International Journal of Medical Microbiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

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

Enterohemorrhagic *Escherichia coli* O157 outer membrane vesicles induce interleukin 8 production in human intestinal epithelial cells by signaling via Toll-like receptors TLR4 and TLR5 and activation of the nuclear factor NF-κB

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ARTICLE INFO

Keywords:
Enterohemorrhagic *Escherichia coli* O157
Outer membrane vesicles
Immunomodulation
Proinflammatory cytokines
Flagellin
Lipopolysaccharide

ABSTRACT

Proinflammatory cytokines play important roles in the pathogenesis of diseases caused by enterohemorrhagic *Escherichia coli* (EHEC) O157, but the spectrum of bacterial components involved in the proinflammatory responses is not fully understood. Here, we investigated the abilities of outer membrane vesicles (OMVs), nanoparticles released by EHEC O157 during growth, to induce production of proinflammatory cytokines in human intestinal epithelial cells. OMVs from both EHEC O157:H7 and sorbitol-fermenting (SF) EHEC O157:H$^-$ induced production of interleukin-8 (IL-8) in Caco-2, HCT-8, and HT-29 intestinal epithelial cell lines. H7 flagellin was the key IL-8-inducing component of EHEC O157:H7 OMVs, whereas cytolethal distending toxin V and O157 lipopolysaccharide (LPS) largely contributed to IL-8 production elicited by flagellin-lacking OMVs from SF EHEC O157:H$^-$. The H7 flagellin-mediated signaling via Toll-like receptor (TLR) 5, and O157 LPS-mediated signaling via TLR4/MD-2 complex, which were followed by activation of the nuclear factor NF-κB were major pathways underlying IL-8 production induced by EHEC O157 OMVs. The proinflammatory and immunomodulatory capacities of EHEC O157 OMVs have pathogenetic implications and support the OMVs as suitable vaccine candidates.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) strains of serotypes O157:H7/H$^-$ are major causes of diarrhea-associated hemolytic uremic syndrome (D+ HUS), which affects mostly children under 5 years and is a leading cause of acute renal failure in childhood (Siegler, 2003; Tarr et al., 2005). Classical non-sorbitol-fermenting (NSF) EHEC O157:H7 strains cause D+ HUS worldwide (Karch et al., 2005; Tarr et al., 2005), whereas sorbitol-fermenting (SF) EHEC O157:H$^-$ (non-motile) strains have emerged as causes of D + HUS in Europe (Buvens et al., 2009; King et al., 2014; Marejková et al., 2013; Mellmann et al., 2008; Orth et al., 2009; Pollock et al., 2010; Vygen-Bonnet et al., 2017). Like other pathogens, EHEC O157 secrete outer membrane vesicles (OMVs), nanoparticles composed of outer membrane, periplasmic, and cytoplasmic components (Bielaszewska et al., 2017). EHEC O157 OMVs carry a cocktail of EHEC O157 virulence factors including Shiga toxin 2a (Stx2a), cytolethal distending toxin V (CdtV), EHEC hemolysin (EHEC-Hly), and flagellin (Bielaszewska et al., 2017; Kolling and Matthews, 1999). The OMVs are internalized by human intestinal epithelial and microvascular endothelial cells, which are the major targets during D + HUS, and deliver the virulence factors intracellularly, hereby causing cell death (Bielaszewska et al., 2017).

OMVs produced by various pathogens exhibit immunomodulatory effects which result from the presence of pathogen-associated microbial patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan, flagellin, and outer membrane porins, within OMVs (reviewed in Ellis and Kuehn, 2010; Kaparakis-Liaskos and Ferrero, 2015). Recognition of the OMV-associated PAMPs by the host pattern recognition receptors such as Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain-containing proteins (NOD1 and NOD2) triggers signaling cascades that ultimately lead to the production of proinflammatory cytokines and chemokines, the key players in innate immunity and modulators of adaptive immune responses (Kaparakis-Liaskos and Ferrero, 2015).

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https://doi.org/10.1016/j.ijmm.2018.06.004
Received 30 April 2018; Received in revised form 26 May 2018; Accepted 16 June 2018

Proinflammatory cytokines play multiple roles in the pathogenesis of D+ HUS (Proulx et al., 2001; Zoja et al., 2010). Patients with D + HUS have increased circulating levels of proinflammatory cytokines compared to patients with uncomplicated EHEC diarrhea (Proulx et al., 2001; Westerholt et al., 2000). High levels of interleukin 8 (IL-8) have been identified as a risk factor for developing D+ HUS (Fitzpatrick et al., 1992; Westerholt et al., 2000) and have been associated with high counts of polymorphonuclear leukocytes (Fitzpatrick et al., 1992), a predictor of a poor disease outcome (Walters et al., 1989). Hence, the ability of EHEC to induce proinflammatory cytokine responses, in particular in intestinal epithelial cells (IECs), which are the first cellular targets encountered by the pathogens during infection, may be a critical step in the pathogenesis of D+ HUS. EHEC O157 bacteria, as well as single bacterial components including purified virulence proteins and O157 LPS, are capable of inducing secretion of proinflammatory cytokines from human intestinal epithelial and other cells (Berin et al., 2002; Guessous et al., 2005; Jandhyala et al., 2010; Miyamoto et al., 2006; Thorpe et al., 1999; Zhang et al., 2012). However, the proinflammatory potentials of EHEC O157 OMVs remain unknown. The aim of this study was to investigate the abilities of EHEC O157 OMVs to induce production of proinflammatory cytokines in human IECs using Caco-2, HCT-8, and HT-29 cell lines as models. Furthermore, we identified OMV components involved in these proinflammatory responses and the underlying signaling pathways.

