Production and verification of human papillomavirus type 18 vaccine in vitro

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Abstract. This study aimed to generate human papillomavirus (HPV) type 18 L1 virus-like particles (VLPs) and investigated the immunity effectiveness in vitro by using the Bac to Bac baculovirus expression system and Ni-NTA purification system to express and purify the VLP. The bio-function and immune response of purified VLP were analyzed by a mouse erythrocyte haemagglutination assay and the IFN-γ ELISPOT. The proteins of interest, primarily located at the nucleus, formed VLP by self-assembly, and had an effective immune activity in vitro. The level of IFN-γ in the specific HPV18 positive group was significantly higher than that of the mixed high-risk HPV infected group and non-HPV infection one in vitro. Our study indicated that HPV18 later protein L1 was efficiently expressed in Bac to Bac baculovirus expression system and the HPV18 L1-VLPs showed immunoreactivity and bio-activity in vitro. Furthermore, the ELISPOT results support the hypothesis that the cell-mediated immunity generated by HPV18 L1-VLP may cross-type among high-risk HPV types.

Introduction

Human papillomavirus (HPVs) belongs to the papillomavirus family, and >120 human types are verified thus far. HPVs are specific for the infection of epithelial cells, including those in the skin, respiratory mucosa and genital tract (1). HPV has been shown to be the etiological agent of many cancers, and persistent infections of HPV types with a high carcinogenic potential are associated with many malignant diseases including head and neck squamous cell carcinomas, esophageal carcinoma, lung cancer, bladder carcinoma as well as genital cancers, especially the development of cervical cancer (2). However, besides surgery and chemotherapy, no specific treatment against HPV infection is available. With the development of molecular biology and immunology, a novel immunotherapy strategy that protects against HPV infection, directly and effectively, and reduces the HPV-related disease burden is needed urgently.

In 1991, Zhou et al (3) reported that the HPV16 L1 capsid protein was synthesized and could assemble by itself into DNA-free virus-like particles (VLPs) in vitro with a certain expression system. VLP is an icosahedral structure and the outer shell, or capsid, contains 72 pentamers, or capsomers of L1, the major capsid protein which has the same conformation epitope as homeotype HPV. VLP was able to induce a strong type-specific humoral response with neutralizing antibodies in vivo. The results suggested that VLP may be one of the best candidate immunogens for HPV vaccine trials.

The association between HPV infection and cervical cancer has been firmly established, and the oncogenic potential of certain HPV types has been demonstrated. Several lines of evidence suggest the importance of the host immune response, especially the cellular immune response, in the pathogenesis of HPV-associated cervical lesions. However, these observations form a compelling rationale for the development of vaccine therapy to combat HPV infection (4).

Many studies on HPV16 VLPs exist, but its bio-function and immune effect have received more and more attention in recent years. Although the second epidemic high-risk type HPV18 is associated with ~20% HPV-related cervical cancer, research focusing on this aspect is rare. For a long time, we simply assumed HPV16 and 18 were the same. It is uncertain whether there is any difference between them or not. It is well-known that the HPV18 infection is related to cervical adenocarcinoma. HPV18 VLPs give knowledge of HPV18's biological characteristics as well as on the occurrence and development mechanisms of cervical adenocarcinoma.

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Abbreviations: HPV, human papillomavirus; IL-2, interleukin-2; IFN-γ, interferon-γ; ELISPOT, enzyme-linked immunospot; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel; CTL, cytotoxic T lymphocyte; PBMCs, peripheral blood mononuclear cells

Key words: human papillomavirus type 18, Bac to Bac baculovirus expression system, virus-like particles, enzyme-linked immunospot
It is estimated that 493,000 new cases of invasive cervical cancer were diagnosed in 2002, 83% of which were in developing countries, e.g. 150,000 new cases in China every year. At the same time, an estimated 273,000 deaths from cervical cancer occur worldwide each year, with over three-fourths of them in developing countries (5,6). However, the costly production and distribution of current VLP vaccines prevents widespread vaccine application in less developed countries. Consequently, due to the high demand in developing countries for the HPV vaccine, we are now producing an HPV prophylactic vaccine of HPV16, 18 and 58 based on L1 VLP. Herein we describe the generation of HPV18 VLPs in detail and demonstrate its functionality for vaccination purposes in vitro, which is fundamental to producing a preventive vaccine for future research.

