Intracellular distribution of recombinant Human Papillomavirus capsid proteins

B. Marigliani1,2, E.A. Kavati1,2, D. Sakauchi1,2, H. B. Oliveira1,3, R. A. Canali1,3, A. A. Sasaki4, J. M. C. Ferreira Jr5, E. Armbruster-Moraes1,6, M. Müller7 and A.M. Cianciarullo*1,2,3
1Laboratory of Genetics, Butantan Institute, Av. Vital Brasil 1500, 05503-900 São Paulo, SP, Brazil
2 Post-Graduation Program Interunits in Biotechnology USP-IPT-IBU, University of São Paulo, SP, Brazil
3Professional Improvement Program, PAP-FUNDAP-SES-SP-Butantan Institute, Brazil
4Laboratory of Mycoserology, Department of Microbiology, Immunology and Parasitology, Federal University of São Paulo-UNIFESP, Rua Botucatu, 862, 04023-062, São Paulo, SP, Brazil
5Laboratory of Immunohematology, Butantan Institute, Av. Vital Brasil 1500, 05503-900 São Paulo, SP, Brazil
6Department of Gynecology and Obstetrics, Clinic Hospital, University of São Paulo, Av. Dr. Enéas de CarvalhoAguiar647, 05403-000 São Paulo, SP, Brazil
7Tumorvirus-Specific Vaccination Strategies Group, German Cancer Research Center - DKFZ, Im Neuenheimer Feld 242, 69120 Heidelberg, Germany
*Correspondence to: amcianciarullo@butantan.gov.br

HPVs (Human papillomaviruses) are associated with several types of cancer including cervical cancer, the second most frequent in women worldwide, caused mainly by HPV16. Presently available HPV vaccines are based on L1, the major capsid protein. For a new generation candidate HPV vaccine, we developed the production of HPV16 L1 and L2 proteins by the same cell so they can self-assemble in a virus-like particle similar to the native HPV capsid. The aim of this study was to map the distribution of recombinant HPV16 capsid proteins in transfected and co-transfected HEK293T cell cultures to evaluate the method for vaccine production, knowing that subcellular localization of capsid proteins has implications on protein purification. Recombinant L1 and L2 were detected in the cell nucleus and cytoplasm by transmission electron microscopy using secondary antibodies conjugated with colloidal gold particles. Their intracellular distribution was analyzed by laser scanning confocal microscopy either, which was also used to confirm flow cytometry data in kinetic curves for establishing the most appropriate moment to harvest transfected cells for protein purification. We have established an efficient system for HPV16 L1L2 production. These capsid proteins are being used to develop a new generation prophylactic vaccine that could be protective against a wide range of HPV.

Keywords Papillomavirus vaccines; virus-like particles; L1 and L2 proteins; capsid proteins; flow cytometry; confocal microscopy; electron microscopy; public health.

1. Introduction

Papillomaviruses can infect the epithelia of humans and other animals, where they are able to induce the formation of benign proliferations. But in some cases, lesions caused by certain types of papillomaviruses undergo malignant transformation. Infection by these types is the most significant risk factor for cancer development. Human papillomavirus (HPV) is a common sexually transmitted virus that can cause genital warts and several types of cancer. High-risk HPV persistent infection can lead to cervical cancer [1], the second most frequent cancer in women worldwide [2]. HPV16 and 18 are responsible for 70% of cervical cancers. Among the types associated with cervical cancer, HPV16 is the most prevalent, present in around 50% of tumor samples. This is also the most common type found in other five HPV-related cancers [3].

All known HPVs are epitheliotropic. Unlike other animal papillomavirus, they do not infect or express their genes in the underlying dermis, the initial infection requires access to cells in the basal layer [4]. The HPV virion is composed of an approximately 8000 base pair genome within an icosahedral 72-capsomere capsid containing 360 copies of the major protein L1 and probably 12 copies of the minor protein L2 [5]. The L2 is not required for capsid formation, although it plays essential roles in the viral infectious entry pathway. Its abundance and arrangement within the virion remain unclear [6].

