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Biochemical and Biophysical Research Communications 351 (2006) 133–139
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Isolation and identification of a cytopathic activity in *Tannerella forsythia*

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Received 30 September 2006
Available online 11 October 2006

Abstract

Interactions between pathogens and host induce human disorders including periodontitis, disintegration of the tooth supporting tissues. *Tannerella forsythia* has been linked to the periodontitis and several cytopathic reagents have been found in the bacterium; however, its contribution to the disease remains unclear. Biochemical approach to explore the cytopathic effect revealed two distinct activities in *T. forsythia* (ATCC 43037) extract; one detaches adherent cells from substratum and another arrests cells at G2. An executor of former activity, forsythia detaching factor (FDF) was identified; its genomic sequence and peptidase activity revealed that FDF is a substantial form of putative PrtH; *prtH* gene was hypothetically identified directly from a DNA fragment of the bacterium and its native product has never been shown. Since FDF was found in the bacterial culture supernatant, its activity implies a contribution to the disintegration of tissues although the mechanism how FDF disturbs cellular anchors remains elusive.

Keywords: *Tannerella forsythia*; Periodontitis; Cytopathic factor; prtH; Tissue disintegration

Periodontitis is an acute or chronic condition that can result in the disintegration of the tooth supporting tissues such as periodontal ligaments and alveolar bone. Among hundreds species of bacteria in the oral cavity, *Tannerella forsythia*, a gram-negative, anaerobic bacterium [1], has long been linked to periodontal disease [2]. Recent reports demonstrated that *T. forsythia* exhibits synergic pathogenicity to the human cells when cultured with other periodontpathic bacteria *in vitro* [3]. Many virulence factors were found in the bacterium [4–9]; however, the pathological features of this bacterium remain unclear. Meanwhile, our previous study demonstrated that sonicated extract from *T. forsythia* contains a protein factor(s) that caused a severe cytopathy on human leukemia cells [10]. This fact implied a role of the cytopathic factor(s), namely CPF(s), contribute, to the pathogenicity of *T. forsythia*.

In this study, we report the identification of a substantial CPF in *T. forsythia*, FDF (registered in tentative name, cytocidal toxin-1 (CCT-1), for GenBank Accession No. AY368075), and would suggest its possible roles in the incidence of the periodontitis.

Materials and methods

Cell culture and assay for cytopathic effect. MA1 cells, derivative of human epidermal carcinoma cell line KB [11], were employed for assays for cytopathic effect (CP). KB and 293T cells were employed to see effects of recombinant FDF. Those cells were cultivated with Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) with 5% CO_2 at 37 °C. To assay CP, growing cells were plated every 5×10^3 cells/100 μl/well into 96-well culture plates. Within 20 h after plating, 10 μl/well of desalted fraction of *T. forsythia* extract was added to the culture in duplicate, and cells were cultivated an additional 72 h. As controls, sequentially diluted cells were plated. The CP was estimated by the WST assay that measures mitochondrial NAD-linked hydrogenase activity as

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.10.012

an improved alteration of MTT assay [12]. Briefly, one-tenth volume of WST-1 solution (Dojindo) was added to the cell culture that was treated with 5 μg of a fraction. The culture was incubated an additional 2 h and absorbance at 450 nm measured by a microplate reader.

Bacterial culture and fractionation of CPF(s). *Tannerella forsythia* (ATCC 43037) was grown in heart infusion broth (Difco) containing hemin (5 mg/l), menadione (1 mg/l), L-cysteine (1%), and *N*-acetylneuraminic acid (15 mg/l) in an anaerobic condition and subjected to prepare the sonicated extract as described previously [10].

The fractionation was started with 150 mg-protein equivalent (hereafter "-protein equivalent" is omitted) of the sonicated extract. It was diluted with phosphate buffer (PB; 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4) and loaded on a diethyl aminoethyl (DEAE)–Sepharose column (diameter (d) = 2.6 cm, height (h) = 45 cm, and bed volume = 140 ml) at flow-rate of 2 ml/min. ÄKTA explorer10S protein purification system (Amersham) was employed to regulate buffer flow and to monitor elution status, and the absorbance units at 254 and 280 nm were recorded automatically. The adsorbed components were washed with 200 ml PB and were eluted by 300 ml linear gradient of NaCl concentrations from 0 to 1 M and collected every 10 ml. Respective fractions were desalted, concentrated, measured protein concentrations, and respective 5 μg of fractions was applied to assay CP on MA1 cells.

For heparin-affinity chromatography, we employed HiTrap Heparin 5 ml column (Amersham). One milligram of sample was loaded to the column and adsorbed materials were eluted by linear gradient of NaCl concentrations from 0 to 2 M within 25 ml. Flow-through fraction (Hep-FT) and adsorbate were fractionated into every 1 ml. CP of each fraction was monitored as stated above.

