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Short communication

High-throughput characterization of lipopolysaccharide-binding proteins using mass spectrometry

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ABSTRACT

Lipopolysaccharide (LPS)-binding proteins interact with LPS in human serum and mediate various immune responses. We describe a high-throughput LPS-binding protein profiling platform for discovering unknown LPS-binding proteins and potential inflammatory mediators. As a pull-down method, the LPS molecules were immobilized onto epoxy beads and then directly incubated with human serum to screen LPS-binding proteins. Through the "untargeted" mass spectrometric approach, potential LPS-binding proteins which elicit various immune responses in human serum were identified by a highly sensitive LTQ Orbitrap Hybrid Fourier Transform Mass Spectrometer (LTQ Orbitrap FT MS). Therefore, this mass spectrometry (MS)-based profiling method is straightforward for screening unknown LPS-binding proteins and provides physiologically relevant binding partners in human serum.

1. Introduction

Lipopolysaccharide (LPS) is the principal proinflammatory unit of the Gram-negative bacterial envelope and is recognized by the Toll-like receptor 4 (TLR4)-MD2 receptor complex [1]. The primary Gram-negative bacterial endotoxin is recognized by immune cells that induce mediators of inflammation [2]. The infection of LPS into the host cell increases the plasma levels of the LPS-binding protein (LBP), which is an acute-phase protein and dramatically amplifies host immune cell responses to LPS by delivery to a plasma membrane-bound CD14 glycoprotein [3–5]. LBP also transfers LPS to a soluble form of CD14, enabling cells, including endothelial cells that do not carry CD14, to become highly LPS responsive [6]. Although the mammalian host interactions with LPS have been established as described earlier, many undiscovered pathways related with other LPS-binding proteins allowing LPS signaling have not yet been identified and are still controversial [7]. Therefore, the screening of other physiologically relevant LPS counterparts from body fluids would be valuable to identify LPS signaling–related pathways.

To screen LPS-binding peptides, several efforts have been made, which may provide possible motifs of promising LPS-binding proteins using biopanning of phage-displayed peptide libraries [8–12]. In previous studies, the homologous peptide sequences against LPS of Gram-negative bacteria had been initially obtained by phage display technology using epoxy beads that introduce a covalent bonding with the hydroxyl group of LPS [9,13]. Although the peptide library screening method is quite applicable to the rapid discovery of new selective LPS-binding ligands such as antimicrobial peptides, the short length of peptide sequences is still restrictive to identify target proteins of LPS *in vivo*.

Here, we describe a rapid and high-throughput LPS-binding protein profiling method for discovering unknown LPS-binding proteins and potential inflammatory mediators through the "untargeted" mass spectrometric approach. As a pull-down manner, the purified LPS molecules were immobilized onto epoxy beads and then incubated with human serum to screen LPS-binding proteins that could be identified by a highly sensitive LTQ Orbitrap Hybrid Fourier Transform Mass Spectrometer (LTQ Orbitrap FT MS). This screening method showed remarkable performance to characterize potential LPS-binding proteins eliciting strong immune responses in humans, which was mediated by human serum including cytokines, TLR4, and other ligands. To the best of our knowledge, LPS-immobilized solid support has not been used for the screening of LPS-binding proteins from human serum previously.

