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Structural modifications of outer membrane vesicles to refine them as vaccine delivery vehicles

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ABSTRACT

In an effort to devise a safer and more effective vaccine delivery system, outer membrane vesicles (OMVs) were engineered to have properties of intrinsically low endotoxicity sufficient for the delivery of foreign antigens. Our strategy involved mutational inactivation of the MsbB (LpxM) lipid A acyltransferase to generate OMVs of reduced endotoxicity from *Escherichia coli* (*E. coli*) O157:H7. The chromosomal tagging of a foreign FLAG epitope within an OmpA-fused protein was exploited to localize the FLAG epitope in the OMVs produced by the *E. coli* mutant having the defined *msbB* and the *ompA*::FLAG mutations. It was confirmed that the desired fusion protein (OmpA::FLAG) was expressed and destined to the outer membrane (OM) of the *E. coli* mutant from which the OMVs carrying OmpA::FLAG are released during growth. A luminal localization of the FLAG epitope within the OMVs was inferred from its differential immunoprecipitation and resistance to proteolytic degradation. Thus, by using genetic engineering-based approaches, the native OMVs were modified to have both intrinsically low endotoxicity and a foreign epitope tag to establish a platform technology for development of multifunctional vaccine delivery vehicles.

1. Introduction

OMVs are spherical lipid bilayer vesicles that are extruded naturally from the OM of Gram-negative bacteria [1]. The size of the membrane vesicles ranges from ~20–200 nm in diameter, and they are composed of characteristically antigenic OM constituents and some periplasmic contents, such as alkaline phosphatase and proteases, and even DNA and virulence factors [1,2]. Interestingly, a proteomic study showed that a number of cytoplasmic proteins also constituted the proteome of *E. coli* OMVs [3]. Thus, OMVs are nanosphere membrane vesicles having intrinsic properties to be a vaccine carrier possessing the native bacterial antigens, as illustrated in Fig. 1. Currently, a new paradigm for vaccine development has been emerging with non-replicating (acellular) vaccine delivery systems characterized by usage of nano/micro-particles, such as virus-like particles [4], liposomal vesicles such as proteoliposomes [5], archaeosomes prepared from the polar membrane lipids of *Archaea* [6], and virosomes composed of some viral envelope lipids [7]. In this respect, OMVs have notable advantages in vaccine development over other lipidic nanoparticles. The native OMVs have a multi-immunogenic capacity to carry a wide spectrum of endogenous antigens, in addition to the natural self-adjuvanticity that is exerted by toll-like receptor (TLR) agonists, such as outer membrane proteins (OMPs), lipoproteins, and lipopolysaccharides (LPS). Beyond that, OMVs are generally superior in enhancing phagocytic uptake, as their surface molecules can be opsonized and/or recognized by the humoral immunity components. Furthermore, OMVs can also be modified by the inclusion of exogenous (synthetic) peptides and specific adjuvants, which will provide the possibility of utilizing the reconstituted OMV as anti-viral or anti-tumor therapeutic vaccines. Incorporation of additional functionality, such as fluorescent dye conjugation, to the surface components of OMVs could make them a more attractive biomaterial for vaccine delivery studies *in vivo*.

In order to use these OMVs as vaccine delivery vehicles, safety issue has to be addressed because native OMVs are composed of fully endotoxic LPS, which provokes excessive secretion of proinflammatory cytokines in humans and animals [8]. In efforts to refine the OMV vector system to be applicable for vaccination, we employed mutational inactivation of the *msbB* gene encoding an acyltransferase catalyzing the final myristoylation step during lipid A biosynthesis [9,10]. It is known that the penta-acylated LPS produced from the *msbB* mutants shows reduced endotoxicity in human cells, compared with normal hexa-acyl lipid A producers [11,12]. In this regard, we hoped to exploit OMVs of

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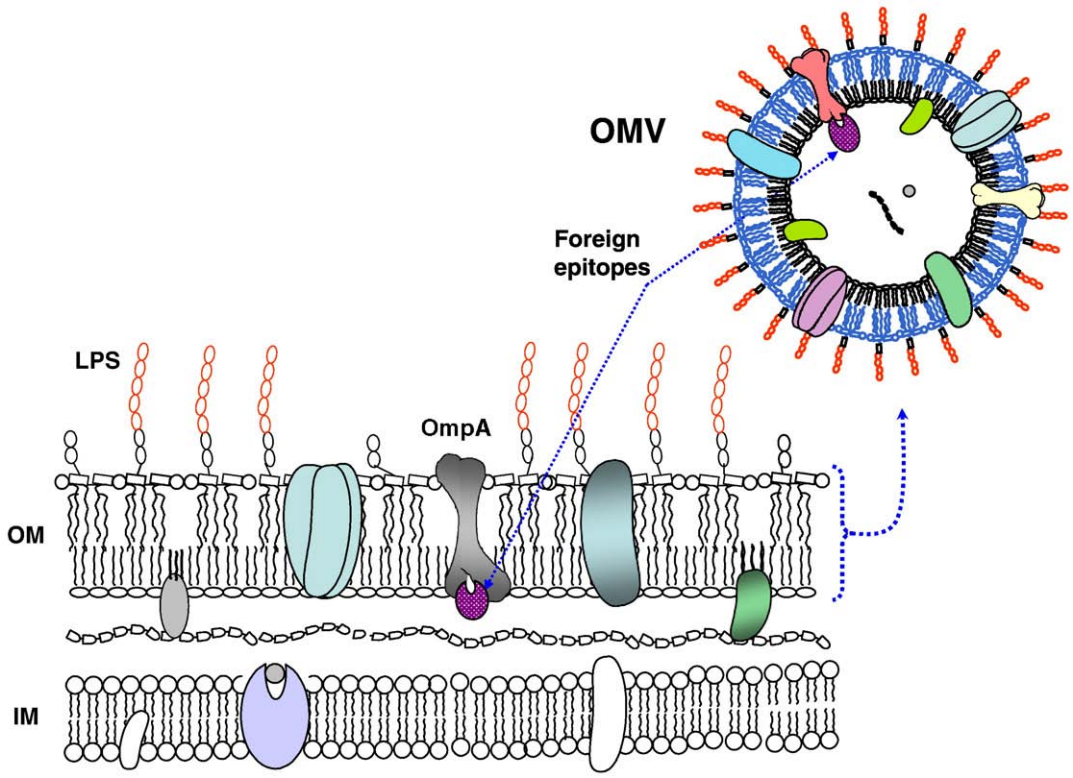

