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Clostridium difficile-derived membrane vesicles induce the expression of pro-inflammatory cytokine genes and cytotoxicity in colonic epithelial cells *in vitro*

Asiimwe Nicholas [a], Hyejin Jeon [a], Gati Noble Selasi [a], Seok Hyeon Na [a], Hyo Il Kwon [a], Yoo Jeong Kim [a], Chi Won Choi [b], Seung Il Kim [b], Je Chul Lee [a, *]

[a] Department of Microbiology, Kyungpook National University School of Medicine, Daegu, Republic of Korea
[b] Drug & Disease Target Team, Korea Basic Science Institute, Daejeon, Republic of Korea

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Clostridium difficile is the most common etiological agent of antibiotic-associated diarrhea in hospitalized and non-hospitalized patients. This study investigated the secretion of membrane vesicles (MVs) from *C. difficile* and determined the expression of pro-inflammatory cytokine genes and cytotoxicity of *C. difficile* MVs in epithelial cells *in vitro*. *C. difficile* ATCC 43255 and two clinical isolates secreted spherical MVs during *in vitro* culture. Proteomic analysis revealed that MVs of *C. difficile* ATCC 43255 contained a total of 262 proteins. Translation-associated proteins were the most commonly identified in *C. difficile* MVs, whereas TcdA and TcdB toxins were not detected. *C. difficile* ATCC 43255-derived MVs stimulated the expression of pro-inflammatory cytokine genes, including interleukin (IL)-1β, IL-6, IL-8, and monocyte chemoattractant protein-1 in human colorectal epithelial Caco-2 cells. Moreover, these extracellular vesicles induced cytotoxicity in Caco-2 cells. In conclusion, *C. difficile* MVs are important nanocomplexes that elicit a pro-inflammatory response and induce cytotoxicity in colonic epithelial cells, which may contribute, along with toxins, to intestinal mucosal injury during *C. difficile* infection.

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1. Introduction

Gram-negative bacteria secrete outer membrane vesicles (OMVs) that are spherical bilayer structures with a diameter of 20–300 nm [1–3]. OMVs secreted from Gram-negative pathogens contribute to bacterial pathogenesis by long-distance transport of virulence factors to host cells, protection of secreted molecules, antimicrobial resistance, and biofilm formation [4–6]. In addition, these extracellular vesicles modulate or stimulate innate immune response [6–8]. Despite the absence of an outer membrane, Gram-positive bacteria, including *Bacillus anthracis* [9], *Bacillus cereus* [10], *Bacillus subtilis* [10], *Clostridium perfringens* [11], *Mycobacterium ulcerans* [12], and *Staphylococcus aureus* [13–15], have been reported to secrete membrane vesicles (MVs) similar to OMVs of Gram-negative bacteria. MVs of Gram-positive bacteria contain virulence factors and toxins, including hemolysin and enterotoxins in *S. aureus* [15,16] and protective antigen, edema factor, and lethal factor in *B. anthracis* [9]. These active toxins are delivered to host cells, inducing host cell pathology *in vitro*. Thus, MVs derived from Gram-positive pathogens may contribute to bacterial pathogenesis *in vivo*, similar to OMVs of Gram-negative pathogens.

Clostridium difficile is an obligate anaerobic, Gram-positive, spore-forming bacterium. This microorganism is a leading causative agent of antibiotic-associated diarrhea, antibiotic-associated colitis, and pseudomembranous colitis [17,18]. Toxigenic *C. difficile* strains produce two toxins, *C. difficile* toxin (Tcd) A and TcdB. The two toxins are largely responsible for the characteristic pathology observed in *C. difficile* infections (CDIs); these toxins modify Rho proteins by monoglucosyltransferase activity, resulting in the collapse of the actin cytoskeleton in host cells [19]. However, TcdA-negative/TcdB-positive variant *C. difficile* strains are increasingly being reported worldwide [20,21]. These variants are capable of causing CDI symptoms through the toxic effects of TcdB alone [22]. In addition to TcdA and TcdB, some *C. difficile* strains have been found to produce a binary toxin (CDT) that exhibit an ADP-

* Corresponding author. Department of Microbiology, Kyungpook National University School of Medicine, 680 Gukchaebosang-Ro, Jung-gu, Daegu 41944, Republic of Korea.
E-mail address: leejc@knu.ac.kr (J.C. Lee).

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ribosyltransferase activity, which is thought to modify actin in a manner that facilitates adherence and colonization of *C. difficile* in the host [19]. Along with cytotoxicity, toxin-mediated inflammatory responses are important to the pathogenesis of CDI [22–24]. Other bacterial factors such as flagella, surface-associated proteins, and culture supernatant have been shown to induce inflammatory responses [25,26]. Since MVs are nanocomplexes that contain many pathogen-associated molecular patterns such as proteins, lipids, DNA, and peptidoglycan, MVs induce a strong inflammatory response [12–14,27]. These results led us to assess whether *C. difficile* MVs play a role in inflammatory responses in intestinal epithelial cells. This study investigated the secretion of MVs from *C. difficile* and the ability of *C. difficile* MVs to induce the expression of pro-inflammatory cytokine genes and cytotoxicity in epithelial cells *in vitro*.

