Separation and structural analysis of lipoprotein in a lipopolysaccharide preparation from *Porphyromonas gingivalis*

Masahito Hashimoto, Yasuyuki Asai and Tomohiko Ogawa

Department of Oral Microbiology, Asahi University School of Dentistry, 1851-1 Hozumi, Mizuho, Gifu 501-0296, Japan

Keywords: Gram-negative bacteria, innate immunity, NF-κB activation, periodontal disease, Toll-like receptor

Abstract

Lipopolysaccharide (LPS) preparations from the periodontopathic bacterium *Porphyromonas gingivalis* (Pg-LPS) are thought to require Toll-like receptor (TLR)2 rather than TLR4, a receptor of *Escherichia coli* LPS (Ec-LPS), for activation of immune cells. However, we previously reported that *P. gingivalis* lipid A, an immunostimulatory principal component of LPS, and its synthetic counterpart activate cells through a TLR4-dependent pathway but not via TLR2. In the present study, a lipoprotein from Pg-LPS (Pg-LP) was shown to be a principal component for TLR2-mediated cell activation. Pg-LP was separated by hydrophobic interaction chromatography followed by preparative electrophoresis and identified by internal peptide sequencing as PG1828, a putative lipoprotein encoded in the *P. gingivalis* genome. The N-terminal structure was characterized as a triacylated lipopeptide using mass spectrometry. Pg-LP, as well as Ec-LPS, was potent in inducing IL-8 production in human gingival fibroblasts. From our results, we propose that Pg-LP is a powerful inflammatory factor of *P. gingivalis*.

Introduction

Lipopolysaccharide (LPS) is an outer membrane component of Gram-negative bacteria that exhibits powerful immunostimulatory and inflammatory activities (1). LPS causes immediate cell activation and release of proinflammatory cytokines by macrophages and is also responsible for systemic inflammation, multiple organ failure and death seen in patients with Gram-negative bacterial sepsis. Recently, Toll-like receptor (TLR)4, with its accessory protein MD-2, was reported to function as a signaling receptor for *Escherichia coli* LPS (Ec-LPS) and LPSs from other types of enterobacterial LPSs (2). Lipid A, a hydrophobic anchor in LPS and essential moiety for the immunostimulatory activities of LPS, has also been shown to be recognized by the receptor. In contrast, a recent study indicated that TLR2 was the primary signal-transducing molecule for an LPS preparation from the periodontopathic bacterium, *Porphyromonas gingivalis* (Pg-LPS) (3). Further, LPSs from some Gram-negative organisms, including *Prevotella intermedia*, *Leptospira interrogans* and *Helicobacter pylori* were reported to activate TLR2-dependent signaling (4–6). These different results are thought to have been caused by differences in the chemical structure of lipid A moieties (7).

We previously demonstrated that highly purified lipid A from *P. gingivalis* (Pg-lipid A) and *P. intermedia*, as well as synthetic counterparts of Pg-lipid A and lipid A from *H. pylori*, activated cells through a TLR4–MD-2-dependent pathway, however, not via TLR2, which was in contrast to the properties of their LPS preparations (8–10). Further, Pg-LPS was reported to antagonize Ec-LPS activity directly via TLR4, suggesting an interaction of Pg-LPS with TLR4 (11). These results indicate that LPS itself may be recognized by TLR4, while a TLR2-activating component might exist in nonenterobacterial LPS preparations.

TLR2 is known to be a predominant signal transducing molecule utilized by peptidoglycan and bacterial lipoproteins (2). Commercially available enterobacterial LPS preparations have been shown to slightly induce a signal via TLR2, in addition to TLR4, however, the activity is the result of contaminants in those preparations, which can be removed by phenol re-extraction (12) and were recently identified as lipoproteins (13). Although it has been reported that nonenterobacterial LPS preparations exhibit activities via TLR2 even after re-extraction (3,4), the component responsible for that activity has not been identified. In the present study, we reassessed the principal component responsible for TLR2-mediated cell activation in Pg-LPS and found it to be a lipoprotein.
Porphyromonas gingivalis lipoprotein

Methods

Bacterial components

Porphyromonas gingivalis strain 381 organisms were grown anaerobically in Gifu anaerobic medium broth (Nissui, Tokyo, Japan) at 37°C for 24 h. Bacterial cells were collected by centrifugation, then washed three times with saline and lyophilized. Pg-LPS was extracted from the lyophilized cells using a phenol–water method (14). Pg-lipid A was isolated by aqueous acetic acid hydrolysis of Pg-LPS followed by successive separation using TLC as previously described (8). Ec-LPS was purchased from List Biological Laboratories.

