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Analysis of immunostimulatory activity of *Porphyromonas gingivalis* fimbriae conferred by Toll-like receptor 2

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ABSTRACT

Bacterial fimbriae are an important pathogenic factor. It has been demonstrated that fimbrial protein encoded by *fimA* gene (FimA fimbriae) of *Porphyromonas gingivalis* not only contributes to the abilities of bacterial adhesion and invasion to host cells, but also strongly stimulates host innate immune responses. However, FimA fimbriae separated from *P. gingivalis* ATCC 33277 using a gentle procedure showed very weak proinflammatory activity compared with previous reports. Therefore, in the present study, biological characteristics of FimA fimbriae were further analyzed in terms of proinflammatory activity in macrophages. Macrophages differentiated from THP-1 cells were stimulated with native, heat-denatured, or either proteinase- or lipoprotein lipase-treated FimA fimbriae of *P. gingivalis* ATCC 33277. Stimulating activities of these FimA fimbriae were evaluated by TNF-α-inducing activity in the macrophages. To clarify the mode of action of FimA fimbriae, anti-Toll-like receptor (TLR) 2 blocking antibody was added prior to stimulation. Weak stimulatory activity of native FimA fimbriae was enhanced by heat treatment and low-dose proteinase K treatment. Higher dose of proteinase K treatment abrogated this up-regulation. The activity of treated FimA fimbriae was suppressed by anti-TLR2 antibody, and more substantially by lipoprotein lipase treatment. These results suggest that lipoproteins or lipopeptides associated with FimA fimbriae could at least in part account for signaling via TLR2 and subsequent TNF-α production in macrophages.

1. Introduction

Porphyromonas gingivalis, a representative periodontopathic bacterium, has a variety of virulence factors such as fimbriae, lipopeptides, lipopolysaccharides, and proteinases called gingipains [1] even though the immunostimulating activity itself is much weaker compared with *Escherichia coli*.

Fimbriae, filamentous appendages that protrude outwards from the bacterial cell surface, play a crucial role in virulence by stimulating bacterial attachment to various host cell or tissue [2]. Although it has been described that two types of fimbrial structures are encoded, one by the *fimA* gene (FimA fimbriae) and another by the *mfa1* gene [3], FimA fimbriae have been studied in greater detail.

Using monoclonal blocking antibodies in monocytes, it has been reported that the inflammatory response to *P. gingivalis* fimbriae is mainly dependent on stimulation of Toll-like receptor (TLR) 2. Likewise, a separate study using anti-TLR2 antibody and human gingival epithelial cells, which predominantly express TLR2, but not TLR4, or CD14, suggested that *P. gingivalis* fimbriae are capable of activating human gingival epithelial cells through TLR2 [4]. TLR2 is reported to be essential for signaling in *Staphylococcus aureus* peptidoglycan, bacterial lipoproteins, lipoteichoic acid, and lipopeptides [5].

It is reported that native major fimbriae were dependent on both TLR4 and especially TLR2 for stimulation of cytokine response, even though CD11/CD18 interaction is required for signaling [6]. In this respect, Ogawa et al. demonstrated that native *P. gingivalis* fimbriae and an active synthetic peptide, ALTTE, induced IL-6 production on human monocytes that is inhibited by anti-CD14, anti-TLR2, or anti-CD11a antibodies but not by anti-CD11b or anti-TLR4 antibodies [7]. On the other hand, Harokopakis et al. showed the ligand binding capacity of CD11b/CD18 occurs through an inside-out signaling pathway involving TLR2 [8].

Thus, whether FimA fimbriae can act as direct ligands for TLR2, and if so, the mode and mechanism of interaction between FimA fimbriae and TLR2 have not been fully elucidated.