Flowcytometry. Cells were collected with PBS containing 2% EDTA and were fixed with PBS containing 4% paraformaldehyde. The cells were pierced with 0.3% Triton X-100 and blocked with 0.25 M Tris–glycine buffer for overnight. The cells were then incubated with a mixture of rabbit anti-human p53 antibody (FL393, Santa Cruz) and mouse anti-human Cyclin B1 antibody (GNS1, SantaCruz) for 1 h and stained with a mixture of Cy5-labeled goat anti-rabbit IgG antibody, fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgG antibody and 4′,6-diamino-2-phenylindol (DAPI). Stained cells were subjected to flowcytometry by BD-LSR multiple laser flowcytometer (BD Biosciences), and fluorescence intensities at FL5 (>380 nm for DAPI), FL1 (530 nm for FITC), and FL6 (>670 nm for Cy5) were analyzed.

Preparation of anti-CP monoclonal antibodies. Anti-CP monoclonal antibodies that were capable of neutralizing cytopathic activity in the DE-fractions (see Results and discussion) were established. Animal experiments were permitted by the Animal Care and Use Committee of Tokyo Medical and Dental University prior to the operation (Permission No. 0040070). Briefly, two mice were immunized with 50 μl of antigen mixture containing 50 μg of either DE-fraction (Fig. 1A) and 25 μl of Freund's complete adjuvant. The activated lymphocytes were collected and fused with mouse myeloma cell line PAI by polyethylene glycol 1500 and cultivated sequentially in HAT- and HT-medium containing 15% FCS until forming colonies. For screening of consequent hybridomas, 0.5 μl of protein G-Sepharose was incubated with 200 μl of hybridoma culture supernatant for 1 h, washed with PBS, and incubated with 5 μg DE-F2. After incubation, respective remainder of DE-F2 was collected and its cytopathic activity to MA1 cells assayed. Six of 584 clones were identified as anti-CP antibody-producing hybridomas.

Electrophoresis and immunoblotting. Respective 1 μg fractions of *T. forsythia* extract were boiled with 2× Laemmli's sample buffer and separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE. Two-step acrylamide concentrations were employed; 8%T (upper), 12%T (bottom), and 2.6%C). The separated proteins were subjected to silver staining or immunoblotting. For immunoblotting, proteins were transferred to polyvinylidene difluoride membrane; the membrane was then blocked with PBS containing 0.5% FCS for 1 h at room temperature; treated with a non-diluted Be-24 hybridoma culture supernatant or rabbit anti-FDF antiserum diluted at 1:5000; stained with alkaline phosphatase labeled secondary antibody. Immunostained bands were visualized by CDP-Star reagents (Amersham).

Gene cloning and preparation of the recombinant protein. *Tannerella forsythia* genomic DNA fragments between 4 and 10 kbps were prepared by *Eco*RI partial digestion and were inserted into λZAP-Express phage vector (Stratagene), and the consequent recombinant phage library was obtained. The 28 kDa fragment of FDF was extracted from a blot and submitted to the N-terminal amino acid sequencing by Edman's degradation method [13]. On the basis of the given amino acid sequence, a degenerative primer (Fig. 1H) was prepared and applied to the polymerase chain reaction (PCR) with T3 primer and *Pfu* DNA polymerase to amplify *fdf* gene fragment; the consequent DNA fragment was sequenced and applied as a probe to pick up corresponding phage clones from the genomic DNA library. The DNA fragment encoding entire *fdf* structural gene was sequenced and was transferred between *Nco*I and *Pml*I sites of pQE-TriSystem His-*Strep* 1 vector (Qiagen) to construct expression plasmid pQE-FDF. The plasmid expresses rFDF tagged with *Strep*- and $(His)_8$-moirties in tandem at C-terminus.

The transgenic *Escherichia coli* strain BL21 (DE3) cells carrying pQE-FDF was cultivated with LB-broth and induced with 400 μM of isopropyl-β-D-thiogalactopyranoside. The bacterial cells were harvested, sonicated with PB buffer containing 400 mM KCl, and centrifuged to obtain cleared lysate. The lysate was loaded to nickel–Sepharose column and the rFDF was eluted by immidazole concentrations between 300 and 500 mM without significant contaminants. The protein was then desalted with PBS, concentrated, and stored at −80 °C.