Separation of immunostimulatory component

Pg-LPS was subjected to hydrophobic interaction chromatography using a method similar to that described previously (15,16). Briefly, Pg-LPS was fractionated with an Octyl Sepharose CL-4B column (Amersham Bioscience, Piscataway, NJ) using 0.1 M of acetate buffer (pH 4.5) with a linear gradient of 1-propanol (15–60%). The fractions were monitored by measuring phosphorus (17) and hexose (18) contents, and also visualized by Tris–glycine SDS–PAGE using a 15% gel with periodic acid-silver (Ag) staining (19,20). TLR-mediated immunostimulatory activities of the fractions were measured using a luciferase assay described below. The selected fractions were then combined, dialyzed and lyophilized to yield an active fraction, which was designated as Pg-AF.

Pg-AF was digested with protease K (1/100, w/w; Takara, Shiga, Japan) for 16 h at 37°C or subjected to phenol re-extraction using a sodium deoxycholate–phenol–water system (12). Lipoprotein in Pg-AF was determined by a monocyte western blotting method (21). Briefly, stimuli were separated by SDS–PAGE using a 15% gel and transferred to a nitrocellulose membrane. The membrane was then cut into 4-mm strips and dissolved in DMSO, and stimuli-coated particles were formed by the addition of PBS. After washing three times with PBS, particle activities were measured using a luciferase assay.

Lipoprotein, eluted at 16 kDa and designated as Pg-LP, was separated using a preparative electrophoresis apparatus AE-6750 (ATTO, Tokyo, Japan) according to the manufacturer’s instructions. Eluates were monitored by SDS–PAGE and those containing lipoprotein were dialyzed against 1 mM of Tris–HCl (pH 8.8), then lyophilized and subjected to acetone precipitation to remove contaminated SDS. Pg-LP was then dissolved in water or 20 mM of octylglycoside and the concentration was estimated with an amino acid analysis using an AccQ-Tag™ method (Waters, Milford, MA). The octylglycoside solution was used for biological assays and the aqueous solution for structural analysis.

Identification of Pg-LP

Pg-LP was digested with trypsin (1/5, w/w; Promega, Madison, WI) or lipoprotein lipase (1/5, w/w; Sigma, St Louis, MO) for 16 h at 37°C. Tryptic digest was subjected to HPLC using a Waters 626-2487 system equipped with a reverse phase Aquapore OD-300 column (100 × 1.0 mm; Perkin Elmer, Norwalk, CT). Digested peptides were eluted using a gradient program (solvent A: 5% acetonitrile, 0.1% TFA, H₂O; solvent B: 70% acetonitrile, 0.085% TFA, H₂O) at a flow rate of 0.05 ml/min, and detected by their absorbance at 210 nm. Isolated peptides were analyzed by N-terminal amino acid sequencing using an automatic peptide sequencer (model 494-cLC; Applied Biosystems, Foster City, CA). Pg-LP was identified by a database search with FASTA using the peptide sequence.

Purification and characterization of lipopeptide

The tryptic digest was also subjected to HPLC using a Waters 600 system equipped with a normal phase YMC-Pack SIL column (150 × 4.6 mm; YMC, Kyoto, Japan). Lipopeptides were eluted using a gradient program (solvent A: chloroform, methanol, water = 65/25/4, v/v/v; solvent B: chloroform, methanol, water = 65/40/4, v/v/v) at a flow rate of 1.0 ml/min, and fractionated into 1 ml portions. The lipopeptide was detected using a luciferase assay. The purified lipopeptide was characterized by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS) on a QSTAR® system equipped with an oMALDI™ ion source (Applied Biosystems) in positive mode. 2,5-Dihydroxybenzoic acid (DHBA) was used as a matrix. Tandem MS (MS/MS) was obtained using the collision-induced dissociation (CID) mode with argon gas.

