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Purification and immunogenicity study of human papillomavirus type 16 L1 protein in *Saccharomyces cerevisiae*

Se Na Kim [a], Hye Sung Jeong [a], Sue Nie Park [b], Hong-Jin Kim [a,*]

[a] *College of Pharmacy, Chung Ang University, 221 Huksuk-Dong, Dongjak-Ku, Seoul 156-756, Republic of Korea*
[b] *Department of Biologics Evaluation, Korea Food and Drug Administration, 231 Jinheungno, Eunpyeong-Gu, Seoul 122-704, Republic of Korea*

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Abstract

Human papillomavirus 16 virus-like particle (HPV16 VLP) vaccines expressed in *Saccharomyces cerevisiae* are under Phase III trial and are expected to be on the market in the near future. We have established a convenient and economical system for the prophylactic study of vaccines derived from HPV16 VLPs, and neutralization tests to standardize HPV serological methodology as a measure of validation. To purify HPV16 VLPs, yeast cells expressing HPV16 L1 protein were cultured and purified on a small scale by ultracentrifugation and size-exclusion and cation-exchange chromatography using open columns. The highly purified HPV16 L1 protein was identified by SDS-PAGE and Western blotting, and electron microscopic analysis confirmed that they self-assembled into VLPs. To test the efficacy of the purified VLPs as a vaccine and their ability to induce humoral immunity, we performed ELISA assays and observed a significant increase in the titer of anti-HPV16 VLPs antibodies in the sera of immunized mice. High anti-HPV16 neutralizing titers were found in the sera of vaccinated mice, as measured by a SEAP-based pseudovirus neutralization assay. These results would be useful in the evaluation of the immunogenicity of HPV vaccine candidates, and provide an international reference standard for HPV serological methods.

Keywords: Human papillomavirus (HPV); Virus-like particle (VLP); Neutralization assay

1. Introduction

Human papillomavirus (HPV) is the second leading cause of cancer deaths in women worldwide, as it is a major risk factor for cervical cancer (Pisani et al., 1993). HPV is a small double-stranded DNA virus containing a circular genome of approximately 8000 base pairs (Pfister and Fuchs, 1994). Native virions of HPV are non-enveloped, 50–60 nm diameter icosahedral structures composed of 72 capsomers, each composed of five L1 molecules (Baker et al., 1991; Trus et al., 1997).

The major capsid protein (L1, ~55 kDa) can self-assembly into virus-like particles (VLPs), which are structurally similar to native HPV virions (Hagensee et al., 1993; Kirnbauer et al., 1992; Rose et al., 1993; Sasagawa et al., 1995; Volpers et al., 1994). VLPs have been shown to induce high-titer virus neutralizing antibody in animal models (Breitburd et al., 1995; Christensen et al., 1996; Kirnbauer et al., 1996; Suzich et al., 1995). Recombinant VLPs have been expressed in animal, insect, yeast and bacterial cells (Kirnbauer et al., 1992; Lowe et al., 1997; Rose et al., 1993; Zhou et al., 1991), and represent the leading candidate vaccines for preventing cervical cancer (Mandic and Vujkov, 2004). However, the yeast expression system offers the advantage of vaccine development that is cost-effective and easy to adapt to large-scale growth in fermenters; in addition, its potential for contamination by toxins or infectious viruses is small compared with bacterial or mammalian expression systems (Cook et al., 1999; Joyce et al., 1999; Neeper et al., 1996).

Currently, the development of VLP vaccines targeted against high-risk HPV types (HPV16 and 18) and low-risk HPV types (HPV6 and 11) is in progress, and quadrivalent HPV L1 VLP vaccines expressed in the yeast system are in Phase III trial and are expected to be available on the market in the near future (Koutsky et al., 2002; Lowy and Frazer, 2003). Hence, standardization of the assays and reference regents for assessing the efficacy of HPV vaccines is greatly needed. Neutralization

* Corresponding author. Tel.: +82 2 820 5613; fax: +82 2 816 7338.
E-mail address: hongjink@cau.ac.kr (H.-J. Kim).

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assays have often been used to evaluate the efficacy of viral vaccine candidates. However, obtaining infectious HPV virions in cell culture systems is difficult. Therefore, an alternative type of *in vitro* assay of HPV neutralization has been developed (Pastrana et al., 2004) that involves the use of pseudovirions consisting of capsids that are formed *in vitro* by expressing the viral L1/L2 protein. However, conflicting results have been obtained in trials using this assay in different laboratories around the world. Therefore, standardization of the assay and the criteria used to evaluate the immune response to this vaccine should make it possible to make comparisons of the outcomes of different trials.

In the present study, we performed an immunogenicity study of a vaccine that was derived from HPV16 VLPs, and neutralization tests to standardize HPV serological methods as a measure of validation. HPV16 VLPs that are made in the yeast expression system were purified by a simple, small-scale three-step purification process, and we assessed their immunogenicity by ELISAs and by SEAP-pseudovirus neutralization assays.