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Increasing evidence suggests proteinaceous substances can be ligands for pattern recognition receptors and stimulate various cells to produce proinflammatory cytokines [9]. The reported cytokine effects of the proteinaceous ligands are similar to the effects of LPS and bacterial lipoproteins. Evidence for proteinaceous ligands of TLRs was derived using recombinant products, purified native molecules, or purified fragments of macromolecules. Since recombinant products are produced by genetically engineered *E. coli*, the final preparations may be contaminated with bacterial products [9] even if the contamination is below detection level by highly sensitive methods. Likewise, purified preparations are also frequently contaminated with bacterial cell-wall products such as LPS and lipoproteins [10].

Although, putative active amino acid motif of FimA fimbriae responsible for TLR2 mediated signaling is indicated [7,11], the facts that FimA fimbriae preparation showed relatively high cytokine-inducing activity and that both TLR1/TLR2 complex and TLR2/TLR6 complex confer FimA fimbriae signaling [12] raised a possibility of the involvement of cell-wall products other than FimA fimbriae. Therefore, the aim of the present study was to further characterize the cytokine-inducing activity of *P. gingivalis* FimA fimbriae.

2. Materials and methods

2.1. Reagents and antibodies

Native FimA fimbriae of *P. gingivalis* strain 33277 were purified as described previously [13] and no detectable LPS contamination was observed (data not shown). Phorbol myristic acetate (PMA), lipoprotein lipase from bovine milk, and proteinase K from *Engyodontium album* were obtained from Sigma–Aldrich (St. Louis, MO). Pam_3Cys-SKKKK x 3HCl (Pam_3CSK4) was purchased from EMC microcollections (Tuebingen, Germany). Blocking mouse anti-human TLR2 antibody and mouse IgG2a antibody (as control) were purchased from eBioscience (San Diego, CA).

Synthetic peptide which have been reported to be active in stimulating macrophages [11] were synthesized by Toray Research Center, Inc. (Tokyo, Japan). As negative controls, the reverse amino acid sequences and an inactive analog peptide in which an alanine was substituted with glycin [7] were also synthesized (Supplementary Table 1).

2.2. Cell preparation and culture

The monocytic cell line THP-1 was maintained in a solution composed of 25 mM Hepes-buffered RPMI 1640 supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin, hereafter referred to as medium. All incubations were carried out at 37 °C in an atmosphere of 5% CO_2 in air.

For the experiments, the cells were incubated in a 96-well culture plate (NUNC, Roskilde, Denmark) at a concentration of 1×10^5 cells/ml in the medium supplemented with 10 ng/ml of PMA to induce differentiation into macrophage-like cells, hereafter referred to as macrophages. After 48 h of incubation, the cells were extensively washed with RPMI 1640, cultured for 12 h further in the medium without FCS, and the medium was then changed to remove the cytokines induced by cell adherence. Finally, various doses of FimA fimbriae, synthetic peptides and Pam_3CSK4 were added to the culture and the cells were stimulated for 6 or 16 h.

2.3. Preparation of FimA fimbriae, and biochemical analysis

To examine whether the activity of FimA fimbriae is in fact attributable to the proteinaceous components, FimA fimbriae were heat-denatured at 95 °C for 10 min or digested by either proteinase K or lipoprotein lipase prior to addition to the culture. FimA fimbriae (500 μg) were treated with 120 or 600 U of the proteinase K in 1 ml of reaction mixture at 37 °C for 12 h. The reactions were then terminated at 95 °C for 10 min. For lipoprotein lipase treatment, the enzyme was reacted at a ratio of 1, 6, or 12 U: 1 μg of FimA fimbriae at 37 °C for 3 h and then the reactions were terminated by heating at 95 °C for 10 min.

For blocking experiment, anti-TLR2 antibody or isotype control was added at 20 μg/ml and preincubated for 30 min prior to the stimulation.

2.4. Cytokine assay

The level of TNF-α in the supernatants of macrophage cultures was determined using commercially available ELISA kits (Thermo Fisher Scientific, Waltham, MA) in accordance with the manufacturer's instructions.

