Molecular Immunology 93 (2018) 47–54

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Full Length Article

Vibrio cholerae OmpU induces IL-8 expression in human intestinal epithelial cells

Jae Seung Yang[a], Jun Ho Jeon[b], Mi Seon Jang[a,f], Seok-Seong Kang[c], Ki Bum Ahn[d,f], Manki Song[a], Cheol-Heui Yun[e], Seung Hyun Han[f,*]

[a] Clinical Research Laboratory, Sciences Unit, International Vaccine Institute, Seoul, Republic of Korea
[b] Division of High-Risk Pathogen Research, Center for Infectious Diseases, Korea National Institute of Health, Cheongju, Republic of Korea
[c] Department of Food Science and Biotechnology, Dongguk University-Seoul, Goyang, Republic of Korea
[d] Research Division for Biotechnology, Korea Atomic Energy Research Institute, Jeongeup, Republic of Korea
[e] Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, Republic of Korea
[f] Department of Oral Microbiology and Immunology, DRI, and BK21 Plus Program, School of Dentistry, Seoul National University, Seoul, Republic of Korea

ARTICLE INFO

Keywords:
Vibrio cholerae
OmpU
IL-8
Adherence
Inflammation
Intestinal epithelial cells

ABSTRACT

Although *Vibrio cholerae* colonizes the small intestine and induces acute inflammatory responses, less is known about the molecular mechanisms of *V. cholerae*-induced inflammatory responses in the intestine. We recently reported that OmpU, one of the most abundant outer membrane proteins of *V. cholerae*, plays an important role in the innate immunity of the whole bacteria. In this study, we evaluated the role of OmpU in induction of IL-8, a representative chemokine that recruits various inflammatory immune cells, in the human intestinal epithelial cell (IEC) line, HT-29. Recombinant OmpU (rOmpU) of *V. cholerae* induced IL-8 expression at the mRNA and protein levels in a dose- and time-dependent manner. Interestingly, IL-8 was secreted through both apical and basolateral sides of the polarized HT-29 cells upon apical exposure to rOmpU but not upon basolateral exposure. rOmpU-induced IL-8 expression was inhibited by interference of lipid raft formation with nystatin, but not by blocking the formation of clathrin-coated pits with chlorpromazine. In addition, rOmpU-induced IL-8 expression was mediated via ERK1/2 and p38 kinase pathways, but not via JNK signaling pathway. Finally, *V. cholerae* lacking *ompU* elicited decreased IL-8 expression and adherence to HT-29 cells compared to the parental strain. Collectively, these results suggest that *V. cholerae* OmpU might play an important role in intestinal inflammation by inducing IL-8 expression in human IECs.

1. Introduction

Vibrio cholerae, a Gram-negative, rod-shaped bacterium, is a causative agent of the life-threatening disease, cholera. The pathogen colonizes the small intestine and activates expression of key virulence factors including cholera toxin (CT) and toxin co-regulated pilus (TCP) (Herrington et al., 1988; Miller et al., 1987). ToxR and TcpP regulate expression of ToxT protein, which in turn activates expression of CT and TCP (DiRita et al., 1991). Thus, ToxR is a primary positive regulator of the virulence cascade of *V. cholerae* (Skorupski and Taylor, 1997). In addition, ToxR activates expression of OmpU, which is important for virulence (Sperandio et al., 1996). *V. cholerae* OmpU is one of the most abundant outer membrane porin proteins comprising a homotrimer of monomeric 38 kDa chains. It also plays a role in the entry of hydrophilic solutes and is selective for cationic ions (Sperandio et al., 1996). In particular, OmpU has gained significant attention because anti-OmpU sera showed passive protection not only against challenge with *V. cholerae* O1 but also against *V. cholerae* O139 (Das et al., 1998). In addition, we have recently shown that *V. cholerae* OmpU is a major immunostimulatory molecule of killed whole cell cholera vaccines by interacting with toll-like receptor 2 (TLR2) (Yang et al., 2015).

