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Production and Prophylactic Efficacy Study of Human Papillomavirus-like Particle Expressing HPV16 L1 Capsid Protein

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To perform the prophylactic study of a vaccine derived from human papillomavirus (HPV) using Balb/c mice, we produced virus like particles consisting of HPV capsid protein L1 which has been reported to induce significant humoral and cellular immunity using various animal model systems. In order to produce HPV16 VLPs, the cDNA of L1 capsid protein in HPV type 16, obtained by polymerase chain reaction, was inserted into yeast expression vector, YEGα-HIR525 under the control of GAL10 promoter. The transformation of YEGα-HPV16 L1 was performed into the yeast *Saccharomyces cerevisiae* Y2805 by the lithium acetate method and the yeast clone expressing the highest level of L1 capsid protein of human papillomavirus type 16 was selected by Western blot analysis using anti-HPV16 L1 antibody. The purification of HPV16 VLP has been performed by the ultracentrifugation and gel-filtration methods. To validate the vaccine efficacy of the purified HPV16 VLPs and investigate the properties of HPV16 VLPs to induce humoral immunity, ELISA assay was performed. A significantly increased production of anti-HPV16 VLP antibodies was observed in sera from immunized mice. The neutralization activity of antibodies in the sera from the vaccinated mice was demonstrated by a rapid and simple assay to detect hemagglutihation inhibition activity.

Key words: Human papillomavirus (HPV), capsid protein, hemagglutination inhibition

Papillomaviruses are non-enveloped, double-stranded DNA viruses containing a circular genome of approximately 8,000 base pairs (Zhou *et al.*, 1992). The association between human papillomavirus (HPV) infection and cervical cancer and the carcinogenesis mechanism of cervical cancer induced by HPV infection have been relatively well demonstrated (Lowy *et al.*, 1994). The development of a prophylactic vaccine to protect against primary HPV infection may therefore be an efficient means to reduce the incidence of cervical cancer in women. Over 80 types of HPV have been identified and the high-risk types of HPV, such as HPV16 and HPV18 are the main causative factor in the pathogenesis of cervical carcinoma (Hagensee *et al.*, 1993). Native HPV has not been sucessfully grown in vitro cell culture systems, thus a vaccine strategy to prevent infection by HPV16 is to use recombinant HPV16 capsid protein (Buonamassa *et al.*, 2002).

Even though HPV16 capsid is composed of major (L1) and minor (L2) protein, recombinant major capsid protein L1, alone is capable of self-assembling capsid protein, (Cho., 2001). The L1 protein has a molecular mass of 55-60 kDa by polyacrylamide gel electrophoresis and is well conserved across types. The viral capsid is built up from pentameric capsomers of L1, with 72 such structures arranged in a icosahedral array (Xiaojiang *et al.*, 2000). Recombinant major capsid protein L1 produces 55nm virus-like particle (VLP) and induces sufficient immune response (Carter, 1991; Schiller & Hidesheim, 2000). Also only L1 gene is introduced in the cells producing the VLPs, this HPV L1 VLPs represent a potentially safe subunit vaccine that does not contain viral oncogenes such as E6 and E7 and is not infectious (Roden *et al.*, 1996). The human papillomavirus type 16 VLP, the first potential vaccine against an HPV16 that causes cervical cancer has passed a strict safety test and is expected to be on the market in the near future (Schiller & Hidesheim, 2000: Evans *et al.*, 2001). Thus, a validation study for the HPV16 L1 recombinant vaccine is required before actual human trials.

In the current study, we produced HPV16 L1 VLPs by cloning the cDNA of L1 capsid protein in HPV16 obtained by polymerase chain reaction and inserted it into

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a yeast expression vector system. We have chosen to express HPV16 L1 VLPs in yeast, *Saccharomyces cerevisiae* because a yeast expression system is relatively safe and highly efficient with yields of purified recombinant proteins. VLPs are purified by ultracentrifugation on sucrose cushion and gel filtration. To validate the vaccine efficacy of the purified HPV16 VLPs and investigate the capacity of HPV VLPs to induce the humoral immunity in Balb/c mouse model system, enzyme-linked immunosorbent assay (ELISA) was performed to measure levels of serum antibodies to HPV16 VLP. Papillomavirus agglutinated mouse red blood cells in a similar manner that virus binds the cell surface receptors to infect cells. Alternatively, the ability of antibodies to inhibit virion interaction with cell surface can be measured by inhibition of VLP-mediated agglutination of mouse RBC. Hemagglutination Inhibition (HAI) assay is a rapid and simple method to measure protective antibody titers after VLP vaccination (Roden *et al.*, 1996) and is regarded as a good surrogate assay for the detection of virus-neutralizing activity of sera from the immumized the mice.

