Identification of a lipoprotein of *Vibrio vulnificus* as an immunostimulant inducing cytokine production of human monocytes

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Identification of a lipoprotein of *Vibrio vulnificus* as an immunostimulant inducing cytokine production of human monocytes

Directed by Professor Soon-Jung Park

The Master's Thesis submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

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This certifies that the Master's Thesis

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The Graduate School

Yonsei University

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Identification of a lipoprotein of *Vibrio vulnificus* as an immunostimulant inducing cytokine production of human monocytes

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Vibrio vulnificus is a gram-negative pathogenic bacterium causing primary septicemia, necrotizing wound infections, and gastroenteritis, especially in immunocompromised humans. Primary septicemia caused by *V. vulnificus* appears to follow a classical septic shock pathway including an overwhelming inflammatory cytokine response resulted in multi-organ failure and death. In this study, I identified a putative lipoprotein (Lpp) encoded by the *lpp* gene as

one of the surface proteins of *V. vulnificus*. Using a mutant *V. vulnificus* in which its *lpp* gene had been knocked out, I found that Lpp is important in production of interferon-γ (IFN-γ) in human peripheral blood mononuclear cells (PBMC). Production of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), but not of IL-4, is induced by the recombinant Lpp (rLpp) in human monocytes. Lipidation of the rLpp protein made in *Escherichia coli* was confirmed by *in vivo* labeling with [^{3}H]-palmitic acid. Experiments using the mutant Lpp unable to be modified by lipidation indicate that lipidation of Lpp is an essential property in cytokine production of human monocytes. A possibility that cytokine production was caused by LPS contamination present in rLpp was excluded in the experiment using polymyxin B-treated rLpp. Role of Toll-like receptor (TLR) 2 in Lpp-induced cytokine production was confirmed by two different *in vitro* assays. Pretreatment of monocytes with antibodies against TLR2 inhibited production of TNF-α and IL-6. TLR2-expressing cells but not TLR4-expressing cells showed a dramatic increase in NF-κB activity. These results suggest a possibility that the Lpp of *V. vulnificus* exerts its function as an immunostimulant to human monocytes by activating TLR2.

Key words : *Vibrio vulnificus*, bacterial lipoprotein, cytokine

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Ⅰ. INTRODUCTION

Vibrio vulnificus, a gram-negative bacterium found commonly in the estuarine environment, has been frequently associated with primary septicemia following the consumption of contaminated shellfish. In over 50% of primary septicemia caused by *V. vulnificus*, the patients died of multi-organ failure as a result of a rapidly progressive shock syndrome[1, 2]. Numerous studies have been undertaken to determine virulence factors involved in the pathogenesis of this

microorganism. *V. vulnificus* produces a number of extracellular products including hemolytic cytolysin[3], elastase[4], and siderophores[5, 6], which may play some role in developing disease. Surface structures such as lipopolysaccharide (LPS)[7, 8] and outer membrane proteins[6] were also studied as a candidate for virulence factors of *V. vulnificus*.

Septic shock is usually resulted from the overproduction and dysregulation of the host cytokine response to invading microorganisms. Inflammation-associated cytokines, such as tumor necrosis factor alpha (TNF-α), interferon-γ (IFN-γ), interleukin-1 (IL-1) and IL-6, play a pivotal role in the host immune response to infection[9]. IFN-γ is a pro-inflammatory cytokine which up-regulates production of other cytokines by both gram-positive and gram-negative bacteria. TNF-α is a principal mediator of acute inflammatory responses mainly to gram-negative bacteria, and is responsible for systemic complication of severe infections, which is produced by activated mononuclear phagocytes. IL-6 is a cytokine that functions in both innate and adaptive immunity, which is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts, and other cells in response to microbes and other cytokines, notably IL-1 and TNF-α. A variety of bacterial products, including LPS, lipoarabinomannans, porins, and capsular polysaccharide, have been identified to elicit or modulate

the release of cytokines from host cells in both *in vivo* and *in vitro* models[10]. Bacterial cell wall components are also known to activate monocytes/macrophages to produce inflammatory cytokines including TNF-α, IL-1, and IL-6. Among bacterial cell wall components, LPS and bacterial lipoprotein (BLP) are recognized as important components in eliciting the systemic inflammatory responses in response to infection. In case of *Neisseria gonorrhoeae*, its initial interaction with mucosal epithelial cells triggers the release of inflammatory cytokines and chemokines including IL-6 and IL-8[11-13], which induce to recruit and activate other immune cells to the site of infection.

A variety of host cell receptors have been implicated in recognition of bacteria or their components. One of them is the toll-like receptor (TLR) family, which play a central role in innate immune defenses[14, 15]. Recently, it has been reported that TLRs with a function of pattern recognition receptors play critical roles in early innate recognition and inflammatory responses by host against invading microorganism[16, 17]. The human TLR family is categorized into at least 10 distinct receptors. Among 10 TLR family members reported, TLR2, TLR4, TLR5, and TLR9 have been implicated in the recognition of different bacterial components. These TLR family members activate nuclear factor kappa B (NF-κB) via interleukin-1 receptor (IL-1R) -associated signal molecules, including

myeloid differentiation protein (MyD) 88, IL-1R-activated kinase, tumor necrosis factor receptor (TNFR) -associated factor 6, and NF-κB-inducing kinase[18]. They are activated in response to microbial component, resulting in subsequent activation of NF-κB[14, 15], a transcription factor involved in the expression of proinflammatory cytokines, chemokines, and adhesion molecules. The ligands recognized by the TLRs are conserved molecular patterns shared by a broad range of pathogens. For instance, TLR4 is a principle signal transducer in the recognition of LPS[15, 19, 20] whereas TLR2 confers responsiveness to BLP/lipopeptides[21, 22, 23, 24], lipoarabinomannan[25], lipoteichoic acid[26], and peptidoglycan[26, 27]. In infected cells by *Mycoplasma pneumouiae*, interruptions of interaction between receptor of host cells and the N-terminal cysteine residue of Lpp results in the reduction of inflammatory response[28].

As a surface protein of *V. vulnificus*, I determined proinflammatory stimulatory activity of the Lpp of *V. vulnificus* in terms its ability to stimulate production of cytokine as IFN-γ, TNF-α, IL-6 in human mononuclear cells. *In vitro* assays using the transfected human embryonic kidney (HEK) 293 cells indicates that TLR2 is required for recognition of the Lpp. Furthermore, I examined whether lipidation of Lpp of *V. vulnificus* is critical in production of proinflammatory cytokine.

Ⅱ. MATERIALS AND METHOD

1. Cultivation of bacteria

Escherichia coli DH5α and BL21 (DE3) were grown in Luria-Bertani (LB [1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5]) at 37°C with shaking. *Vibrio vulnificus* ATCC 29307 was cultured in LBS broth (1% tryptone, 0.5% yeast extract, 2% NaCl, pH 7.5) at 30°C with shaking. The bacterial growth was monitored by measuring its absorbance at 600 nm.