DNA sequencing

A DNA fragment for a lipoprotein, PG1828, of P. gingivalis strain 381 was obtained by PCR using synthesized primers (forward, 5'-CGCTGAAATGAGACTCTCAAG-3'; reverse, 5'CCATGCAAGAAGTTCGAGA-3'), designed according to the DNA sequence of P. gingivalis W83 (22), and cloned into pGEM-T (Promega). The construct was transformed into an E. coli DH5α strain and the sequence of the fragment was determined with an automated DNA sequencer ABI PRISM® 3100-Avant (Applied Biosystems) using a BigDye® Terminator v3.1 Cycle Sequencing Kit.

Immunobiological assays

Ba/F3 cells stably expressing p55LgkLuc, an NF-κB/DNA binding activity-dependent luciferase reporter construct (Ba/kB), murine TLR2 and the p55LgκLuc reporter construct (Ba/mTLR2) and murine TLR4/MD-2 and the p55LgκLuc reporter construct (Ba/mTLR4/mMD-2) were kindly provided by Dr K. Miyake (Institute of Medical Science, University of Tokyo, Japan). NF-κB-dependent luciferase activity in these cells was determined as described previously (8,23).

Human gingival fibroblasts were obtained according to a method similar to that described previously (8). The tissue sample was collected after receiving written informed consent under a protocol approved by the Institutional Review Board of Asahi University. Cells were stimulated with the indicated doses of the test specimens in culture medium supplemented with 10% FBS for 24 h at 37°C. Following incubation, culture supernatants were collected and analyzed using an ELISA kit for secreted IL-8 (Genzyme Teche, Minneapolis, MN) and results determined using a standard curve prepared for each assay.

Results and Discussion

It was previously reported that LPS preparations from enterobacterial species were separated into subfractions.
according to their hydrophobicity using hydrophobic interaction chromatography (15). Thus, in the present study, Pg-LPS was first subjected to hydrophobic interaction chromatography to attempt subfractionation. In our analysis for phosphate and hexose contents, two peaks were observed in the elution profile, as shown in Fig. 1(A). Most of the phosphate and hexose probably originating from contaminated nucleic acid and polysaccharide emerged in the void volume of the column, and the remaining contents were eluted as a second peak at a propanol concentration of 60% (by volume). SDS-PAGE profiles of the fractions showed that the second peak between fractions 104 and 140 contained LPS molecules (Fig. 1B). In contrast to the broad LPS peak, NF-κB activation through TLR2 was observed only between fractions 104 and 112 (Fig. 1C). This finding suggested that an immunostimulatory component could be separated from the other components. Thus, the active fractions were combined and designated as Pg-AF.

We next searched for an active component in Pg-AF. Pg-AF was resolved by SDS–PAGE and analyzed by monocyte western blotting. In the silver-stained gel, LPS bands were displayed at 10, 12, 14, 16 and 17 kDa, as well as in ranges of 18–40 kDa, whereas only a single major band was found at 16 kDa in the CBB-stained gel (Fig. 2A). In the monocyte western blotting analysis, TLR2-mediated NF-κB activation was mainly detected in a molecular mass range of 14–18 kDa (Fig. 2A). SDS–PAGE results of proteinase K-digested Pg-AF revealed no CBB-positive band at 16 kDa, while NF-κB activity was detected at the front of the dye, however, not in the area of 16 kDa (Fig. 2B). These observations indicated that the protein