Results and discussion

Fractionation of CPF(s) from the sonicated extract of T. forsythia

Since our previous result demonstrated that the sonicated extract of *T. forsythia* contains obvious activity of CPF(s) [10], we started from the extract to seek components of CPF(s). The extract was first fractionated with DEAE–Sepharose column; CPF(s) were eluted in split fractions at NaCl concentrations of 120–300 mM (DE-F1) and 400–500 mM (DE-F2) (Fig. 1A). Cytopathic activities in both fractions induced similar alterations of the morphology and the cell cycle status of MA1 cells (Fig. 1B and C); those induced G2 arrest with accumulated Cyclin B1- and p53-levels and sub-G1 populations, and consequently revealed that both fractions contain common G2-arrest inducing factor (G2AIF) as a component of CPF(s). The feature of this G2AIF implied the cytolethal distending toxin (CDT) which is commonly found in gram-negative pathogens including *Haemophilus ducreyi* [14] and *Actinobacillus actinomycetemcomitans* [15]. However, unlike CDT treated epithelial cells, the majority of MA1 cells treated with either DE-fraction detached rather than spread on the substratum (Fig. 1B). The fact suggested that the both fractions contained what detached target cells in addition to the G2AIF.

The CPF(s) in either of DE-fraction was separated in flowthough (Hep-FT) and adsorbed (Hep-BF) fractions by the heparin–Sepharose column (Fig. 1D). The Hep-BF contained a few detectable bands of proteins (Fig. 1G, lane 3) and detached the target cells (Fig. 1E) whereas the Hep-FT contained multiple proteins (data not shown) and distended the target cells along with an axis (Fig. 1E). Since DE-fractions induced mixed morphological changes in

