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Pro-inflammatory cytokine production from normal human fibroblasts is induced by *Tannerella forsythia* detaching factor

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Background and Objective: *Tannerella forsythia* is a periodontal pathogen. Recently, we have reported that the cytopathic component of *T. forsythia* contains two distinct factors. One arrests the cell cycle at the G2 phase and the other, named forsythia detaching factor, detaches adhesion-dependent immortalized human cells. In this study, we investigated the biological function of forsythia detaching factor using human normal fibroblasts.

Material and Methods: A recombinant forsythia detaching factor, reported previously, was used. TIG-3 cells, cultured in the absence or presence of forsythia detaching factor, were lysed and the supernatant was analyzed by western blotting with polyclonal forsythia detaching factor antibodies. The cells were subsequently fractionated to isolate the cytoplasmic, mitochondrial and remaining fractions. In order to measure the activity of mitochondria using nicotinamide adenine dinucleotide-linked reductase, the water-soluble tetrazolium method was used. The mitochondrial oxidative membrane potential was estimated by measuring the oxidization-dependent fluorogenic conversion of dihydrotetramethylrosamine using flow cytometry. The concentration of interleukin-8 in the culture supernatant was assayed using a Human IL-8 ELISA kit.

Results: Forsythia detaching factor-treated cells detached from the substratum and aggregated from 3 to 24 h. Then, the detached cells resumed adhesion and proliferated after 48 h. The western blot analysis revealed that most forsythia detaching factor *trans*-located into the mitochondrial fraction. Forsythia detaching factor suppressed the nicotinamide adenine dinucleotide-linked reductase activity in a dose-dependent manner and consequently increased the mitochondrial oxidative membrane potential. The production of interleukin-8 was reinforced in forsythia detaching factor-treated cells at 72 h through an increase of the mitochondrial oxidative membrane potential.

Conclusion: The forsythia detaching factor might be involved in the virulence of *T. forsythia* through induction of the pro-inflammatory cytokine interleukin-8.

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Periodontitis is an infectious disease that leads to the inflammatory destruction of periodontal tissues. Among more than 300 species of bacteria in the oral cavity, *Porphyromonas gingivalis*, *Treponema denticola and Tannerella forsythia* have been implicated in the disease as the 'red complex' (1,2). 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 (3). The role of *P. gingivalis* and *T. denticola* is well established in periodontitis development or progression. However, a few putative virulence factors of *T. forsythia* have been identified, such as a trypsin-like protease (4), a sialidase (5), a cell-surface-associated and secreted BspA protein (6), a hemagglutinin (7), components of the bacterial S-layer (8), methylglyoxal production (9) and an apoptosis-inducing activity (10). Recently, we have reported that a protein factor from *T. forsythia* (cytopathic factor) induced apoptotic cell death in a human leukemia cell line (10). Biochemical investigation of the cytopathic effects revealed that a cytopathic component contained two distinct factors (11). One arrested the cell cycle at the G2 phase and the other detached adhesion-dependent human transformed cell lines, such as KB and 293T, almost irreversibly from the substratum, while its removal allowed recovery of adhesion without remarkable delay in cell proliferation. The latter was named 'forsythia detaching factor' (GenBank accession no. AY368075, registered under the tentative name: *cytocidal toxin 1*, *cct-1*). The genomic sequence analysis indicated that forsythia detaching factor is identical to the native form of a previously reported putative cysteine protease, PrtH, the *prtH* gene having been identified directly from a DNA fragment of this bacterium (4). In other words, the *prtH* gene was part of the forsythia detaching factor coding gene (the *fdf* gene) and forsythia detaching factor might be the functional protein in *T. forsythia*.

In order to analyze the biological feature of forsythia detaching factor in periodontitis lesions, normal fibroblasts (human embryonic fibroblast TIG-3 cells) were used as the target in this study.

Material and methods

Preparation of the recombinant forsythia detaching factor and dose definition

The recombinant forsythia detaching factor used in this study has been reported previously (11). Briefly, the expression plasmid, pQE-forsythia detaching factor, 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 forsythia detaching factor was eluted by imidazole concentrations between 300 and 500 mM without any contaminants. The purified protein was desalted with a HiPrep 26/10 Desalting spin column (Amersham Biosciences, Piscataway, NJ, USA), concentrated and stored at −80°C. In all experiments, the amount of forsythia detaching factor was adjusted to the culture dish surface area rather than the volume of the medium to enhance the reproducibility of the effects of forsythia detaching factor. In some experiments, forsythia detaching factor was heated at 60°C for 15 min. All of the experiments in this study were conducted at least three times, in triplicate. Unless otherwise stated, only the results obtained of one representative experiment are shown. The experimental values are the means ± standard deviation of triplicate wells. Statistical analysis was performed using the Student's *t*-test, and the significance level was set as $p < 0.05$.