2.5. Statistical analysis

All experiments were performed in triplicate wells for each set of conditions and repeated at least twice. Results were expressed as means ± standard deviation (SD). Data were analyzed by paired or unpaired *t*-test. A probability value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of native FimA fimbriae, synthetic peptides and Pam_3CSK4 on the production of TNF-α in macrophages

Stimulatory effect of native FimA fimbriae on the TNF-α production was very weak and the effect was observed only at a concentration of 10 μg/ml. Because of previous reports regarding the active motif within native FimA fimbriae in stimulating macrophages [11], we tested the stimulatory activity of the synthetic peptide having exactly the same amino acid sequence that induced positive response in the previous study (peptide No. B459) together with 2 negative control peptides (peptide No. B460 and B461). As shown in Fig. 1, none of the peptides demonstrated stimulatory activity even at a concentration of 100 μg/ml. The activity of native FimA fimbriae was 10,000 times weaker than that of Pam_3CSK4, which is known to be TLR2 agonist (Fig. 1). Because of the very weak stimulatory activity, antagonistic effect of FimA fimbriae on Pam_3CSK4 stimulation was examined. However, no such effect was observed (data not shown).

3.2. Effect of heat-denature and enzyme treatment of FimA fimbriae on TNF-α production by macrophages

If the proteinaceous components are responsible for the TNF-α-inducing activity, degradation of the protein may result in lowering the activity. To test this hypothesis, we denatured native FimA fimbriae by heating at 95 °C for 10 min or proteinase K digestion prior to the stimulation. Fig. S1 shows protein profile of the native, heat-denatured, and proteinase K-treated FimA fimbriae. There was no significant difference in electrophoretic profile between native and heat-denatured FimA fimbriae, though several minor bands with lower molecular weights appeared in heat-denatured sample. Digestion with 120 U/ml of proteinase K at 37 °C degraded 41 kDa major FimA fimbriae into 25, 23, and 12 kDa fragments. Increased concentration of proteinase K was further digested and 41 kDa native FimA fimbriae disappeared after digestion with 600 U/ml of proteinase K at 37 °C (Supplementary Fig. 1).

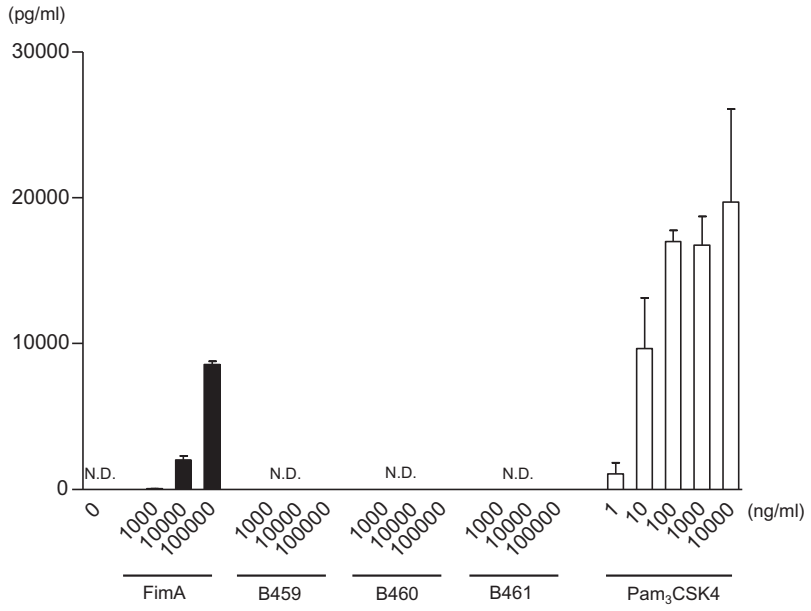

Fig. 1. Stimulatory effect of native FimA fimbriae of *P. gingivalis* and Pam_3CSK4 on TNF-α production. Macrophages were unstimulated or stimulated with various doses of FimA fimbriae, synthetic peptides and Pam_3CSK4 for 16 h, and human TNF-α induction in culture supernatants was analyzed by ELISA. Results are shown as means ± SD. ND: not detected.