Materials and Methods

Cloning of YEGα-HIR525-HPV16L1

The yeast strain used as the host for the expression of HPV16 L1 and yeast vector were *S. cerevisiae* Y2805 and YEGα-HIR525, kindly provided by H. Kang (Korea Research Insitute of Bioscience and Biotechnology, Daejon, Korea). Yeast expression vector YEGα-HIR525 contains the GAL10 promotor. The *EcoRI/SalI* HPV16L1 gene was obtained by double polymerase chain reaction, using the pBAC-HPVL1L2 vector kindly provided by J. T. Schiller (National Cancer Institute, Bethesda, USA) as a template. For the cloning of YEGα-HPV16 L1, it was necessary to eliminate an *EcoRI* site within the HPV16 L1 gene. For this, vector pBAC-HPV16 L1L2 was used as a template in a PCR using forward primer, 5'-ACA-TTC-TAT-GAA-CTC-CAC-TAT-TTT-GG-3' and reverse primer, 5'-GTC-GAC-TTA-CAG-CTT-ACG-TTT-TTT-GCG-3'. The forward primer eliminates the *EcoRI* site (GAA-TTC to GAA-CTC). The PCR products were purified and used as the reverse primer in a second PCR with the forward primer 5'-GTT-TTC-ATG-TCT-TGG-CTG-CCT-G-3'. The second PCR products were cloned into the PCR-Blunt II TOPO Cloning Kit (Invitrogen, Carlsbad, CA) and transformed into the *E. coli* strain (DH5α). Identity and size of the target plasmid DNA was confirmed by isolation of plasmid DNA from DH5α and its characterization by restriction enzyme digestion with *EcoRI* and *Sal I* followed by agarose gel electrophoresis. The restriction enzyme digested plasmid was ligated into the YEGα-HIR525 vector digested with the same enzymes and transformed into the yeast strain.

Expression of HPV16L1 protein in yeast

S. cerevisiae Y2805 strain was transformed with YEGα-HPV16 L1. Baffled flasks were inoculated with pre-cultured cells in SD-URA, a synthetic complete medium without uracil, and incubated with vigorous shaking at 30°C. For expression of HPV16 L1 protein from GAL10 promoter, YPDG (1% yeast extract, 2% peptone, 1% glucose, 1% galactose) was used as an expression medium for 48 h.

Western blot analysis

After harvesting the cells, the cell pellets were broken with glass beads, and the resulting clarified cell lysates were evaluated for the expression of HPV16 L1 by Western blot. analysis samples containing total cellular protein were electrophoresed on 10% acrylamide gels under reducing and denaturing conditions and transferred onto PVDF membranes (Millipore, Bedford, USA). The HPV16 L1 protein was detected using the mouse anti-HPV16 L1 (Chemicon Inc., Temecula, CA) as the primary antibody and goat anti-mouse IgG-HRP conjugate as the second antibody. The membranes were processed using a chemiluminescent ECL Detection Kit (Amershampharmacia, Inc. Buckinghamshire, UK).

