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Novel TLR4-antagonizing peptides inhibit LPS-induced release of inflammatory mediators by monocytes ☆

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Abstract

Toll-like receptor 4 (TLR4) has become a new target for combating Gram-negative bacterium-induced sepsis. In this study, we screened peptides that can interact with TLR4 from a random 16-peptide library using yeast two-hybrid system and performed functional identification for the obtained peptides. We got two positive clones out of 1.28×10^7 transformants. The peptides were sequenced and synthesized. Protein sequence comparison confirmed that the two peptides had no homologous proteins. The two peptides were found to significantly inhibit LPS-induced NF-κB activation in HEK-293 cells that were transfected with TLR4 cDNA, LPS-induced IκBα (IκB alpha) phosphorylation and NF-κB activation in monocytes, and release of IL-1, IL-6, and TNF-α by monocytes. We further confirmed that the No. 9 peptide could bind to TLR4 extracellular domain, but the No. 24 peptide could not, suggesting that two novel peptides were identified as the antagonists of TLR4, which significantly inhibited the effects of endotoxin in vitro. The No. 9 peptide may function through binding to TLR4 extracellular domain. Our findings suggest a promising countermeasure against Gram-negative bacterium-induced sepsis.

Keywords: Toll-like receptor 4; Yeast two-hybrid system; Interaction; Peptide; Endotoxin; Monocyte

As a common complication of severe trauma, shock, and major operations, sepsis is a major cause of deaths because it leads to septic shock, and even multiple organ failure. Unfortunately, no specific sepsis-combating measures have been developed yet [1–3]. Approximate one-half of all sepses in clinical settings owe it to Gram-negative bacteria. Endotoxin, a component of Gram-negative bacteria, has been shown to be a major pathogenic factor of Gram-negative bacterium-associated sepsis. Endotoxin makes macrophages, neutrophils, and other inflammatory cells to release cytokines, such as IL-1, IL-6, and TNF-α, in great amounts, which may render inflammatory reactions out of control, ultimately resulting in sepsis, septic shock, or multiple organ failure syndrome [4,5]. Based on the cascade reactions described above, researchers have been striving to develop anti-inflammatory measures against inflammatory mediators. Although such a strategy may suppress excessive inflammatory reactions in body and then alleviate damages to target organs, its effect is usually unsatisfactory, because the complex cascade reactions between multiple inflammatory mediators dwarf the one-for-one countermeasures. In addition, the clinical effect of some analogues or antagonists of LPS seems dim, although researches

☆ *Abbreviations:* TLR4, Toll-like receptor 4; LPS, lipopolysaccharide.

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are still under way [6,7]. Therefore, it is urgent to search for new sepsis-combating measures.

TLR4 is a newly discovered receptor for LPS and also a key molecule for signal transduction [8–10]. Researches have shown that mice with TLR4 gene knockout are completely irresponsive to LPS. Mice with mutations in TLR4 gene (C3H/HeJ) are highly tolerated with endotoxin. These findings suggest that TLR4 may be used as a novel target for the prevention and treatment of Gram-negative bacterium-induced sepsis [11–13]. In this study, we screened peptides that could interact with TLR4 and performed preliminary functional identifications for the obtained peptides by using yeast two-hybrid system and the technique of peptide library.

Materials and methods

Strains, plasmids, random peptide library, and reagents. Escherichia coli strains DH5α and HB101 were preserved by our institute. LPS (0111B4, Lot No. 69H4157) was purchased from Sigma Chemical (St. Louis, MO). The yeast two-hybrid kit, random MATCHMAKER peptide library, pCMV-β-galactosidase, and culture medium for yeast were purchased from Clontech. PfuDNA polymerase, restrictive endonucleases, ligases, and high-fidelity PCR kit were purchased from Takara (Japan). 5-Bromo-4-chloro-3-indolyl-β-galactoside, poly(dI–dC), acrylamide, and *N*,*N*′-methylene bisacrylamide were purchased from Sigma (USA). T4-polynucleotide kinase was purchased from Promega. TLR4 plasmid was a kind gift from Beutler B (The Scripps Institute, La Jolla, CA, USA). Fugene 6 transfection kit was purchased from Roche Molecular Laboratories. pCMV-β-galactoside plasmid was purchased from Gene, β-galactoside ELISA detection kit was from Roche, and ELISA kit for NF-κB activity was from Active Motif (USA). TLR4 McAb [14] and TLR4 extracellular domain protein [15] were prepared at our institute. IκBα phosphorylation immunoblot kit was purchased from New England Biolabs. Chemoluminescence kit was purchased from NEC. *N*-Ethyl-*N*′-(diethyl-aminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Thermo. Biointeraction real-time analyzing system was the product of IAsys Plus (Affinity Sensor, USA). ELISA kits for IL-1, IL-6, and TNF-α were purchased from Clontech, and the standard preparations of IL-1, IL-6, and TNF-α were from Genzyme (USA).

