Changes in Gene Expression in Human Epithelial and Mast Cells in Response to Vesicles from *Klebsiella pneumonia* ATCC 13883

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Received: 19 November 2018 / Accepted: 14 December 2018 / Published online: 28 December 2018
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Abstract We investigated alterations in the expression of immune-related genes in epithelial cells and mast cells treated with outer membrane vesicles (OMVs) derived from *Klebsiella pneumoniae* (KpOMVs). Previous studies have shown that OMVs contain substances that enable their delivery to host cells and induce an immune response. Our results indicate an increase in expression of genes such as *IL-8*, *IL-1b*, *MIP-1α*, *HMOX1*, *HSPA1A*, and *IL-24* in epithelial cells and mast cells treated with KpOMVs. The pathogenicity of KpOMVs was confirmed by measuring the changes in the expression of these immune-related genes.

Keywords *Klebsiella pneumoniae* · Immune related genes · Outer membrane vesicles · Pathogen · Toxin

Membrane vesicle trafficking and accumulation are important for intercellular communication [1]. Numerous bacteria, including *K. pneumoniae*, produce and secrete outer membrane vesicles (OMVs) under physiological conditions [2]. Bacterial OMVs regulate intercellular exchanges including cell–cell signaling, DNA and protein transfer, and cell death. OMVs deliver many effector

molecules to host cells [3, 4], and act as vectors for vaccines and facilitate new modes of drug delivery [5].

OMVs contain outer membrane proteins, lipopolysaccharides (LPS), cell–cell signaling molecules, and other virulence factors. Gram-negative bacteria produce OMVs containing virulence factors such as the heat-labile toxin and cytolethal distending toxin [6]. Virulence mechanisms are associated with proteins involved in the acquisition of trace elements, extracellular proteins with lytic functions, and protein complexes that contribute to their secretion. These proteins promote host colonization and saprophytic activities [7]. Toxic elements and different pathogen-related molecular systems in OMVs targeted to host cells cause host cell pathology and regulate host immune reaction.

Klebsiella pneumoniae causes respiratory infections, leading to significant morbidity and mortality. Clinical isolates of *K. pneumoniae* exhibit multi-antimicrobial agent resistance, causing therapeutic complications during clinical treatment [8, 9]. This microorganism produces numerous virulence factors, including capsular polysaccharides [10], LPS [11, 12], siderophores, and adhesins; however, factors cytotoxic to host cells have not been identified. Cytotoxicity of *K. pneumoniae* does not have a direct association with bacterial adherence to epithelial cells [13]. Therefore, we hypothesized that the extracellular virulence complexes defined by Straus [14] may be *K. pneumoniae* OMVs that trigger cytotoxicity in host cells.

OMVs manufactured during bacterial growth are important and closely related to the viability and infections of bacteria in the host [15]. Mechanisms underlying vesicle development and protein targeting to OMVs, and OMV functions remain unclear. OMV production of *K. pneumoniae* ATCC 13883 were examined by liquid chromatography-tandem mass spectrometry (LC–MS/MS). In

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Table 1 PCR primers used for quantitative real-time RT-PCR

