic antimicrobials. Smoking status was also recorded for each patient and it was noted whether this included cigarettes. Subjects in neither group had severe systemic diseases that may affect the immunological response. A summary of the clinical characteristics of the study population and sampling

Table 1a
Clinical characteristics of healthy subjects and patients with periodontitis.

	Healthy subjects n = 10	Patients with periodontitis n = 20	*P* Value
Age (years)	28.3 ± 1.3	50.0 ± 12.4*	<0.01
Gender (male:female)	9:1	13:7	–
Number of teeth	27.2 ± 1.7	25.8 ± 3.6	NS
BOP (% of sites)	0.12 ± 0.25	46.2 ± 26.5*	<0.01
PD (mm)	1.70 ± 0.16	3.82 ± 1.26*	<0.01
CAL (mm)	1.70 ± 0.16	4.84 ± 1.56*	<0.01

Data are expressed as mean ± standard deviation.
BOP: bleeding on probing, PD: probing depth, CAL: clinical attachment level.
* There were significant differences between groups regarding age, BOP, PD and CAL (Mann-Whitney *U*-test; $P < 0.01$).