2. Materials and methods

2.1. Bacterial strains

The *C. difficile* reference strain ATCC 43255 (NTCC 11080, VPI 10463) from the American Type Culture Collection (Manassas, VA, USA) and two clinical isolates, *C. difficile* 7725 and 8082, obtained from the diarrheal stool of hospitalized patients at the Kyungpook National University Hospital in Daegu, Korea, were used in this study. *C. difficile* ATCC 43,255 was ribotype 087, toxinotype 0, and positive for the *tcdA* and *tcdB* genes, but negative for the *cdtA* and *cdtB* genes [28]. Bacteria were anaerobically cultured in an anaerobic chamber (Sheldon Manufacturing Inc., Cornelius, OR, USA) at 37 °C on brain-heart infusion (BHI) medium (BD Biosciences, San Jose, CA, USA) supplemented with sodium taurocholate (10% w/v, Sigma-Aldrich, St. Louis, MO, USA), L-cysteine (10% w/v, Sigma-Aldrich), hemin in 1 M sodium hydroxide (10% v/v, Sigma-Aldrich), and vitamin K1 in 95% ethanol (0.02% v/v, Sigma-Aldrich).

2.2. Cell culture

Two human epithelial cell lines, Caco-2 cells, originating from a heterogeneous colorectal adenocarcinoma, and HEp-2 cells, originating from a laryngeal carcinoma, were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were grown in Dulbecco's modified Eagle medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine, 1000 U/ml penicillin G, and 50 μg/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. Confluent cells were seeded in 6-well plates for the cytokine gene expression assay and flow cytometric analysis.

2.3. Purification of MVs

C. difficile MVs were prepared from bacterial culture supernatants as previously described [13,14]. Briefly, bacteria were anaerobically cultured at 37 °C in 500 ml of BHI broth to the late log phase. Bacterial cells were removed by centrifugation at 6000 × *g* for 20 min at 4 °C. The culture supernatants were filtered using the QuixStand Benchtop System (GE Healthcare, Amersham, UK) with a 0.2-μm hollow fiber membrane (GE Healthcare) and then concentrated by ultrafiltration with a 100-kDa hollow fiber membrane (GE Healthcare). MVs were collected by ultracentrifugation of the concentrated samples at 150,000 × *g* for 6 h at 4 °C and resuspended in a small volume of phosphate-buffered saline. The protein concentration of MVs was determined using a modified BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). The purified MVs were streaked onto blood agar and BHI plates to check for sterility and then stored at −75 °C until use.

2.4. Identification of proteins in C. difficile MVs

Proteins associated with MVs of *C. difficile* ATCC 43255 were identified using one-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometry (1-DE-LC-MS/MS) as previously described [14]. All MS and MS/MS spectra were acquired using the LCQ-Deca ESI ion trap mass spectrometer in the data-dependent mode. The MS/MS spectra were used to search *C. difficile* ATCC 43,255 proteins in the NCBI non-redundant database (http://www.ncbi.nlm.nih.gov/) using MASCOT software (Matric Science, Boston, MA, USA). A proteomic analysis was performed in triplicate with different samples. Proteins identified in all three experiments were analyzed. The locations of proteins were predicted using a subcellular location prediction program, Cello version 2.5 (http://cello.life.nctu.edu.tw/). The exponentially modified protein abundance index (emPAI) was generated using MASCOT software [29]. Proteins identified in *C. difficile* MVs were classified according to Gene Ontology (GO) functions using DAVID Bioinformatics Resources 6.7 (https://david.ncifcrf.gov).

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The cultured bacterial cells were lysed by sonication (Branson Ultrasonics Corp., Danbury, CT, USA). Proteins in the culture supernatant were precipitated with 20% trichloroacetic acid. The bacterial lysate, culture supernatants, and purified MVs corresponding to 10 μg of protein were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (1 M Tris HCl [pH 6.8], 10% SDS, 1% bromophenol blue, glycerol, and β-mercaptoethanol) and boiled for 10 min. The proteins were separated by 10% SDS-PAGE, and the gels were stained with Coomassie G-250 (Bio-Rad, Hercules, CA, USA).

2.6. RNA isolation and quantitative real-time polymerase chain reaction of pro-inflammatory cytokine genes

The expression levels of genes encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH), interleukin (IL)-1β, IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 were determined by quantitative real-time PCR (qPCR) as previously described [7,8]. Caco-2 cells were treated with different concentrations of *C. difficile* ATCC 43255 MVs (1, 5, and 10 μg/ml) for 3 h. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized by the reverse transcription of 2 μg of total RNA using oligo dT primers and M-MLV reverse transcriptase in a total reaction volume of 40 μl (Fermentas, Lithuania). Gene transcripts were quantified using Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) using an ABI PRISM 7500 Real-Time System (Applied Biosystems). The amplification specificity was evaluated by a melting curve analysis. Fold changes in gene expression were calculated using the comparative Ct method ($2^{-\Delta\Delta C_T}$) [30], and sample transcript levels were normalized to GAPDH expression levels. Each experiment was performed in triplicate.

2.7. Flow cytometric analysis

Caco-2 and HEp-2 cells were seeded at a concentration of 2.5×10^5 cells/ml in 6-well plates and then treated with different concentrations (1, 5, 10, and 20 μg/ml) of *C. difficile* ATCC 43255 MVs for 24 h. Cells were treated with 40 μg/ml of cyclophosphamide as a positive control for cell death. Cells were stained with propidium iodide (PI; BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. The samples were assessed in a FACSCalibur flow cytometer (BD Biosciences) by plotting the PI

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