Intestinal epithelial cells (IECs) primarily function as a first-line defense system against intestinal microbes and provide signals for acute inflammatory responses through production of proinflammatory mediators at infection sites. Although intensive studies have been conducted on *V. cholerae*, less is known about the molecular mechanisms of *V. cholerae*-induced inflammatory responses because cholera has long been considered as a non-inflammatory disease (Farthing, 1997). However, recent works showed proinflammatory responses including infiltration

* Corresponding author at: Department of Oral Microbiology and Immunology, DRI, and BK21 Plus Program, School of Dentistry, Seoul National University, Building 86 (Room 304), 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Republic of Korea.
E-mail address: shhan-mi@snu.ac.kr (S.H. Han).

https://doi.org/10.1016/j.molimm.2017.11.005
Received 31 July 2017; Received in revised form 5 November 2017; Accepted 7 November 2017

of polymorphonuclear leukocytes, upregulation of lactoferrin, tumor necrosis factor-α, serum interleukin (IL)-6, and macrophage inhibitory protein-2 during natural cholera infection and in a pulmonary infection animal model (Fullner et al., 2002; Mathan et al., 1995; Qadri et al., 2004). Of these inflammatory mediators, Sarkar et al. reported increased expression of IL-8 upon *V. cholerae* infection in epithelial cells (Sarkar and Chaudhuri, 2004).

IL-8 is produced at the early stage of infection by a variety of cells, including epithelial cells, endothelial cells, fibroblasts, keratinocytes, and macrophages (Hoffmann et al., 2002). It plays an important role in the recruitment of both innate and adaptive immune cells, such as neutrophils and lymphocytes, to infection sites (Huber et al., 1991). IL-8 gene expression is tightly regulated at the transcriptional level via mitogen-activated protein kinases (MAPKs) including p38 kinase, c-Jun-N-terminal kinase (JNK), and extracellular-regulated kinase 1/2 (ERK1/2) (Hoffmann et al., 2002). Subsequently, stimulation of MAPK cascades activates transcription factors such as nuclear factor (NF)-κB, NF-IL6, and activating protein (AP)-1, all of which are involved in transcriptional activation of IL-8 (D'Angio et al., 2004). Notably, NF-κB is essential for IL-8 expression whereas NF-IL6 and AP-1 are likely to induce maximal gene expression (Hoffmann et al., 2002).

Although OmpU has been identified as a major outer membrane protein and important immunostimulating molecule of *V. cholerae* in immune cells including murine macrophages and human monocytes (Sakharwade et al., 2013; Yang et al., 2015), little is known about its potential effect on human IECs to promote inflammatory responses. In the present study, we investigated induction of IL-8 expression by recombinant OmpU (rOmpU) together with the underlying molecular mechanisms in a human IEC line, HT-29.

2. Materials and methods

2.1. Reagents and chemicals

Nystatin, chlorpromazine, and polymyxin B (PMB) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). U0126, PD98059, SB203580, SB202190, SP600125, and JNK inhibitor I were obtained from Calbiochem (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin) were obtained from Hyclone (Logan, UT, USA). Antibodies specific to ERK1/2, phospho-ERK1/2 (p-ERK1/2), p38 kinase, phospho-p38 kinase (p-p38), JNK, phospho-JNK (p-JNK), and horseradish peroxidase (HRP)-labeled anti-rabbit IgG were purchased from Cell Signaling Technology (Beverly, MA, USA). HRP-labeled anti-mouse IgG and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Sigma-Aldrich Inc., respectively. All other reagents were purchased from Sigma-Aldrich Inc. unless otherwise stated.

