Interaction of HPV16L1L2 VLP with stem cells CD34+/CD117+ of the human amniotic fluid

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The detection of HPV DNA in the amniotic fluid of pregnant women raises the discussion around the possibility of alternative contamination pathways. The presence of distinct cell types in the amniotic fluid, including stem cells, has been described. The HPV capsid is composed of two structural proteins called L1 and L2, used in vaccine development. This study investigates the possibility of the HPV16 L1L2 VLP interaction with stem cells from human amniotic fluid. For this purpose, cell samples were obtained from amniotic fluid by transabdominal amniocentesis and interaction assays using immunofluorescence methods were analyzed by confocal microscopy, confirming this possible interaction. The possibility of HPV infection of stem cells from the hematopoietic precursor lineage could give support to the hematological pathway as a route of transmission, mainly in the mother-fetal transmission hypothesis.

Keywords HPV; VLP; L1 and L2 viral capsid proteins; stem cells; human amniotic fluid; cellular interaction; light microscopy; confocal microscopy; electron microscopy; public health.

1. Introduction

During pregnancy, the maternal-fetal fluid balance is critical, and the amniotic fluid is essential for fetal growth and development. Placenta is the interface between mother and fetus and has a key role in a successful pregnancy [1].

The amniotic fluid provides the fetus with a source of water, protects from physical trauma, allows for normal movements critical for anatomic development, and contributes to the development of the fetal organs like lungs [2].

The amniotic fluid obtained in early second trimester of pregnancy, around 15th weeks, contains different cell types (AFCs). The majority of the cells are comprised of differentiated cells originated from desquamated fetal skin, such as fibroblasts. The minor part, approximately 1% of the cells in amniotic fluid, is categorized as stem cells. These amniotic fluid stem cells are found in a precursor state [3-5], while the differentiated cells of amniotic fluid can be readily reprogrammed to a pluripotent stem cell stage [6]. These stem cells isolated from human amniotic fluid were named hAFSCs (human amniotic fluid stem cells) [3].

The Oct4 is a specific marker for embryonic stem cells. Over 90% of the hAFSCs express this marker, which has been associated with undifferentiated state maintenance and embryonic stem cells and germ cells pluripotency [7]. The hAFSCs also express SSEA4, CD34, CD117, CD105, SOX-2 and Nanog which are described as markers of embryonic stem cells, although not exclusively [8].

The Human papillomavirus (HPV) represent one of the most complex groups of pathogenic viruses, with around 120 types fully characterized [9]. They can be classified according to their potential for the development of neoplasia. The high-risk group is represented mainly by HPVs types 16, 18, 31 and 33, while in the low-risk group HPV 6 and 11 are the most significant [10, 11].

The HPV icosahedral capsid is composed of two structural proteins, L1 and L2. In the worldwide, HPV16 L1 VLP (virus-like particles) has been utilized in the prophylactic vaccine development due to its capacity to induce high immune response [12, 13] and the high stability of IgG anti-L1 in serum over time [14]. L2 can induce a low-titer of antibodies to a wide-range of divergent Papillomavirus types and species, suggesting the capacity to generate a pan HPV vaccine [15-17].

The oncogenesis of high-risk HPVs acts changing the cellular cycle of infected cells, leading to uncontrolled development, immortality and malignancy [18, 19]. Thus, they are considered the central causes of cervical cancer, and have recently been associated with head and neck, skin and lung cancers [20-22].
HPVs are exclusively intracellular pathogens, with a replication cycle dependent of the cell machinery [11]. The infection preferentially occurs in epithelial cells, however the presence of viral DNA has been observed in organic fluids as plasma [23, 24] and breast milk [25]. In addition, HPV’s DNA was found in tissues like peripheral blood [26, 27], umbilical cord blood [28] and placenta [19, 28]. Other studies have demonstrated the presence of viral DNA in amniotic fluid cells [29, 30].

The HPV presence in the amniotic fluid before the birth has generated a discussion around the possibility of the newborn contamination [31, 32]. Then, it was confirmed that caesarean born do not protected children from the mother-fetal transmission, suggesting the prenatal HPV transmission [33]. The newborn infection can induce recurrent respiratory papillomatosis (RRP), characterized by the periodic growth of benign lesions in the superior digestive tract, causing chronic dyspnoea, cough, and, in several cases, complete obstruction [34, 35].