Materials and methods

Cloning of HPV18 L1 and the construction of recombinant bacmid HPV18L1. The HPV18 L1 full-length gene was amplified by polymerase chain reaction (PCR) from plasmid PBR322-HPV18 including the complete gene of HPV18, which was kindly provided by Professor H. zur Hausen (Heidelberg University, Germany). PCR was performed using a high-fidelity DNA polymerase (Invitrogen, USA) in a 50 μl reaction mixture with the primers: L1, forward 5'CGGAAATTCGCACTTTACACATTACTTCCTGG 3' and reverse 5'CAGGAAACAGCTATGAC3'. For these sequences, the EcoRI and HindIII restriction sites are underlined. The reaction conditions were: degeneration at 94˚C, 30 sec; annealing 52˚C, 45 sec; extension 72˚C, 60 sec and 35 reaction cycles. The final PCR product was added with poly (A) tail and ligated to the T-easy vector (Promega, USA), producing the T-HPV18 L1 plasmid.

E. coli DH5α competent cells were transformed with ligation. Penicillin-resistant clones were screened for the presence of the L1 gene by PCR, restriction endonucleases were cleaved and the products electrophoresed in an ethidium bromide-stained 1% agarose gel. The correct sequence was confirmed by DNA sequencing (Invitrogen). Purified T-HPV18 L1 plasmid was cleaved with EcoRI and HindIII restriction endonucleases and ligated to the pFastBac Donor vector (Invitrogen), which was previously digested with EcoRI and HindIII, producing the pFastBac-HPV18 L1 plasmid.

After the recombinant pFastBac donor plasmid was determined by DNA sequencing to be correct, the pFastBac-HPV18 L1 was transformed into DH10BAC (containing bacmid and helper plasmid) for transposition into the bacmid. All transposition steps are according to the instruction manual.

PCR analysis of recombinant bacmid. PCR was carried out to confirm that the gene of interest had been transposed to the bacmid. The pUC/M13 amplification primers used in the study are directed at the sequences on either side of the miniAT TTn7 site within the lacZa-complementation region of the bacmid (7). If transposition has occurred, the size of the PCR product produced by these primers should be 2,300 bp plus the size of the insert. The primers were: sense, 5'GTTTTTTCCCGTC ACGAC3' and anti-sense 5'CAGGAAACAGCTATGAC3'. After incubation at 93˚C for 3 min, 25-35 cycles of PCR was performed as follows: 94˚C for 45 sec, 55˚C for 45 sec and 72˚C for 5 min.

Cell culture and the transfection of recombinant baculovirus. Monolayer cultures of SF-9 cells (kindly provided by the Wuhan Institute of Virology Chinese Academy of Sciences, China) which were derived from pupal ovarian tissue of the fall armyworm, Spodoptera frugiperda, by Vaughn in 1977, using the packaging cells in the Bac to Bac baculovirus expression system, were grown at 27˚C in Grace's insect medium supplemented with 10% fetal calf serum (Gibco/BRL, USA). The cells were transfected with recombinant bacmid HPV18 L1 by Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Viruses were harvested from the cell culture medium at 72 h post-transfection. Amplification of the viral stocks and viral plaque assay was carried out according to the instruction manual (7). The cells were infected at a multiplicity of infection (MOI) of 15-20 with the recombinant baculoviruses encoding the L1 protein. The cells were then collected at 48-72 h post-infection. The protein expression from isolated virus stocks was determined by Western blot analysis (see below).

SDS-PAGE electrophoresis and Western blot analysis. The cells were harvested 48-72 h after infection, lysed by boiling in SDS sample buffer and electrophoresed on 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). Proteins were stained with 0.25% Coomassie blue or transferred to a nitrocellulose membrane. The membrane was blocked for 1 h with 5% skim milk at 37˚C and incubated with the 1:500-diluted monoclonal antibody anti-HPV18 L1 (Santa Cruz, USA) overnight at 4˚C. The secondary antibodies were then added at a 1:1000 dilution. The immunoreactive bands were visualized by the horseradish peroxidase ECL system (Amersham Pharmacia Biotech, Braunschweig, Germany) according to the manufacturer's instruction.