2. Materials and methods

2.1. Isolation and purification of OMVs and OMV doses used in experiments

OMVs were isolated from EHEC O157 strains 5791/99 (NSF O157:H7 from D+ HUS patient), 493/89 (SF O157:H$^-$ from D+ HUS patient), and 493/89Δ*stx*$_{2a}$ (a spontaneous *stx*$_{2a}$-negative derivative of 493/89), and purified with OptiPrep (Sigma-Aldrich, Taufkirchen, Germany) density gradient ultracentrifugation as described previously (Bielaszewska et al., 2017). The total protein concentrations in purified OMVs were determined with Roti-Nanoquant reagent (Carl Roth, Karlsruhe, Germany), concentrations of LPS with LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Bonn, Germany), and concentrations of virulence factors (Stx2a, EHEC-Hly, CdtV, and H7 flagellin) using calibration curves generated from purified proteins (Bielaszewska et al., 2017). The spectra of OMV-associated virulence factors and the OMV concentrations of total proteins, virulence factors, and LPS used in experiments are shown in Table 1. OMVs from strain TA153 (*E. coli* MC1061 harboring *cdt*V operon from strain 493/89 in SuperCos I) and the respective vector control TA154 (*E. coli* MC1061 harboring SuperCos I) were described earlier (Bielaszewska et al., 2017).

2.2. Purified O157 LPS, H7 flagellin, Stx2a and EHEC-Hly

Purified O157 LPS was purchased from BioTrend Chemikalien (Cologne, Germany). H7 flagellin was isolated from strain 5791/99 by the procedure described for H4 flagellin (Kunsmann et al., 2015). Purified Stx2a and EHEC-Hly were prepared as described earlier (Aldick et al., 2007; Bauwens et al., 2011) and biological activities of the preparations were confirmed by Vero cell assay (Bauwens et al., 2011) and a microtiter hemolytic assay (Aldick et al., 2007). Protein concentrations in purified H7 flagellin, Stx2a, and EHEC-Hly were 550 µg/ml, 2.8 mg/ml, and 30.5 µg/ml, respectively.

2.3. Antibodies and inhibitors

Antibody against *E. coli* H7 flagellin (prepared in rabbits) was described previously (Bielaszewska et al., 2017). Anti-TLR4 neutralizing antibody, anti-TLR5 neutralizing antibody, and the respective rat IgG control were from InvivoGen (San Diego, CA, USA). Phospho-NF-κB p65 (Ser536) antibody (rabbit) was from Cell Signaling Technology (Leiden, The Netherlands), and anti-nuclear matrix protein p84 antibody (rabbit) was from Abcam (Cambridge, UK). Alkaline-phosphatase-conjugated goat anti-rabbit IgG was from Dianova (Hamburg, Germany). Polymyxin B was from Sigma-Aldrich, and NF-κB inhibitors SN50 [6-amino-4-(4-phenoxyphenylethylamino) quinazoline] and TPCK (N-α-p-tosyl-l-phenylalanine chloromethyl ketone) were from Calbiochem (San Diego, CA, USA) and Sigma-Aldrich, respectively.

2.4. Cell cultures

Human colonic adenocarcinoma cell lines Caco-2 and HT-29 (ACC 169 and ACC 299, respectively), and human colorectal adenocarcinoma cell line HCT-8 (ATCC CCL-244) were used in passages 24 to 30, 17 to 22, and 10 to 19, respectively. Caco-2 cell line was cultured in Eagle minimal essential medium with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 1% nonessential amino acids. HCT-8 cell line was cultured in RPMI 1640 with 10% FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate, and HT-29 cell line in McCoy's 5 A with 2 mM L-glutamine and 10% FCS. Cell culture media and reagents were from Lonza (Cologne, Germany).

2.5. Cytokine production induced by OMVs

Caco-2, HCT-8, and HT-29 cells were grown until confluence in 96-well plates and incubated for 24 h with purified OMVs from strains 5791/99, 493/89, or 493/89Δ*stx*$_{2a}$ (for OMV concentrations of the total proteins, virulence factors, and LPS see Table 1). Cell culture supernatants were analyzed for the presence of 12 cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17 A, interferon-γ, tumor necrosis factor-α, and granulocyte macrophage colony-stimulating factor) using

Table 1
Origins of OMVs and concentrations of OMV-associated total proteins, virulence factors, and LPS used in experiments.

OMVs from strain	Strain characteristics[a]	Relevant components (ng/ml) in OMVs used for experiments[b]					
		Total protein	Stx2a	CdtV	EHEC-Hly	H7 flagellin	O157 LPS
5791/99	O157:H7 (NSF)	450	51	38	2	73	962
493/89	O157:H$^-$ (SF)	450	51	38	0	0	965
493/89Δ*stx*$_{2a}$	O157:H$^-$ (SF)	450	0	38	0	0	965
TA153	*E. coli* MC1061 harboring *cdt*V-*ABC*$_{493/89}$ in SuperCos I	398	0	38	0	0	0
TA154	*E. coli* MC1061 harboring SuperCos I	365	0	0	0	0	0

[a] NSF, non-sorbitol-fermenting; SF, sorbitol-fermenting; H$^-$, nonmotile.
[b] Stx2a, Shiga toxin 2a; CdtV, cytolethal distending toxin V; EHEC-Hly, EHEC hemolysin; LPS, lipopolysaccharide.