Purification of HPV16L1 protein

Three liters of YPDG medium were inoculated with S. cerevisiae strain Y2805 harboring plasmid YEGα-HPV16 L1 and grown with shaking at 30°C for 48 h. The cells were harvested, and the pellets were frozen at -70°C. All subsequent steps were carried out at 4°C. Cell pellets were thawed and resuspended in ice-cold 100 ml break buffer (20 mM sodium phosphate, pH 7.2, 100 mM NaCl, 1.7 mM EDTA). We added phenylmethylsulfonyl fluoride (PMSF), pepstatin A, and leupeptin. Cells were transferred into the chamber of a Bead-Beater (Biospec Products, Barflesville, USA) together with glass beads (Sigma, St Louis, USA). Cells were lysed for 5 min and cleared by centrifugation at 5,000 g for 10 min at 4°C. All cleared supernatant was layered onto a cushion of 45% sucrose in the break buffer and 0.01% Tween-80 and HPV16 L1 were added. The proteins were pelleted by ultracentrifugation at 24,000 rpm for 4 h in a Beckman SW 28 rotor. The pellets were resuspended in break buffer and clarified by centrifugation at 5,000 g for 10 min. All supernatant was fractionated at room temperature by size-exclusion chromatography on a 530 ml column of sephacryl-1000 resin (Amershampharmacia, Inc. Buckinghamshire, UK). The running buffer for this column was 10 mM sodium phosphate, pH 7.2, 150 mM NaCl, 0.01% Tween-80. Fractions were analyzed by Western blot for the presence of HPV16 L1 protein, using the anti-HPV16L1 mouse monoclonal antibody.

Electron microscopy

The purified HPV16 L1 VLPs were observed to carbon-

coated grids and negatively stained with uranyl acetate. A Zeiss transmission electron microscope at an instrumental magnification of x 50,000 was used for visualization.

Immunization of Balb/c mice with VLPs

Groups of female, 6-8 week-old Balb/c mice were immunized by subcutaneous injection with 30 purified yeast-derived HPV16 L1 VLPs adsorbed to Freund's complete adjuvant. The mice were boosted with the same amount of VLPs adsorbed to Freund's incomplete adjuvant at weeks 3 and 5. Control mice were inoculated only with the adjuvant. At two weeks after injection, mice were bled and sera were collected and stored at -20°C.

ELISA (enzyme-linked immunosorbant assay)

The presence of antibodies was determined by ELISA using baculovirus-derived HPV16 L1 VLPs kindly provided by J. T. Schiller (National Cancer Institute, Bethesda, USA). ELISA plates were coated with 200 ng of VLPs in the coating buffer (carbonate buffer, pH 9.6) at 4°C overnight, washed three times with washing buffer (0.01% Tween-20 in PBS), and blocked with 10% skim milk in washing buffer for 30 min at 37°C for 1 h. Unbound antibodies were removed by washing and goat anti-mouse IgG-HRP conjugate was added. When the incubation time was over, peroxidase conjugates were added. The color reaction was stopped by adding 2.5 M H_2SO_4 and absorbance was recorded at 450 nm.

Neutralization assay (hemagglutination inhibition assay)

Fresh blood was obtained from Balb/c mice, collected in the same volume of Alsever's solution (Sigma, St. Louis, USA) and RBCs were separated by centrifugation at 2500 rpm for 10 min, the supernatant was discarded and the cells were washed with phosphate-buffered saline containing 1mg of bovine serum albumin per ml (1% B-PBS). The erythrocyte pellet was suspended in 1% B-PBS to the final 0.5% concentration. Prepared sera were diluted two-fold serially in 1% B-PBS and dispensed into 96 well round-bottom plates. Yeast-derived HPV16 L1 VLPs were added to an equal volume of sera and incubated at 37°C for 1h. Finally, the prepared RBC were dispensed and incubated at 4°C for 3 h.

Results and Discussion

Recombinant expression of HPV16 L1 proteins in yeast

Yeast expression vector plasmid YEGα-HIR525 was used to express HPV16 L1 protein harboring galactose-inducible promoter GAL10 and the yeast auxotrophic marker, uracil expression gene (ura3) as the selective marker (Fig. 1A). YEGα-HPV16 L1 was constructed by cloning L1 gene (1.5 kb) in the YEGα-HIR525 (6.5 kb) vector. YEGα-HPV16 L1 was transformed and expressed in the *S. cerevisiae*