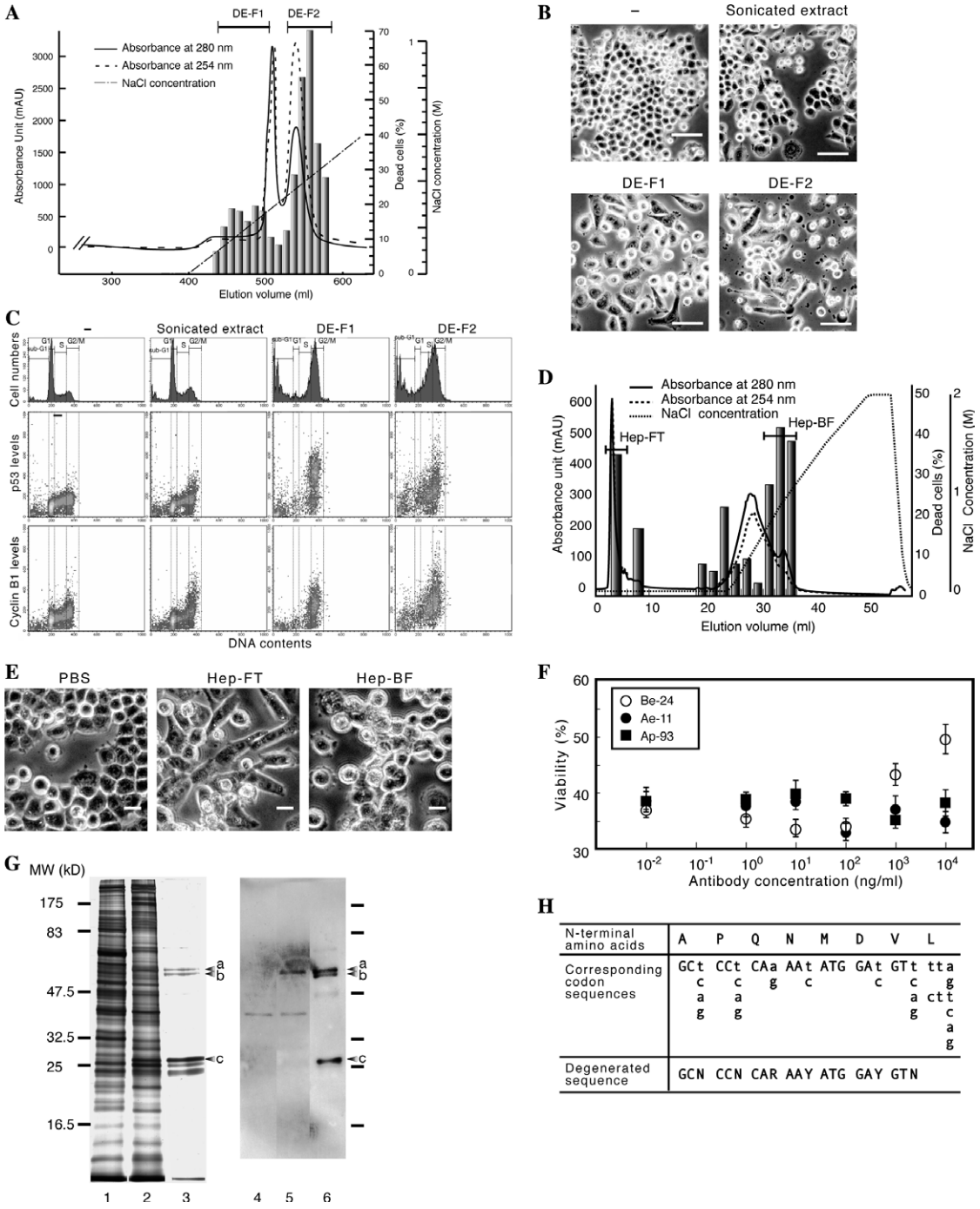

Fig. 1. Fractionation of CPFs. (A) The sonicated extract of *T. forsythia* was applied to DEAE–Sepharose column and elution profiles of absorbance at 280 and 254 nm were recorded. Cytopathic activities (CP) of respective fractions were monitored (solid bar). (B) Morphological change of MA1 cells incubated with sonicated extract (25 μg/ml) or either DE-fractions (0.5 μg/ml). Open bars: 50 μm. (C) CPF(s) arrested MA1 cells in late S to G2 phases and induced cell death. The cell cycle status and both Cyclin B1 and p53 levels were observed by flowcytometry at 48 h of the treatment. (D) CPFs were separated into Hep-FT and Hep-BF. A DE-fraction was separated by heparin–Sepharose column and CP in 1 μg of fractions were monitored (solid bars). (E) A CPF in Hep-FT distend cells along an axis while another CPF in Hep-BF detached cells from substratum. KB cells plated in 35-mm dishes were incubated with 0.5 μg of indicated fraction for 48 h. Bars: 10 μm. (F) Monoclonal antibody Be-24 partly neutralized CP in DE-F2. Fresh medium containing 1 μg/ml DE-F2 was incubated with indicated amount of purified antibodies. After removal of antibodies, 100 μl of respective media was applied to cultivate 2×10^3 MA1 cells in 96-well plates. CP was assayed at 72 h of cultivation. (G) Identification of FDF. Ten micrograms of sonicated extract (lanes 1 and 4), DE-F2 (lanes 2 and 5), and Hep-BF (lanes 3 and 6) were separated by SDS–PAGE and subjected to silver staining (lanes 1, 2, and 3) or immunoblotting (lanes 4, 5, and 6). Arrowheads indicate band positions revealed with Be-24. (H) Designing degenerative upstream primer for detected peptide sequence. The thickest band detected at 28 kDa by immunoblotting (shown by arrowhead c in G) was submitted to N-terminal amino acid sequencing. Consequent octa-peptide sequence is represented in the top.

the target cells (Fig. 1B), these observations suggested that the components of CPF(s) were separated by heparin column; however, Hep-BF seemed not to induce cell death by 48 h at 0.5 μg/ml while 0.5 μg/ml Hep-FT seemed to induce significant populations of dying cells.

To ask whether the activity in the Hep-BF was ascribed to a component of CPF, we probed proteins in Hep-BF by Western blotting using a series of anti-CP neutralizing monoclonal antibodies. Among the antibodies, only Be-24 (Fig. 1F) revealed distinct bands in the Hep-BF (Fig. 1G). The band at highest molecular size was also detected in DE-F2, and consequently this band was estimated as a native CPF component and designated as forsythia detaching factor (FDF). Next, the major band detected by Be-24 in the Hep-BF at 28 kDa was subjected to the amino acid sequencing and consequent octa-peptide: Ala-Pro-Gln-Asn-Met-Asp-Val-Leu was revealed as a part of the protein. Based on this sequence, a degenerated upstream primer for cloning was prepared (Fig. 1H).

Molecular cloning of the fdf gene

The genomic DNA library of *T. forsythia* was first applied to amplify a *fdf* gene fragment by PCR with the degenerative primer and the T3 primer that resides in the vector. A major PCR product of approximately 0.95 kbps was validated by DNA sequencing and was applied as a probe to isolate entire DNA fragment of *fdf* gene. The consequent genomic DNA fragment was approximately 2.3 kbps. DNA-sequence analyses revealed that the putative open reading frame (ORF) encoding the octa-peptide sequence of 1611 bps started from 804 bps upstream;