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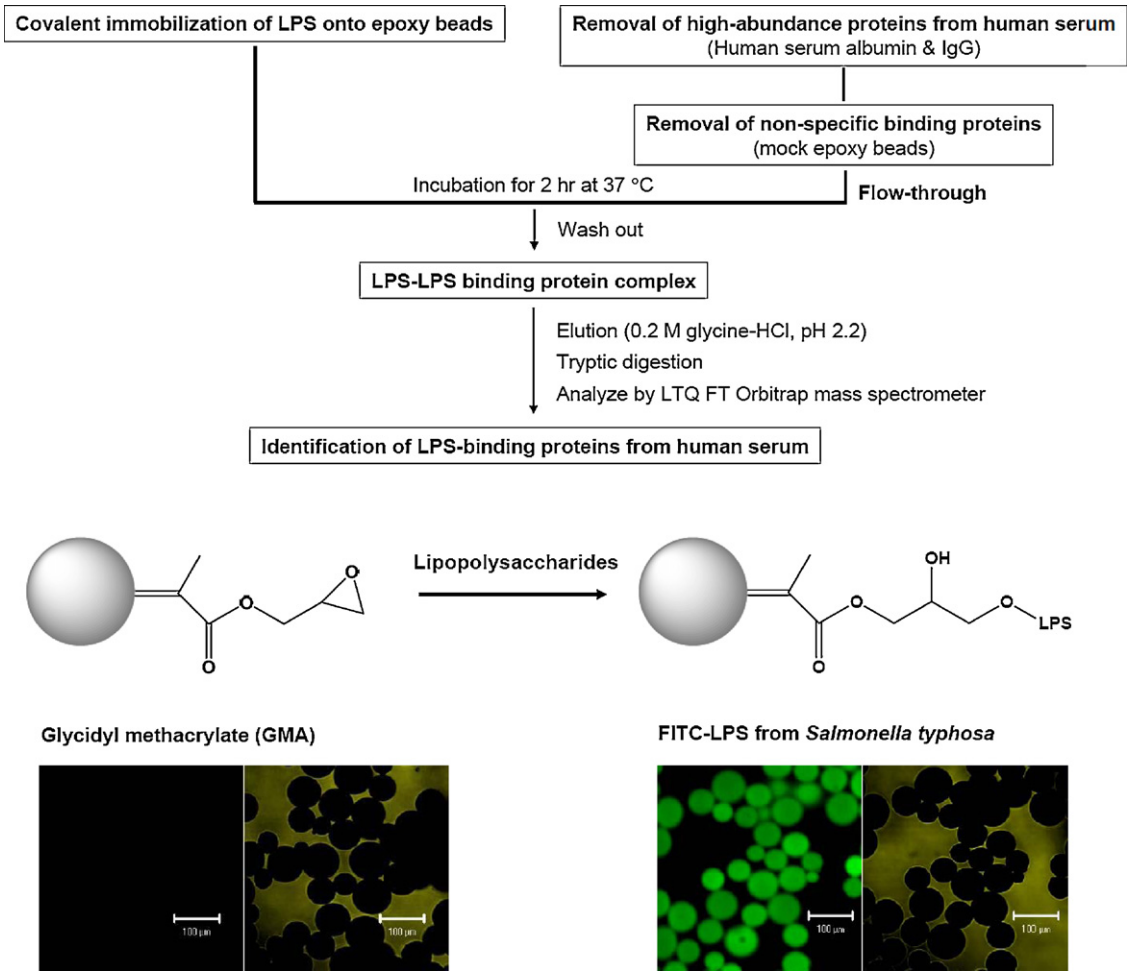

Fig. 1. (a) Experimental workflow of LPS-binding protein profiling from human serum *via* "untargeted" mass spectrometric approach. (b) Covalent immobilization of LPS molecules onto glycidyl methacrylate beads including epoxy groups. The one-step coupling method was verified by using FITC-conjugated LPS from *Salmonella typhosa*.

2. Materials and methods

2.1. Preparation of LPS-conjugated beads

The glycidyl methacrylate beads including epoxy groups were prepared as described in previous reports [9,13]. Fluorescein isothiocyanate (FITC)-LPS from *Salmonella typhosa* and unlabeled LPS from *S. typhosa* and *Pseudomonas aeruginosa* were purchased from Sigma–Aldrich (St. Louis, MO, USA). To immobilize the LPS molecules, the epoxy beads (50 mg), presuspended in 0.02 M NaOH (400 μl), were coincubated with LPS (100 μl of 0.2 M NaOH, 10 mg/ml in endotoxin-free water) at room temperature overnight on an orbital shaker. After washing with distilled water, β-mercaptoethanol (10 μM) was mixed to the LPS-immobilized beads for 2 h at room temperature to defunctionalize the residual epoxy groups on the beads. Then, the beads were washed with distilled water and were stored at 4 °C for the next use. The LPS conjugation onto epoxy beads was verified by FITC-LPS from *S. typhosa* and a confocal laser scanning microscope (LSM5 Pascal, Carl Zeiss, Germany).

2.2. LPS-binding protein screening from human serum

Albumin and the major subclasses of gamma-globulin (IgG) of human serum (40 μl, obtained from a healthy male donor, 41 years, at the Seoul National University College of Medicine), were removed using a ProteoSeek Albumin/IgG Removal Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instruction. After blocking with 1% skim milk for 30 min at room temperature, the human serum was incubated with LPS unconjugated beads at 37 °C for 1 h and the unbound fraction was transferred to LPS-conjugated beads. Each LPS-immobilized bead (20 mg) and serum (100 μl) was incubated at 37 °C for 2 h, and the supernatant was discarded. After mild washing with PBS buffer, the bound proteins were eluted from the beads by elution buffer (0.2 M glycine–HCl, pH 2.2) for 30 min and then neutralized by a neutralizing buffer (1 M Tris(hydroxymethyl)aminomethane–HCl, pH 9.1). For the proteomic analysis of the eluted proteins, tryptic digestion was proceeded by adding 10 μl of trypsin (1 μg/μl, sequencing grade, Promega, Madison, WI) into each sample, which was then incubated at 37 °C for 16 h. The digested sample was desalted using SepPak C-18 cartridge (Waters, Milford, MA), dried, and resolved by ultra-pure water for further LTQ mass spectrometric analysis.