As shown in Fig. 2, TNF-α production by macrophages was significantly augmented by heat-denatured FimA fimbriae compared with native protein. Unexpectedly, treatment with low-dose (120 U/ml) of proteinase K for 12 h up-regulated the TNF-α-inducing activity of FimA fimbriae. Since it was expected that this curious finding might have derived from insufficient digestion by the enzyme, further degradation by higher dose of the enzyme was carried out. As a result, the up-regulated stimulatory effect was abolished by these treatments (Fig. 2A). There was no significant difference between different stimulation times on TNF-α production except for heat-denatured FimA fimbriae (Fig. 2B).

3.3. Effect of anti-TLR2 antibody on TNF-α induction in macrophages stimulated with heat-denatured or proteinase K-treated FimA fimbriae

Since denaturation or degradation of FimA fimbriae significantly up-regulated the stimulatory effect on TNF-α production by macrophages, conformational change or appearance of receptor-binding site of FimA fimbriae was expected to account for these effects. To clarify whether the enhanced stimulatory effect is attributable to TLR2 ligand binding activity, anti-TLR2 blocking antibody was added to the cultures prior to addition of the stimulants. Anti-TLR2 antibody significantly suppressed up-regulation of TNF-α-inducing activity by heat-denatured or proteinase K-treated FimA fimbriae (Fig. 3). However, no such effect was observed for anti-TLR4 and anti-TLR5 antibodies (Supplementary Fig. 2).

3.4. Effect of lipoprotein lipase treatment on the TNF-α-inducing activity of heat-denatured FimA fimbriae

Most, if not all, the TLR2 ligands are lipid antigens and anti-TLR2 blocking antibody suppressed the TNF-α-inducing activity of FimA fimbriae. These observations led us to speculate the biological activity of FimA fimbriae could at least in part be due to lipoproteins

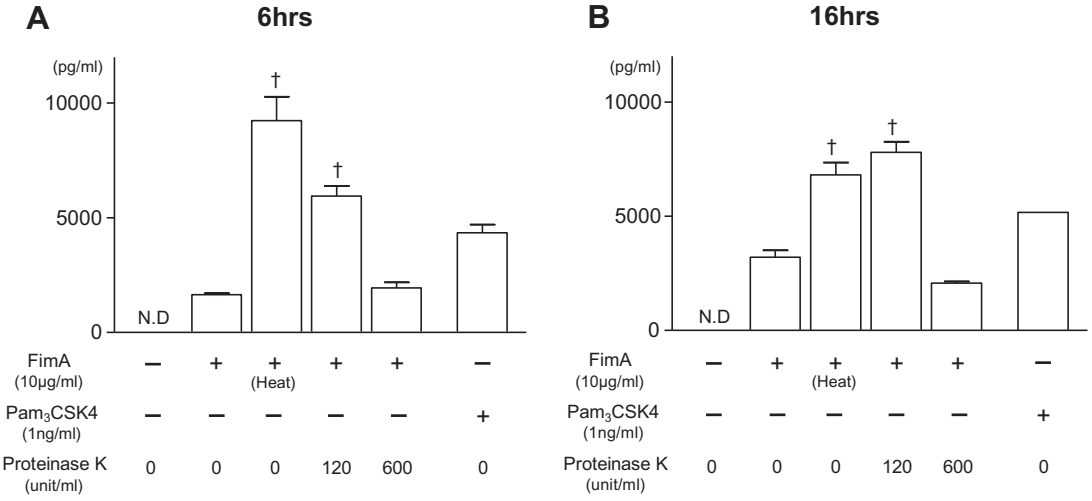

Fig. 2. Heat treatment and proteinase K digestion of native FimA fimbriae up-regulate TNF-α production in macrophages. Native FimA fimbriae were heat-denatured at 95 °C for 10 min or digested with proteinase K under indicated conditions. THP-1-derived macrophages were stimulated with either native or treated FimA fimbriae for 6 (A) or 16 h (B) and TNF-α induction in culture supernatants was analyzed by ELISA. Results are shown as means ± SD. Significant differences are shown ($^{\dagger}P < 0.01$, paired *t*-test).