2. Preparation of PBMC and monocytes

Human peripheral blood mononuclear cells (PBMCs) were prepared from buffy coat of healthy donors by Ficoll density-gradient centrifugation (Amersham, Pharmacia, Piscataway, NJ), and resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Culture of 1×10^5 PBMCs were performed in 96-well plates (Falcon, Lincoln Park, NJ). Monocytes (>95%) were isolated by countercurrent elutriation from peripheral blood as described elsewhere[29]. Monocytes (1×10^5 or 5×10^5) were cultivated in 96-well or 24-well flat-bottom microtiter plates, respectively. Cells

were cultured for different periods with or without stimuli (lysates of *V. vulnificus* or the rLpp). For blocking experiments, cells were preincubated for 1h with medium containing 10 μg/ml anti-human TLR2 monoclonal neutralizing antibody (BioLegend, San Diego, Calif.) or medium alone. To neutralize any residual LPS remaining during the purification of recombinant protein, polymyxin B (Sigma, St. Louis, Mo) was added to the rLpp at a concentration of 20 μg/ml, prior to being incubated with the prepared monocytes.

3. Cultivation of cell lines

HEp-2 cell line (ATCC CCL 23) derived from human epithelial cells was cultured in RPMI- 1640 containing 10% fetal bovine serum (HyClone, Logan UT), 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. The HEK 293 cells (ATCC CRL 1573) were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Karsruhe, Germany) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

4. Purification of outer-membrane proteins from *V. vulnificus*

V. vulnificus ATCC 29307 was cultured until it reached to the stationary phase, and then harvested by centrifugation. Cell pellets resuspended in 10 mM Tris-HCl (pH 7.4) were disrupted by sonication, and then centrifuged at 10,000 ×g for 20 min. The resulting supernatant was ultracentrifuged at 100,000×g for 1 h, and then the pellet fraction was resuspended in 7 mM EDTA containing 1% (vol/vol) sodium lauryl sarkosinate. The mixture was incubated at 37°C for 30 min. After anther round of ultracentrifugation, the pellet was resuspended in 10 mM Tris-HCl (pH 7.4).

5. Expression and purification of recombinant Lpp

A 810-bp DNA fragment containing the *lpp* gene was amplified from genomic DNA of *V. vulnificus* ATCC 29307 by PCR with two primers, lipoF (*Nco* I) (5'-CATGCCATGGCTATGAAATTTAGCCTTAAAGG-3') and lipoR (*Hin*d Ⅲ) (5'-CCCAAGCTTCCAGCCTTTTACTACGCC-3'). The resultant *lpp* DNA was cloned into an expression vector pET-28b (Novagen, Darmstadt, Germany), to produce pET*lpp*. *E. coli* BL21(DE3) carrying pET*lpp* was grown to OD_{600}=0.6, and further incubated with 1 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h. The histidine-tagged rLpp overexpressed

as described above was subsequently purified by using an Ni^{2+}-nitrilotriacetic acid affinity column according to the manufacturer's instruction (Qiagen, Valencia, CA).

Using two primers, mlipo F (5'-CATGCCATGGATGGGCGAAAAAGCGACTGAC-3') and mlipo R (5'-CCCAAGCTTCCAGCCTTTTACTACGCC-3'), a part of *lpp* gene losing the lipidation site (69-bp) was amplified from genomic DNA of *V. vulnificus* ATCC 29307 and cloned into an expression vector pET-28b. Recombinant Lpp without lipidation site was overexpressed and purified as described above.

6. Western blot analysis

Purified rLpp (100 μg) was mixed with 0.5 ml of complete Freund's adjuvant (Sigma), and inoculated intraperitoneally into a specific pathogen-free rat (CrjBgi:CD[SD]IGS, 7-week-old, female). Two additional immunizations were performed with the same amount of Lpp protein mixed with incomplete Freund's adjuvant (Sigma) 2 and 4 weeks after the primary immunization. A week after the third immunization, serum was obtained from the immunized rat, and used for western blot analysis.

Bacterial lysates were prepared in a buffer containing 50 mM Tris-HCl (pH

6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 20% glycerol, separated in 10% SDS-polyacrylamide gels (SDS-PAGE), and transferred to a nitrocellulose membrane (Millipore, Bedford, Mass.). The membranes were incubated with polyclonal antisera (diluted 1/5,000 to 1/20,000) in a blocking solution (phosphate-buffered saline [PBS] with 5% skim milk and 0.05% Tween 20), washed three times with PBS containing 0.1% Tween 20 (PBS-T), and incubated with anti-rat or anti-mouse immunoglobulin G coupled to alkaline phosphatase (AP). Being washed three times with PBS-T, the immunoreactive bands were visualized by using the nitroblue tetrazolium (NBT)-5-bromo-4-chloro-3-indolyl phosphate (BCIP) system (Promega, Madison, Wis.).

7. Determination of lipidation of Lpp protein

In vivo labeling of Lpp with [^{3}H]-palmitic acid was performed as described by Theisen *et al*.[30]. Two *E. coli* strain, DH5α or BL21, carrying the indicated plasmid (pET28b or pET*lpp*), were grown to mid-log phase, and the incubation was continued for 12 h in the presence of 50 μCi of [^{3}H]-palmitic acid (Amersham, Pharmacia, Piscataway, NJ) either with or without 1 mM IPTG. After the labeling was stopped with 10% trichloroacetic acid (wt/vol), the cells

were resuspended in a boiling buffer (2% SDS, 50 mM Tris-Cl, pH 8.0), and then boiled for 10 min. Cell extracts were prepared by sonication, and then phase-partitioned at 37°C with an addition of 5% Triton X-114. The pellet was washed three times in a radioimmunoprecipitation buffer (1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-Cl, pH 8.0) before being resuspended in a sample loading buffer. An equal amount of each fraction was subjected to a 10% SDS-PAGE, and transferred to polyvinylindene fluoride membranes (PVDF; Milipore, Bedford, MA, USA). The membrane was treated with Amplify solution (Amersham, Pharmacia, Piscataway, NJ) and exposed to preflashed Hyperfilm-MP (Amersham) for 2 week at -70°C.

8. Flow cytometry

To measure the cytotoxicity of *V. vulnificus*, the viability of HEp-2 cells was examined using propidium iodide (PI), which only stains nucleic acids of dead cells with disrupted membranes. Flow cytometric analysis to estimate the percentage of cells stained with PI was performed on at least 5,000 cells from each sample by fluorescence-activated cell sorting (FACScan; BD Biosciences, Franklin Lakes, NJ). Briefly, HEp-2 cells were incubated with various *V. vulnificus* strains (wild-type, *Δlpp* mutant, or *Δlpp* mutant with the

complemented plasmid containing the wild-type *lpp* gene) for 15 min.

9. Measurement of cytokines

PBMCs and monocytes in 96-well plates (1×10^5 cells/well) were co-incubated with culture medium, bacterial lysates, or rLpp at a concentration from 10 to 10,000 ng/ml for 18 h. Cell-free supernatants were then collected by centrifugation, transferred to new tubes, and stored at -70°C until analysis. The levels of each cytokine in the supernatants were determined using enzyme-linked immunosorbent assays (BD Biosciences, NJ or Biosource, Camarillo, CA).