![Fig. 1. Fractionation of TLR2-activating component in a P. gingivalis LPS preparation. (A) Elution profile of the P. gingivalis LPS preparation from an Octyl Sepharose CL-4B column. (B) SDS–PAGE profiles of the fraction from the Octyl Sepharose column. The gel was visualized by silver stain. (C) NF-κB activation of the fraction from the Octyl Sepharose column using Ba/kB, Ba/mTLR2 and Ba/mTLR4/mMD-2 cells.](image-url)
at 16 kDa was a primary TLR2-mediated immunostimulatory molecule in the *P. gingivalis* LPS preparation. We also investigated the effect of phenol re-extraction (12) on the protein. Following re-extraction, a 16 kDa band was still found in the CBB-stained SDS–PAGE gel and NF-jB activity was observed at 14–18 kDa (Fig. 2C). Further, the phenol re-extraction did not affect the activity of Pg-AF (Fig. 2D). These results show that the protein at 16 kDa was not removed by phenol re-extraction, in agreement with previous reports (3,4).

The protein was then separated by preparative electrophoresis. TLR2-mediated NF-kB activation activity of the protein was reduced ~10-fold by trypsin digestion (Fig. 3A), while most of the activity was abrogated by lipoprotein lipase digestion. These results suggested that the protein is a lipoprotein and it was designated as Pg-LP. To identify Pg-LP, the N-terminal amino acid sequencing was examined by Edman degradation. However, no amino acid was detected, suggesting that the amino group of the N-terminal amino acid was blocked. Thus, internal peptide sequencing was performed. Pg-LP was digested with trypsin and subjected to reverse phase HPLC (Fig. 3B). The sequences of the isolated peptides were TEEAADXTAMDXATLVE for peak A and TEEAADXTAMDXATLV for peak B, where X represents an unidentified amino acid. In a database search with FASTA using the peptide sequence, the peptide had a high sequence similarity to a putative lipoprotein, PG1828 (accession no. NP_905926), encoded in the *P. gingivalis* W83 genome (22).

The DNA sequence of *P. gingivalis* strain 381 PG1828 (accession no. AB164391) and the deduced amino acid sequence (Fig 3C) were also consistent with the sequence of Pg-LP. An open reading frame of PG1828 encoded 72 amino acids. Since the sequence contains a typical prokaryotic lipoprotein signal peptide at its amino terminus, it is expected that the peptide is cleaved off and then the resulting N-terminal cystein is lipidated. Thus, the mature lipoprotein containing 52 amino acids attached by lipid moiety was expected to have a molecular mass of ~6 kDa. Since the isoelectric point of mature PG1828 was estimated to be 3.7, the lesser amount of SDS bound to the protein was considered responsible for the anomalously low electrophoretic mobility (24).

![Fig. 2](image-url)

**Fig. 2.** Separation of TLR2-activating component in the Pg-AF. (A) NF-kB activation of the fraction of Pg-AF separated by SDS–PAGE using Ba/mTLR2 cells. The gel was visualized by silver and CBB stains. (B) NF-kB activation of the fraction of proteinase K-digested Pg-AF separated by SDS–PAGE using Ba/mTLR2 cells. (C) NF-kB activation of the fraction of re-extracted Pg-AF separated by SDS–PAGE using Ba/mTLR2 cells. (D) NF-kB activation of Pg-AF and re-extracted Pg-AF using Ba/mTLR2 cells.

Since a bacterial lipoprotein has been shown to possess N-terminal acylated S-(2,3-dihydroxypropyl)cysteine (25), the N-terminal structure of Pg-LP was characterized. The tryptic digest was subjected to normal phase HPLC (Fig. 4A) and the active component was eluted only between fractions 8 and 10. Further, the MALDI-TOF-MS spectra of the component showed mainly two pseudomolecular ions [M+H]+ at m/z 1411.0 and 1425.0, though weak signals at m/z 1397.0 and 1439.0 were also observed. In the MS/MS spectra of the precursor ion at m/z 1425.0 (Fig. 4B and C), C-terminal y ions were observed at m/z 547.3, 433.2, 346.2, 218.1 and 147.1, which identified a sequence, NSQAK. The N-terminal b ion at m/z 878.7 corresponds to triacylated S-(2,3-dihydroxypropyl)cysteine, while ions at m/z 840.5 and 854.6 show monoacylated...
dehydroalanyl NSQAK formed by β-elimination of the 2,3-diacyloxypropylthio group. MS/MS spectra of the other signals (data not shown) correspond to the same lipopeptide, except for the acyl constituent. Since the sequences of the lipopeptides agreed with the putative N-terminal structure of Pg-LP, Pg-LP was shown to be a lipoprotein. Further, since triacylated lipopeptide is reported to activate cells via TLR2 (26), Pg-LP is a TLR2-dependent immunostimulatory component.