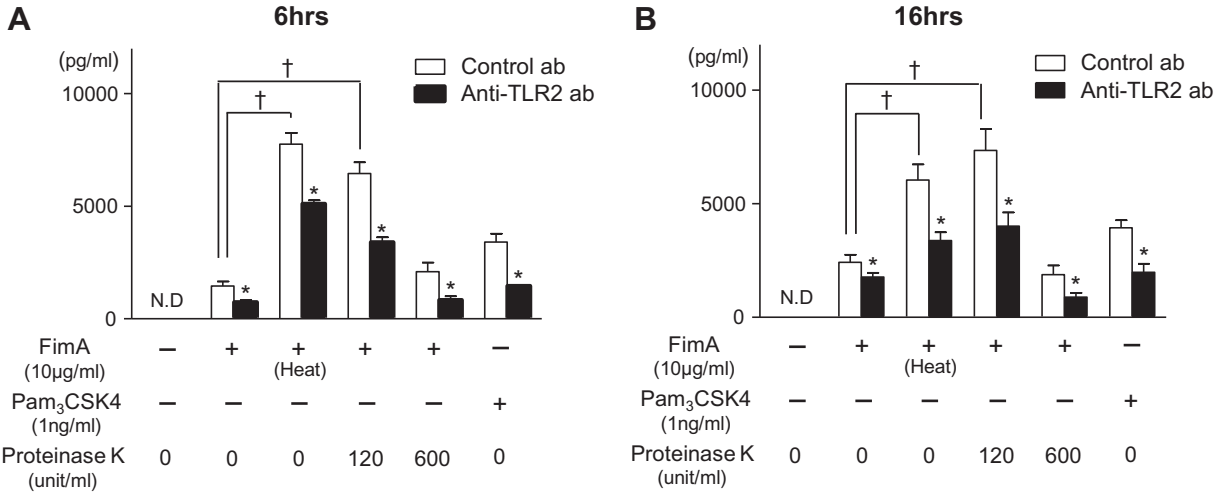

Fig. 3. Effects of anti-TLR2 antibody on the production of TNF-α in macrophages stimulated with native and treated FimA fimbriae. Macrophages were cultured in the presence of anti-TLR2 antibody or control antibody at a concentration of 20 μg/ml, and then either stimulated with the indicated stimulants for 6 (A) or 16 h (B) or left unstimulated. TNF-α induction in culture supernatants was analyzed by ELISA. Results are shown as means ± SD. Significant differences are shown ($^{\dagger}P < 0.01$, paired *t*-test; $^{*}P < 0.01$, unpaired *t*-test).

or lipopeptides bound to FimA fimbriae. To test this possibility, heat-denatured FimA fimbriae were digested with various doses of lipoprotein lipase. As shown in Fig. 4, increasing concentration of lipoprotein lipase decreased the production of TNF-α in the culture supernatant in a dose-dependent manner, irrespective of presence or absence of heat-denaturation prior to enzyme treatment.

3.5. Interleukin-1 receptor-associated kinase (IRAK)-M induction in macrophages by FimA fimbriae and its relation to TNF-α expression

In our previous study, we found that LPS from *P. gingivalis* preferentially up-regulated IRAK-M in macrophage. To examine the effect of FimA fimbriae on the expression of IRAK-M and its relation to TNF-α expression, macrophages were stimulated with native and heat-denatured FimA fimbriae and FimA fimbriae did not induce significant up-regulation of IRAK-M expression in macrophages (Supplementary Fig. 3).