Construction of pGBKT7 DNA-BD/TLR4. The yeast two-hybrid plasmid pGBKT7 DNA-BD/TLR4 was constructed. PCR was performed with TLR4 cDNA as a template using specific primers: 5′-GGAATTCCATATGGAGCTGAATTTCTTAC-3′ (sense primer, *Nde*I site is underlined); 5′-AACGACGTCTCAGATAGATGTT GCTTC-3′ (antisense primer, *Pst*I site is underlined). Amplification was carried out by using the following cycle: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, reassociation at 60 °C for 50 s, extension at 72 °C for 1.5 min (32 cycles), and a final extension step of 72 °C for 10 min. The resulting products were stored at 4 °C. The amplification products and pGBKT7 DNA-BD plasmid vectors were digested with *Nde*I and *Pst*I, and ligated with T4 ligase. The transformants were extracted, digested, and identified by sequencing. After comparisons with the sequences listed in GenBank, the transformants were confirmed to be the right ones.

Preparation of competent yeasts and transformation of peptide library plasmids. The procedures were carried out according to the instructions of the manufacturer of yeast two-hybrid system (Clontech) (Cat# PT3061 and Cat# PT3024-1). Large-scale transformations were performed using electroporation [16].

Identification of autoactivation of TLR4 bait protein and detection of its toxicity on yeasts. Constructs of pGBKT7 DNA-BD/TLR4 were transformed in small amounts into AH109 yeasts. The transformation liquid was smeared on SD/-Trp or SD/-His plates. After culture for 3–4 days at 30 °C, growth status of the colonies was observed. Trp^+ colonies were selected for β-galactosidase detection.

Detection of expression of pGBKT7 DNA-BD/TLR4 fusion protein in yeasts. Proteins were extracted from AH109 yeasts (genotype: *MATα,trp1-901,leu2-3,112,ura3-52,his3-200,gal4Δ,gal180Δ, LYS2::GAL1$_{uas}$-GAL1$_{TATA}$-HIS3,GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2,URA3::MEL1$_{uas}$-MEL1$_{TATA}$-lacZ*), yeasts transformed with pGBKT7 DNA-BD plasmids, and yeasts transformed with pGBKT7 DNA-BD/TLR4 plasmids by using the urea/SDS method. Protein samples were subjected to SDS–PAGE. One-half of the resulting gel was used for detection of expression of the fusion protein by Coomassie brilliant blue staining. The remaining gel was electrotransferred onto a PVDF filter; Western blotting was performed with mouse anti-human TLR4 McAb as a first antibody (diluted by a ratio of 1:200) and with goat anti-mouse IgG/HRP as a second antibody (1:2000); then chemoluminescence was used to detect the expression of the fusion protein.

Amplification of AD-peptide library plasmids. According to the instructions of the manufacturer of the peptide library plasmid, titer of peptide library was measured. Peptide library culture and density of culture capsules for amplification were determined by the measured titer and volume of peptide library. After 24–36 h of culture at 30 °C, and when the colonies were near to fusion, the colonies were collected and plasmids were extracted with alkaline lysis and purified with polyoxyl.

Peptide library screening with full-length TLR4 as bait. In each batch, 50 SD/-Leu/-Trp/-His plates were prepared. Meanwhile, efficiency of transformation and number of transformed clones were determined using SD/-Leu/-Trp plates to ensure the magnitude of screened peptide library. After 3–5 days of culture at 30 °C, His^+ transformants with a growth diameter of more than 2 mm were selected and inoculated on SD/-Leu/-Trp/-His/-Ade plates. Then His^+, Ade^+, and $lacZ^+$ colonies were screened.

Analysis of AD-peptide plasmids with suspected interaction using reverse screening. DNA-BD/TLR4 plasmids were eliminated from questionable positive clones with cycloheximide (CHX), and thus only yeasts containing AD-peptide library plasmids were allowed to grow. A small amount of AD-peptide library plasmids were extracted and transformed into *E. coli* HB101 bacterial cells. After amplification, plasmids were extracted again. The resulting AD-peptide library plasmids were preliminarily screened. β-Galactosidase detection was performed to determine the presence or absence of autoactivation of AD-peptide/AH109.

Elimination of false positive clones by using yeast mating. To eliminate false positive clones of yeasts containing AD peptide, yeast-mating experiments were performed. pGBKT7 DNA-BD plasmids, pGBKT7 DNA-BD/TLR4 plasmids, and pGBKT7 Lam plasmids were transformed into Y187 yeasts (genotype: *MATα,ura3-52, his3-20,ade2-101,trp1-901,leu2-3,112,gal14Δmet-gal180Δ,URA3:GAL1$_{uas}$-gal$_{TATA}$-lacZ*) in small amounts, respectively. Meanwhile, pGBKT7 AD plasmids and pGADT7 AD/peptide plasmids were transformed into AH109 yeasts. Yeast-mating experiments were performed according to Table 1 to detect expression of lacZ and His, with pCL-1 as the positive control and pGBKT7 Lam as the negative control. If only the results were the same with Table 1, a true positive clone was considered; otherwise, false positive clones were considered.

Specific two-hybrid between pGBKT7 DNA-BD/TLR4 plasmids and AD-peptide library plasmids. To validate the interaction and to eliminate false positive clones as thoroughly as possible, pGBKT7 DNA-BD/TLR4 plasmids and AD-peptide library plasmids were re-transformed into Y187 yeasts for specific two-hybrid to detect the activity of lacZ and His expression, with pCL-1 and T+p53 as the positive controls and with Lam as the negative control.