So, can the presence and possible HPV interaction with amniotic fluid cells, including stem cells, carry the virus or its DNA? Trying to answer this question, the present study investigates the possibility of HPV VLP interaction with stem cells from human amniotic fluid.

2. Experimental procedures

All recommendations of the National Biosafety Law (CTNBio) and Bioethics were respected. This work was judged and approved by Human Research Ethical Committee from Real e Benemérita Associação Portuguesa de Beneficência – Hospital São Joaquim with the Protocol number: 369-08.

2.1. Amniotic fluid achievement and culture

The amniotic fluid samples were obtained from volunteers between the 15th and 19th weeks of gestation, by transabdominal ultrasound-guided amniocentesis (approximately 10-15 mL), from clinical recommendation. The samples were centrifuged at 1200 rpm for 20 minutes. The supernatant was discarded and the pellet was resuspended in 5-10 mL of DMEM (Dulbecco’s Modified Eagle’s Medium, Cultilab, Sao Paulo, BR) supplemented with 20% fetal bovine serum (FBS, Cultilab, Sao Paulo, BR) – D20. The cells were cultivated in tissue culture plastic flasks (TPP®) at 37°C and 5% of CO2. The medium was replaced every 3-5 days. When cultured cells reached 50-80% confluence, they were washed in PBS (phosphate-buffered saline) and detached using trypsin (Solution Trypsin/EDTA 2.5g/L, Cultilab, Sao Paulo, BR). Then, cells were counted, the cell viability was analyzed and they were platted on glass coverslips for interaction assays and immunofluorescence.

2.2. Rosenfeld method

The cells were cultivated on coverslips, fixed in 2% paraformaldehyde (PFA) and stained with the Rosenfeld’s dye (0.97g Giemsa, 0.53g May-Grünwald, 1L methanol), a modified Giemsa-May-Grünwald formulation [36]. Then, they were washed in PBS; diluted dye in distilled water (1:1) was added for 10 minutes, followed by washing in distilled water. Coverslips were placed on a slide for microscopy analysis in Leica DMI. Images were obtained by 20 x / 0.30 objective lens, with digital camera LG.

2.3. Interaction assay

The VLPs were produced as described in [37] and the interaction assays were based in [38]. Cultured cells of the amniotic fluid were washed in PBS, HPV16 L1 VLPs were added and incubated for 4 hours at 37°C with 5% CO2 in DMEM medium without FBS or antibiotics, followed by further wash and then prepared for immunofluorescence assay.

2.4. Immunofluorescence

Cultured cells were washed 3 times in PBS and fixed with 2% PFA in PBS for 1 hour at 4°C. Then, they were washed in PBS and incubated with PBS+5% BSA for 1 hour. Then, cells were washed and incubated with primary antibodies previously diluted in PBS containing 0.01% Tween 20 and 0.5% BSA, pH 8.0, for 1 hour at room temperature, followed by washings and incubation with secondary antibodies diluted in PBS containing 0.01% Tween 20 and 1.5% BSA, pH 7.2, for 1 hour under slow agitation at room temperature. Finally, samples were washed and-round glass coverslips were mounted onto slides with Mowiol and kept at 4°C until confocal laser scanning microscope examination. All analysis was conducted using a Zeiss LSCM 510 Meta.

2.5. Transmission electron microscopy

Samples were adsorbed onto carbon-coated grids and negatively stained with 2% uranyl acetate. Grids were allowed to air-dry prior to examination by Zeiss EM 109 transmission electron microscope operated at 80 kV. Micrographs were taken at various magnifications [39].
2.6. Ultrastructural immunocytochemistry
Ten-microliter drops containing the purified HPV16 L1L2 VLPs were adhered on 2% parlodion and carbon-coated nickel grids for 10 min at room temperature. Then, they were incubated for 1 h at room temperature in a humid chamber with a primary mouse monoclonal neutralizing antibody against L1 protein from HPV16 (#C65315M, Biodesign International), and a primary rabbit antibody against L2 protein from HPV16 (provided by Prof. Dr. R.B.S. Roden, from Johns Hopkins University School of Medicine, Baltimore, MD, USA), diluted in PBS / 1.5% BSA / 0.01%Tween 20, pH 7.2.