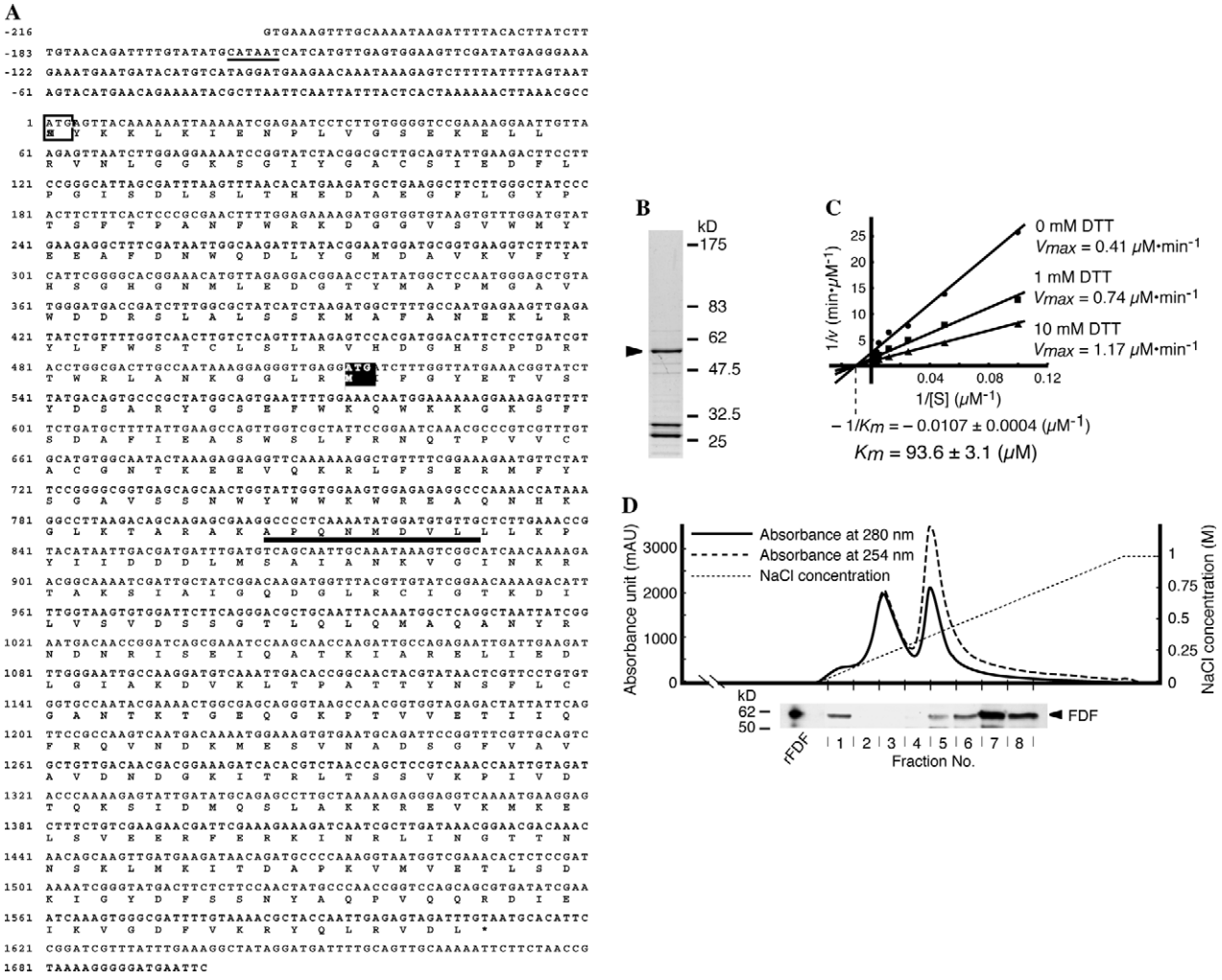

Fig. 2. Identification of the *fdf* gene and its native product FDF in the culture supernatant of *T. forsythia*. (A) Genetic sequence of *fdf* gene. Open box, putative translation initiation codon estimated by the molecular size of native FDF; closed box, putative initiation codon of *prtH* gene; thick underline, chemically determined octa-peptide sequence from the isolated protein. (B) Purified rFDF protein expressed in *E. coli*. One microgram of purified rFDF separated by SDS–PAGE was visualized by Coomassie brilliant blue. Arrowhead: intact rFDF. (C) Catalytic activity of rFDF to Z-VGR-pNA. Various concentrations of Z-VGR-pNA in the assay buffer (50 mM Tris–HCl, 100 mM NaCl, pH 7.5) were catalyzed by 50 μg/ml rFDF in the presence of indicated concentrations of DTT at 30 °C. Absorbance at 405 nm was monitored and the results in initial 4 min were employed to estimate the reaction velocities. (D) Native FDF protein was detected in the bacterial culture supernatant. Proteins in 2.5 L culture supernatant of *T. forsythia* were precipitated by ammonium sulfate and applied to a DEAE–Sepharose column. Eluted fraction of 25 μg was applied for immunoblotting, respectively.