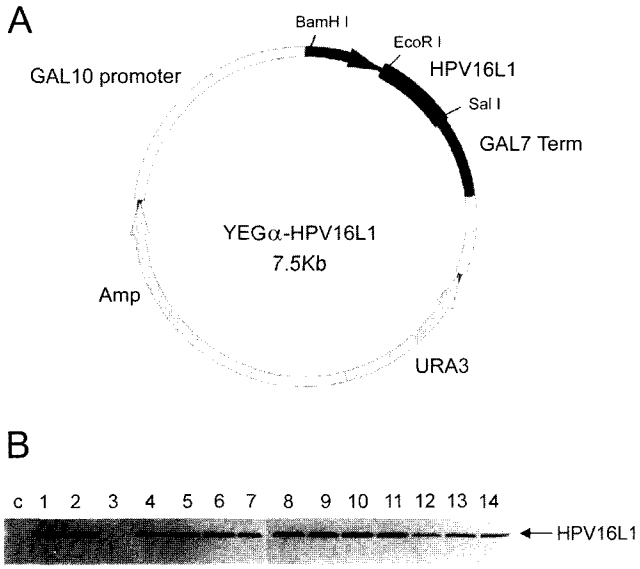

Fig. 1. Construction of YEGα-HPV16 L1. (A) Yeast expression vector plasmid for HPV16 L1 gene. (B) Expression of HPV16 L1 in *S. cerevisiae*. Yeast clones harboring the YEGα-HPV16 L1 were cultured in YPDG for 24 h. The samples were fractionated on 12% SDS-polyacrylamide gel, blotted on PVDF membrane, and reacted with the HPV16 L1 monoclonal antibody. C stands for the control yeast cell extracts.

Y2805. Figure 1B shows the results of a Western blot analysis of total cell extracts prepared from the recombinant *S. cerevisiae* Y2805 using a mouse anti-HPV16 L1 antibody (Camvir-1). In all cases, a 55-60 kDa HPV16 L1 specific bands were detected and we chose the yeast clone expressing a high-level of HPV16 L1 protein. The expression of HPV16 L1 in the yeast clone we had chosen was analyzed by Commassie blue staining and Western blot analysis after incubation for 0, 12, 24, 36, 48, 60, 72 h. in a shake-flask culture to optimize incubation time for high expression of HPV16 L1 (Fig. 2A). It demonstrated that the optimum time for high expression of HPV16 L1 was 48 h (Fig. 2B). The level of expression of HPV16 L1 was visualized better by Western blot analysis than Commassie blue staining.

Purification of HPV16 L1 protein

To purify HPV16 VLP, the cell lysates of the yeast clone expressing the highest level of HPV16 L1 was prepared by mechanical disruption followed by ultracentrifugation at 5 cm cushion of 45% and size-exclusion chromatography. After 45% sucrose cushion ultracentrifugation, the white pellet could be detected visually and was analyzed by Western blot using HPV16 L1 monoclonal antibody (data not shown). The pellet was resuspended and size-exclusion chromatography was performed using sephacryl-1000 resin (Pharmacia). Individual fractions of the peak, shown in the Fig. 3A, were collected and analyzed for the

