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Characterization and evaluation of the immune responses elicited by a novel human papillomavirus (HPV) therapeutic vaccine: HPV 16E7-HBcAg-Hsp65 fusion protein

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A B S T R A C T

Human papillomaviruses (HPV), particularly HPV16, are associated with most cervical cancers. Currently, although prophylactic vaccines have been developed, there is still an urgent need to develop therapeutic HPV vaccines. In this study, a novel fusion protein, HPV 16 E7-HBcAg-Hsp65 (VR_{111}), with the goal of increasing anti-HPV16 cellular immunity was developed. VR_{111} was analyzed using SDS-PAGE, western-blotting, capillary isoelectric focusing (cIEF), analytical ultracentrifugation (AUC) and dynamic light scattering (DLS). Gamma interferon (IFN-γ) secretion assay was performed by enzyme-linked immunospot (ELISPOT) and ELISA to test their ability to induce cellular immune response. Significant correlation between ELISPOT and ELISA was observed ($r = 0.8680$, $p < 0.0001$). It was shown that VR_{111} could induce a significant increase in E7-specific $CD8^+$ T cell responses. Humoral immune response was also observed. The antibody titer levels were measured by ELISA. These results indicated that VR_{111} was a promising therapeutic vaccine for treatment of cervical cancer with possible therapeutic potential in clinical settings.

1. Introduction

Cervical cancer is the second most common cause of women cancer mortality in the world (Tomatis, 1990). Human papillomaviruses (HPV), particularly HPV-16, are associated with most cervical cancers (Bosch et al., 1995) and head and neck cancers. Studies have shown that HPV-16 is the predominant subtype of both cancers, accounting for 46–63% of cervical cancer cases and 90% of head and neck cancer cases (Gillison et al., 2000; Muñoz et al., 2004; Yan et al., 2009). A prophylactic HPV vaccine strategy would be the most effective in adolescents and young adults (Koutsky, 1997; Grunbaum et al., 2004; Agosti and Goldie, 2007). Recently, an HPV quadrivalent (types 6, 11, 16 and 18) vaccine was approved for immunization of girls and young women (9–26 years) and a bivalent (type 16, 18) vaccine was approved for use in females (10–45 years) (Gardasil, 2006; Cervarix, 2007).

Therapeutic vaccines are based on the induction of cellular immunity directed against cells presenting viral antigens. The goal of therapeutic vaccines is the clearance of infected cells and the rejection of HPV-associated lesions and tumors. So far, E6 and E7 seem to be the most promising target molecules for therapeutic vaccines because they are constitutively expressed in cervical cancer cells (Meneguzzi et al., 1991; zur Hausen, 2002). Several therapeutic vaccines against HPVs are currently being developed. Many studies have provided evidence that heat shock protein 65 (Hsp65) can elicit potent specific cellular adaptive immune responses cytotoxic T-cell effectors (Cho et al., 2000; Weigel et al., 2002; Daniel et al., 2005; Li et al., 2006). Hsp65 is thus an effective carrier for heterologous peptide epitopes for therapeutic vaccines against cancer or chronic infectious diseases. Pre-clinical data demonstrated the ability of Hsp fusion proteins to induce antigen-specific cytotoxic T lymphocytes, Type 1 cytokines and anti-tumor immunity. HspE7, a fusion of full length E7 antigen from HPV 16 onto full length Hsp65, is in clinical development for treatment of diseases caused by HPV (Chu et al., 2000; Liu et al., 2007). The core antigen of hepatitis B virus (HBcAg) is extremely immunogenic, and functions as both a T-cell-dependent and a T-cell-independent antigen. Therefore, HBcAg is a promising candidate target for therapeutic vaccine (Yang et al., 2007; Wang et al., 2011).

In this study, a novel fusion protein, HPV 16 E7-HBcAg-Hsp65 (VR_{111}), was developed by fusing HPV 16 E7 and HBcAg sequence to Hsp65 sequence in *E. coli* expression system. VR_{111} was then characterized and its immune responses were assessed by ELISPOT assay and ELISA.

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2. Materials and methods

2.1. Expression and purification of VR_{111} in E. coli

VR_{111} was expressed by IPTG induction at high levels in *E. coli* SE1 expression system under the control of the T7 promoter. VR_{111} seed culture fluid was grown with shaking at 30 °C over night in LB medium. A series of ideal fermentation conditions was selected to use in a 30 l fermentor and the expression of fermentation attained 1 g/l. The supernatants before and after induction were analyzed using SDS-PAGE and western blotting.

VR_{111} was purified to >95% with multiple-step purification process. VR_{111} bulk contained 0.8 mg/ml fusion protein in 50 mmol/l phosphate buffer (pH7.5). Each 0.5 ml dose of VR_{111} product contained 240 μg fusion protein and 450 μg of aluminum adjuvant.

2.2. cIEF assay

The capillary isoelectric focusing (cIEF) profiles were generated on a Convergent BioScience iCE280 Analyzer (Convergent Bioscience, Toronto, Canada) using a FC-coated cIEF cartridge. The sample contained 0.1 mg/ml VR_{111} bulk, 8 M urea, 4% pH 3–10 Pharmalyte, 1% methylcellulose and 0.05% *pI* marker standards (*pI* markers 4.65 and 6.61). Focusing was performed in two stages: initial focusing was performed for 1 min at 1500 V and final focusing was completed after 2 min at 3000 V with detection at 280 nm (Zhou, 2013).

