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Outer membrane vesicles from pathogenic bacteria initiate an inflammatory response in human endothelial cells

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ABSTRACT

Introduction: Gram-negative bacteria release outer membrane vesicles (OMVs) during growth that contain various membrane components involved in eliciting an inflammatory response, including lipopolysaccharide and virulence factors. However, little is known about the role of OMVs in sepsis. The objective of this study was to determine how OMVs, derived from *Escherichia* (*E.*) *coli*, elicit the cellular responses involved in activating the inflammatory cascade, and to determine whether additional virulence factors in pathogenic OMVs augment the inflammatory response.

Methods: Human umbilical endothelial cells were inoculated with OMVs from non-pathogenic *E. coli* (npOMV) or pathogenic *E. coli* (pOMV) and analyzed for adhesion protein synthesis, cytokine production, and necrosis factor (NF)-κB translocation.

Results: Flow cytometry demonstrated that human umbilical vein endothelial cells exposed to npOMV or pOMV significantly increased expression of E-selectin and intercellular adhesion molecule, with a large population of cells demonstrating increased expression of both proteins. Interleukin-6 levels were significantly elevated by 4 h after exposure to npOMV and pOMVs. NF-κB translocation to the nucleus was shown to be induced by npOMV and pOMVs. However, the role of additional virulence factors associated with pOMVs remains undefined.

Conclusions: Both npOMVs and pOMVs are capable of initiating the inflammatory cascade in endothelial cells. OMVs trigger NF-κB translocation to the nucleus, resulting in up-regulation of adhesion molecules and cytokines, presumably for the recruitment of leukocytes. By eliciting an inflammatory response, OMVs could facilitate the transition from a localized infection to a systemic response, and ultimately sepsis.

1. Introduction

Sepsis remains one of the leading causes of mortality in critically ill patients [1]. It is a syndrome that presents over a continuum, beginning as a systemic inflammatory response that can progress to multisystem organ failure, potentially ending with death [1–4]. During an infection, complex interactions between host inflammatory cells are needed to prevent bacteria from spreading systemically. However, if host defenses become overwhelmed, bacteria

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are able to enter systemic circulation. Circulating bacteria elicit inflammatory changes that alter blood flow, which ultimately cause metabolic derangements leading to cell death [5].

All Gram-negative bacteria, including *Escherichia* (*E.*) *coli*, constitutively produce outer membrane vesicles (OMVs) [6,7]. OMVs are secreted portions of the bacterial outer membrane and were first seen in electron micrographs over 40 y ago [8]. They are produced throughout the bacterial growth cycle and range in size from 50–250 nm in diameter [9]. OMVs are heterogeneous and primarily contain outer membrane components reflective of the parent bacterial cell, including proteins, lipids, virulence factors, and lipopolysaccharide (LPS) [10–12]. In addition, they have been shown to contain constituents of the periplasm and cytoplasm [9]. OMVs are believed to be involved in DNA transfer, cellular communication, and toxin delivery, but their precise role in infection and sepsis has not been fully elucidated [13,14].

OMVs have been shown to initiate a systemic inflammatory response that can lead to death in animal models [15,16]. However, the mechanisms involved in this response have yet to be elucidated *in vitro*. OMVs contain LPS, a potent stimulator of the inflammatory system, which plays a role in septic shock [6,17]. LPS binds to Toll-like receptor (TLR)-4 causing necrosis factor (NF)-κB, a transcription factor responsible for the up-regulation of inflammatory mediators, to become activated and translocated to the nucleus [18–20].

The endothelium lines the circulatory system and plays an important role in the regulation of hemostasis and inflammation [21]. Endothelial expression of adhesion proteins, including intercellular adhesion molecule (ICAM) and E-selectin, lead to leukocyte activation and adhesion [22,23]. ICAM, which facilitates adhesion and transmigration of leukocytes, is constitutively expressed at low levels by the endothelium and is up-regulated following infection [24,25]. Conversely, E-selectin, which is involved in tethering and rolling is produced *de novo* in response to infectious and inflammatory stimuli [25,26]. During infection, local endothelial activation leads to changes in vasomotor tone and a shift towards a procoagulant state, and leukocyte activation is increased [27]. In contrast, endothelial dysfunction in sepsis leads to systemic vascular permeability, insufficient perfusion, and organ damage [28,29].

Two important cytokines involved in the endothelial inflammatory response are interleukin (IL)-6 and tumor necrosis factor (TNF)-α. They are responsible for activation of endothelial and inflammatory cells and facilitate the recruitment of leukocytes to the site of injury. IL-6 is necessary for initiating an effective endothelial response against an infection. Endothelial IL-6 production is induced by a variety of stimuli, including bacteria, LPS, and TNF-α [30–32]. TNF-α has been shown to activate the endothelium and can cause changes in endothelial permeability, and even cause apoptosis [33]. Importantly, IL-6 and TNF-α levels have been shown to have prognostic implications in those with sepsis [34–37].

The severity of sepsis correlates with the degree of endothelial activation and, thus, allows endothelial cells to serve as a model for research into the biochemical changes seen in sepsis [38,39]. This study used an endothelial cell model to determine the potential of OMVs to initiate the inflammatory cascade without the participation and interaction of other inflammatory cells. Additionally, this study sought to determine whether the endothelial inflammatory response is amplified by the additional virulence factors found in OMVs from pathogenic bacteria.

2. Materials and methods

2.1. OMV isolation

OMVs were isolated from two separate strains of *E. coli*. The nonpathogenic OMVs (npOMVs) were derived from MK8A44, an *E. coli* Nlpi mutant with a hypervesiculation phenotype [40]. The pathogenic OMVs (pOMV) were derived from a clinical isolate of an enterotoxigenic *E. coli* [(ETEC), ATCC 43886; Manassas, VA]. Using a previously published protocol, OMVs were isolated from MK8A44 or ETEC cultures [16,40]. Briefly, a 500-mL volume of Luria-Bertani broth (Fisher Scientific, Fair Lawn, NJ) was inoculated with an *E. coli* strain and cultured overnight at 37°C. After incubation, whole cells were removed from cultures by centrifugation (10,000 × *g*) and ultrafiltration (0.45 μm). OMVs were collected from the cleared supernatant by centrifugation (15,404 × *g*) and resuspended in phosphate-buffered saline (PBS) supplemented with sodium chloride to a final concentration of 0.2 M. The OMV suspension was again filtered (0.45 μm) to ensure whole bacterial cell removal. The procedure was repeated until an adequate volume of pooled OMVs for each strain for all experiments was obtained.

2.2. Vesicle purity

Each preparation of isolated vesicles was cultured overnight on agar at 37°C. Plates were monitored for colony formation to ensure that each preparation was free of whole cell contamination.

In addition, each vesicle preparation was imaged with atomic force microscopy (AFM). To prepare AFM samples, OMVs were diluted 1:1 in deionized water before applying 30 μl of the suspension to freshly cleaved mica [41]. The sample was allowed to dry in air at ambient temperature. After rinsing with deionized water to remove residual buffer salts, the sample was dried with a stream of nitrogen gas and imaged in non-contact mode using an Agilent 5500 AFM (Agilent Technologies, Inc., Santa Clara, CA). Images were collected at a rate of approximately 6 μm/second at a resolution of 512 points/line. After verification that OMV preparations were free of whole cells contaminants, preparations were pooled into a single stock.

2.3. Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell and grown in Endothelial Growth Media2 (PromoCell, Heildelberg, Germany). Passages three through eight were used for all experiments. Media and reagents used in the culturing of HUVECs tested negative for LPS contamination using a standard limulus amoebocyte