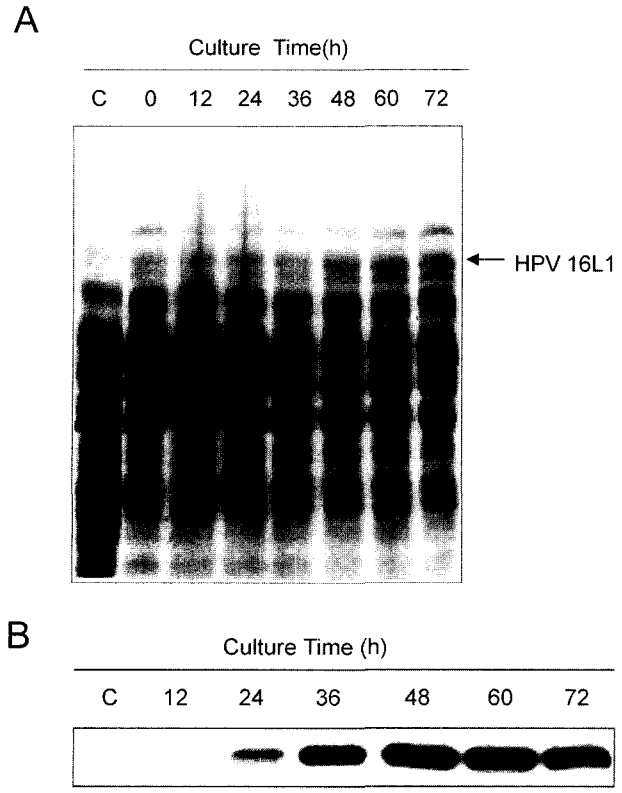

Fig. 2. Expression of HPV16 L1 in *S. cerevisiae*. The level of HPV16 L1 expression in *S. cerevisiae* transformanant harboring YEGα-HPV16 L1 were analyzed by Commassie blue staining (A) and Western blot (B) after 0, 12, 24, 36, 48, 60, 72 h incubation in the shake-flask culture.

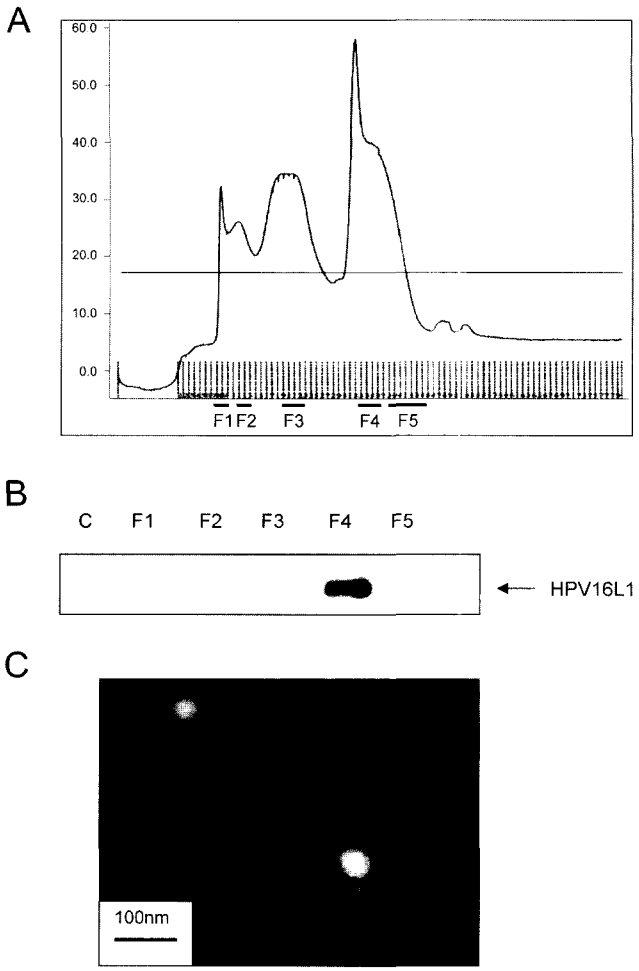

Fig. 3. Purification of HPV16 L1 virus-like particle. (A) Elution profile of the size-exclusion column of Sephacryl-1000 resin, F1-Fraction Number 8, 9; F2- Fraction Number 12,13; F3-Fraction Number 19,20,21; F4-Fraction Number 32,33,34; F5-Fraction Number 35,36,37,38,39,40. (B) Fractions were analyzed by Western blot to check the purified HPV16 L1. (C) HPV16 L1 VLPs in fraction 4 were examined by transmission electron microscopy (Zeiss, EM900). VLPs were negatively stained with uranyl acetate on a carbon coated copper grid.

presence of HPV16 L1 by HPV16 L1 specific Western blot. As shown Fig. 3B, HPV16 L1 VLPs were detected in fraction 4 at a density of about 0.5 mg/ml which was measured by Bradford assay. Electron microscopy analysis of the enriched fraction reveals that the HPV16 L1 VLPs assembly form was detected in fraction 4 (Fig. 3C). The average diameter of the spherical particle was about 50 nm, which was identical to HPV VLPs reported previously (Schiller & Lowy, 1996; Rossi *et al.*, 2000).