Fig. 1. Proposed model for OMV production from genetically engineered *E. coli* O157:H7. The inactivation of MsbB activity and foreign epitope fusion to the OM-integral β-barrel domain of OmpA can generate OMVs composed of mainly penta-acylated LPS and the C-terminally truncated OmpA tagged with a foreign epitope, as illustrated.

low endotoxicity from an *E. coli* O157:H7 *msbB* mutant to establish their potential for vaccination.

We reasoned that incorporation of a foreign epitope into the OMV could be achieved by chromosomal tagging with DNA constructs containing the foreign epitope DNA conjoined with homologous DNA arms to be fused with *ompA* gene; the foreign epitope expressed with OmpA in fusion should be targeted to the OM to result in the spontaneous inclusion within the OMVs. If the foreign epitope in OMVs that have reduced endotoxicity could be generated in the *msbB* background harboring mainly penta-acylated LPS molecules, then these OMVs would be desirable vaccine delivery vehicles because the foreign epitope is physically linked to TLR agonistic adjuvants.

OmpA is one of the most abundant OM proteins in typical enteric Gram-negative bacteria such as *E. coli* and *Salmonella* [13]. In addition to its abundance, the well-characterized two-domain structure of OmpA is advantageous to be fused with any sort of soluble foreign proteins. The crystal structure of OmpA showed that the N-terminal 171 residues adopt the β-barrel domain, which traverses the OM with eight anti-parallel β-sheet segments, exposing four external loops on the bacterial surface [14]. The remaining C-terminal portion of OmpA protein was shown to reside in the periplasm [14]. A truncated OmpA containing the N-terminal β-barrel domain but lacking as much as 132 amino acids from the periplasmic C-terminal domain was conferring the truncated OmpA insertion and the OM stability [15].

Accordingly, by construction of an OmpA fusion with the FLAG epitope as a model of the foreign epitope tagging in the OMV of the *msbB* mutant of *E. coli* O157:H7, we successfully modified OMVs intrinsically to be utilized as multifunctional vaccine delivery vehicles.

2. Materials and methods

2.1. Creation of msbB mutants in E. coli O157:H7

E. coli O157:H7 strain Sakai [16] was used as parental strain for creation of gene-specific mutations by using one-step PCR mutagenesis method [17]. Accordingly, the parental *E. coli* stain was transformed with pKD46, a temperature-sensitive plasmid carrying the Red recombinase system from the λ bacteriophage under the control of the arabinose-inducible pBAD promoter. The Red recombinase system mediates the replacement of the target chromosomal sequence with an antibiotic resistance cassette obtained by PCR amplification using primers carrying homologies to the vicinity on the gene targeted for disruption. The O157 *E. coli* carrying pKD46 was transformed by electroporation with the PCR product generated from a PCR with the template plasmid pKD3 as described previously [17]. Transformants were plated in Luria–Bertani (LB) agar, containing the appropriate antibiotics (chloramphenicol (Cm) 10 μg/ml; ampicillin (Amp) 100 μg/ml), and mutants were confirmed by PCR and DNA sequence analysis.

As previously reported, *E. coli* O157:H7 strains possess two homologous *msbB* genes: a chromosomal *msbB1* and a plasmid-encoded *msbB2* gene [18]. In order to inactivate MsbB activity, the two *msbB* genes have to be disrupted at the same time. The sequential double *msbB* mutations have been achieved in *E. coli* O157:H7 by curing of pKD46 and introduction of pCP20, leading to site-specific excision of the integrated Cm cassette in either single *msbB* mutant (Δ*msbB1* or Δ*msbB2*). Thus, the other *msbB* gene targeting followed up with each single *msbB* mutant, resulting in two separate mutants for the *msbB1*/*msbB2* genes in the *E. coli* O157:H7. The resulting two mutants were designated Sakai-DM1 for the *msbB1*/*msbB2* sequence and Sakai-DM2 for the *msbB2*/*msbB1* sequence. Since there was no phenotypic variation between these two Sakai-DM mutants, Sakai-DM1 renamed as Sakai-DM (Supplementary Table 1) was chosen for further OMV production studies.

2.2. Construction of Sakai-DM mutants carrying the ompA::FLAG and pagP::FLAG fusions

As illustrated in Fig. 2A, intact *ompA* gene was cloned into pBlueScript-II SK vector to construct a template plasmid for the *ompA*::FLAG gene fusion assisted by the pKD46-encoding λ-Red recombinase. At first, a PCR amplicon called mega-primer DNA was