HPV16 L1 has been used in vaccine development worldwide, due to its capacity to induce high immune response. L2 can induce a low titer of antibodies, although to a wider range of papillomavirus types and species than L1 [7]. When using a vaccine made of HPV16 L1 and adjuvant, there is evidence of cross-protection against other phylogenetically related HPV types [8]. Nowadays there are two prophylactic vaccines available to protect against HPV: the quadrivalent yeast-produced Gardasil (HPV16/18/6/11) by Merck, and the bivalent insect cell-produced Cervarix (HPV16/18) by GlaxoSmithKline. They are highly immunogenic, well tolerated, safe and highly effective [8,9]. Both vaccines are based on the major capsid protein L1, that self-assembles into VLPs (virus-like particles) structurally similar to the native virion [10,11]. VLPs are non-oncogenic, non-infectious and unable to replicate. Current VLP vaccines induce type-restricted antibody responses to conformational L1 epitopes [12].
Currently available prophylactic vaccines have demonstrated up to 100% efficacy against persistent infection and HPV-associated diseases [8,9], although they are type-restricted and expensive, require refrigeration, multiple doses and intramuscular injection [13]. Thus, there is a search for a broader-spectrum vaccine to prevent HPV infection. Studies in animal models have demonstrated that the minor L2 protein, specially the amino terminus, can induce protective virus-neutralizing antibodies [14,15]. Besides being less immunogenic than L1, immunization with L2 in animal models can provide protection mediated by neutralizing antibodies [16]. As L2 has the capacity to generate potent and broad-spectrum human papillomavirus neutralizing antibodies, it could be used to produce second-generation vaccines to help reduce the HPV-related diseases specially in world areas where they are a significant cause of morbidity and mortality [17].

Knowing that the subcellular localization of proteins has implications on protein purification, the aim of the present work was to map the distribution of the recombinant L1 and L2 HPV16 capsid proteins expressed in transfected and co-transfected human embryonic kidney cells in order to evaluate this method for the production of a new generation prophylactic HPV vaccine prototype.

2. Experimental procedures

All recommendations of the National Biosafety Law (CTNBio) for activities involving Genetically Modified Organisms – GMOs were respected. All experiments in the present study were approved by Butantan Institute Institutional Biosafety Committee (CIBio).

2.1 Plasmid vectors

The DNA plasmid vectors pUF3/L1h and pUF3/L2h were provided by Prof. Dr. Martin Müller from DKFZ-Heidelberg, Germany. Both eukaryotic expression vectors carry codon-modified HPV16 structural genes improved for efficient translation in human cells and are placed under the control of the human cytomegalovirus promoter (pCMV). Nucleotide maps of the plasmids used in this work can be found in our previous work [18].

Escherichia coli DH5α (Invitrogen, Carlsbad, CA) was used for plasmid vectors amplification. E. coli was transformed according to a standard protocol. Briefly, plasmid vectors were added to quimiocompetent E. coli and incubated on ice for 30 min, at 42°C for 2 min and on ice again for 5 min. After this, 450 μl of LB broth (1% tryptone, 0.5% yeast extract, 1% sodium chloride, pH 7.0) were added, followed by incubation at 37°C for 90 min. Aliquots of 100 μl were plated onto LB agar plates (LB broth, 1.5% agar, 100 μg/ml ampicillin) and incubated at 37°C for 18 h. Positive clones (ampicillin resistant) were inoculated in 3 ml LB broth and cultured at 250 rpm agitation for 18 h at 37°C. Amplified plasmid DNA was extracted using AxyPrep™ Plasmid Miniprep Kit (Axygen, CA), following the manufacturer's instructions. Plasmid DNA concentration was measured using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, MA).

2.2 Cell culture and protein expression

Human embryonic kidney 293T (HEK293T), a cell line carrying an integrated copy of the simian virus 40 large T antigen genome, was purchased from ATCC (American Type Culture Collection, Rockville, MD). Cells were grown in 75 cm² sterile tissue culture flasks (TPP®, Switzerland) at 37°C in D10. D10 is composed of 10 ml DMEM (Dulbecco Modified Eagle Medium, Cultilab, São Paulo, BR) supplemented with 10% foetal bovine serum (Cultilab, São Paulo, BR). When cells reached 80% confluence, with medium renewal every 4 days, they were subcultured, stocked or transfected.

For subculturing, cells were rinsed three times in PBS (phosphate-buffered saline) to remove all traces of serum that contains trypsin inhibitor. For detaching the adherent layer and making it into a single-cell suspension suitable for subculturing, we added 1 ml of trypsin (Trypsin/EDTA Solution 2.5 g/L, Cultilab, São Paulo) to flask and placed it at 37°C for 10 min. After observing detached cells under an inverted microscope, we added 2 ml of complete medium and aspirated the cells by gently pipetting. Cells were counted in a Neubauer chamber and appropriate aliquots were added to new culture flasks. For stocking, cells were preserved in liquid nitrogen in complete medium supplemented with 10% (v/v) DMSO for cryoprotection.