10. Luciferase assay

HEK 293 cells (5×10^5) were transfected with 1.0 μg of pFLAG-TLR2, 1.0 μg of pNF-κB-luc, 1.0 μg of pcDNA 3.1 (+) - MyD 88 and 0.5 μg of pCH110 in 24-well plates. As a negative control, plasmid pFLAG-CMV (Invitrogen, San Diego, CA) was transfected instead of pFLAG-TLR2. For another set, pFLAG-TLR4 was used to transfected to HEK 293 cells along with pNF-κB-luc and pCH110. After 24 h, the transfected cells were stimulated with rLpp or with LPS. After an additional 20 h-incubation, the cells were lysed and assayed for

luciferase activity using a Luciferase Reporter Assay System (Promega). Normalized reporter activity is expressed as the luciferase activities divided by the activities of β-galactosidase of the same transfected cells.

11. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Student's *t*-test (SYSTAT program, SigmaPlot version 9; Systat Software Inc.) was used to evaluate the statistical significance of the results.

Ⅲ. RESULTS

1. Localization of the Lpp of *V. vulnificus*

We examined the location of the Lpp protein in *V. vulnificus* (Fig. 1). Lysate of *V. vulnificus* was fractionated into the soluble, inner membrane, and outer membrane fractions by a series of centrifugations and treatment with sodium lauryl sarkosinate. These fractions were then subjected to SDS-PAGE and analyzed by western blot using polyclonal sera specific to the rLpp.

In all of the three fractions of *V. vulnificus*, Lpp was detected as a protein of 30 kDa, which is an expected size of the *lpp* ORF made of 270 amino acids. Despite each sample contained the same amount of protein, the intensity of the Lpp band appears stronger in the outer membrane. Therefore, Lpp is one of the outer membrane proteins in *V. vulnificus*.

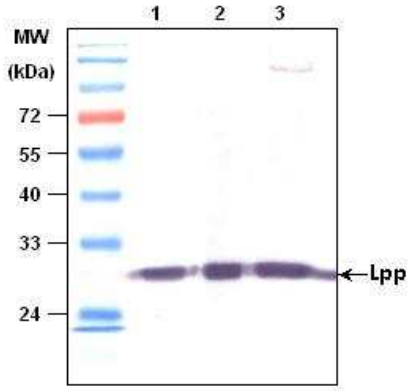


Fig. 1. Localization of the Lpp of *V. vulnificus*. Western blot analysis of various protein fractions of *V. vulnificus* with polyclonal antibodies against rLpp. Soluble proteins (lane 1), inner membrane proteins (lane 2), and outer membrane proteins (lane 3) of *V. vulnificus*.

2. Confirmation of *Δlpp* mutant *V. vulnificus* and the *Δlpp* mutant *V. vulnificus* carrying the complementation plasmid with the wild-type *lpp* gene

To examine the functional role of Lpp, a *lpp* deletion mutant *V. vulnificus* was constructed, (H.-S. Yang and K.-H. Lee, unpublished results). In addition, a plasmid containing the wild-type *lpp* gene was added to this *lpp* knockout *V. vulnificus* to examine if the mutant phenotype can be restored to wild-type (H.-S. Yang and K.-H. Lee, unpublished results).

Three *V. vulnificus* strains, wild-type, *Δlpp* mutant, and *Δlpp* mutant with the wild-type *lpp* gene, were examined with respect to their intracellular level of Lpp (Fig. 2). A strong immunoreactive band of 30 kDa was detected in western blot of the wild-type lysates with polyclonal antibodies against Lpp, whereas it was missing in the lysates of the *Δlpp* mutant *V. vulnificus*. When the wild-type *lpp* gene was added back to the *Δlpp* mutant *V. vulnificus*, the Lpp band was appeared, indicating that expression of Lpp was restored in the complementation strain.

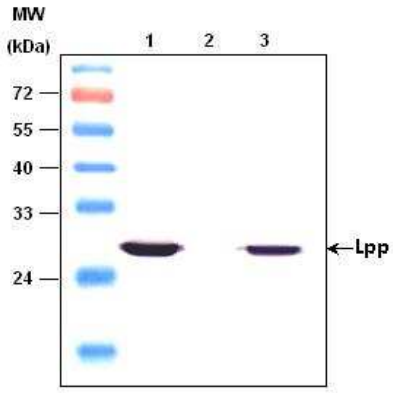


Fig. 2. Confirmation of expression of Lpp in the *Δlpp* mutant *V. vulnificus* and the *Δlpp* mutant with a complemented plasmid containing wild-type *lpp* gene. Western blot of *V. vulnificus* strains using antibodies specific to the rLpp. Each lane contains 20 μg of cell lysates prepared from wild-type *V. vulnificus* ATCC 29307 (lane 1), *Δlpp* mutant (lane 2), and the *Δlpp* complemented with wild-type *lpp* gene (lane 3).

3. Lipidation of the rLpp

Because the deduced amino acid sequence of Lpp protein contains a sequence identical to the consensus sequence [Leu-Ala(Gly)-Ala-Cys] for lipid modification in *E. coli*[31], I asked whether the Lpp is modified by lipidation as suggested from its amino acid sequences. Four different *E. coli* strains, DH5α with pET28b, BL21 with pET28b, DH5α with pET*lpp*, and BL21 carrying pET*lpp*, were grown in presence of [^{3}H]-palmitic acid, and examined for their radiolabeled protein(s). Two of the four strains, DH5α with pET*lpp*, and BL21 carrying pET*lpp*, were cultured in the absence or in the presence of 1 mM IPTG to confirm that expression of the radiolabled protein was induced by IPTG. Each lysate was subdivided into the aqueous, detergent, and insoluble pellet fractions with the treatment of Triton X-114, and analyzed by SDS-PAGE and antoradiography. A protein labeled with [^{3}H]-palmitic acid was detected as 30 kDa only in the lysates prepared from BL21 with pET*lpp* grown in the presence of IPTG (Fig. 3). This result indicates that the rLpp was lipidated by [^{3}H]-palmitic acid. A property of lipid-containing protein was confirmed in strong radioactivity observed in the detergent phase of IPTG-induced BL21 with pET*lpp*. Thus, the *V. vulnificus lpp* gene product is lipid-modified in *E. coil*.

