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Variation among *Staphylococcus aureus* membrane vesicle proteomes affects cytotoxicity of host cells

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ABSTRACT

Staphylococcus aureus secretes membrane-derived vesicles (MVs), which can deliver virulence factors to host cells and induce cytopathology. However, the cytopathology of host cells induced by MVs derived from different *S. aureus* strains has not yet been characterized. In the present study, the cytotoxic activity of MVs from different *S. aureus* isolates on host cells was compared and the proteomes of *S. aureus* MVs were analyzed. The MVs purified from *S. aureus* M060 isolated from a patient with staphylococcal scalded skin syndrome showed higher cytotoxic activity toward host cells than that shown by MVs from three other clinical *S. aureus* isolates. *S. aureus* M060 MVs induced HEp-2 cell apoptosis in a dose-dependent manner, but the cytotoxic activity of MVs was completely abolished by treatment with proteinase K. In a proteomic analysis, the MVs from three *S. aureus* isolates not only carry 25 common proteins, but also carry ≥60 strain-specific proteins. All *S. aureus* MVs contained δ-hemolysin (Hld), γ-hemolysin, leukocidin D, and exfoliative toxin C, but exfoliative toxin A (ETA) was specifically identified in *S. aureus* M060 MVs. ETA was delivered to HEp-2 cells via *S. aureus* MVs. Both rETA and rHld induced cytotoxicity in HEp-2 cells. In conclusion, MVs from clinical *S. aureus* isolates differ with respect to cytotoxic activity in host cells, and these differences may result from differences in the MV proteomes. Further proteogenomic analysis or mutagenesis of specific genes is necessary to identify cytotoxic factors in *S. aureus* MVs.

1. Introduction

Staphylococcus aureus causes a wide spectrum of infections in humans, from superficial cutaneous infections to deep systemic infections, such as pneumonia, endocarditis, osteomyelitis, and bacteremia [1,2]. The wide spread of methicillin-resistant *S. aureus* and the emergence of vancomycin resistance are of great concern in clinical settings [3–5]. The extensive range of diseases caused by *S. aureus* is likely associated with virulence factors, which include structural components, enzymes, and toxins [6–8]. However, the secretion of these virulence factors from bacteria and their delivery to host cells or tissues are not fully understood. Pathogenic bacteria have specialized secretion systems for the transport of toxins, exoenzymes, and other virulence factors to the extracellular environment or host cells [9–11]. In addition, extracellular vesicles produced by pathogenic bacteria play a role in the delivery of virulence factors to host cells. Gram-negative bacteria secrete outer membrane vesicles (OMVs), which contribute to the delivery of virulence factors to host cells and subsequently induce host cell pathology [12–17]. Gram-positive bacteria also produce and secrete membrane vesicles (MVs) into the extracellular milieu during growth [18–24]. Thus, MVs secreted from *S. aureus* may be associated with the development or progression of diseases.

Clinical *S. aureus* isolates produce MVs during both *in vitro* culture and *in vivo* mouse infection [18,19,21,22]. *S. aureus* MVs interact with the cytoplasmic membrane of host cells via a

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cholesterol-rich membrane microdomain and subsequently deliver their component staphylococcal protein A (SPA) to host cells [18]. Many virulence-associated factors, including protease, enzymes, and toxins, have been identified in *S. aureus* MVs based on proteomic analyses [18,19]. Consistent with these observations, *S. aureus* MVs induce cytotoxicity in host cells *in vitro* and induce atopic dermatitis-like skin inflammation in a murine model [18,21]. Interestingly, α-hemolysin (Hla) has been identified as a cytotoxic factor in *S. aureus* MVs [22]. Cytotoxicity has been observed in HeLa cells treated with 30 μg/ml Hla-containing MVs purified from *S. aureus* 8325-4 and in HEp-2 cells treated with 50 μg/ml MVs purified from *S. aureus* 06ST1048 [18,22]. Although the host cells were different, the concentration of *S. aureus* MVs necessary for the induction of cytotoxicity differed between these two studies. The cytotoxicity of host cells induced by MVs derived from different *S. aureus* strains has not yet been reported. To further understand the cytotoxic activity of *S. aureus* MVs in host cells and to identify cytotoxic factors associated with *S. aureus* MVs, we purified MVs from different clinical *S. aureus* isolates and compared their cytotoxic activity in host cells. Furthermore, we analyzed the proteomes of *S. aureus* MVs to identify common or strain-specific proteins among the MVs from different *S. aureus* isolates. We report here that MVs from clinical *S. aureus* isolates differ with respect to cytotoxic activity in host cells, and these differences may result from differences in the MV proteomes.