For HPV16 capsid proteins expression, pre-plated HEK293T cells were transfected with pUF3/L1h or co-transfected with pUF3/L1h and pUF3/L2h using GeneJuice® Transfection Reagent (Novagen®) according to the manufacturer's instructions. The transfection medium was removed 4 h post-transfection and 10 ml of D10 was added. In order to map the intracellular distribution of the L1 and/or L2 expressed proteins, transfected cells we analysed by confocal and electron microscopy 48 h post-transfection. We also performed western blotting analysis to confirm the HPV16 capsid proteins expression.
2.3 Confocal Laser Scanning Microscopy (CLSM)
Transfected HEK293T cells were washed three times in 1 ml PBS, fixed in 2% paraformaldehyde at 4°C for 1 h, washed again three times in PBS and stocked at 4°C. For immunofluorescence, cells were blocked with 5% BSA (Bovine Serum Albumin) in PBS for 1 h, washed three times in PBS and then incubated with specific primary antibody diluted in 0.5% BSA and 0.05% Tween 20 in PBS for 1 h at room temperature. The primary antibodies used were anti-HPV16 L1 (BD Pharmingen®) and anti-HPV16 L2 (provided by Prof. Dr. R. B. S. Roden, from Johns Hopkins University School of Medicine, Baltimore, MD, USA). After being washed in 1% BSA in PBS, cells were incubated with the respective secondary antibody diluted in 1.5% BSA and 0.01% Tween 20 in PBS for 1 h at room temperature. The secondary antibody used for L1 was Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes®, OR), for L2 detection we used Alexa Fluor® 633 goat anti-rabbit IgG. Then, the cells were washed in PBS, mounted on a slide in Mowiol (Calbiochem, CA) with PI (propidium iodide) for nuclear stain, and kept at 4°C protected from light until microscopic examination. LSCM was also used to confirm kinetic curves obtained by cytometry. All confocal analyses were conducted using a Zeiss LSM 510 Meta laser scanning microscope.

2.4 Transmission Electron Microscopy (TEM)
Non-transfected HEK293T cells were washed in PBS, pre-fixed with 2.5% glutaraldehyde, washed in 0.1 M phosphate buffer, post-fixed with 1% osmium tetroxide, rinsed in phosphate buffer, 0.85% NaCl and distilled water, incubated in 2% aqueous uranyl acetate, dehydrated in ethanol series (30%, 50%, 70%, 95% and 100%), ethanol-acetone (1:1), 100% acetone, infiltrated with acetone-Epon series (2:1, 1:1, and 1:2), embedded in pure Epon resin overnight at 4°C and in fresh Epon in gelatine capsules at 60°C for 72 hours to polymerization. Thin sections were mounted on carbon pre-coated grids and post-stained with uranyl acetate followed by lead citrate. The same protocol was used in our previous work [18].

For ultrastructural immunocytochemistry, transfected and co-transfected HEK293T cells were washed in PBS, washed in 0.1 M phosphate buffer, fixed in 3.5% sucrose, 4% paraformaldehyde, 1% glutaraldehyde in phosphate buffer, washed in phosphate buffer and in PBS. Then, cells were dehydrated in ethanol series (50%, 70%, and 80%), pre-embedded in 2:1 LR White acrylic resin and 70% ethanol, embedded in 100% LR White for 1 h, fresh resin overnight, embedded in fresh resin in gelatine capsules and allowed to polymerize at 60°C for 48 h. Blocks thin sections were mounted on carbon pre-coated grids, incubated with the same specific primary antibodies used in LSCM, secondary antibodies conjugated with colloidal gold particles (Sigma-Aldrich®) for immunolabeling of L1 (10 nm) and L2 (5 nm) and stained with 2% uranyl acetate. All TEM analyses were performed in MET Zeiss EM 109 operated at 80kV.

2.5 SDS-PAGE and Western Blotting
For SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis), samples were diluted in SDS-loading buffer (10:1), boiled for 5 min, centrifuged, separated by gel electrophoresis in 10% polyacrylamide gel, stained with Coomassie blue or transferred to nitrocellulose membranes. For western blotting, membranes were blocked overnight in 0.05% Tween 20, 5% non-fat milk in PBS, incubated with anti-L1 and anti-L2 for 1 h at room temperature. Then, membranes were washed in PBS and incubated for 1 h with peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin (IgG, Sigma-Aldrich®) diluted 1:10,000 in 5% non-fat milk in PBS. The L1 and L2 proteins were detected using Novex® ECL HRP Chemiluminescent Substrate Reagent Kit (Invitrogen).