Fibroblast culture

TIG-3 cells are human embryonic fibroblasts (12). The cells were maintained at ≈40% confluence in Dulbecco's modified Eagle's medium, supplemented with 8% fetal calf serum, at 37°C in 5% CO_2.

Cell lysate preparation and immunoblotting

TIG-3 cells, cultured in the absence or presence of forsythia detaching factor and heated forsythia detaching factor, were washed with cold phosphate-buffered saline twice and lysed with lysis buffer (400 mM NaCl, 50 mM HEPES-Na, pH 7.0, 1 mM EDTA, 0.1% Nonidet P-40 and 0.5% protease inhibitor cocktail against proteases, comprising: chymotrypsin, 1.5 μg/mL; thermolysin, 0.8 μg/mL; papain, 1 mg/mL; pronase, 1.5 μg/mL; pancreatic extract, 1.5 μg/mL; and trypsin, 0.002 μg/mL (Roche, Basel, Switzerland). The lysates were clarified by centrifugation at 15,000 *g* for 5 min and the protein concentration of the supernatant was determined using a bicinchoninic acid assay reagent (Sigma). The samples containing 10 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to poly(vinylidene) difluoride membranes. Immunoblotting was performed as previously described (11). Rabbit anti-forsythia detaching factor was used at a 1 : 5000 dilution. Animal experiments were approved by the Animal Care and Use Committee of Tokyo Medical and Dental University before the operation (Permission no. 0060114).

Analyses for mitochondrial function: the levels of the oxidative membrane potential and interleukin- 8 production

The lysates were prepared from TIG-3 cells that were cultured in 3 × 10 mm dishes in the absence or presence of forsythia detaching factor. The lysates were subsequently fractionated to isolate the three types of fractions – cytoplasmic, mitochondria and the remaining debris containing nuclei – by using the Mitochondria Isolation Kit (Pierce, Hudson, OH, USA), according to the manufacturer's instructions. To assay the nicotinamide adenine

dinucleotide-linked reductase activity of mitochondria, the water-soluble tetrazolium method (13) was performed as described previously. Briefly, a one-tenth volume of water-soluble tetrazolium-8 solution (Dojindo, Tokyo, Japan) was added to the cell culture in 96-well plates and incubated for an additional 2 h. Reduced nicotinamide adenine dinucleotide (NADH) is generated from nicotinamide adenine dinucleotide by the reaction of dehydrogenases. The amount of the yellow-colored formazan dye generated by dehydrogenases in cells was measured by the absorbance at 450 nm. The mitochondrial oxidative membrane potential was estimated by measuring the oxidization-dependent fluorogenic conversion of dihydrotetramethylrosamine (MitoTracker Orange CM-H2TMRos; Invitrogen, Carlsland, CA, USA), which specifically binds mitochondrial membranes. Briefly, TIG-3 cells cultured in the presence of various concentrations of forsythia detaching factor were further incubated with 150 μM MitoTracker Orange CM-H2TMRos (Invitrogen) for 30 min. The cells were subsequently fixed with 4% formaldehyde at 37°C for 20 min, blocked with 0.5% fetal calf serum and subjected to flow cytometry to measure the mean value of the filters for detecting emitted light at an intensity at 585 nm. The relative value was then calculated by comparing it to the corresponding value of the nonforsythia detaching factor-treated culture. The concentration of interleukin-8 in the culture supernatant was assayed using the Human interleukin-8 ELISA kit (Biosource, Nivelles, Belgium) according to the manufacturer's instructions.