2. Materials and methods

2.1. Expression of HPV16 L1 protein in yeast

Recombinant HPV16 L1 protein was expressed as previously described (Park et al., 2002). *Saccharomyces cerevisiae* strain EGY48 not transformed with the HPV16 L1 gene was used as a negative control. EGY48 was grown with shaking at 30 °C for 24 h in YPD medium, which consists of 1% yeast extract, 2% peptone and 2% glucose (DIFCO Laboratories, USA).

2.2. Purification of HPV16 L1 protein

To purify intracellular HPV16 L1 protein, cells were harvested and lysed (Park et al., 2002). Cleared lysates were layered onto cushions of 45% sucrose in breakage buffer (20 mM sodium phosphate, pH 7.2, 100 mM NaCl, 1.7 mM EDTA) and pelleted by ultracentrifugation at 25,000 rpm for 10 h. The pellets were resuspended in 1 ml breakage buffer with 0.01% Tween-80 (Shi et al., 2005) and fractionated at room temperature by size-exclusion chromatography on a 1.0 cm × 100 cm Glass Econo-column (Bio-Rad Laboratories Inc., USA) that was loaded with Sephacryl^{TM} S-1000 resin (Amersham Pharmacia Biotech, Sweden). The running buffer for this column was 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 0.01% Tween-80. Fractions were collected into glass tubes and analyzed by SDS-PAGE and Western blotting for the presence of HPV16 L1 protein. The fractions of highest purity were pooled, equilibrated against binding buffer (20 mM Tris, pH 7.2, 100 mM NaCl, 0.1 mM EDTA, 5% glycerol, 15 mM 2-mercaptoethanol) and applied to P-11 cationic phosphocellulose (Whatman, UK) in a 0.8 cm × 4 cm Poly-Prep chromatography column (BIO-RAD Laboratories Inc., USA) that had previously been equilibrated with binding buffer at 4 °C. Following a wash with a one-column volume of washing buffer (binding buffer plus 0.25 M NaCl), HPV16 L1 protein was eluted with elution buffer (binding buffer plus 1 M NaCl), and fractions were analyzed using SDS-PAGE and Western blotting.

2.3. Western blotting analysis

Samples containing HPV16 L1 protein were electrophoresed on 12.5% acrylamide gels under reducing and denaturing conditions and transferred onto PVDF membranes (Q-Biogene, USA). The protein was detected using mouse anti-HPV16 L1 (Camvir-1; Chemicon International Inc., USA) as primary antibody and goat anti-mouse IgG–HRP conjugate (Sigma, USA) as secondary antibody. The membrane was visualized using Western blotting luminol reagent (Santa Cruz Biotechnology, USA).

2.4. Electron microscopy

Purified HPV16 L1 was dialyzed against PBS at 4 °C for 3 h, absorbed to carbon-coated grids and negatively stained with 2% phosphotungstic acid. TEM was performed using a TEM200CX transmission electron microscope at final magnifications of 41,000×.

2.5. Immunization of BALB/c mice with HPV16 VLPs

The immunogenicity of the purified HPV16 VLPs was tested in BALB/c mice. Twenty female 6-week-old BALB/c mice, purchased from ORIENT Co., Korea, were maintained in an air-conditioned room and supplied with sterile chow and water, and used when they reached 7 weeks of age. They were divided into four groups; 15 were immunized by subcutaneous injection of 5 μg purified HPV16 VLPs adsorbed to Freund's complete adjuvant (Sigma, USA), and five controls were inoculated with adjuvant only. After 3 weeks, all the mice were boosted twice with the same amount of VLPs adsorbed to Freund's incomplete adjuvant (Sigma, USA). Ten days later, they were bled from their tails, and sera were collected and stored at −20 °C.

2.6. Enzyme-linked immunosorbent assays (ELISAs)

HPV16 VLP-specific antibody was assayed by ELISA. The ELISA plates were coated overnight with 100 ng/well of purified VLPs in PBS at 4 °C, washed three times with washing buffer (PBS-T; 0.05% Tween-20 in PBS), and blocked with 2% BSA in PBS-T for 1 h at room temperature. Unabsorbed proteins were removed by washing, and serially diluted mouse sera were added in diluent buffer (0.3% BSA in PBS-T). The plates were then incubated at 37 °C for 1 h. Thereafter, they were washed with PBS-T, goat anti-mouse IgG–HRP conjugate was added to the wells, and incubation continued for a further hour at 37 °C. Unbound secondary antibody was removed by washing and bound antibody was stained with substrate—that is, one tablet of *o*-phenylendiamine (Sigma, USA) in 25 ml of phosphate–citrate buffer, dissolved with a capsule of sodium perborate (Sigma, USA) in 100 ml of dH_2O. The color reaction was stopped by adding 3 M H_2SO_4 and absorbance was recorded at 492 nm.