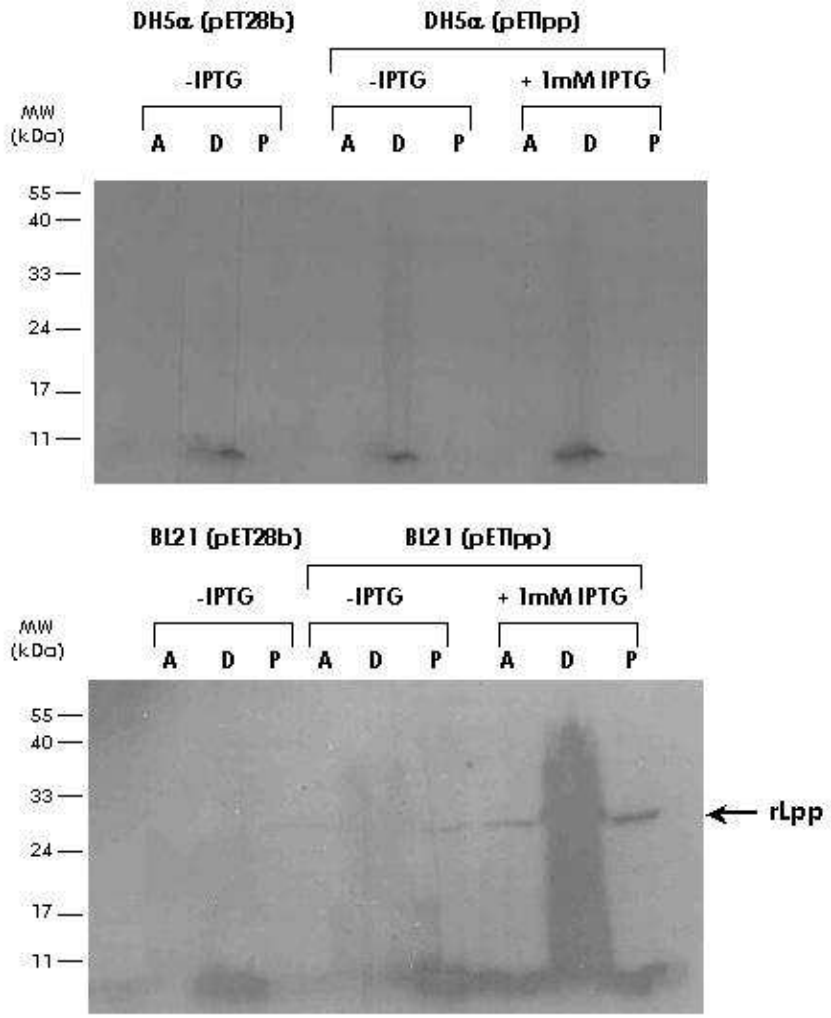


Fig. 3. Autoradiograph of the rLpp extracted with Triton X-114 from *E. coli* cells labeled with [^{3}H]-palmitic acid. *In vivo* labeling of Lpp with [^{3}H]-palmitic acid was performed as described by Theisen *et al*.[30] Two *E. coli* strains,

DH5α or BL21, carrying the indicated plasmid, were grown to mid-log phase, and the incubation was continued for 12 h in the presence of [^{3}H]-palmitic acid. Cell extracts were prepared by freezing and thawing, and then phase-partitioned at 37°C with an addition of Triton X-114 (5%). For two strains, DH5α (pET*lpp*) and BL21 (pET*lpp*), 1 mM IPTG was added with [^{3}H]-palmitic acid at the same time. An equal amount of protein in each fraction was subjected to a 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was treated with Amplify solution (Amersham) and exposed to X-ray film for 2 weeks. A: aqueous phase, D: detergent phase, P: pellet. [^{3}H]-labeled rLpp was indicated with an arrow.

4. Role of Lpp in cytotoxicity of *V. vulnificus*

The ability of the *lpp* knockout mutant to kill the host cells was compared with that of wild-type *V. vulnificus* (Fig. 4). When HEp-2 cells were not exposed to *V. vulnificus*, only 3% of them were dead as determined by PI-staining and FACS analysis. Upon a 15 min-incubation with wild-type *V. vulnificus* with multiplicity of infection (MOI) of 5, 73% of HEp-2 cells were stained by PI, whereas *lpp* knockout mutant showed lower percentage of PI-straining, 42% under the same conditions. When the *lpp* knockout mutant carried the wild-type *lpp* gene, the percentage of PI-stained cells was increased to 54%.

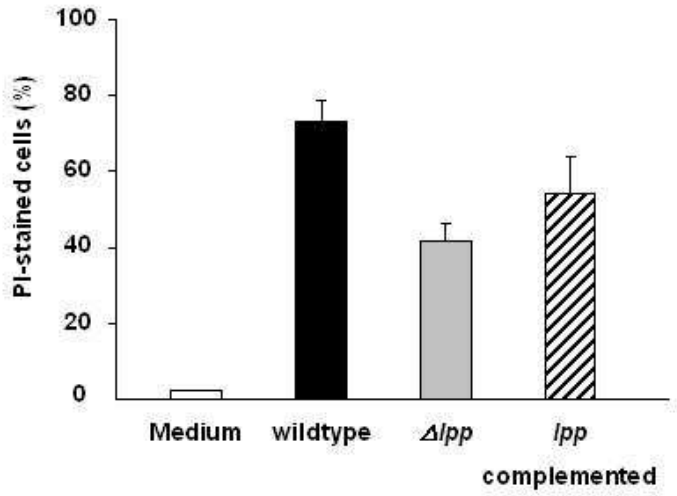


Fig. 4. Role of *V. vulnificus* Lpp protein in cytotoxicity to the HEp-2 cells.

HEp-2 cells (4×10^5) were incubated with live *V. vulnificus* at MOI of 5. Fifteen minutes after incubation, viabilities of HEp-2 cells were determined by staining them with 5 μg/ml propidium iodide (PI), and analyzing of a flow cytometry.

5. Cytokine production of human PBMC induced by cell extracts of various *V. vulnificus*

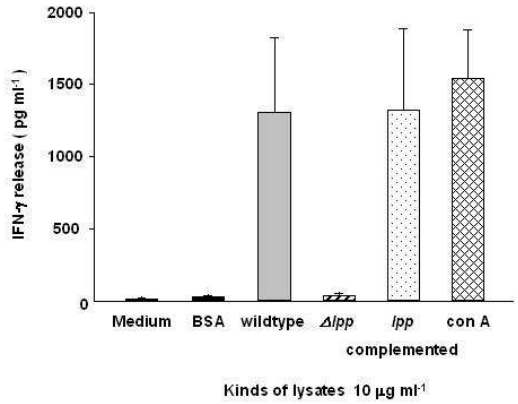
I examined a possibility that Lpp of *V. vulnificus* may serve as an immunostimulant to induce cytokine production by human cells. As expected, I incubation of human PBMC with the lysates prepared from wild-type *V. vulnificus* resulted in significant production of IFN-γ (1,300 pg/ml) by human PBMC (Fig. 5A). On the other hand, PBMC treated with the same amount of lysates derived from the *lpp* knockout mutant did not induce formation of IFN-γ at all (41 pg/ml). This level of IFN-γ is comparable with those of the control cells, such as PBMC treated with medium or BSA (13 or 32 pg/ml, respectively). Dramatic increase in IFN-γ production was observed in PBMC stimulated with the lysates of the *Δlpp* strain when it carried the complementation plasmid with the wild-type *lpp* gene (1,300 pg/ml). PBMC treated with conconavalin A (con A) at 10 μg/ml was served as a positive control to show the level of IFN-γ at 1,500 pg/ml.