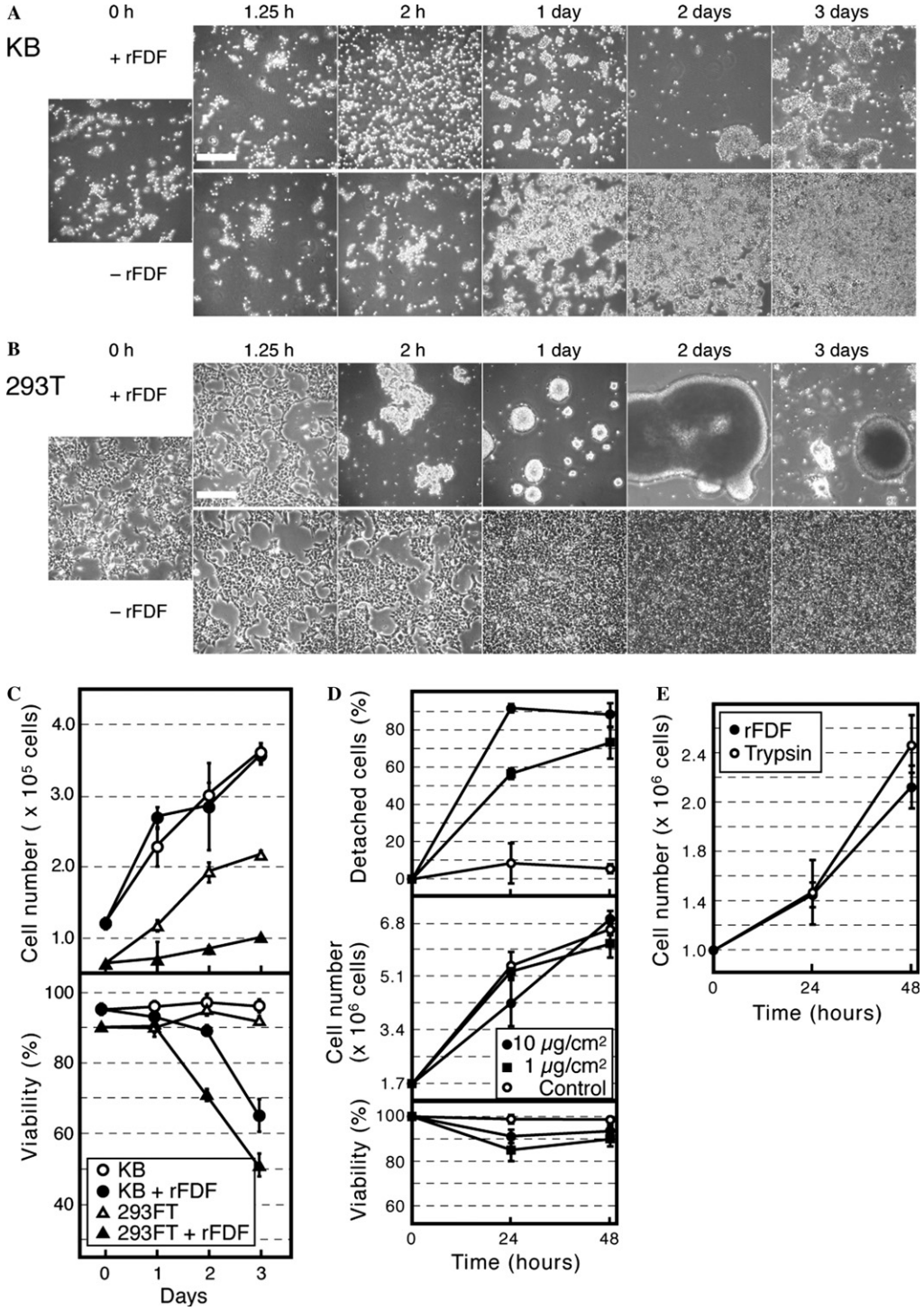

Fig. 3. Characterization of rFDF. (A) Morphological alterations of KB cells induced by rFDF. A hundred thousands of KB cells growing in 60-mm dishes were treated with four ml of either fresh medium (−rFDF) or the medium containing 10 μg/ml (i.e., 1.8 μg/cm^2) of purified rFDF (+rFDF). Photographs were taken at indicated period whereas the observed number of detached and/or aggregated cells in the limited fields did not reflect the average density in the culture. Open bar: 200 μm. (B) Morphological alterations of 293 T cells induced by rFDF. 293T cells were similarly treated with rFDF, and morphological changes were recorded as described. (C) Growth of KB and 293T cells in the presence or absence of rFDF. KB and 293T cells were similarly cultivated and treated as stated above, and checked cell numbers and viabilities by trypan-blue exclusion assay in triplicate at the indicated times. Error bars: standard deviations. (D) rFDF detaches target cells in dose dependent manner whereas it affects viability little. Adherent KB cells were cultivated with indicated amounts of rFDF in the 24-well culture plate and both adherent and detached cells were counted, respectively. (E) Recovery of adhesion potential of the target cells. The detached KB cells treated with 10 μg/cm^2 rFDF for 48 h in (D) were collected and replated with fresh medium, and counted to compare with regularly replated cells. No significant numbers of detached cells were left at 24 and 48 h after plating and thus the numbers of adherent populations were plotted.