2.2. Bacterial strains and construction of ompU mutant

Vibrio cholerae El Tor O1 Inaba (T19479) was kindly provided by Prof. Jan Holmgren (Gothenburg University, Sweden). The *ompU* gene was inactivated by deletion of 832 bp out of 1053 bp ORF using polymerase chain reaction (PCR)-mediated linker mutagenesis, as described previously (Milton et al., 1996). PCR was carried out to amplify the 5′ amplicon of the *ompU* gene using OMPU001F (5′-GCCGCATGCCACGC GCTGTCATTGGAAC-3′) and OMPU001R (5′-GCCGACGGATCCATGGA TTCCGTCAGCGTAAGCG-3′). For amplification of the 3′ amplicon of *ompU*, OMPU002F (5′-CATGGATCCGTCGGCACTACTTCAAGCCAAACT TCC-3′) and OMPU002R (5′-CGGGAGCTCATCCAGCCACAAATCGC CCT-3′) primer pairs were used. Linker and *Bam*HI restriction sites were added to primer OMPU001R and OMPU002F to link the 5′- and 3′-amplicons of *ompU*. PCR was then carried out with primer OMPU001F and OMPU002R using two fragments as templates to obtain a fusion fragment of 1310 bp. The resulting fusion product containing deleted *ompU* was cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and the neomycin phosphotransferase gene (*nptI*) from pUC4 K (Amersham, Piscataway, NJ, USA) was then inserted into the *Bam*HI restriction site. A *Sph*I/*Sac*I-digested fragment of 2563 bp containing 5′ amplicon, *nptI* gene, and 3′ amplicon was cloned into corresponding sites of suicide vector pCVD442 (Donnenberg and Kaper, 1991). The resulting plasmid was used to delete the *ompU* gene on a chromosome of *V. cholerae* O1 Inaba by allelic exchange (Philippe et al., 2004) using the *Escherichia coli* SM10 λ pir strain as conjugal donor, as previously described (Milton et al., 1996). To inactivate bacteria, both wild-type (WT) and *ompU*-deficient (*ΔompU*) strains were cultured in Luria-Bertani (LB) broth at 37 °C for 2–3 h. Inactivated bacteria were prepared by incubating cells in 0.6% formaldehyde per OD_{600} for 5 h with stirring. Cells were washed and resuspended in PBS, and bacteria were confirmed to be completely killed on LB agar plate.

2.3. Expression and purification of recombinant OmpU

The gene encoding OmpU was amplified with forward primer 5′-GGCCGCGGATCCGGGACAATAAATTAGGACTTAATAAGAT-3′ and reverse primer 5′-CCGGGCCTCGAGGAAGTCGTAACGTAGACCGATA-3′ from genomic DNA of *V. cholerae* O1 Inaba. The PCR product was digested with *Bam*HI/*Sal*I and cloned into corresponding sites of pET21d (+) (Novagen, Madison, WI, USA), a 6 histidine-tagged portion of the C-terminal region. Expression of rOmpU was induced with 0.5 mM isopropyl β-D-thiogalacto-pyranoside in *E. coli* BL21 (DE3). rOmpU was purified using Ni-NTA metal resin (Clontech, Mountain View, CA, USA) and solubilized with buffer containing 6 M urea. Then, the protein was refolded by removing urea with step-wise dialysis (sequential decrease of urea from 6 M, 4 M, 2 M, 1 M, 0.75 M, 0.5 M, 0.25 M, 0.1 M, and then, finally to 0 M) in a cellulose membrane (Spectra/Por® 6, cutoff 1000 Da). The purity was over 95% estimated by SDS-PAGE analysis, as described previously (Yang et al., 2015). The rOmpU concentration (1.1 mg/ml) was determined by BCA assay (Pierce, Rockford, IL, USA), and endotoxin content (272 EU/ml) in rOmpU was measured using Pierce LAL chromogenic endotoxin quantitation kit.

2.4. Cell culture

HT-29 cells were obtained from the American Type Culture Collection (Mannas, VA, USA) and cultured in DMEM supplemented with 10% heat-inactivated FBS and antibiotics (100 unit/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a 5% CO_2 incubator. HT-29 cells were pre-treated with 50 μg/ml of PMB to block residual endotoxic activity that might possibly remain in rOmpU. To prepare confluent and polarized cells, HT-29 cells were grown on polycarbonate transwell filters (6.5 mm diameter, 0.4 μm pore size, Costar, Corning, NY, USA) and allowed to achieve confluence (ca. 3 weeks) (Woo et al., 2013). To confirm cell polarization, the electrical resistance (150–200 Ω/cm^2) of trans-epithelial cells was measured by EVOM2 Epithelial Volt/Ohm-meter (World Precision Instruments, Sarasota, FL, USA). Then, the cells were treated with rOmpU (50 μg/ml) either on the apical or basolateral side of the cell layer for 48 h. The culture media were collected from the apical and basolateral compartments to measure IL-8 secretion using ELISA.

2.5. Measurement of cell viability

Viability of cells was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)-based cytotoxicity assay as described previously (Cheon et al., 2008). Briefly, MTT reagent (0.5 mg/ml) was added to each well containing cells and further incubated at 37 °C CO_2 incubator for 1 h. Then, the MTT solution was removed and dimethyl sulfoxide was added to dissolve the cell. Absorbance was measured at 570 nm, with the correction wavelength at 650 nm using a microplate reader (Molecular Devices, Sunnyvale, CA,