Pg-LP activates NF-κB via TLR2, but not TLR4/MD-2, in a similar fashion to Pg-LPS, whereas Pg-lipid A stimulates TLR4/MD-2 (Fig. 5). In addition to NF-κB activation, Pg-LP activated human gingival fibroblasts to induce IL-8 production (Fig. 6), and this activity was significantly stronger than those of Pg-LPS. Further, Pg-lipid A was less potent in inducing IL-8 production than Pg-LPS. Since Pg-lipid A is a highly purified version lipid A considered to be an essential moiety for activity of the LPS molecule from Porphyromonas gingivalis (8), Pg-LP rather than the LPS molecule itself is considered to be a potent immunostimulatory component in Pg-LPS. Porphyromonas gingivalis is known to be a suspected periodontopathic bacteria and is frequently isolated from periodontal pockets of patients with periodontal diseases (27), thus Pg-LP may be a virulence factor of the inflammation process in diseases. Further, the IL-8 production-inducing activity of Pg-LP is comparable to that of Ec-LPS. Therefore, we propose that Pg-LP is an inflammatory factor of P. gingivalis.

Escherichia coli lipoprotein was readily extracted with LPS as a mixture using the standard extraction methods and separation of lipoprotein from the associated LPS was achieved by phenol re-extraction using sodium deoxycholate.
Porphyromonas gingivalis lipoprotein

Fig. 5. TLR2-mediated cell activation by Pg-LP. Ba/xB, Ba/mTLR2 and Ba/mTLR4/mMD-2 cells were stimulated with Pg-LP (1 ng/ml), Pg-LPS (100 ng/ml), Ec-LPS (1 ng/ml) and Pg-lipid A (100 µg/ml) for 4 h, and luciferase activities were measured.

Fig. 6. IL-8 production in human gingival fibroblasts induced by Pg-LP. Cells were stimulated with the indicated doses of Pg-LP, Pg-LPS, Pg-lipid A and Ec-LPS for 24 h, and IL-8 production was determined by ELISA. The results are presented as the mean ± SEM. When an individual result is presented, it is representative of at least three independent experiments.

(12,13). Sodium deoxycholate causes dissociation of lipoprotein from LPS and the released lipoprotein becomes dissolved in the phenol layer, however, we found that Pg-LP could not be separated from Pg-LPS by the same re-extraction protocol. In this case, sodium deoxycholate may cause dissociation of the complex, however, the released Pg-LP is considered to be soluble in the aqueous phase due to its acidic nature.

The biological activities of lipoprotein prepared from Bacteroides forsythus, which is closely related to P. gingivalis, have recently been reported (28). Although the chemical structure has not been elucidated, the glycoprotein is considered to be distinguished from LPS-associated lipoproteins like Pg-LP. From our results, we concluded that a lipoprotein from a P. gingivalis LPS preparation represented a principal component involved with TLR2-mediated cell activation. In addition to those from P. gingivalis, other LPS preparations from non-enterobacterial organisms have been reported to activate a TLR2-dependent signaling pathway (4–6). Thus, attempts to detect TLR2-activating lipoproteins in LPS preparations are important.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research Priority Area (No. 16017299) of the Ministry of Education, Culture, Sports, Science and Technology, Japan. We are grateful to Mr Y. Shionoya of Applied Biosystems Japan for the amino acid sequencing and Mr M. Benton for his critical reading of the manuscript.

Abbreviations

Ec-LPS Escherichia coli LPS
LPS lipopolysaccharide
Pg-AF active fraction from Pg-LPS
Pg-LP lipoprotein from Pg-LPS
Pg-LPS LPS preparation from Porphyromonas gingivalis
TLR Toll-like receptor

References


