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Production and purification of virus-like particles of different enterovirus subtypes as vaccines

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ABSTRACT

Enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16), both belonging to enterovirus species A, are the major etiological agents for hand-foot-and-mouth-disease that causes severe neurological complications and death in young children. Since prevalent EV-A71 subgenotypes change, developing bivalent vaccines composed of the epidemic EV-A71 subgenotype and CV-A16 is desirable. Virus-like particles (VLPs) of enteroviruses can be produced by co-expressing P1 polyprotein and 3CD protease and are promising vaccine platforms, but VLP production and purification issues are rarely addressed. To produce VLPs of different enterovirus subtypes as vaccines, we exploited the *flash*BAC GOLD™ system to generate 4 baculoviruses expressing P1 and 3CD derived from different prevalent enterovirus subtypes (EV-A71 C2, C4 and B5 and CV-A16). By fine-tuning the strategies to infect High Five™ cells, we achieved high level production of VLPs derived from EV-A71 C2, C4, B5 and CV-A16, reaching 195, 85, 157 and 173 mg/L, respectively. We further purified and characterized these 4 subtypes of VLPs. Immunization of mice with VLPs of subtypes that were predominantly prevalent over the last decade (C4 and CV-A16), alone or in combination, successfully induced humoral and cellular immune responses. These data demonstrated the potential of such baculovirus/insect cell platform for timely, swift and mass production of VLP vaccines against epidemic enterovirus strains.

1. Introduction

Hand-foot-and-mouth-disease (HFMD) generally results in mild and self-limiting illness but may cause severe neurological complications and even death in infants and young children under 5 years of age. HFMD outbreaks have occurred in Asian Pacific countries (*e.g.* China, Taiwan and Vietnam) over the past 2 decades [1], and HFMD epidemic is particularly serious in China, where > 10 million HFMD cases and 3046 fatalities are reported from 2008 to June 2014 [2]. More than 90% of HFMD arises from the infection of enterovirus A, among which enterovirus A71 (EV-A71) and coxsackievirus A16 (CV-A16) are the major etiological agents [2,3]. EV-A71 infection accounts for 70% of severe HFMD cases and 90% of HFMD-related deaths [2]. Conversely, CV-A16 infection also causes severe cases and death [1] and co-infection of CV-A16 and EV-A71 provokes serious complications and enhances the chance of genetic recombination [4], thus entailing the need to develop EV-A71 and CV-A16 vaccines.

EV-A71 are primarily classified into 11 subgenotypes (A, B1∼B5, C1∼C5). Currently, 3 inactivated virus vaccines against the most prevalent EV-A71 subgenotype (C4) have been issued new drug certificates and production licenses in China [4]. These approved vaccines induce broad cross-neutralization activity against other prevalent EV-A71 genotypes such as C2, C5, B4 and B5 [5–7], but do not provide sufficient protection against different enterovirus species such as EV-A16 [1,5,6,8,9]. Meanwhile, inactivated CV-A16 virus vaccine induces immune responses and confers protection against CV-A16 virus infection [10,11]. However, the production yield of EV-A71 [12] and CV-A16 [13,14] vaccines are low.

Enteroviruses have an RNA genome that can be translated into a polyprotein, and the viral P1 polyprotein is cleaved by 3CD protease into individual structural proteins. By mimicking the natural process, we developed virus-like particle (VLP) vaccines by constructing recombinant baculoviruses to co-express P1 and 3CD

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derived from EV-A71 C2 genotype [15]. In infected insect (Sf-9) cells, the expressed P1 polyprotein is cleaved by 3CD into structural proteins (VP1, VP3 and VP0) and spontaneously assemble into VLP. The EV-A71 VLP is fairly stable [16] and capable of inducing long-lasting protective immune responses in mice [17–19] and monkeys [20], hence rendering VLPs a promising HFMD vaccine platform [21]. Such P1/3CD co-expression strategy has been adopted to produce VLPs of EV-A71 C4 genotype or CV-A16 using the baculovirus/insect cell [11,22–27], *Sacharyomyces cerevisiae* [28–30] and *Pichia pastoris* [31,32] expression systems.