2.3. LTQ ESI-MS/MS analysis

The LPS-binding proteins were analyzed using a nanospray LTQ FT Orbitrap mass spectrometer (Thermo Electron Corp., USA) in positive ion mode. The analytical column for nanospray ion source was made by a P-2000 laser puller (Shutter Instrument, Novato, CA, USA) to create a 5-μm tip [14]. The samples were preloaded to C18 (Agilent, USA)-packed spray tip by a home-made high-pressure

Table 1
Identification of LPS from *Pseudomonas aeruginosa* and *Salmonella typhosa* binding proteins using LTQ FT Orbitrap mass spectrometer.

LPS binding protein	LPS from *Pseudomonas aeruginosa*		LPS from *Salmonella typhosa*	
	Max. XCorr (charge)	Del CN	Max. XCorr (charge)	Del CN
CD40-ligand: tumor necrosis factor (ligand) superfamily member 5				
QGFYYIYTQVTFCSNR	3.06(2)	0.32	2.76(2)	0.21
Chemokine (C-X-C motif) ligand 2: GRO2 oncogene				
LLRVALLLLLVAASRR	3.61(3)	0.21	3.12(3)	0.26
Brevican isoform: hyaluronan-binding protein				
WTFLSRGR	2.03(2)	0.21	2.04(2)	0.16
Programmed cell death 8 (apoptosis-inducing factor) isoform 2				
ISGLGLTPEQK	2.74(3)	0.11	2.80(3)	0.10
Human toll-like receptor 4				
VEKTLLRQQVELYR	–	–	2.52(3)	0.15
Neural cell adhesion molecule				
DGNPFYFTDHRIIPSNNSGTFR	–	–	2.63(3)	0.16
L chain L, crystal structure of CD142				
TVAAPSVFIFPPSDEQLK	–	–	3.08(2)	0.20
von Willbrand factor				
LRISQK	–	–	2.01(2)	0.15
Complement component 8, gamma polypeptide				
PASPISTIQPKANFDAQQFAGTWLLVAVGSACR	2.55(3)	0.28	–	–
T-cell receptor alpha chain				
SIYNQEGK	–	–	2.36(2)	0.13
T-cell receptor beta chain				
NIQYFGAGTRLSVLEDLKNVFPPEVAVFEPK	2.56(3)	0.33	–	–
LGCVSLCQQLLSRGAWKHHIFWR			2.57(3)	0.15
Human toll-like receptor 8				
GNKLLFLTDSLSDFTSSLRTLLLSHNR	2.59(3)	0.29	–	–

Note: Max. XCorr is the highest XCorr value (>1.5, 2.0, 2.5 for singly-, doubly-, and triply-charged peaks respectively) of the identified peptide. DelCN is the difference in magnitude between the peptide fit with the highest Xcorr and the peptide fit with the second best Xcorr.

bomb with a pressure of 1 MPa of nitrogen gas. The loaded sample was eluted at a flow rate of 300 nl/min *via* a mobile-phase gradient from 0% B buffer to 100% B buffer using an Ultimate 3000 2D-nano LC (LC packing, Netherlands). Buffer A was prepared with water/acetonitrile (98/2 (v/v)) and 0.1% formic acid, and buffer B with water/acetonitrile (20/80 (v/v)) and 0.1% formic acid. For the MS/MS fragmentation, 35% of normalized collision energy and 2 Da of isolation width were used. 5 scan events were performed. The first full scan with LTQ was followed by the second full scan with FT Orbitrap; then, three data-dependent MS/MS analyses were performed. The heated capillary was maintained at 200 °C and the spray voltage was 1.50 kV. The maximum ion collection time was set to 10 ms and three microscans were averaged per scan.