2.6 Flow Cytometry
For establishing the most appropriate moment to harvest transfected cells for protein purification, we analysed the kinetic of the HPV16 L1 protein expression by flow cytometry. At each determined post-transfection time (0, 12, 24, 36, and 48 h) cells were rinsed in PBS, detached with 1 ml trypsin, resuspended in 2 ml D10, centrifuged at 800 rpm for 5 min at 4°C and rinsed in PBS. For fixation and permeabilization, cells were incubated for 15 min in 1% PFA, rinsed in PBS, incubated for 30 min in 0.1% Triton X-100 at room temperature and rinsed in PBS. For immunofluorescence cells were incubated with 3% BSA, 0.05% Tween 20 in PBS for 30 minutes, rinsed in PBS, incubated with the primary antibody anti-HPV16 L1 diluted in 3% BSA for 1 h, rinsed, incubated with the secondary antibody anti-mouse Alexa Fluor® 488 diluted in 3% BSA for 1 h and finally rinsed for analysis. We used BD FACSCanto™ II cytometer and data were analysed employing BD FACS Diva software.

3. Results and Discussion
The HEK293T cell lineage was selected for its high transfectability and because these cells constitutively express the large T-antigen of SV40. Thus, transfected plasmids containing the SV40 origin are replicated to a high copy number and, therefore, are highly expressed.
3.1 HPV16 L1 expression kinetic

The kinetic expression of HPV16 L1 protein study showed that the most appropriate moment to harvest the cells for protein purification is 12 h post-transfection, when 77.30% of the cells were expressing the L1 protein (Fig. 1). This result was confirmed by CLSM (Fig. 2), where the highest expression could be seen at 12 and 24 h post-transfection. After these experiments we decided to harvest the cells 12 h post-transfection for subsequent protein purification purposes.

![Fig. 1 HPV16 L1 kinetic expression. The highest expression is observed 12 h post-transfection.](image1)

![Fig. 2 HPV16 L1 kinetic expression by confocal laser scanning microscopy. Nuclear region in red (PI), L1 protein in green (Alexa Fluor 488). HEK293T non-transfected cells (A). Post-transfection times: 3 h (B), 12 h (C), 24 h (D), 36 h (E), and 48 h (F).](image2)

3.2 HPV16 L1 and L1L2 expression by CLSM

Highly efficient transfection of HEK293T by lipofection resulted in more than 85% of the cells expressing the proteins of interest. The L1 and L2 proteins were detected in the cell nucleus and cytoplasm of transfected and co-transfected cells (Fig. 3).
Fig. 3 HPV16 L1 and L1L2 expression by confocal laser scanning microscopy. Non-transfected HEK293T cells, nuclear region in red (PI) (A). HEK293T cells transfected with L1 plasmid, nuclear region in red (PI) and L1 proteins in green (Alexa Fluor 488) (B and C). 293T cells co-transfected with L1 and L2 plasmids (D and E). L1 in green, L2 in red (Alexa Fluor 633) (D). L1 in green, L2 in blue, and nuclear region in red (PI) (E).

3.3 HPV16 L1 and L1L2 expression by TEM

Firstly, we analyzed the non-transfected HEK293T cellular morphology by conventional method for transmission electron microscopy (Figure 4A). Secondly, we transfected and co-transfected HEK293T cells and observed the distribution of the recombinant L1 and L2 proteins expressed. Both proteins could be seen in the cell nucleus and cytoplasm. It was also possible to observe VLP clusters (Figure 4B-D).
Recombinant HPV16 L1 and L2 proteins were successfully expressed in HEK293T human cell cultures. Proteins were detected by TEM in nucleus and cytoplasm of transfected and co-transfected cells. It was also possible to indentify immunolabeled capsid-similar regions containing both L1 and L2 proteins, indicating that these proteins are self-assembling into VLPs made of the two structural HPV16 proteins. The localization of L1 and L2 in the same cellular regions was confirmed by CLSM. Detection of HPV capsid proteins in the cell nucleus and cytoplasm is in accordance with previous findings [18–20].

4. Conclusion

We successfully established an efficient system of HPV16 L1L2 VLP production for the development of a new generation prototype of HPV prophylactic vaccine. This candidate vaccine could embody public health programs, especially in developing countries where the prevalence of HPV-related cancer is a significant cause of morbimortality.

5. Perspectives

We are now testing different methods for purification of HPV16 VLPs made of L1 and L2 proteins. This prototype vaccine will be formulated with a natural adjuvant and tested in animals in order to evaluate its capacity to induce immune responses with antibodies specific for HPV16 and also for other types.

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