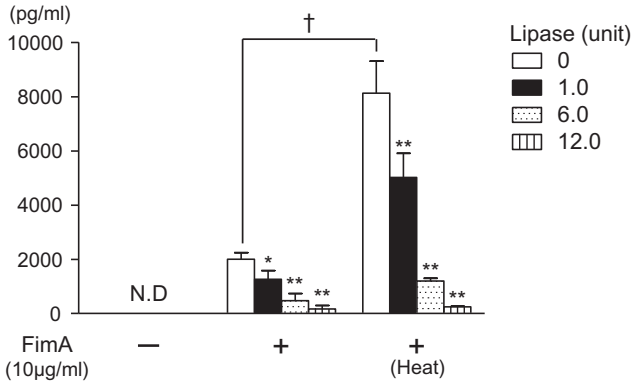

Fig. 4. Effects of lipoprotein lipase treatment on the production of TNF-α in macrophages stimulated with heat-denatured FimA fimbriae. Native or heat-denatured FimA fimbriae were digested with lipoprotein lipase at indicated concentrations at 37 °C for 3 h and then the reactions were terminated by heating at 95 °C for 10 min. THP-1-derived macrophages were stimulated with treated FimA fimbriae for 16 h and TNF-α induction in culture supernatants was analyzed by ELISA. Results are shown as means ± SD. Significant differences are shown ($^{\dagger}P < 0.01$, paired *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$, unpaired *t*-test).

4. Discussion

Adhesion and proliferation are important steps for bacteria to establish infection in host tissues [14,15]. Adhesion involves substantial machinery. *P. gingivalis* possess fimbrial protein that is encoded by *fimA* gene, which plays important roles in adhesion and invasion to various cells [16]. FimA fimbriae also act as an immunostimulatory component that enable induction of proinflammatory cytokines in monocytes/macrophages [6,7,17], epithelial cells [4], and endothelial cells [18,19].

Immunostimulatory activity of *P. gingivalis* is known to be much lower than that of *E. coli*. We previously demonstrated that *P. gingivalis* LPS induced up-regulation of IRAK-M, a negative regulator of TLR signaling pathway, in THP-1-derived macrophage more robust than *E. coli* LPS [20]. In addition, it is reported that *P. gingivalis* can, by manipulating host response, evade TLR-mediated immunity dependent on the exploitation of chemokine receptors, such as CXCR4 [21]. These findings let us speculate that major pathogenic component, FimA fimbriae, which are reported to act as TLR2 ligands, are also involved in the escape mechanism of this bacterium from host immune surveillance system. However, FimA fimbriae did not induce significant up-regulation of IRAK-M expression in macrophages. The difference of IRAK-M-inducing activity between LPS and FimA fimbriae could be due to difference of biological activities. Since Pam_3CSK4 in the present study and peptidoglycan as reported in the previous study [22] could induce IRAK-M, difference in the receptor usage may not be account for weak IRAK-M-inducing activity of FimA fimbriae. Moreover, and quite unexpectedly, the ability of TNF-α induction by FimA fimbriae used in the present study was much lower compared with not only other TLR2 ligands such as Pam_3CSK4, but also native or recombinant FimA fimbriae, as reported in other studies [6,12,17] in which the activity of their FimA fimbriae was more than 10 times higher than that of our FimA fimbriae. One explanation for these discrepancies is the difference in the procedures for purification of FimA fimbriae. FimA fimbriae preparations used in this study were prepared without sonication to avoid contamination of other cell membrane components, whereas others used sonication as a first step of purification [23]. Since almost all the cell membrane components are considered to be liberated by sonication, contamination by authentic TLR ligands in the preparation is highly likely even though the quantities would be very small.

Our study demonstrated that TNF-α production in macrophages was significantly up-regulated by FimA fimbriae after heat treatment. Digestion with low-dose proteinase K also enhanced TNF-α-inducing activity of FimA fimbriae. These results suggest that the active motif was exposed after denaturalization or degradation of proteinaceous component. Although the mechanisms for the up-regulation of proinflammatory activity are yet to be resolved, it is possible that proteinase K treatment may have dissociated accessory proteins from native FimA fimbriae. These minor fimbriae are reported to be associated with FimA fimbriae, and minor fimbriae-deficient mutants are more potent inducers of NF-κB activation and TNF-α production compared with wild-type [24].