Despite the promise of VLPs as EV-A71 and CV-A16 vaccines, the product yield issues are rarely addressed. Furthermore, although current EV-A71 vaccines elicit antibodies that cross-neutralize heterotypic subgenotypes [5,6,18,19], the molecular epidemiology of EV-A71 changes by spontaneous genomic mutations and recombination between circulating EV-A71 subgenotypes. As such, selection of only one subgenotype may not be sufficient to confer protection against other EV-A71 subgenotypes [33]. Therefore, development of bivalent or polyvalent vaccines composed of EV-A71 subgenotypes homologous to the circulating EV-A71 strain(s) and CV-A16 is desired, and capability of producing VLPs of different EV-A71 subgenotypes and CV-A16 at high levels is crucial.

In this study, we aimed to produce and purify enterovirus VLPs of 4 different subtypes, including EV-A71 subgenotypes (C2, C4 and B5) and CV-A16. For high level production, we adopted a new strategy by constructing recombinant baculoviruses using a new *flash*BAC^{TM} vector to express P1 and 3CD under the control of polyhedrin and CMV promoter, respectively [34]. We also modified the infection schemes to elevate the production yield of C4 and CV-A16 VLPs and further purified these 4 subtypes of VLPs. Immunization of mice with VLPs of subtypes that were highly prevalent over the last decade (C4 and CV-A16), alone or in combination, successfully induced humoral and cellular immune responses. These data demonstrated the potential of such baculovirus/insect cell platform for timely, swift and mass production of VLP vaccines derived from circulating enterovirus strains.

2. Materials and methods

2.1. Cell culture and preparation of recombinant baculoviruses

For baculovirus production, Sf-9 cells were cultured in TNM-FH medium (Sigma) containing 10% fetal bovine serum (FBS) at 27°C. For VLP production, High Five^{TM} cells (Invitrogen) were cultured in shaker flasks at 27°C in serum-free Sf-900 II medium (Invitrogen).

Recombinant baculovirus BacF-P1-C3CD co-expressing P1 and 3CD derived from EV-A71 C2 genotype was constructed previously using *flash*BAC GOLD^{TM} (Oxford Expression Technologies, UK) system [34]. Other recombinant baculoviruses co-expressing P1 and 3CD, under the control of the *polh* promoter (P_{polh}) and CMV promoter (P_{CMV}), respectively, were generated in a similar fashion using pBacPAK8-polh/CMV as the backbone plasmid. To generate pBacPAK8-polh/CMV, the *Kpn*I, *Sac*I, *Eco*RI, *Sma*I and *Not*I sites of pBacPAK8 plasmid (Clontech) were removed by *Kpn*I/*Not*I digestion, followed by Klenow fragment fill-in and DNA ligation to generate pBacPAK8-ΔKSESN plasmid. pFastBAC DUAL (Invitrogen) was amplified by inverse PCR and ligated to generate a plasmid pFastBAC-Δpolh/p10 without P_{polh} and P_{CMV}. The fragment encoding the multiple cloning sites and poly A regions of pFastBAC-Δpolh/p10 was digested with *Sna*BI and *Avr*II and cloned into pBacPAK8-ΔKSESN by *EcoR*V/*Xba*I to yield pBacPAK8-Δpolh/p10 plasmid. Finally, a DNA fragment encoding the P_{polh} and P_{CMV} was PCR-amplified from pBac-P1-C3CD plasmid [18] and cloned into pBacPAK8-Δpolh/p10 plasmid by *Xho*I/*Stu*I to generate pBacPAK8-polh/CMV backbone.