Cell	Gene name	Forward primer (5' → 3')	Reverse primer (5' → 3')	Product size (bp)
HMC-1	<i>GAPDH</i>	TGGAAGGACTCATGACCACA	TTCAGCTCAGGGATGACCTT	163
	<i>CYP1A1</i>	TGATAAGCACGTTGCAGGAG	AAGTCAGCTGGGTTTCCAGA	199
	<i>SCG2</i>	AATCAAGGCAGCTCTCAGGA	CTGCCTTTTCTTGTTGAGG	154
	<i>IL24</i>	CAACTGCAACCCAGTCAAGA	TCTGCATCCAGGTCAGAAGA	157
	<i>SC4MOL</i>	GATGCTTTGGTTGTGCAGTC	CCAAATGGAGCCTGAAACTC	121
	<i>IL1B</i>	GGGCCTCAAGGAAAAGAATC	TTCTGCTTGAGAGGTGCTGA	205
	<i>HMGCS1</i>	GTGGCAGAAAGAGGGAAAATG	AAAGGCTTCCAGGCCACTAT	175
	<i>PNCK</i>	TGAGGGCCAGCTATGAGTTT	TTCTTCCGGATCTGCTCACT	199
	<i>HBZ</i>	GAGGACCATCATTGTGTCCA	GATGTCGTCGATTTCGCTTCA	211
	<i>CAPN5</i>	CTCATCAGTGCCTCCATCAA	GCGGATCATGTCCAACCTTCT	165
	<i>ALDH5A1</i>	CAGCTGATGTTGGGTTAGCA	AAGCCCCCGTAACACACATA	223
	<i>IL8</i>	GTGCAGTTTTGCCAAGGAGT	CTCTGCACCCAGTTTTCTT	196
HEp-2	<i>EGR1</i>	TGACCGCAGAGTCTTTTCTT	TGGGTTGGTCATGCTCACTA	203
	<i>IL8</i>	GTGCAGTTTTGCCAAGGAGT	CTCTGCACCCAGTTTTCTT	196
	<i>DUSP5</i>	GGAGGCCTTCGATTACATCA	AGGGCTCAGTGTCTGCAAAT	181
	<i>CTGF</i>	TACCAATGACAACGCCTCCT	ATGTCTTCATGCTGGTGACAG	173
	<i>IER3</i>	CCAGCCAAAAGGCTTCTCTT	CAGATTAAAGGGCTCGAGGA	159
	<i>DNAJB1</i>	AAGAAGCAAGATCCCCCAGT	GCTGGAATGTTGTTGGAGGT	227
	<i>HMOX1</i>	CTTCTTCACCTTCCCCAACA	AGCTCCTGCAACTCCTCAAA	165
	<i>PTGS2</i>	TGAGCATCTACGGTTTGCTG	TGCTTGTCTGGAACAACCTGC	158
	<i>HSPA1A</i>	CGACCTGAACAAGAGCATCA	AAGATCTGCGTCTGCTTGGT	213
	<i>CDH15</i>	CAGATCAAGTCGGACAAGCA	GCAAACGCTCTTAGCCTGAA	173
	<i>DNAJB9</i>	ATGGCTACTCCCCAGTCAAT	GTGGTACTTCATGGCCAACT	162
	<i>GMPPB</i>	GAAGGAAATGAAGGCACAGG	ACGTCACTGTTGAGGACGAA	150
	<i>HYOU1</i>	TCCAAAGGCTACGCTACGTT	AGAACCATGCCCAACACTTC	186
	<i>ISG20</i>	AGATCCTGCAGCTCCTGAAA	TGTTCTGGATGCTCTTGTGC	207
	<i>PDIA4</i>	CAAGCGTTCTCTCCAATTC	CGGACTGCTCGATCATGTAA	179
	<i>SDF2L1</i>	ACCTCTGTGTTCTGTCAAGTCA	GCTTGATGAAGATGCCTTCC	130
	<i>SYVN1</i>	CCAACATCTCCTGGCTCTTT	CAGGATGGCATACTCAAAGC	152

the same way we identified 159 proteins associated with OMV [16]. The intracellular location of the identified proteins was investigated, showing that KpOMV was enriched in the outer membrane protein but lacked the inner membrane protein as previously described [2, 3].

Klebsiella pneumoniae ATCC 13883, HEp-2 cells, and HMC-1 cells were cultured [16]. KpOMVs were extracted from bacterial culture media supernatants according to a previous report [16]. HEp-2 and HMC-1 cells were seeded at 1.5×10^5 cells in each 6-well cell culture dishes and incubated at 37 °C, 5% CO₂ for 24 h. The cells were incubated with 20 µg/ml KpOMVs for 24 h, and subsequently washed thrice with phosphate-buffered saline. Total RNA was purified using Trizol (Invitrogen Life Technologies, Carlsbad, USA) and an RNeasy column (Qiagen, Valencia, USA), respectively, according to the protocol. Following DNase I digestion, RNA purity was

evaluated by determining the OD_{260/280} ratio (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, USA) and kept at – 80 °C until the next experiment.

The amplification of the total RNA was carried out using Ambion Illumina RNA Amplification Kit (Ambion, Austin, TX, USA) and purified to obtain biotinylated cRNA. Following purification of cRNA, concentration of samples was measured with NanoDrop (ND-1000 spectrophotometer; Wilmington, DE, USA).

The cRNA samples labeled with the human 8-expression bead array (Illumina, Inc., San Diego, CA, USA) were hybridized at 58 °C for 16–18 h. The array signals were captured by Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK). Array data was extracted, processed, and analyzed using Illumina GenomeStudio v2009.2 (Gene Expression Module v1.5.4).

Fig. 1 Chip-based gene expression analysis. Heat map analysis of genes present on both chips. Genes in HEP-2 cells (a) and HMC-1 cells (b) whose expression increased or reduced upon exposure of OMVs are shown

Hybridization quality and general chip performance were checked by visual examination of raw scanned data and internal quality control checks. The raw data was obtained by Illumina GenomeStudio v2009.2. Array data were selected based on a p value < 0.05 in at least 50% samples. Selected values were log-transformed and standardized by the quantile method. Fold-changes in expression were used to compare control and test samples.