Furthermore, enhanced TNF-α-inducing activity of heat-denatured FimA fimbriae was deteriorated by lipoprotein lipase or higher dose of proteinase K treatments prior to stimulation. These observations indicate that exposed active fragment(s) or molecule(s) that stimulates macrophages could be lipoproteins or lipopetides. Lipoproteins are found in more than 400 proteins from all kinds of bacteria [25], including *P. gingivalis* [26]. They are anchored to the cellular membrane via lipid chains attached to conserved N-terminus and induce strong proinflammatory responses from macrophages [14].

The above speculation is further supported by the fact that anti-TLR2 blocking antibody effectively suppressed TNF-α-inducing activity of heat-denatured as well as native FimA fimbriae. Therefore, TLR2 is thought to confer the signaling of FimA fimbriae. This was further confirmed by the findings obtained from blocking experiment using anti-TLR4 and anti-TLR5 antibodies. However, this does not necessarily imply that proteinaceous component is involved in the TLR2 signaling. Previous studies have shown that TLR2 is the main receptor recognizing lipoproteins and lipopeptides. Triacylated lipoproteins are recognized by the TLR1/TLR2 complex [27,28], but diacylated lipopeptides, lacking the amide-bound lipid chain, can activate both the TLR1/TLR2 and TLR2/TLR6 complexes [29,30]. It is reported that both TLR1/TLR2 and TLR2/TLR6 are important in the transcription of proinflammatory cytokines. However, CD14 is much more important and a critical molecule in this response [12]. Contrary to that report, the effect of CD14 is less pronounced compared with TLR2 in IL-6 production of monocytes stimulated with FimA fimbriae [7]. The discrepancy between these studies could be due to the different methods of preparation of FimA fimbriae, in which contaminated lipoproteins or lipopetides are variable. Previous studies demonstrated that presence of triacylated lipopeptides in the LPS preparation of *P. gingivalis* is responsible for the immunostimulatory activity via TLR2 [31].

Biogenesis and assembly of FimA fimbriae have recently been clarified. Shoji et al., demonstrated by using N-terminal amino acid sequencing of the prosequences, treatment of *P. gingivalis* cells with globomycin, an inhibitor for lipoprotein-specific signal peptidase, amino acid substitution of the cysteine residue of the prosequence of fimbrillin, and [^{3}H]-palmitic acid labeling, that the FimA fimbriae are matured through lipoprotein precursor forms [32]. They also clarified that Arg-gingipain is involved in the processing on the surface to yield the mature forms. However, whether the lipoprotein precursor forms were present in our preparation of FimA fimbriae is again not known.

The results of our study suggested that the TNF-α-inducing activity of FimA fimbriae could be attributable to a lipoprotein component that is inapparent in native form of FimA fimbriae, though the structural relationship of the lipoprotein with FimA fimbriae is not known. However, there are reports that epitopes of FimA fimbriae for immunostimulatory activities were determined using synthetic peptides covering the entire length of the subunit of FimA fimbriae [11,33]. The results of both of these studies suggest that similar active motif can stimulate CD14–TLR2 complex, despite the fact that the former study underscored the role of CD14, while the latter study highlighted the role of TLR2. Overall, these results suggest that peptide rather than lipopeptide directly stimulates the cells via pattern recognition receptors. However, we were unable to confirm the active motif of FimA fimbrie in spite of using exactly the same peptide. Although the reason(s) for this discrepancy remained unsolved, the question was again raised as to whether proteinaceous ligands can activate TLR signaling.

Most TLR2 ligands are lipopeptides or lipoproteins, and *P. gingivalis* FimA fimbriae are only exceptional proteinaceous ligands of bacterial origin reported so far. Our study demonstrated that the activity of native FimA fimbriae was suppressed by TLR2 antibody, which is concordant with other studies [7,12,17]. However, the activity of native FimA fimbriae itself, which is far less than that of previous reports, became dramatically increased after denaturation or degradation by heat treatment or enzymatic digestion, respectively.

Information regarding lipoproteins of *P. gingivalis* is limited, and how the lipoproteins associate with plasma membrane or whether the lipoproteins bind or associate with FimA fimbriae is not known. Further studies are clearly needed to clarify whether FimA fimbriae are associated with lipoprotein, and if so, the biochemical structures.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.06.040.

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