In a subsequent experiment, formation of IFN-γ by PBMC stimulated with *V. vulnificus* lysates was systematically examined by treating them with various concentration of the bacterial lysates ranged from 0.5 to 10 μg/ml (Fig. 5B). While the secretion of IFN-γ in PBMC treated with wild-type lysates was

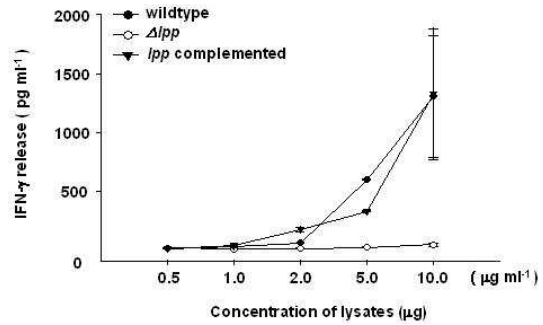
increased in a dose-dependent manner, no distinct formation of IFN-γ was detected in PBMC stimulated by the *Δlpp* lysates at any concentration. A similar pattern of IFN-γ formation was observed in PBMC when they were exposed to the lysates of the *Δlpp* complemented with the *lpp* gene wild-type.

The next question was whether the purified rLpp was able to induce IFN-γ by PBMC (Fig. 5C). Indeed, the rLpp also induced IFN-γ production by human PBMCs to a level of 1,800 pg/ml at concentration of 10 μg/ml.

A


2000
1500
1000
500
0
IFN-γ release (pg ml-1)
Medium
BSA
wildtype
Δlpp
lpp
complemented
con A
Kinds of lysates 10 μg ml-1

B


wildtype
Δlpp
lpp complemented
2000
1500
1000
500
0
IFN-γ release (pg ml-1)
0.5
1.0
2.0
5.0
10.0
(μg ml-1)
Concentration of lysates (μg)

C

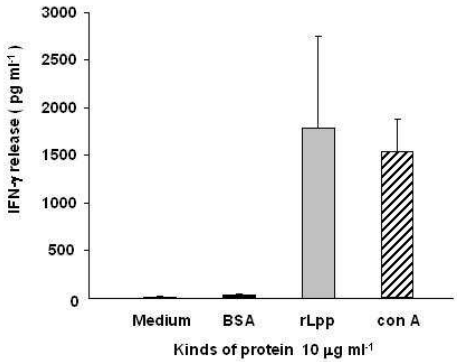

3000
2500
2000
1500
1000
500
0
IFN-γ release (pg ml-1)
Medium
BSA
rLpp
con A
Kinds of protein 10 μg ml-1

Fig. 5. Cytokine production of human PBMC induced by cell extracts of various *V. vulnificus*.

(A) Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation of heparinized blood over a Ficoll-Hypaque density gradient at 1,600 rpm for 30 min. PBMC (1×10^5) were then incubated with bacterial lysates equivalent to 10 μg of the total protein prepared from three different kinds of *V. vulnificus* (wild-type, *lpp* knockout mutant, and *Δlpp* complemented with the *lpp* gene) for 18 h, and the concentration of IFN-γ in the supernatant was determined using Human IFN-γ ELISA kit (BD biosciences). PBMC exposed to conconavalin A was included as a positive control for IFN-γ production. (B) PBMC (1×10^5) were incubated with various concentration of bacterial lysates prepared from three different kinds of *V. vulnificus* (wild-type, *lpp* knockout mutant, and *Δlpp* complemented with the *lpp* gene) for 18 h, and the concentration of IFN-γ in the supernatant was determined using Human IFN-γ ELISA kit (BD biosciences). (C) PBMC (1×10^5) were incubated with the recombinant Lpp for 18 h, and the concentration of IFN-γ in the supernatant was determined using Human IFN-γ ELISA kit (BD biosciences).

6. Cytokine profiles of monocytes stimulated with rLpp of *V. vulnificus*

To assess a role of *V. vulnificus* Lpp in the cytokine production of monocytes, I prepared human monocytes, and then treated them with various concentrations of rLpp. As a positive control for the cytokine production by monocytes, LPS was used to stimulate monocytes in the same manner. Production of TNF-α (Fig. 6A) and IL-6 (Fig. 6B) by these rLpp-treated monocytic cells were determined at 18 h post-stimulation. Increasing concentrations (0.01-1.0 μg) of rLpp resulted in a dose-dependent formation for TNF-α and IL-6 by monocytes. In response to increased amount of LPS, monocytes displayed increased formation of these two cytokines.

I then investigated whether Lpp may function as an immunostimulant specifically to produce a certain set of cytokines or ubiquitously to produce most of cytokines. Measurement of IL-4 by rLpp-stimulated monocytes indicated that they were unable to produce IL-4 at all (Fig. 6C).

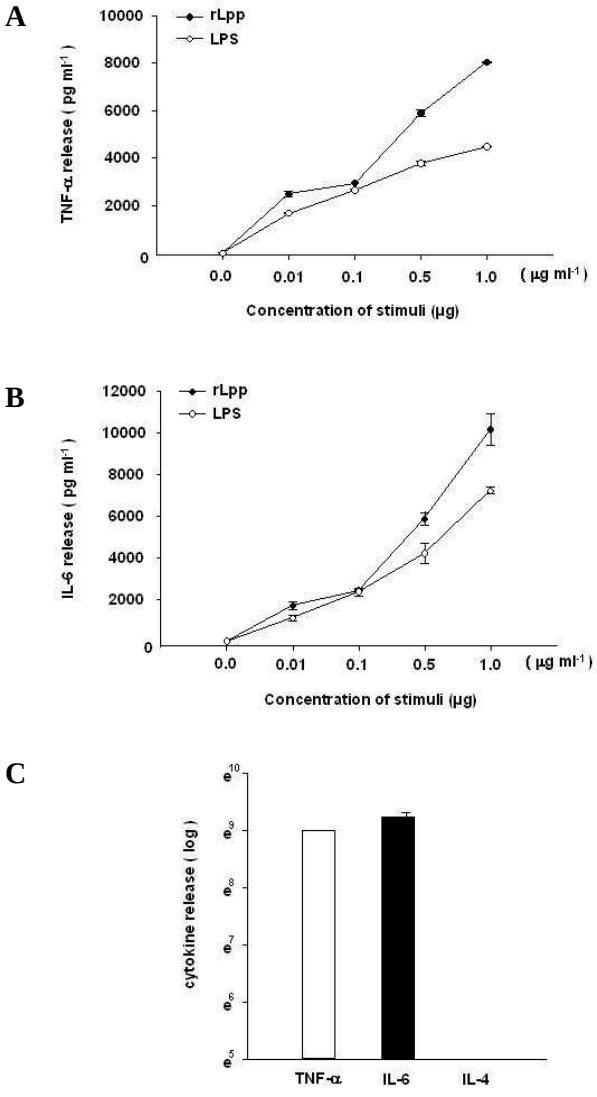


Fig. 6. Production of cytokines in monocytes induced by rLpp of *V. vulnificus*. Confluent monocytes (1×10^5 ml^{-1}) were exposed to various concentrations of rLpp ranging from 0.01 to 1.0 μg. Monocyte cells treated with

medium and LPS of *E. coil* were used as a negative and positive control for cytokine production, respectively. Cytokine concentrations were measured using Human TNF-α ELISA kit and Human IL-6 ELISA kit (Biosource).