Results

Forsythia detaching factor detached human normal fibroblasts

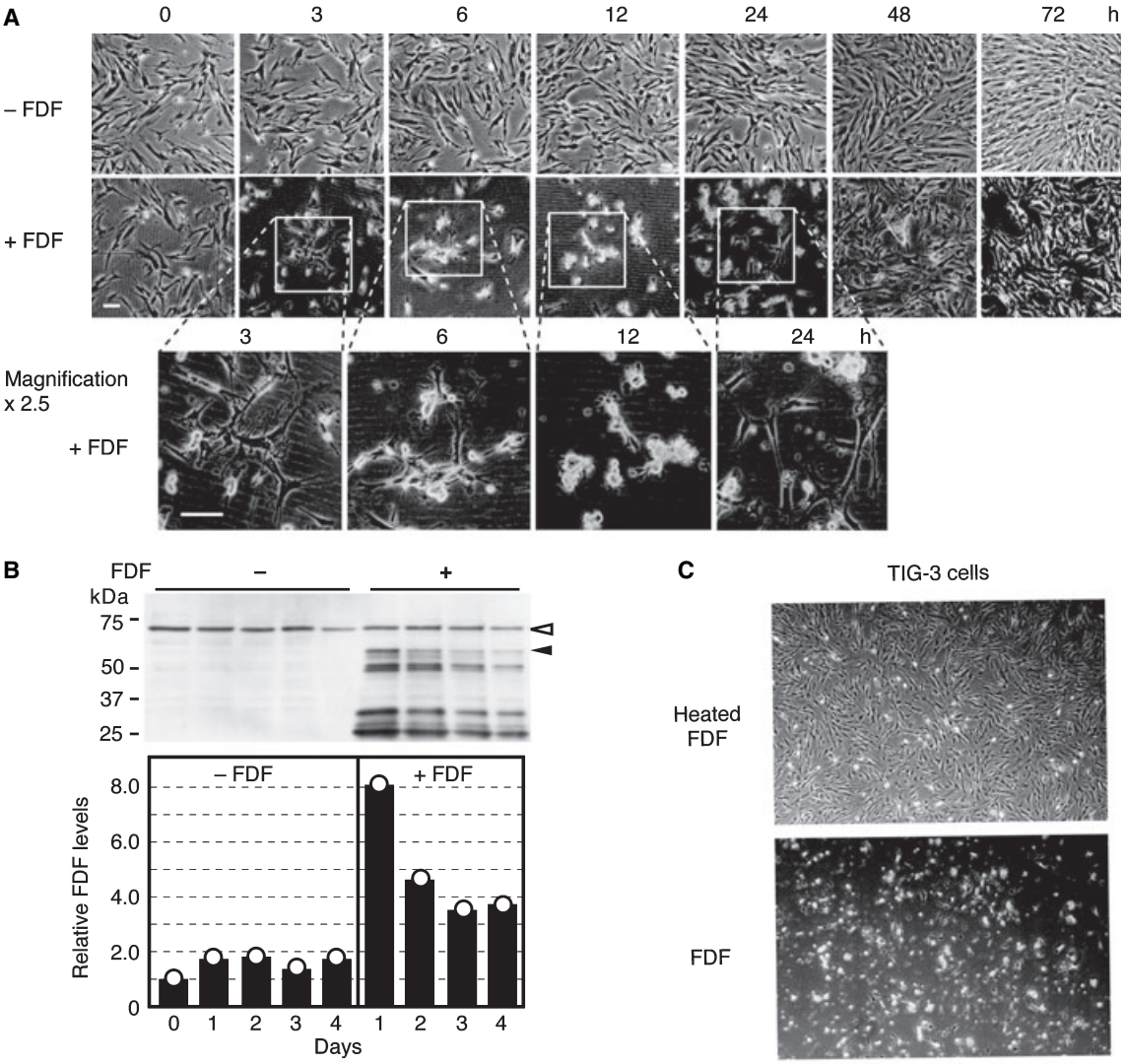
In order to examine whether forsythia detaching factor detaches normal human cells without an effect on proliferation or survival, human diploid fibroblast TIG-3 cells were treated with forsythia detaching factor. Forsythia detaching factor-treated cells displayed the generation of xenomorphic particles and aggregates 3–24 h after the addition of forsythia detaching factor (Fig. 1A). The detached cells resumed adhesion at 48 h and increased in number gradually. To investigate the stability of forsythia detaching factor, western blot analysis with anti-forsythia detaching factor was carried out in forsythia detaching factor-treated cells. The intensity of bands of nonspecific 70 kDa protein (open arrow) was identical, indicating that the same amount of protein was loaded in each well. Forsythia detaching factor (60 kDa; closed arrow) was detected in the lysates prepared from forsythia detaching factor-treated cells on days 1–4 (Fig. 1B). These results demonstrate that forsythia detaching factor detached target cells from the substratum, and, moreover, contributed to the cellular mechanisms maintaining their survival and proliferative potentials. No significant effect was observed 24 h after treatment of cells with heated forsythia detaching factor (Fig. 1C). The 60-kDa form of forsythia detaching factor (closed arrow) was not found in the lysates prepared from cells treated for 24 h with heated forsythia detaching factor (Fig. 1D). As the activities of forsythia detaching factor were lost by heating at 60°C for 15 min, forsythia detaching factor is probably a heat-labile factor composed of protein.