The EV-A71 C2 virus was described previously [15]. The EV-A71 C4, B5 and CV-A16 viruses used in this study were provided by Taiwan Center for Disease Control (Table 1). The authentic enteroviruses were propagated in RD cells [19], and the viral RNAs were reverse transcribed into cDNAs. The P1 gene of each subtype was amplified by PCR with *Mss*I at the 5′ end and *Not*I at the 3′ end. The 3CD gene of each subtype was PCR-amplified with flanking *Avr*II/*Nru*I sites. The P1 and 3CD genes of the same subtype (EV-A71 C4, B5 or CV-A16) were sequentially cloned into pBacPAK8-polh/CMV by *Mss*I/*Not*I and *Avr*II/*Nru*I treatment, respectively, to generate transfer plasmids pBacPAK8-C4-P1-C3CD, pBacPAK8-B5-P1-C3CD or pBacPAK8-CVA16-P1-C3CD. The transfer plasmid was co-transfected with *flash*BAC GOLD^{TM} DNA into Sf-9 cells to generate recombinant baculoviruses BacF-C4-P1-C3CD, BacF-B5-P1-C3CD or BacF-CVA16-P1-C3CD for the production of VLPs derived from EV-A71 C4, B5 and CV-A16 subtypes. These viruses were propagated to passage 2 by infecting Sf-9 cells and titrated by end-point dilution [35].

2.2. Single clonal baculovirus selection by limiting dilution

To select baculovirus clones, limiting dilution method was used [36]. Briefly, the parental baculovirus was 10-fold serially diluted to 10^{-9}. Sf-9 cells were seeded into 96-well plates (1×10^4 cells/well, 100 µl) and infected by the serially diluted virus (10^{-6} to 10^{-9} dilution, 100 µl/well, two plates per dilution). After incubation at 27°C for 10 days, the number of wells with cytopathic effect (CPE) was recorded. When < 10% of wells in the 2 plates were infected by a virus dilution, the wells were regarded as infected by single clonal baculovirus. The infected culture supernatants in these wells were harvested individually, propagated and tittered by infecting Sf-9 cells as described above.

2.3. VLP production, purification and characterization

VLPs were produced in 1 liter shaker flasks (working volume = 250 ml) by infecting High Five^{TM} at 4×10^6 cells/ml and MOI 3, or at 2×10^6 cells/ml and MOI 0.1. Extracellular VLPs in supernatants were harvested at 6 days post-infection (dpi) by centrifugation (12,000 × g for 30 min) and purified by a two-column chromatographic process [37] using the fast flow liquid chromatography (FPLC) with minor modification. Briefly, the supernatant was concentrated by tangential flow filtration (TFF) with a 1000 kDa cut-off membrane (Sartorius) and loaded into a hydroxyapatite chromatography (CHT^{TM} Ceramic Hydroxyapatite, Type I, 40 μm, Bio-Rad). The VLP-containing flowthrough was collected and concentrated again by TFF with a 300 kDa cut-off membrane (Sartorius). The VLP was purified again by size exclusion chromatography (Sephacryl^{TM} S-400 HR, GE Healthcare, UK). The VLP-containing fractions were collected, concentrated and buffer-exchanged by Spin-X® UF concentrator (100 K MWCO, Corning) with 100 mM sodium phosphate (NaPi) buffer (pH 6.5). The purified VLP samples were aliquoted and stored at −80°C until analysis. The total protein concentration of purified VLP samples was quantified by Coomassie Plus (Bradford) Assay Kit (Thermo Scientific). The presence of VP0, VP1, and VP3 in the VLP was detected by SDS-PAGE and the purity was calculated by densitometry. The concentration of purified VLP samples was defined as the total protein concentration × the VLP purity. The VLP morphology was observed by transmission electron microscopy (TEM) as described [16].

2.4. Western blot

Western blot analysis of VLP was performed as described [37]. The primary antibodies were rabbit anti-VP1 polyclonal antibody for VP1 detection, rabbit anti-VP3 polyclonal antibody for VP3

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