To exclude a possibility that cytokine production by rLpp-stimutated monocyte was caused from the residual contamination of LPS in the purified rLpp, the rLpp was pretreated with polymyxin B, which is an amphipathic LPS binding molecule, and therefore neutralizes LPS, and then used as a stimulant for monocytes (Fig. 7). As a control, LPS was also pretreated with polymyxin B before it used for activation of monocytes. As expected, the ability of polymyxin B-treated LPS was dramatically attenuated in cytokine production by monocytes. On the other hand, preincubation of rLpp with polymyxin B induced cytokine production by monocytes as quite efficiently as rLpp without treatment of polymyxin B, clearly demonstrating that rLpp is free of LPS contamination and the cytokine production in rLpp-exposed monocytes is derived from endogenous activity of Lpp of *V. vulnificus*.

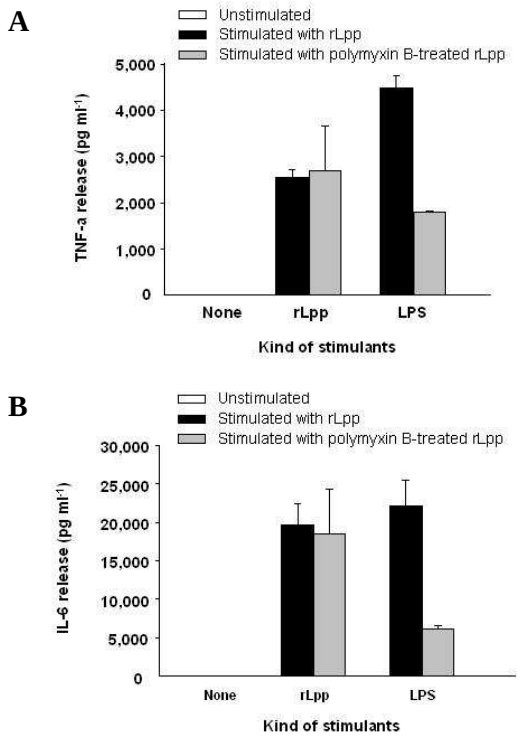


Fig. 7. Effect of polymyxin B on cytokine production by rLpp-treated monocytes. Monocytes (5×10^5) were seeded in 24-well culture plates, and the exposed to rLpp at a concentration of 1.0 μg/ml. LPS of *E. coli* was used as a positive control to see the effect of polymyxin B. Polymyxin B was added at a concentration of 20 μg/ml to neutralize residual LPS which may remain during the purification of rLpp. Following an 18 h-exposure to the rLpp, the culture supernatants were collected, and then assayed for cytokine production by ELISA.

7. Role of TLR2 in cytokine production by rLpp-stimulated monocytes

The next experiment was performed to determine if the Lpp of *V. vulnificus* activates human monocytes via interaction with TLR2, which is a well-known receptor for Lpp of *Mycobacterium*[32]. Monocytes were pretreated with antibodies specific to TLR2 present on the surface of human monocytes prior to being stimulated with rLpp (Fig. 8). As a control, monocytes were also treated with isotype IgG control instead of TLR2 antibodies, and then stimulated with rLpp in an identical way. Only the cells treated with TLR2 antibodies showed a significant decrease in production of both TNF-α and IL-6. In contrast, the anti-TLR2 antibody was ineffective in inhibition of cytokine production by LPS-stimulated monocytes. Therefore, this result suggests that Lpp of *V. vulnificus* may act as an immunostimulant via its interaction with TLR2.

In vitro system was also constructed in which TLR2 or TLR4 is expressed along with their adaptor protein, MyD88. As a control, another set of HEK 293 cells were transfected with pFLAG-CMV1, the vector for TLR2- or TLR4-expressing plasmids. Since a luciferase reporter for NF-κB activities was included in the transfection, luciferase activities were measured without or with stimulation of rLpp (Fig. 9). Without rLpp-stimulation, any of HEK 293 cells did not show dramatic activities of NF-κB. Upon an incubation with rLpp, the

HEK 293 cells transfected with pFLAG-TLR2 demonstrated a significant increase in NF-κB activities.

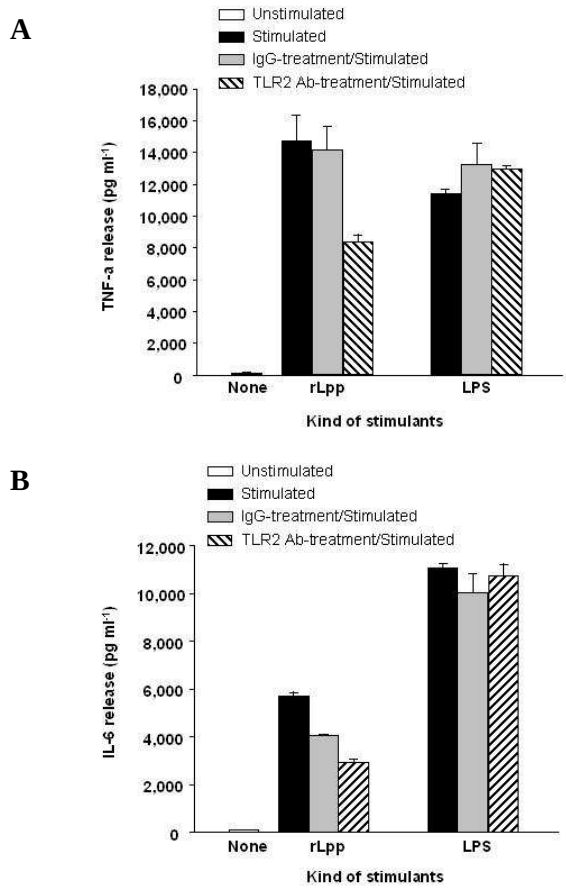


Fig. 8. Influence of TLR-neutralizing antibodies cytokine production by rLpp-stimulated monocytes. Human monocytes were preincubated for 1 h either with anti-TLR2 antibodies TL2.1 (BioLegend), or with the relevant isotype control. The cells were then stimulated with rLpp (1.0 μg/ml) of *V. vulnificus* for 18 h, and the culture supernatants were assayed for cytokine production by ELISA. Monocytes pretreated with antibodies specific to TLR2

was also stimulated with LPS instead of rLpp, and then assayed for cytokine productions.

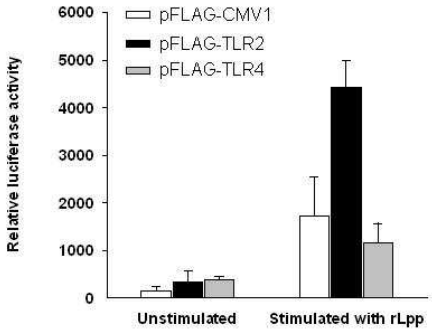


Fig. 9. Role of TLR2 in rLpp-induced activation of NF-κB. A total of 5 × 10^5 HEK 293 cells were transfected with 1 μg of pFLAG-TLR2, 1 μg of pNF-κB-luc, 1 μg of pcDNA 3.1 (+) - MyD 88 and 0.5 μg of pCH110 in 24-well plates. As a negative control, plasmid pFLAG-CMV (Invitrogen) was transfected instead of pFLAG-TLR2. For another set, pFLAG-TLR4 DNA was used to transfected to HEK 293 cells instead of pFLAG-TLR2. After 24 h, transfected cells were stimulated with rLpp. After additional 20 h-incubation, the cells were lysed and assayed for luciferase activity using a Luciferase Reporter Assay System (Promega). Normalized reporter activity is expressed as the firefly luciferase activities divided by activities of β-galactosidase of the identical sample.