Forsythia detaching factor affects the mitochondrial function

Forsythia detaching factor bound to the cells for 4 d. In order to determine whether forsythia detaching factor reaches a particular organelle following cell entry, the localization of internal forsythia detaching factor in the TIG-3 cells was evaluated by the fractionation of cell lysates. Western blot analysis showed that almost the intact size of forsythia detaching factor was detected in both the mitochondrial and debris fractions, but not in the cytoplasmic fraction (Fig. 2).

In order to evaluate the effects of forsythia detaching factor on the mitochondrial function, the activities of the nicotinamide adenine dinucleotide-linked reductase in forsythia detaching factor-treated cells were analyzed by the water-soluble tetrazolium assay. The reductase activities began to be suppressed within 12 h of treatment with forsythia detaching factor, and the activity of the cells treated with 2 $\mu g/cm^2$ of forsythia detaching factor was decreased to one-third of that of the control at 48 h. The reduction rate of the activity in cells treated with 2 $\mu g/cm^2$ of forsythia detaching factor was twice that of the cells treated with 1 $\mu g/cm^2$ of forsythia detaching factor at 48 h (Fig. 3A). The extent of suppression was gradually reinforced by 72 h, while the cells started to reattach to the substratum and to proliferate at

Fig. 1. Forsythia detaching factor detached TIG-3 cells and exhibited stable association with the cells. (A) TIG-3 cells were cultured in the absence (–FDF) or presence (+FDF) of 2 $\mu g/cm^2$ of forsythia detaching factor. Photographs were captured with either a 4× (upper two rows) or a 10× (bottom row) objective lens at the center area of the dishes. Bar: 30 μm. (B) TIG-3 cells were divided into two groups. The first and second groups were cultured in the absence or presence of 1 $\mu g/cm^2$ of forsythia detaching factor. The respective groups of cells were collected at the indicated period of culture and subjected to immunoblotting to detect forsythia detaching factor using image-analysis software IMAGE J, version 1.3.7t, for Mac. The results were further calculated by setting a value of forsythia detaching factor at time 0 as 1.0. (C) TIG-3 cells were cultured in the presence of 2.5 $\mu g/cm^2$ of heated forsythia detaching factor and unheated forsythia detaching factor for 24 h. (D) Lane 1: identification of purified recombinant forsythia detaching factor protein; 0.5 μg of sonicated extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to immunoblotting. Lanes 2, 3 and 4: TIG-3 cells in the absence (lane 2) or presence of 2.5 $\mu g/cm^2$ of heated forsythia detaching factor (lane 3) and unheated forsythia detaching factor (lane 4) for 24 h; 10 μg of sonicated extract was subjected to immunoblotting. FDF, forsythia detaching factor.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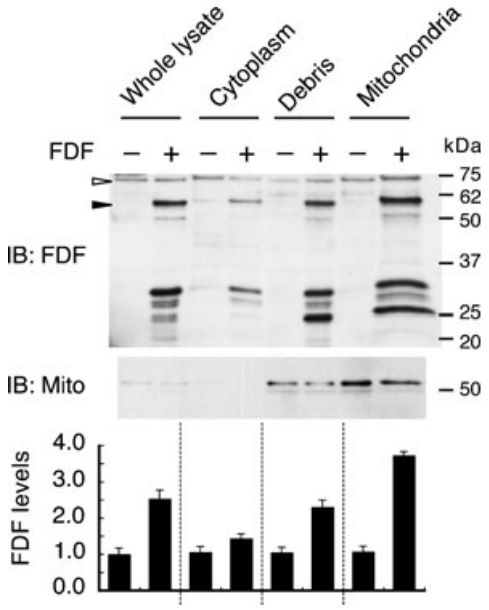

Fig. 2. TIG-3 cells were cultured in the absence or presence of 1 μg/cm^2 of forsythia detaching factor for 2 h, after which the mitochondrial fraction was extracted. Fractions of 10 μg were immunoblotted with the indicated antigens. FDF, forsythia detaching factor; IB, immunoblotting; Mito, mitochondrial antigen.