2.3. Analytical ultracentrifugation (AUC) and dynamic light scattering (DLS)

The sedimentation velocity analysis was performed using an Optima XL-I system (Beckman Instruments, PaloAlto, CA) with optical absorbance detection at 280 nm. For the constant speed method, VR_{111} bulk of 380 μl was placed in the sample compartment of a double-sector centerpiece (400 μl of buffer was placed in the reference compartment) and centrifuged at 10,000 rpm for at least 3 h at 20 °C. The data were analyzed to calculate the sedimentation velocity by DCDT+ software.

DLS analysis provided information on the average size, sizedistribution and polydispersity of VR_{111}. Light scattering measured the diffusion of molecules in solutions. DLS results were collected in 1.0 ml samples in DTS0012 cell using a Zetasizer Nano system (Malvern Instruments, Malvern, U.K.). Measurements were collected at 25 °C in the automatic mode.

2.4. Measurement of IFN-γ by ELISPOT assay and ELISA

2.4.1. Immunization of C57BL/6 mice with VR_{111}

Experiments with animals were performed in accordance with the guidelines of the institutional committee for the care and use of experimental animals. Female 6 to 8-week-old C57 BL/6 mice were purchased from Shanghai Laboratory Animal Research Center, China. All mice were maintained under specific-pathogen-free conditions and were immunized at 6–8 weeks of age. Seven groups of 8 mice each were vaccinated by intraperitoneal inoculation two times on day 0 and day 14 with 30, 10, 3.3, 1.1, 0.37, 0.12 μg/VR_{111} and adjuvant as negative.

2.4.2. IFN-γ ELISPOT assay

ELISPOT was performed using mouse IFN-γ kit from BD bioscience (San Diego, USA). Briefly, splenocytes lymphocytes were isolated from immunized mice 4 week after priming injection. Spleens were obtained from distinct mice and splenocytes lymphocytes were obtained by EZ-sep Mouse Lymphocyte Separation medium (Dakewe, Beijing, China). According to the ELISPOT protocol, the plate was coated with anti-IFN-γ capture antibody in 100 μl of PBS per well. After overnight incubation at 4 °C, the wells were washed with PBS and blocked for 1 h with culture medium containing 10% FBS. Freshly isolated splenocytes were prepared from immunized mice. Splenocytes were plated at 6×10^5 cells per well with 10 μg/ml of E7 (49-57Db) (Cat#:51582,GL Biochem, Shanghai, China), or no peptide, in a final volume of 200 μl and then incubated for 36–48 h at 37 °C/5% CO_2. Spot numbers of IFN-γ were analyzed with a fully automated computer assisted video imaging analysis system, CTL-ImmunoSpot® S5 Versa Analyzer (Cellular Technology, Ohio, USA).

2.4.3. IFN-γ ELISA

Splenocytes were plated at 6×10^5 cells per well with 10 μg/ml of E7 peptide and then incubated for 3 days at 37 °C/5% CO_2. IFN-γ concentration was quantified from cell supernatants using a specific ELISA according to the manufacturer''s instruction (Cat#:3321-1H-6,Mabtech AB, Stockholm, Sweden). Data were expressed as IFN-γ released in pg/ml ± S.D.

2.5. Cellular immune response

Seven groups of 5 mice each were vaccinated by intramuscular inoculation or intraperitoneal inoculation two times on day 0 and day 14 with 30, 10, 3.3 μg/VR_{111},10 μg/VR_{111} bulk, 10 μg/E7 and 10 μg/Hsp E7, respectively. Negative control groups of 5 mice were injected with adjuvant on day 0 and day 14. The cell-mediated immune response was tested by the ELISPOT assay as described

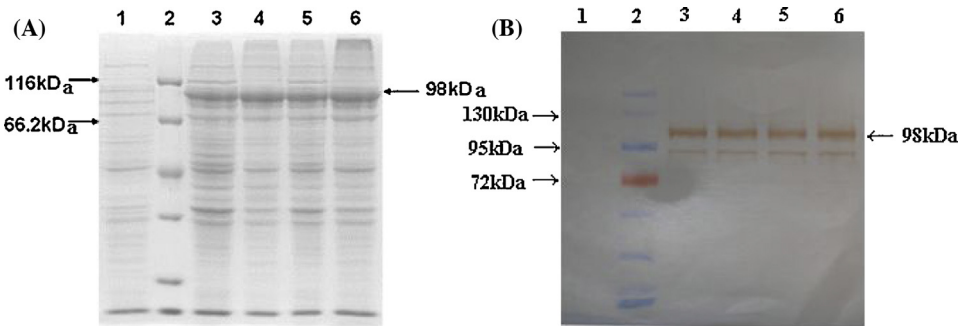

Fig. 1. Analysis of the expression of VR_{111} by SDS-PAGE (A) and Western blotting (B). Lane 1 in A and B, supernatant before induction; Lane 2 in A and B, molecular size marker; Lane 3 in A and B, supernatant after induction 1 h; Lane 4 in A and B, supernatant after induction 2 h; Lane 5 in A and B, supernatant after induction 3 h; Lane 6 in A and B, supernatant after induction 4 h; arrows at the left indicate the molecular weight of molecular size marker; arrows at the right indicate the position of the VR_{111}(~98 kDa).