Purification of HPV18 L1 VLPs. HPV18 L1 VLPs were purified under native conditions with Ni-NTA His•Bind Resin (Novagen, Germany). All procedures followed the protocols in the Ni-NTA His•Bind Resin handbook. The infected cells were lysed, mixed with bind buffer and added to the Ni-NTA resin. After being bound, the lysates were washed three times with washing buffer to wash off the unbound protein. Then the L1 protein was eluted with elute buffer. The purified VLPs were sterilized by filtering and made into protein powder for convenience of dilution.

Electron microscopy. Cells were prepared for electron microscopy as previously described (8). Briefly, cells (pre-purification) were fixed in 2% paraformaldehyde 0.1% glutaraldehyde in PBS. After fixation, the cells were washed in Sabatini's solution and post-fixed with 1% osmium tetroxide. The samples were then passed through a graded alcohol series, infiltrated with acetone and embedded in Epon 812. Ultrathin (60 nm) sections were cut with a diamond knife on an MT2 Ultramicrotome (Sorval), stained with uranyl acetate and lead citrate, mounted on 300-mesh grids and examined with a Philips electron microscope (EM, model EM 400T) (Royal...
Haemagglutination assay. The conformational intact VLPs can agglutinate mouse erythrocytes efficiently (9). Erythrocytes from C57BL/6 mice were drawn behind the eyeball, anti-coagulated by heparin sodium and harvested by centrifugation. The erythrocytes were washed with PBS and resuspended at 1% (v/v) in PBS containing 1 mg/ml bovine serum albumin (BSA). Serial dilutions of purified VLPs were prepared in PBS containing 1 mg/ml BSA and mixed with an equal volume of a 1% (v/v) suspension of erythrocytes in PBS. A 100 μl aliquot of the mixture was added to each well of a 96-well plate. After incubation at 4°C for 3 h, the plates were photographed. PBS containing 1 mg/ml BSA was regarded as the negative control and erythrocytes alone as the blank control (10).

IFN-γ enzyme-linked immunospot (ELISPOT) assay, human subjects and isolation PBMCs. Blood samples were collected from 20 out-patients of the Department of Obstetrics and Gynecology of Tongji Hospital (affiliated to Tongji Medical College of Huazhong University of Science and Technology, China). The experimental protocols had previously been reviewed and approved by the Institute Review Boards of Tongji Hospital. Patients did not receive any treatment such as surgery, radiation or chemotherapy, except for cervical biopsy before the collection of PBMCs. The PBMCs were prepared with Ficoll-Paque solution (Amersham Biosciences, Uppsala, Sweden) for separation of the lymphocytes according to a method previously described (11). After centrifuging the blood/Ficoll-Paque gradients at 800 g for 30 min, the layer containing the lymphocytes was transferred to another centrifuge tube and washed in PBS (12).

Detection and typing of the HPV genome. To confirm that HPV infection was present in the cervical keratinocytes, cervical cells were collected with a cervical brush and subjected to tests by the Hybrid Capture II system (Digene, USA) according to the manufacturer's instructions. After undergoing denature, hybridization, capture, detection and analysing, the samples which were infected with 13 high-risk HPV were obtained without HPV typing. Individual HPV typing was confirmed by HPV DNA gene array. Briefly, hybridized arrays were scanned on a STORM 860 phosphorimager/fluorimager (Amersham Biosciences, UK). The resulting images were analyzed with the array vision software 8.0 rev 4 (Imaging Research Inc, Canada) and data from the duplicate spots were normalized using gene spring software version 5.0 (Silicon Genetics, USA) (13).

Two patients confirmed as having HPV18 infection by HPV gene array were used as the positive group, 13 patients infected with high-risk HPV as proved by the Hybrid Capture II system without HPV typing served as the mixed high-risk group, while five healthy women without HPV infection were the negative group.