it encodes 537 amino acids (aa) and 60,536.76 Da of molecular mass was consistent with the native FDF (Fig. 1G). The fact suggested the first ATG of the ORF to be a translation start site. Although no Shine–Dalgarno sequence was identified within the upstream sequence, the Pribnow-box-like sequence CATAAT was found at 159 bps upstream of the first ATG (Fig. 2A). Unexpectedly, a part of the *fdf* gene was consistent with putative *prtH* gene (GenBank Accession No. AB001892) [7] except 5′ 513 bps. Contrary to the *fdf* gene, the *prtH* gene of *T. forsythia* was directly cloned from the genomic DNA-library under the objective to find putative cysteine protease and no native form has been shown. Therefore, we assumed that the *prtH* is a part of the *fdf* gene and the FDF is the authentic gene product. To ask whether FDF retains an activity of PrtH, we examined the catalytic activity of recombinant FDF (rFDF) (Fig. 2B) to a synthetic peptide *N*-benzoyl-Val-Gly-Arg-*p*-nitroanilide (Z-VGR-pNA); a reported substrate for PrtH [7]. In the presence of the substrate at 200 μM, the catalytic activity of rFDF was detected in higher concentrations than 10 μg/ml, and the activity was increased in the presence of DTT. The reaction velocity of 50 μg/ml rFDF was estimated by the initial 4 min of reactions for various concentrations of the substrate in the presence or absence of 1 and 10 mM DTT. Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) were estimated by the Lineweaver–Burk plot. Consistent $K_m = 93.6 \pm 3.1$ μM was obtained regardless of the presence of DTT while respective V_{max} was varied (Fig. 2C). Therefore, the reducing condition may rather accelerate the catalytic activity of rFDF than the affinity between rFDF and the substrate.

Next we asked whether FDF was considered to be a substantial CPF or an internal enzyme of the bacterium. To detect the FDF in the low concentration, we immunized rabbits with rFDF and obtained high-affinity anti-FDF antisera. These antisera clearly reacted with the native FDF in the bacterial culture supernatant; it eluted in corresponding fractions to DE-F1 and DE-F2 from a DEAE–Sepharose column (Fig. 2D), and consequently showed that FDF is a candidate of the extracellular CPF.

rFDF induced detachment of human adherent cells

In order to seek a function of FDF to the human cells, purified rFDF was added to the cell culture. Human embryonic fibroblast 293T cells and KB cells were cultivated with growth media containing 10% FCS in the presence or absence of 10 μg/ml (equivalent of 1.8 μg/cm^2) rFDF, and morphological alterations were monitored in the center area of the dishes. As expected from the nature of Hep-BF, both cells detached within 2 h and kept adrift more than 48 h while some population formed aggregates (Fig. 3A and B). This fact may suggest that FDF rather targets cell-matrix adhesion than cell–cell interaction. rFDF also caused distinct growth suppression in 293T cells but in KB cells (Fig. 3C). Viabilities of those cells were obviously decreased by three days of treatment. The contradiction between the distinct loss of viability and the little affected proliferation of both cells after two days of treatment suggested a possible induction of abortive proliferation; a stimulated growth that is ill associated with maturation in every cell cycle. The discrepancy between 293T and KB cells suggested that the growth inhibition and the start of cell death depend on the nature of target cells and thus these may not be the direct effect of FDF. It is possible that FDF disturbs cell adhesion machinery including the focal adhesion complex and induces subsequent downstream signaling that affects the cell growth and integrity.

Next, we asked whether those detached cells lost adhesion potential irreversibly. KB of 1×10^6 cells were plated in 24-well plates (i.e., 5.2×10^5 cells/cm^2) and randomly selected 2-wells of cells were counted on the next day. Remaining wells were further cultivated with 500 μl of fresh media containing 0, 3.8, and 38 μg/ml (equivalent of 0, 1, and 10 μg/cm^2) rFDF, respectively. Populations of both detached and adherent cells were counted in triplicate at 24 and 48 h of treatment. The detached populations were raised with the increase of rFDF; however, the viability and growth rate of those cells were little affected within 48 h again (Fig. 3D). Subsequently, detached cells from the culture treated with 10 μg/cm^2 rFDF were collected and replated at 1×10^6 cells/well in 24-well culture plate with fresh medium without rFDF. Regularly trypsinized cells were plated in same density and both cultures were counted at 24 and 48 h after the plating. The rFDF treated cells attached to the substratum and continued growth without significant difference to trypsinized cells. Therefore, FDF treated cells retain intact potential to express adhesion molecules; FDF may target extracellular adhesion machinery to detach target cells.