3. Results and discussion

In the first step of the workflow, LPS was immobilized onto the epoxy beads through a covalent bonding as described in previous studies (Fig. 1). The covalent immobilization of the LPS was validated by the confocal microscopic imaging of LPS-FITC from *S. typhosa*. After overnight incubation at the highly basic condition (0.5 M NaOH), the fluorescence signal of LPS-FITC from *S. typhosa* was saturated onto the epoxy beads. In this condition, LPS is partially hydrolyzed but the antigenic or the protein-binding potency of LPS was not changed [15]. These results directly show that the bait molecules have been successfully immobilized onto solid support for the next pull-down experiment *via* covalent bonding chemistry. To remove the nonspecific binding proteins, the LPS-unconjugated bead was preincubated with human serum. The flow-through of the human serum was subsequently transferred to each LPS from *P. aeruginosa* and LPS from *S. typhosa*-immobilized bead. After isolation of the LPS-binding proteins from human serum, the bound proteins were tryptic digested, purified, and analyzed using LTQ Orbitrap FT MS with positive mode ionization of nanospray MS/MS that provides high mass accuracy and sensitivity to identify low-abundant LPS-binding proteins in human serum. In addition, the nonspecific binding fraction was also tryptic digested and analyzed by LTQ Orbitrap FT MS. The LPS-binding proteins in human serum were identified with SEQUEST software. As expected, most of the nonspecific bound proteins were high-abundant proteins in human serum such as undepleted albumin, apolipoproteins, and transferrin (data not shown). In contrast to peptide library screening methods, this global LPS-binding protein profiling method enables to characterize directly target proteins from human serum in an unbiased manner.

Recently, we had developed a rapid glycan immobilization technique through a 4-hydrazinobenzoic acid (HBA)-functionalized bead. It is then applied to the high-throughput screening of glycan-binding proteins from human serum using LTQ Orbitrap FT MS [16]. Although the pull-down assay was based on the solid-phase screening method, high-potential glycan-binding proteins closely related with immunological rejection in xenotransplantation could be characterized. Moreover, most of the identified proteins were low-abundant proteins in human serum, which means that the highly sensitive mass spectrometry is adapted from a solid-phase binding protein profiling strategy introduced earlier.

The highly sensitive mass spectrometric method enabled us to characterize several promising LPS-binding proteins that are known to play a role in invasive bacterial infection and to upregulate following LPS administration. In fact, potential LPS-binding proteins were identified by phage display technology using heptamer/dodecamer/cyclic heptamer peptide library against LPS-immobilized epoxy beads from the previous studies [9,13]. From the identical sequences (AWLPWAK, YTFFEPLSSLTR, CDGPFKLLC), Toll-like receptors (TLR) 9 and 2 were annotated using the SwissProt database. Although the member of TLR family plays a fundamental role in pathogen recognition and activation of innate immunity related with Gram-positive and Gram-negative bacteria, we had to characterize physiologically and pathologically relevant LPS-binding proteins such as other TLR family (i.e., TLR4), cytokines, and CD antigens. First of all, the identification of human TLR4 is

one of the most promising and physiologically relevant data in this solid-phase pull-down assay. TLR4 plays a key role in the innate immune system to recognize the infection by Gram-negative bacteria [17]. The pattern recognition receptor makes a complex with MD-2 and directly mediates to LPS-induced inflammatory responses through intracellular signaling pathway [18]. The direct interaction between TLR4 and LPS molecules is still controversial, but this study has shown a possibility of the direct recognition of them. Recently, the affinity (K_D = 14.1 μM) of LPS for TLR4, which was expressed in insect cells, was measured using surface plasmon resonance (SPR) [19]. Although the interaction is relatively very weak, it could be stronger in the circumstance of TLR4/MD-2 complex *in vivo*. Second, the CD40-ligand was identified in both *P. aeruginosa* and *S. typhosa* as a LPS-binding protein. The identified CD40-ligand has showed a significantly higher increase in plasma levels after LPS stimulation [20]. This result indicates that the interaction has a potential to mediate both proinflammatory and prothrombic immune responses. As shown in Table 1, several proteins related to innate or adaptive immune responses and upregulated plasma proteins following LPS administration were recognized by the LPS-immobilized beads.

4. Conclusions

The global LPS-binding protein profiling method described here could identify physiologically and immunologically promising target ligands such as TLR4, CD40-ligand, complement component, T-cell receptors, and von Willebrand factor from complex human body fluids. The one-step covalent conjugation of the LPS molecules is highly stable and enables a solid-phase-based pull-down assay. Moreover, the use of highly sensitive mass spectrometry allows one to identify low-abundant proteins in human serum with an "untargeted" manner. Although several LPS-binding proteins are proposed that may participate in immune signaling by bacterial LPS, the biologic functions of these proteins should be identified by additional immunological experiments. In the future, we plan to apply this platform to discover the interaction between various polysaccharides and their signaling proteins inducing various cell–cell or cell–host adhesion and recognition.

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