2. Materials and methods

2.1. Bacterial strains and cell culture

S. aureus M060 was isolated from a nasal swab of a hospitalized patient with staphylococcal scalded skin syndrome (SSSS) at the Changwon Fatima Hospital in Changwon, Korea. This isolate produced exfoliative toxin A (ETA). Two *S. aureus* isolates, 01ST93 and 06ST1048, were obtained from the blood of hospitalized patients at the Kyungpook National University Hospital in Daegu, Korea. *S. aureus* 03ST17 was isolated from the skin lesions of atopic dermatitis at the Kyungpook National University Hospital. Three *S. aureus* isolates, 01ST93, 03ST17, and 06ST1048, were supplied by the National Culture Collection for Pathogens-Kyungpook National University Hospital (Daegu, Korea). Four *S. aureus* isolates used in this study were resistant to oxacillin, i.e., they were methicillin-resistant *S. aureus*. Bacteria were maintained on blood agar plates and grown in Luria–Bertani (LB) broth. *Escherichia coli* Top10 and BL21 (DE3) were grown on LB agar plate or in LB broth. When necessary, kanamycin (50 μg/ml) was added to the culture medium. Two eukaryotic cell lines, HEp-2 and COS-7, were purchased from the Korean Cell Line Bank (Seoul, Korea) and 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.

2.2. Purification of MVs

S. aureus MVs were prepared from bacterial culture supernatants as previously described [18,19]. Briefly, bacteria were grown in 500 ml of LB broth to reach late log phase at 37 °C, with shaking. 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 concentrated by ultrafiltration with a 100-kDa hollow fiber membrane (GE Healthcare). The MV fractions were collected by ultracentrifugation of the concentrated samples at 150,000 × *g* for 3 h at 4 °C and resuspended in a small volume of phosphate-buffered saline (PBS). The protein concentration of MVs was determined using a modified BCA assay (Thermo Scientific, Waltham, MA, USA). The purified MVs were streaked onto blood agar plates to check for sterility and stored at −75 °C until use.

2.3. Treatment of S. aureus MVs with proteinase K

Purified *S. aureus* MVs were treated with 0.1 μg/ml proteinase K (Fermentas, St. Leon-Rot, Germany) for 1 h or 3 h at 50 °C for MV protein degradation. Intact MVs and proteinase K-treated MVs were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie G-250 (Bio-Rad, Hercules, CA, USA).

2.4. Identification of proteins in S. aureus MVs

Proteins in the MVs derived from *S. aureus* M060 and 03ST17 were identified using one-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometry (1-DE-LC-MS/MS) as previously described [18]. 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 searched with MASCOT software (Matric Science, Boston, MA, USA) against *S. aureus* TW20 proteins from the NCBI non-redundant database (http://www.ncbi.nlm.nih.gov/) and a decoy sequence database. The locations of proteins were predicted using a subcellular location prediction program, Cello version 2.5 (http://cello.life.nctu.edu.tw/). Because the decoy sequence database for *S. aureus* TW20 did not contain all known *S. aureus* toxins, the MS/MS spectra were examined using the decoy sequences of 23 selected toxins: staphylococcal enterotoxin A (SEA), SEB, SEC, SED, SEE, SEG, SEH, SEI, SEJ, SEM, SEN, SEO, ETA, ETB, ETC, leukocidin S-Panton Valentine toxin (LukS-PV)/LukF-PV, LukE-LukD, LukM, Hla, β-hemolysin (Hlb), δ-hemolysin (Hld), γ-hemolysin (Hlg), and toxic shock syndrome toxin (TSST). The exponentially modified protein abundance index (emPAI) was generated using MASCOT software [25].

2.5. SDS-PAGE and western blot

The cultured bacterial cells, culture supernatants, and purified MVs were resuspended in 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. Proteins in the culture supernatant were precipitated with ammonium sulfate. The samples corresponding to 10 μg of protein were separated using 12% SDS-PAGE, and the gels were stained with Coomassie G-250. The proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and analyzed by western blotting using a sheep polyclonal anti-ETA antibody (Abcam, Cambridge, MA, USA), a rabbit polyclonal anti-staphopain A (ScpA) antibody (Sigma–Aldrich, St. Louis, MO, USA), or a rabbit polyclonal anti-Hld antibody (Sigma–Aldrich). The membrane was incubated with a secondary antibody coupled to horseradish peroxidase and was developed using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

2.6. Genomic DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was purified from *S. aureus* isolates cultured in LB broth using a genomic DNA preparation kit (SolGent, Seoul, Korea). The extracted DNA was used as a template for the amplification of toxin genes by PCR. The specific primers for toxin genes including *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *she*, *sei*, *sej*, *sem*, *sen*, *seo*, *eta*, *etb*, *lukS-PV*/