Conclusion

Our data showed that CPFs produced by *T. forsythia* include at least two different factors, G2AIF and FDF. Whereas G2AIF has remained to be identified, we isolated and identified FDF. Since FDF was found in the bacterial culture supernatant, its function to detach adherent cells implicates its possible role in the disintegration of the subgingival tissue that may result in an incidence of periodontitis. How FDF disturbs cellular adhesion remains to be elucidated.

Acknowledgments

This work was supported by following grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology; Grant-in-Aid for Scientific Research (B) (2) #14370601 for T.N., Grant-in-Aid for Scientific Research (C) (2) #14571978 for S.A., and Grant-in-Aid for Exploratory Research #12877303 for N.T. We thank Takashi Ohuchi for advices on hybridoma preparation.

References

[1] A.C. Tanner, M.N. Strzempko, C.A. Belsky, G.A. McKinley, API ZYM and API An-ident reactions of fastidious oral gram-negative species, J. Clin. Microbiol. 22 (1985) 333–335.

[2] S.S. Socransky, A.D. Haffajee, M.A. Cugini, C. Smith, R.L. Kent Jr., Microbial complexes in subgingival plaque, J. Clin. Periodontol. 25 (1998) 134–144.

[3] C. Bodet, F. Chandad, D. Grenier, Inflammatory responses of a macrophage/epithelial cell co-culture model to mono and mixed infections with *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, Microbes Infect. 8 (2006) 27–35.

[4] M.F. Maiden, A. Tanner, P.J. Macuch, Rapid characterization of periodontal bacterial isolates by using fluorogenic substrate tests, J. Clin. Microbiol. 34 (1996) 376–384.

[5] P.H. Braham, B.J. Moncla, Rapid presumptive identification and further characterization of *Bacteroides forsythus*, J. Clin. Microbiol. 30 (1992) 649–654.

[6] H. Ishikura, S. Arakawa, T. Nakajima, N. Tsuchida, I. Ishikawa, Cloning of the *Tannerella forsythensis* (*Bacteroides forsythus*) *siaHI* gene and purification of the sialidase enzyme, J. Med. Microbiol. 52 (2003) 1101–1107.

[7] T. Saito, K. Ishihara, T. Kato, K. Okuda, Cloning, expression, and sequencing of a protease gene from *Bacteroides forsythus* ATCC 43037 in *Escherichia coli*, Infect. Immun. 65 (1997) 4888–4891.

[8] A. Hasebe, A. Yoshimura, T. Into, H. Kataoka, S. Tanaka, S. Arakawa, H. Ishikura, D.T. Golenbock, T. Sugaya, N. Tsuchida, M. Kawanami, Y. Hara, K. Shibata, Biological activities of *Bacteroides forsythus* lipoproteins and their possible pathological roles in periodontal disease, Infect. Immun. 72 (2004) 1318–1325.

[9] N. Higuchi, Y. Murakami, K. Moriguchi, N. Ohno, H. Nakamura, F. Yoshimura, Localization of major, high molecular weight proteins in *Bacteroides forsythus*, Microbiol. Immunol. 44 (2000) 777–780.

[10] S. Arakawa, T. Nakajima, H. Ishikura, S. Ichinose, I. Ishikawa, N. Tsuchida, Novel apoptosis-inducing activity in *Bacteroides forsythus*: a comparative study with three serotypes of *Actinobacillus actinomycetemcomitans*, Infect. Immun. 68 (2000) 4611–4615.

[11] T. Nakajima, N. Ohi, T. Arai, N. Nozaki, A. Kikuchi, K. Oda, Adenovirus E1A-induced apoptosis elicits a steep decrease in the topoisomerase IIα level during the latent phase, Oncogene 10 (1995) 651–662.

[12] M. Ishiyama, H. Tominaga, M. Shiga, K. Sasamoto, Y. Ohkura, K. Ueno, A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet, Biol. Pharm. Bull. 19 (1996) 1518–1520.

[13] C. Rochat, H. Rochat, P. Edman, Some S-alkyl derivatives of cysteine suitable for sequence determination by the phenylisothiocyanate technique, Anal. Biochem. 37 (1970) 259–267.

[14] X. Cortes-Bratti, C. Karlsson, T. Lagergard, M. Thelestam, T. Frisan, The *Haemophilus ducreyi* cytolethal distending toxin induces cell cycle arrest and apoptosis via the DNA damage checkpoint pathways, J. Biol. Chem. 276 (2001) 5296–5302.

[15] T. Sato, T. Koseki, K. Yamato, K. Saiki, K. Konishi, M. Yoshikawa, I. Ishikawa, T. Nishihara, p53-independent expression of p21(CIP1/WAF1) in plasmacytic cells during G(2) cell cycle arrest induced by *Actinobacillus actinomycetemcomitans* cytolethal distending toxin, Infect. Immun. 70 (2002) 528–534.