48 h, even in the presence of higher concentrations of forsythia detaching factor (Fig. 1A). These results show that forsythia detaching factor suppressed the nicotinamide adenine dinucleotide-linked reductase activity in a dose-dependent manner, but did not disturb the upstream metabolic pathways, such as the tricarboxylic acid cycle.

Induction of interleukin-8 production following accumulation of the oxidative membrane potential in mitochondria

To investigate the effect of the suppression of the nicotinamide adenine dinucleotide-linked reductase activity, we studied the oxidative membrane potential in mitochondria of forsythia detaching factor-treated cells. The oxidative level of the cells treated with 2 μg/cm^2 of forsythia detaching factor was about three times and twice higher than that of the control at 48 and 72 h, respectively (Fig. 3B). Moreover, the levels of 2 μg/cm^2 of forsythia detaching factor-treated cells were significantly higher than those of 1 μg/cm^2 of forsythia detaching factor-treated cells at 48 and 72 h. These results indicate that forsythia detaching factor treatment caused a distinct increase in the mitochondrial oxidative membrane potential in a dose-dependent manner.

In order to analyze the production of interleukin-8 in the cells treated with forsythia detaching factor, the concentration of interleukin-8 in the culture supernatant was assayed by enzyme-linked immunosorbent assay. There was no difference in interleukin-8 production between control cells and cells treated with forsythia detaching factor (1 or 2 μg/cm^2) for 48 h. However, the interleukin-8 concentration of the cells treated with 2 μg/cm^2 of forsythia detaching factor was significantly higher than that of cells treated with 1 μg/cm^2 of forsythia detaching factor and of control cells at 72 h (Fig. 3C).

Discussion

In this study, we demonstrated that forsythia detaching factor induced the detachment and subsequent reattachment of cells, and that it also reinforced the production of the pro-inflammatory cytokine, interleukin-8, by increasing the mitochondrial oxidative membrane potential. In general, adhesion-based signals for the maintenance of integrity are thought to be important for nonlymphoid normal cells. The proliferation and survival without adhesion-based signaling occurred only when the cells were transformed malignantly (14,15). Forsythia detaching factor detached target cells from the substratum 3–24 h after treatment. Interestingly, however, it also contributed to the cellular mechanism maintaining their survival and proliferative potential. The intact size of forsythia detaching factor was detected by western blotting in the cells treated for 4 d. This refutes the notion that the survival and proliferation of forsythia detaching factor-treated cells occurred by the degradation of forsythia detaching factor. As no significant effect was observed when cells were treated with heated forsythia detaching factor (data not shown), forsythia detaching factor was assumed to be composed of protein.

Whereas transformation of a proto-oncogene renders fibroblasts resistant to anoikis (anoikis is apoptosis induced by loss of adhesion to the extracellular matrix), nontransformed fibroblasts (normal fibroblasts) in suspension undergo cell cycle arrest and cell death (16). It is well documented that proteases from *P. gingivalis* can induce cell death in fibroblasts (17,18). Wang *et al.* reported that after 24 h in the presence of a *P. gingivalis* protease, human gingival fibroblasts lost attachment and started rounding, and that none of the rounded cells proliferated. In our study, almost all the detached cells proliferated, even when they were isolated and returned to culture (data not shown). Forsythia detaching factor detached from the substratum; however, it also contributed to the cellular mechanism that maintains cellular survival and proliferative potential of fibroblasts in suspension. It is possible that the proliferation in forsythia detaching factor-treated cells might be up-regulated by factors for cell growth and survival or by down-regulation of the cell death signals.

As forsythia detaching factor-treated cells proliferated following detachment from the substratum, it is suggested that forsythia detaching