HPV16 VLP vaccination induces anti-L1 IgG antibodies

To determine if vaccination of the yeast-derived HPV16 L1 VLPs would specifically prime a humoral response to the baculovirus-derived HPV16 L1 VLPs, IgG antibodies were analyzed by ELISA in sera from mice immunized with the yeast-derived HPV16 L1 VLPs subcutaneously after, first, second, and third immunizations. Sera diluted from 1:500 to 1:8000 were added to baculovirus-derived HPV16 L1 VLPs pre-coated well of microtiter plate and bound IgGs were detected by HRP conjugated goat anti-mouse IgG. As shown in Fig. 4A, all sera from mice immunized with HPV16 L1 VLPs developed HPV16 specific IgGs. IgG titers were increased according to the subsequent injection number. The antibody response detected in sera from twice-boosted mice were significantly higher than that observed in primed and one time-boosted mice and revealed still high antibody titers at 8000 fold diluted sera compared with that in primed or first immunized mice. Sera from control mice which were not vaccinated with HPV16 L1 VLPs did not contain HPV16 VLP specific IgGs.

IgG antibodies from immunized mice have hemagglutination inhibitory activity/ neutralizing capacity

It was reported that papillomavirus could agglutinate mouse erythrocytes (Roden *et al.*, 1996). Alternatively, the ability of antibodies to inhibit virion interaction with the cell surface can be measured by inhibition of VLP-mediated agglutination. Thus hemagglutination inhibition (HAI) assay is regarded as a good surrogate assay for the detection of

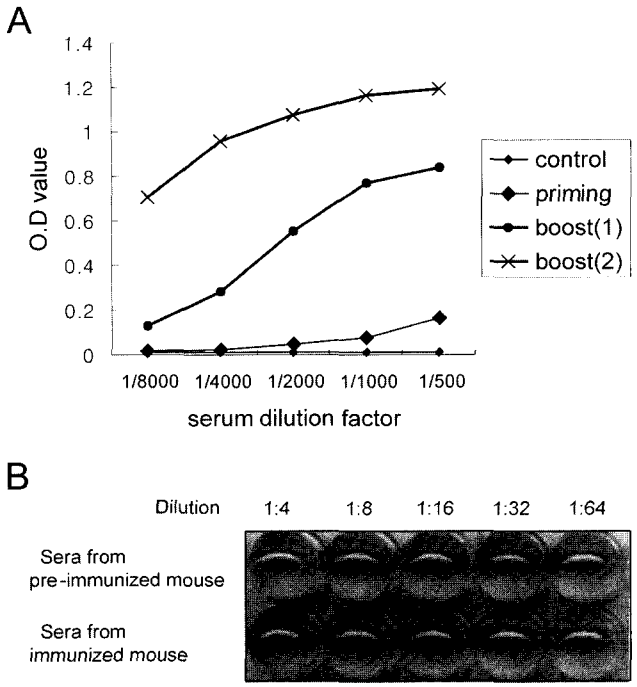

Fig. 4. Prophylactic efficacy of HPV16 VLP. (A) Presence of IgG antibodies in sera from mice vaccinated subcutaneously was detected by ELISA. Absorbance values corresponding to non-immunized mice (control) and mice immunized one time (priming), two times [boosting (1)], three times [boosting (2)] were measured 2 weeks after immunization, (B) Inhibition of HPV16 L1 VLPs induced hemaggultination. Sera from mice vaccinated subcutaneously with HPV16 L1 VLPs were serially diluted and mixed with the VLPs before reacted with mouse erythrocytes. Sera from pre-immunized mice were used as negative control.

virus-neutralizing activity of immunized sera. The proper amount of VLPs necessary for complete agglutination with mouse erythrocytes was determined by performing hemagglutination test. For HAI assay, sera from preimmunized mice and mice immunized twice were used. Sera were precleared to exclude nonspecific hemagglutination activity by heating for 30 min at 56°C and twofold serially diluted in PBS. As shown in Fig. 4B, hemagglutination inhibition activity appeared in sera from immunized mice, which was significantly higher at 1:4, 1:8, 1:16 dilution than sera from preimmunized mice. Basal HAI activity found in sera from preimmunized mice and immunized mice at low dilution might not be induced by specific antibodies in the sera but due to the degradation of the papillomavirus receptor proteins on mouse erythrocytes, which caused the erythrocytes to clump. The results demonstrated that antibodies in sera from HPV16 L1 VLP vaccinated mice have neutralizing capacity.

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