IFN-γ ELISPOT assays (12,14). HPV18-specific T-cell responses were quantified by ELISPOT assay according to the manual of the human IFN-γ ELISPOT kit (U-CyTech, The Netherlands). Briefly, fresh PBMCs were plated onto 96-well plates that had been pre-coated with 0.5 g of anti-IFN-γ monoclonal antibody (BD Biosciences, USA). PBMCs were added at a concentration of 1,000,000 cells per well in a volume of 100 μl of Lympho-spot no-serum medium (U-CyTech) with antibiotics (50 U/ml of penicillin-streptomycin). The concentration of the HPV18 L1 VLPs was 10-100 μg/ml in the Lympho-spot medium. Plates were incubated overnight at 37°C and 5% CO2. The wells containing PBMCs alone in the Lympho-spot medium were used as negative controls. The wells containing PBMC and phorbol 12-myristate 13-acetate (PMA) served as positive controls. Triplicate experimental wells and quadruple control wells were used. The number of spots per well was counted using an automated ELISPOT plate reader (BioReader 4000, Germany), and the number of specific T cells was calculated by subtracting the negative control values.

Statistical analysis. Statistical analysis and graphical presentation were performed by SPSS13.0 and Sigma Plot 10.0. The results are given as the mean ± standard error of the mean (SEM). Statistical analysis of significance was based on one-way ANOVA. P<0.05 was considered statistically significant.

Results

PCR analysis of the recombinant bacmid. As shown in the Gene Bank, the standard length of HPV18 L1 (X05015) is 1,700 bp. Therefore, the PCR product produced by the pUC/M13 primer should be 4,000 bp (2,300 plus 1,700) if the construction was successful (7). Fig. 1 shows that a band of nearly 4,000 bp was found at lane 3, proving the correct recombinant. Furthermore, 48-72 h after infection, the SF-9 cells became rounded, swelled, had a stronger refraction than the uninfected cells and shed gradually. The SF-9 cells were harvested, the cDNA was extracted and used as a PCR template, and then amplified with an HPV18 L1 gene-specific primer. The PCR product was ~1,700 bp the length of HPV18
L1 (lane 2). The above indicated that the infected Sf-9 cells contained recombinant bacmid including the HPV18 L1 gene.

Expression and purification of the recombinant HPV-18 L1 protein. The Sf-9 cells were infected with high-titer recombinant baculoviruses, and 72 h later, the cell lysates were analyzed by SDS-PAGE electrophoresis and Western blot analysis. As shown in Fig. 2, SDS-PAGE electrophoresis demonstrated a novel 63-kDa protein seen from the Coomassie blue-staining SDS-PAGE electrophoresis. Lane 1: uninfected Sf-9 cells as the blank control; lanes 2 and 3: the unpurified HPV18 L1 protein; lanes 4 and 5: the partly purified HPV18 L1; lanes 6 and 7: the purified HPV18 L1 protein under the native conditions and lane 8: (Mr) markers (170, 130, 100, 70, 55, 40, 35 and 25 kDa). The arrowhead on the right indicates the position of recombinant L1 (-63 kDa).

These structures were distinguished from baculovirus inclusions seen in the cross-section in that the VLP arrays were not surrounded by a membrane-like structure and individual particles which had a greater diameter (16).

To prove the structure of the purified recombinant L1 proteins in vitro, the purified protein was prepared as previously described (8) and examined by TEM. Fig. 3C and D show the particles in detail. All these results suggested that the HPV18 L1 protein can self-assemble into VLP-like structures successfully in vitro, which established the crucial basis for the development of the HPV18 vaccine.

Haemagglutination assay. The haemagglutination assay is regarded as a surrogate method of evaluating the capacity of antibody-mediated neutralization (9). To determine if HPV18 L1 VLP-induced antibodies in the sera had a neutralizing capacity, haemagglutination assay was performed. Successful haemagglutination by VLPs was obtained from 50 to 400 ng of pure VLP protein per 100 μl, suggesting that native and properly folded VLPs maintained antigenicity in vitro (Fig. 4).