8. Role of lipid moiety of *V. vulnificus* Lpp in the cytokine production by monocytes

Mutant rLpp was made, which lost 23 amino acid residues in the amino-terminal portion of the protein. Since the missing region of Lpp is suggested as important residues for lipidation, this mutant rLpp is expected to be unable to be modified by lipidation. Monocytes were stimulated with the mutant rLpp at a various concentration ranged from 0. 001 to 1 μg/ml, and then assayed for their ability to produce TNF-α and IL-6 (Fig. 10). While wild-type rLpp is potent in cytokine production by monocytes, ability of the mutant rLpp in cytokine production by monocytes was significantly affected. However, formation of cytokine was observed in monocytes stimulated with high concentration of the mutant rLpp.

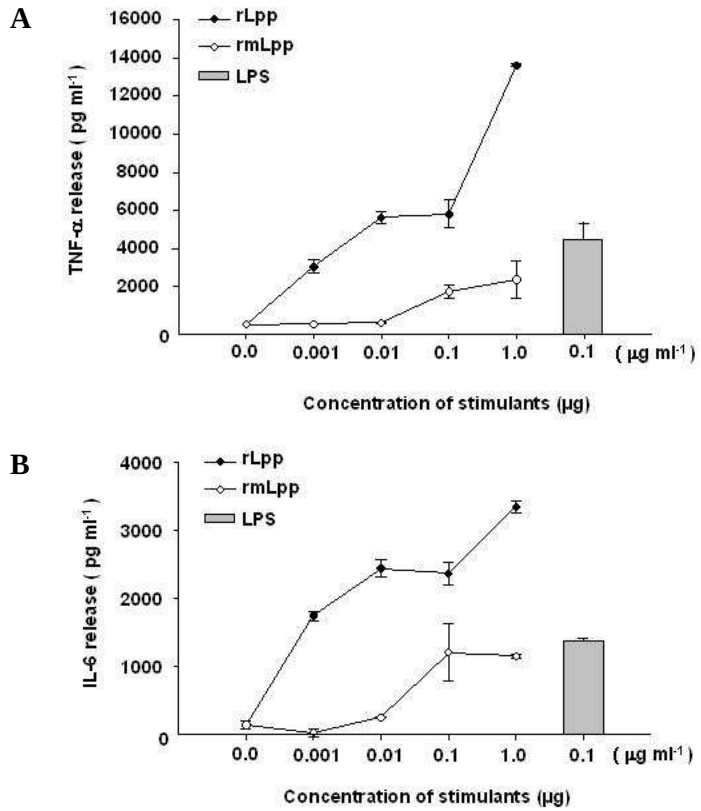


Fig. 10. Effect of lipid moiety of Lpp for the cytokine production. Monocytes were seeded at 5×10^5 cells/ml and exposed either to rLpp or mutant rLpp. LPS was used as a positive control for induction of cytokine production. Following an 18 h exposure to the rLpp or mutant rLpp, culture supernatants were collected and assayed for cytokine production by ELISA.

IV. DISCUSSION

The innate immune system of humans recognizes microbial pathogens via action of its highly conserved pattern recognition receptors, which can be associated with bacterial structures. Molecules recognized by these receptors are synthesized exclusively by microorganisms including LPS, peptidoglycans, lipotechoic acids, and bacterial lipoproteins (BLP)[33]. Especially, lipoproteins (Lpps) of *Mycobaterium tuberculosis* are known to activate a variety of host cells including monocytes, macrophages, and neutrophils to produce cytokine production[31, 32]. In Lpp is characterized by a unique NH_2-terminal lipo-amino acid, *N*-acyl-*S*-acylglyceryl cysteine. While LPS is produced only by gram-negative bacteria, Lpp is produced by the complete spectrum of bacterial pathogens[33], and is one of the abundant proteins in certain gram-negative bacteria (~700,000 molecules/cell)[33]. One of Lpps in *V. vulnificus* was identified as a surface protein having the antigenic property (Han, Y.-S., and S.-J. Park, unpublished results). Therefore, I examined whether the Lpp is able to stimulate the human cells to produce cytokine. Since intact *V. vulnificus* has a strong cytotoxicity to various types of human cells (Kim, W.-H. and S.-J. Park, unpublished results), it is impossible to measure cytokine production by

challenge with intact *V. vulnificus*. Thus, bacterial lysate or rLpp was used in all the experiments. Indeed, lysates of wild-type *V. vulnificus* trigger cytokine production by human PBMC, but the lysate of *V. vulnificus* lacking Lpp is completely defective in induction of cytokine production (Fig. 5A, B). This result supports an idea that Lpp of *V. vulnificus* is a major immunostimulant.

V. vulnificus is known to cause fatal septicemia especially in immunocompromised humans. My data suggest the potent immunostimulating activity of *V. vulnificus* Lpp on human monocytes. The clinical progression of systemic inflammatory response from sepsis to severe sepsis has been documented[34]. Spirochaetal microorganisms that lack LPS initiate considerable host injury by Lpps that exist on their cell surfaces[35], and there is evidence that they act as major proinflammatory agonists during bacterial infection[31].

The *V. vulnificus* Lpp of 30 kDa induces monocytes to produce TNF-α and IL-6 in a manner analogous to LPS (Fig. 6). Therefore, it is postulated that a cell surface receptor may transduce the signal for this 30 kDa Lpp as TLR4 functions to transduce the LPS signal. TLRs, a group of well-known receptors of the innate immune response, contain a cytoplasmic domain that is homologous to the signaling domain of the IL-1 receptor, which can activate a signaling pathway leading to activation of NF-κB, and subsequent transcription

of cytokine genes[35, 36, 37]. *Mycobacterium* Lpp has been shown to trigger host defense mechanisms primarily through TLR-2 by Lpp-mediated IL-12/NO production by monocytes/macrophages and Lpp-induced apoptosis of monocytes[22, 38]. I here demonstrate that TLR2 is involved in *V. vulnificus* Lpp-initiated response of human monocytes using two different assays. That is, cytokine production in Lpp-stimulated monocytes is blocked by antibodies against TLR2 (Fig. 8), and *V. vulnficus* Lpp induces activation of NF-κB in a HEK 293 cell line through TLR2 (Fig. 9).

Although our data indicate that TLR2 is the receptor responsible for *V. vulnificus* Lpp-mediated immune response, it does not exclude a role for other TLRs in this process. In *Mycoplasma* spp., acylated Lpps are abundant cell surface antigen, and many putative Lpp-encoding genes are identified in the *Mycoplasma* genomes[39]. One of prevalent forms is triacylated Lpps, in which three fatty acids are bound to its N-terminal cysteine residue: two in a diacylglyceride that is linked via a thioether bond to the sulfhydryl group (diacylglyceryl modification); and one to the amine group (*N*-acylation). The study on Lpps of *M. gallisepticum* and *Mycoplasma mycoides* indicates the presence of diacylated and triacylated forms of Lpps[40]. In TLR1-deficient mice, N-palmitoyl-(S)-[2,3-bis(palmitoyloxy)-(2RS)-propyl] (Pam3)/Cys-Ser-Lys 4

(CSK4) [Pam_3CSK_4] containing three acyl chains failed to induce cytokines, whereas macrophage-activating lipopeptide from *Mycoplasma fermentans* (MALP-2) containing two acyl chains could induce cytokines[41]. Therefore, it is thought that triacylated lipoproteins are recognized by combined action of TLR1 and TLR2, whereas diacylated lipoproteins are recognized by TLR2 in conjunction with TLR6. Future investigation will be performed to unveil the roles of TLR1 and TLR6 for *V. vulnificus* Lpp-induced activation of human cells.