K-means analysis and hierarchical clustering were performed to measure similarity visualization of discriminately expressed genes was performed by the R statistical language v. 2.6.1. Biological ontology-based gene expression was conducted by the Panther database (<http://www.pantherdb.org>).

HEP-2 and HMC-1 cells were plated in a cell culture dish of 6 well at a density of 5×10^5 cells each well and treated with 1 μ g KpOMVs for 24 h. Total RNA was isolated using Trizol (Invitrogen Life Technologies), and real-

time PCR was carried out with an ABI PRISM 7500 cycler (Toyobo, Japan), using PCR conditions as per cycler commands with an annealing temperature of 60 °C. The Oligonucleotides were fabricated by Primer 5.0 and synthesized by Invitrogen (Table 1). The amount of cDNA was quantified by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. In all samples, we analyzed the expression of genes of interest based on the proportion of their expression to that of GAPDH using the formula: relative expression = $2^{-(\Delta C_t \text{ sample} - \Delta C_t \text{ control})}$, with $\Delta C_t = (C_t \text{ gene}) - (C_t \text{ GAPDH})$. All experiments were repeated three times to increase accuracy.

Gene expression, determined via the fluorescence signal on a DNA chip, revealed that expression of 20 HEP-2 and 38 HMC-1 genes increased following KpOMV exposure (Fig. 1a, b). Gene expression analysis via qRT-PCR in HEP-2 and HMC-1 cells treated with KpOMVs revealed increased expression of several genes. The expression level

Fig. 2 Analysis of gene expression by real-time PCR (RT-PCR). RT-PCR based quantification of changes in the expression of 17 selected genes from HEp-2 cells (a) and HMC-1 cells (b). Data are expressed as fold-changes with respect to *GAPDH* expression

of proinflammatory cytokine genes, *IL-8*, *IL-1 β* , and *MIP-1 α* , in HEp-2 exposed to KpOMVs was previously demonstrated [17]. Changes in expression of other immune response-related genes were detected. Upon OMV exposure, the expression of *IL-8*, *CTGF*, *DNAJB1*, *HMOX1*, and *HSPA1A* was increased in HEp-2 (Fig. 2a), while the expression of *CYP1A1*, *SC4MOL*, *IL-24*, *IL-1B* and *ALDH5A1* was increased in HMC-1 (Fig. 2b).

Immune response is initiated by the interaction among OMV (produced by bacterial colonization or infection), neighboring epithelial cells, and immune cells. The host immune response is synergistically regulated by the OMV component. Of the OMVs, LPS is the most abundant host immunostimulatory component [18]. We confirmed the outer membrane proteins and the porins, including OmpA, OmpC, and OmpX, and predicted that they were associated with immune response and leukocyte chemotaxis induction [16]. LPS uptake by macrophages is enhanced by OmpA, and microvascular endothelial cell involvement by *E. coli* is caused by OmpA. OMVs also contain immune-activating pathogen-associated molecular patterns, including outer membrane proteins, peptidoglycans, and flagellins [19, 20]. These interact with host cells and promote inflammatory responses to gram-negative pathogens [3].

We also confirmed increased expression of immune-related genes, *IL-8*, *IL-1 β* , *MIP-1 α* , *HMOX1*, *HSPA1A*, and *IL-24*, in human cell lines exposed to OMVs. *IL-8* gene expression, which was high in HEp-2 (Fig. 2a), induces phagocytosis by neutrophils and other granulocytes by

activating the neutrophil chemotactic factor. *HMOX1* up-regulates *IL-10* and *IL-1RA* expression to induce anti-inflammatory immune response [21]. *IL-24*, a member of the *IL-10* family of anti-inflammatory cytokines, exerts pleiotropic effects on immune regulation and inflammation [22]. Thus, KpOMVs induce the expression of several genes in host cells and contribute to bacterial infection in host cells.

We found many potential folliculate proteins that play a role in the elimination of virulent elements, the invasion of phages, the removal of competing organisms, the transfer of genetic substance and proteins to other bacteria, the targeting of host cells, and the regulation of host immune responses. The genetics and pathogenesis of *K. pneumoniae* have been well established. The current study provides a holistic view of gram-negative bacterial OMVs and mechanisms underlying their biogenesis and activities in multi-drug resistant pathogenic bacteria. These findings may facilitate development of efficient vaccines and improve our understanding of infection mechanisms used by pathogenic bacteria, including *K. pneumoniae*, *N. meningitides*, and *P. aeruginosa*.

Acknowledgements This research was supported by the Basic Science Research Program, through the National Research Foundation of Korea (NRF), funded by the Ministry of Science ICT & Future Planning (NRF-2014R1A1A1002349).

Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest (commercial or otherwise) to declare regarding this study.

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