IFN-γ ELISPOT. Using HPV18 L1, VLPs pulsed the PBMCs in vitro with a concentration from 10, 20, 40, 80 to 100 μg/ml. We evaluated the presence of a detectable immune response by determining the presence of IFN-γ-producing cells after VLPs and PBMCs were co-incubated for 36 h by means of the IFN-γ ELISPOT. A significant difference occurred in the number of spot-forming CD8+ T cells (SFC: spot-forming cells) in response to HPV18 infected patients in a dose-dependent manner. The average numbers of IFN-γ-secreting T cells of the wells in each group are listed in Table I in different VLP concentrations by the mean ± standard error of the mean (SEM). As shown in Table I and Fig. 5D, the SFCs...
per 10^6 PBMCs in the HPV18 positive group with each VLP concentration were much greater in number than those in the mixed high-risk and negative groups, which had a high statistical significance (P<0.05). In the mixed high-risk group, fewer T-cell responses of IFN-γ secretion were identified and nearly no responses in the negative group. This study showed that the purified HPV18 L1 VLP proteins may serve as targets for HPV18-specific CD8+ T-cell responses.

Discussion

Globally, cervical cancer is the second most common cause of poor survival from cancer in women, with an estimated 510,000 newly diagnosed cervical cancer cases, counting for nearly 10% of all cancers (17). Furthermore, in developing countries, cervical cancer is often the most common cancer in women. Thus, cervical cancer is a critical public health problem and an overall disease burden for women throughout the world, especially in less developed countries. The fact that infection with certain HPV types is now recognized as a necessary intermediate step, the genesis of cervical cancer has led to new research fronts for the prevention of HPV-
related diseases (18,19). Research on the safety and efficacy of candidate prophylactic vaccines against HPV have shown very promising results with nearly 100% efficacy in preventing the development of persistent infections and cervical pre-cancerous lesions. The Gardasil vaccine produced by Merck that protects against the most common disease-causing HPV types (HPV6, 11, 16 and 18) was allowed to be used in clinical application by the American Food and Drug administration (FDA) in 2006 (20). In 2007, the American Cancer Society (ACS) developed guidelines for the use of the prophylactic quadravalence HPV vaccine. They address the use of prophylactic HPV vaccines, including who should be vaccinated and at what age, as well as a summary of policy and implementation issues (21). Therefore, generating our own vaccine is essential.

In this investigation, we used the L1 major capsid protein of HPV18 was expressed in the Bac to Bac baculovirus system efficiently and had a good bio-activity and immunity in vitro. In addition, it was proven with TEM that the purified recombinant L1 protein spontaneously assembled into a variety of aggregates, mainly particles with morphological characteristics in vitro similar to those of the VLPs. These observations confirmed that L1 has an intrinsic capacity for self-assembly and other papillomavirus proteins are not required for VLP formation in the baculovirus system (16).

Certainly, there are many kinds of HPV vaccines including protein, peptide, DNA and vector-based. We chose VLP-based vaccine, mainly because it is easy to produce on a large scale, is safe and highly immunogenic. As discussed previously, the L1 VLP is almost identical morphologically and antigenically to the infectious virus particle (22). Furthermore, we chose Bac to Bac baculovirus expression system for the following reasons: i) High levels of heterologous gene expression are often achieved compared to other eukaryotic expression systems, particularly for intracellular proteins. In many cases, the recombinant proteins are soluble, post-translationally modified and easily recovered from infected cells late in the infection when the host protein synthesis is diminished; ii) the cell lines used for the Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) propagation grow well in suspension cultures, permitting the production of recombinant proteins in large-scale bioreactors; iii) expression of the hetero-oligomeric protein complexes is achieved by simultaneously infecting cells with two or more viruses or by infecting cells with recombinant viruses containing two or more expression cassettes and iv) baculoviruses have a restricted host range, limited to specific invertebrate species. They are safer to work with than most other mammalian viruses since they are non-infectious to vertebrates (23).

In this investigation, we used the Ni-NTA purify system which is based on the theory that the 6xhis-tag in the N terminal of the proteins of interest can be bonded with the Ni-NTA resin, and then, eluted from the resin. Purified VLP was thus obtained without high centrifugalization and other traditional steps. This would be convenient and consistent with a large scale production.