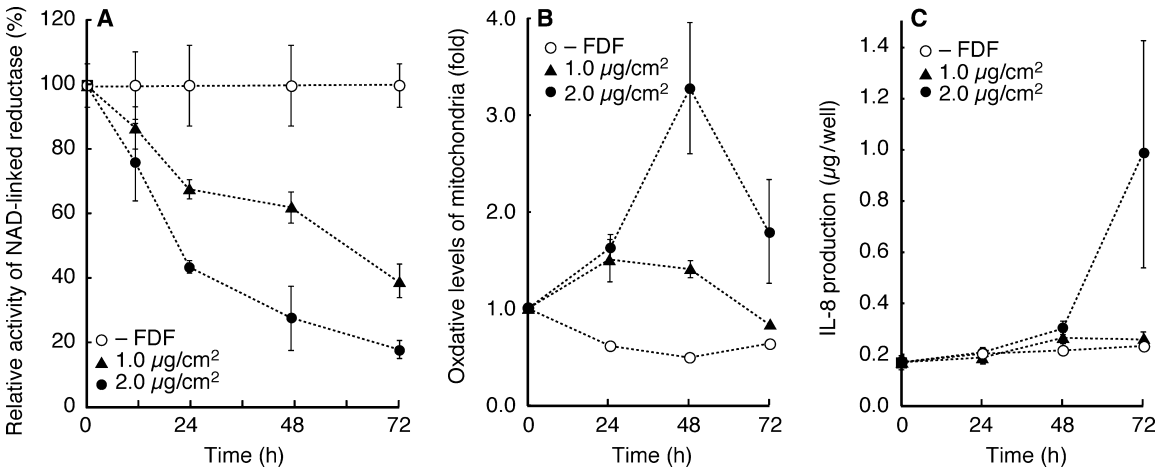

Fig. 3. (A) TIG-3 cells were cultured in the presence of various amounts of forsythia detaching factor, and the relative activities of nicotinamide adenine dinucleotide-linked reductases were assessed in triplicate by the water-soluble tetrazolium assay at the indicated times. Every result was compared with nonforsythia detaching factor-containing cultures plated at various densities, and the relative activity was calculated. Error bars show the respective standard deviations. (B) TIG-3 cells were cultured in the presence of the indicated amount of forsythia detaching factor, and the mitochondrial oxidative potentials were assayed every 24 h in duplicate by monitoring the fluorogenic conversion of dihydrotetramethylrosamine. Error bars show the respective standard deviations. (C) The remaining culture supernatants from the previous analysis were used to assess interleukin-8 concentrations. Error bars show the respective standard deviations. FDF, forsythia detaching factor; NAD, nicotinamide adenine dinucleotide.

factor enters cells with an affinity for particular organelles and regulates intracellular signaling. The majority of the intact size forsythia detaching factor was detected in both the mitochondrial and debris fractions, but not in the cytoplasmic fraction. These results demonstrated that forsythia detaching factor entered the TIG-3 cells and most of the internalized forsythia detaching factor translocated to mitochondria. The analyses of the effect on mitochondrial function revealed that forsythia detaching factor suppressed the nicotinamide adenine dinucleotide-linked reductase activity in a dose-dependent manner. The nicotinamide adenine dinucleotide-linked reductase transfers oxygen to the electron transport chain during energy production. Suppression of its activity induces the reduction of oxygen consumption; in other words, the increase of the oxidative potential in mitochondria. In this study, the production of interleukin-8 was reinforced in the cells treated with forsythia detaching factor through an increase of the mitochondrial oxidative membrane potential. Hwang *et al.* reported that the accumulation of reactive oxygen species occasionally accompanies cellular pro-inflammatory responses (19). We measured the reactive oxygen species concentration in KB cells (a human epidermal carcinoma cell line) treated with forsythia detaching factor (data not shown). In that study, the concentration of reactive oxygen species (6.5 μM) in the cells treated with 2 μg/cm^2 of forsythia detaching factor was more than four times higher than that of the control (1.5 μM) at 48 h. Furthermore, it is reported that oxidative potential stimulates the production of cellular reactive oxygen species (20). It might therefore be suggested that forsythia detaching factor induced interleukin-8 production by a higher concentration of reactive oxygen species following the suppression of the nicotinamide adenine dinucleotide-linked reductase activity. *P. gingivalis*, *Treponema denticola* and *T. forsythia* stimulated the secretion of pro-inflammatory cytokines, chemokines, prostaglandin E2, and matrix metalloproteases-9 (21) in a mixed culture of macrophage and epithelial cells. This study provides evidence for the possibility of interleukin-8 induction activity of *T. forsythia* itself in fibroblasts. Interleukin-8 plays a pivotal role in the recruitment and activation of neutrophils and monocytes in various experimental models of inflammation (22). Therefore, forsythia detaching factor is one of the important virulence factors of *T. forsythia* and it may contribute to the initiation, prolongation and amplification of the inflammatory response in periodontal tissue.

In conclusion, our data indicate that forsythia detaching factor does not disturb the upstream metabolic pathways because neither cell proliferation nor cell viability were inhibited after 48 to 72 h of incubation, whereas forsythia detaching factor induced the production of the pro-inflammatory cytokine, interleukin-8, through suppression of the nicotinamide adenine dinucleotide-linked reductase activity. Therefore, forsythia detaching factor probably has a role in the initiation, prolongation and amplification of the inflammatory response in periodontal tissue.

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