In many known Lpps with immunological activity, the portion of protein responsible for its is located in the NH_2-terminal triacylated lipopeptide region[42, 43, 44, 45, 46, 47, 48, 49]. Activation through TLR2 by Lpps was dependent on fatty acyl moieties because deacylated forms of OspA and *T. pallidum* lipopeptide had no activity[22]. When I observed the effect of lipid moiety of *V. vulnificus* Lpp, the importance of lipidation was also confirmed from less cytokine production from human monocytes triggered by nonlipidated Lpp (Fig. 10). However, nonlipidated form of *V. vulnificus* Lpp still retains some residual activity to induce cytokine production by monocytes when high concentration of Lpp is treated. It raises a possibility that protein portion of *V. vulnificus* Lpp also contributes minor function as an immunostimulant.

A recent report showed a significant increases in the levels of proinflammatory

cytokines TNF-α, IL-1β and IL-6 in the serum from *V. vulnificus* infected patients compared to healthy individuals, confirming a important role for dysregulation of the cytokine response in pathogenesis of *V. vulnificus*[50]. This study identified the Lpp as a bacterial component responsible for activation of human monocytes to produce cytokine, and found that it acts through TLR2 of the host cells. Based on these data, I expect that TNF-α and IL-6, the cytokines measured in this study, appeared to be useful as monitoring parameters for the prognosis of the disease. Because monocyte-derived proinflammatory cytokines, such as TNF-α and IL-6, have been well known to play a pivotal role in the pathogenesis of septic shock, many investigators have to realize the usefulness of the proinflammatory cytokines as prognostic parameters of individual septicemic patients. In addition, the Lpp of *V. vulnificus* capable of interacting with monocytes, would be a potential molecule in the development of new agents for prevention and therapy of *V. vulnificus* infection.

Ⅴ. CONCLUSIONS

1. The *lpp* gene of *V. vulnificus* encodes a membrane-associated protein, which is modified with lipidation.

2. Reduced cytotoxicity of the *Δlpp* knockout mutant *V. vulnificus* against HEp-2 cells indicates that Lpp play a role in pathogenesis of *V. vulnificus*.

3. Induced formation of TNF-α and IL-6 in the Lpp-treated monocytes shows an ability of the Lpp protein as an immunostimulant.

4. Based on the attenuated ability of non-lipidated form of Lpp in cytokine production by monocytes, the lipid moiety of Lpp is essential in its function as an immunostimulant.

5. Results obtained by two different assays, neutralization of cytokine production by TLR2 antibodies and induced cytokine production in TLR2-expressed cells by Lpp, clearly indicates that Lpp of *V. vulnificus* triggers cytokine production by monocytes via the interaction with TLR2.

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ABSTRACT (IN KOREAN)

인체세포에서 사이토카인 생성을 유도하는 *Vibrio vulnificus* lipoprotein 작용

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구 성 영

패혈증 비브리오균 (*Vibrio vulnificus*) 은 그람 음성의 미생물로 음식물 섭취를 통하여 위장염과 같은 소화기관의 감염과 피부 접촉으로 인한 감염으로 인체에 침입하여 괴사성 상처감염을 일으키고 패혈증을 유발한다. 패혈증 비브리오균에 의한 패혈증은 전형적인 패혈증성 쇼크, 그리고 극심한 염증성의 사이토카인 반응을 나타낸다.

본 연구에서는 패혈증 비브리오균의 표면 단백질 중 하나인 lipoprotein이 숙주 내에서 innate inflammation을 유도하는 지를 조사하고자 하였다.

패혈증 비브리오균의 lipoprotein을 생성해내는 *lpp* 유전자를 제거한 돌연변이를 만들어 연구에 사용함으로써, 이 단백질이 인체세포에서 염증반응에 관여하는 사이토카인 중의 하나인 Interferon-ɣ (IFN-ɣ)가 유도되는데 있어 중요하다는 것을 알았다. 이를 바탕으로 하여, 패혈증 비브리오균의 lipoprotein에 대한 숙주세포 내에서의 반응을 조사하기 위하여 재조합단백질을 합성하였다. [^{3}H]-palmitic acid 을 이용하여 박테리아 내에서 lipoprotein에 표지함으로써, native form의 lipoprotein의 특성 중의 하나인 lipidation 되는 과정을 재조합단백질 역시 거치게 된다는 것을 확인하였다. 합성된 재조합단백질을 이용하여 인체세포에 반응시킨 결과 염증반응에 관여 하는 tumor necrosis factor-α (TNF-α)와 Interleukin-6 (IL-6)이 생성되지만 IL-4는 생성되지 않는다는 것을 알았다.

Lipoprotein은 아미노기 말단 (NH_2-terminal)에 lipo-amino acid 즉 N-acyl-S-acylglyceryl cysteine이라는 특이한 부분을 가지고 있다. 본 연구에서는 lipoprotein의 이런 특징적인 구조가 인체세포에서의 면역반응을 일으키는데 중요하게 작용하는지에 대해서도 조사하였다. 그 결과 lipidation되지 않는 lipoprotein은 lipidation된 lipoprotein에 비해 인체세포에서 사이토카인 생성을 유도하는 능력이 많이 저하되는 것을 확인하였다. 이로서 단백질의 lipid 부분이 사이

토카인 생성을 유발하는데 있어 중요한 역할을 한다는 사실을 알 수 있었다.

Lipoprotein이 인체세포와 반응할 때, Toll-like receptor (TLR)를 통하여 일어나는 지의 여부를 조사하였다. TLR2 항체를 이용하여 인체세포에 재조합 lipoprotein으로 자극시키기 전에 TLR2 항체를 처리한 세포에서는 TNF-α 와 IL-6의 생성이 억제되는 것을 확인하였다. 그리고 재조합 lipoprotein에 의해 유도되는 사이토카인 생성에 있어 TLR2의 역할을 좀 더 확인하기 위하여 TLR2가 발현되는 세포와 TLR4가 발현되는 세포를 이용하여 NF-κB의 활성도를 측정한 결과, TLR2가 발현되는 세포에서만 재조합 lipoprotein에 반응시켰을 때, NF-κB의 활성도가 높게 측정되는 것을 확인하였다. 이러한 결과는 패혈증 비브리오균의 lipoprotein이 인체세포에서 TLR2를 활성화시켜 면역자극제로서의 기능을 할 것이란 기대를 가능하게 한다.

핵심되는 말: *Vibrio vulnificus*, bacterial lipoprotein, cytokine