Furthermore, others have reported that a successful immune response to genital HPV infections is characterised by strong, local cell-mediated immunity (CMI) that is associated with lesion regression and protection against further infection with the same genotype of HPV (24). However, few reports on HPV-specific cytotoxic lymphocyte (CTL) responses in humans have been published (25-27). In our investigation, we studied HPV18 CTL responses to test the VLPs immune reactions on patients, which would provide strong support for clinical use. As was reported (28), the T-cell-mediated protection from viral infection as well as control of the tumor is thought to be promoted by type 1 cytokine responses. In general, type 1 Th cells (CD4 or 8) which express IL-2, IFN-γ and TNF-α/β are cytotoxic. However, type 2 Th cells express IL-4, -5, -6, -10 and -13, and provide efficient help for B-cell activation. Consistent with this view, IL-2- and IFN-γ-producing T cells are believed to promote the development of cell-mediated immunity against HPV-associated diseases. This study focused mainly on IFN-γ producing in HPV18 VLPs induced immune response.

In this investigation, we showed efficient detection of HPV18-specific IFN-γ-producing autologous T cells after stimulations with HPV-18 L1 VLPs using an IFN-γ ELISPOT assay (Fig. 5 and Table I). The ELISPOT assay is a recently developed technique for detecting cytokine expression at the single-cell level for special antigen-stimulated CD8+ T cells. Routinely used techniques such as ELISA and RT-PCR, do not provide information regarding the frequencies of cytokine-producing cells, in contrast to sensitive methods such as ELISPOT. We utilized ELISPOT immunological methods to quantitate the concentration of IFN-γ and the number of IFN-γ-secreting T lymphocytes in normal and HPV-infected populations (Fig. 5 and Table I).

The results of our investigation demonstrated that PBMCs pulsed with HPV18 VLPs were able to induce HPV18-specific cytotoxic CD8+ T-cell immune responses for HPV18-infected populations dose-dependently. In the mixed high-risk group without HPV typing, we also found that some immune responses occurred. It has been assumed that there are HPV18 positive patients in the mixed HPV infection group without HPV typing or, as discussed before, highly homologous VLPs such as HPV-6/11 (29), -31/33, -18/45 (30) and -16/31 (31) share one or more cross neutralization epitopes. Another hypothesis is that the cell-mediated immunity generated by L1 VLPs is cross-neutralising and not type-specific which is not the same as the immunodominant neutralising antibodies as the evidence showed before (30).

In 2002, Combina et al (32) reported that the immunization of mice with HPV L1 VLPs indicated the existence of cross-neutralization between types and cross-reactivity occurrence. They hypothesized that an HPV vaccine composed of the most frequent types (HPV16 and 18) also protected against related but less frequent types (HPV31, 33, 45 and 58). It is still uncertain whether this is sufficient to offer cross-protection in vivo. Therefore, further investigation is required. However, in the negative group, fewer reactions were detected. Our explanation is that, although infected populations had not previously been infected by HPV18, they were still able to generate HPV18-specific CTL.
responses from the endogenous L1-specific CD8+ T lymphocytes (28) or the non-specific reaction.

In general, there were more HPV18-specific CD8+ T-cell precursors in the PBMCs of the HPV18 infected populations compared with the mixed high-risk and negative groups. These HPV-specific CD8+ T-cell precursors replicated and responded when they encountered the HPV L1 VLPs (28). A large case study will be needed to investigate this behavior in vitro and in vivo.

HPV18 VLPs may be used in a variety of ways to increase our understanding of HPV18 biology and mechanism. For example, they can be employed to study the HPV18 capsid formation, identify and characterize the HPV18 cell receptor and be considered as critical candidates for HPV18 vaccine development. HPV18 VLPs may provide abundant reagents with which to carry out immunologic and mechanism studies not only of cervical adenocarcinoma, but of other HPV-related diseases as well (16).

In conclusion, vaccination against HPV infection may reduce the risk thereof, thereby decreasing the incidence of cervical cancer. Our study on HPV18 VLPs provides a powerful foundation for the production of HPV18 vaccine and treatment of HPV18-related diseases.

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