After rinsing in PBS/1% BSA, samples were incubated for 30 min at room temperature in a humid chamber with a secondary antibody goat anti-mouse IgG complexes with 10-nm gold particles (Sigma), for L1 and goat anti-rabbit IgG complexes with 5 nm gold particles (Sigma) for L2, diluted in PBS/0.5% BSA/0.05% Tween 20, pH 8.0, rinsed in PBS and distilled water, contrasted with 2% uranyl acetate for 5 min, and examined with a Zeiss EM 109 transmission electron microscope operated at 80 kV [40].

3. Results and discussion

3.1. Amniotic fluid cells culture
In primary culture it was possible to observe different cell types: fibroblast-like cells and blast cells similar to stem cells. The cultivation period was about 20-25 days (Fig. 1a-b). Apparently, the AFCs were efficiently adapted to in vitro growth, and demonstrated high rate of viability.

By staining with Rosenfeld’s dye, it was possible to identify different morphological cell types present in the amniotic fluid cultured cells (Fig. 1c). Fibroblast-like cells with basophilic cytoplasm, whereas the blast stem cells-like was characterized by acidophilic cytoplasm and large basophilic nuclei.

![Figure 1](image_url) – Amniotic fluid cells cultivated by 10 days (a) and 25 days (b) in DMEM medium supplemented with 20% FBS, at 37°C and 5% CO2. (c) The Rosenfeld method show differences in the morphology between both cellular types present in cultured amniotic fluid cells, Fibroblast-like cells (blue arrow) and blast stem cells-like (red arrow).

3.2. Production and immunolabeled of HPV16 L1L2 VLPs
The HPV16 L1L2 VLPs were produced in HEK293T cells expressing the vectors pUF3/L1h and pUF3/L2h transiently transfected. The expression was highly efficient and the VLPs were clarified and quantified as shown in [37]. Ultrastructural immunocytochemistry was performed to confirm the expression of L1 and L2 proteins.
3.3. hAFSCs and VLPs interaction

The cultured amniotic fluid cells were challenged by interaction tests with VLPs to verify this hypothesis. As shown in the next figures, the interaction is possible and was confirmed in immunofluorescence analyzed by confocal microscopy. This interaction can occur with L1 (Fig. 3) and L2 (Fig. 4) proteins.

Once confirmed the interaction between amniotic fluid cells and VLPs of HPV16, additional analyses were performed to observe what kind of cells in the amniotic fluid interacts with VLPs. For this, immunofluorescence assays were carried out with stem cells specific markers like CD34, CD117, Oct4, and SSEA4.

The CD34⁺, CD117⁺, Oct4⁻ and SSEA4⁺ stem cells interacted with the HPV16 L1L2 VLPs and the internalization was confirmed by LSCM (Fig. 5 and 6; 7 and 8; 9 and 10; 11 and 12, respectively). The negative controls confirmed the specificity of the secondary antibodies used, confirming the quality of the tests and the expression of the antigens analyzed.
Figure 5 – Analysis of L1 VLPs internalization in CD34+ stem cell hematopoietic progenitor (a) L1 detected by primary antibody anti-L1 (Biodesign), followed by secondary antibody FITC anti-mouse IgG 
(BD, green). Figure 6 – Study of the internalization of L2 VLPs in CD34+ stem cells. (a) L2 was labeled with primary antibody, followed by secondary antibody AlexaFluor® 488 anti-rabbit (Invitrogen, green). In figures 5 and 6, (b) CD34 receptor detected by respective primary antibody (Caltag), followed by secondary antibody AlexaFluor® 647 anti-mouse IgG (Invitrogen, red); (c) transmitted light channel; (d) corresponds to the overlap of the images, except the channel of transmitted light.

Figure 7 – Analysis of L1 VLPs internalization in CD117+ stem cell hematopoietic progenitor (a) L1 detected by primary antibody anti-L1 (Biodesign), followed by secondary antibody FITC anti-mouse IgG 
(BD, green). Figure 8 – Internalization of L2 VLPs by CD117+ stem cells hematopoietic progenitor. (a) L2 was labeled with primary antibody, followed by secondary antibody Alexa Fluor® 488 anti-rabbit (Invitrogen, green). In the figures above, (b) CD117 receptor detected by primary antibody (Caltag), followed by secondary antibody Alexa Fluor® 594 (Invitrogen, red). In both figures, (c) transmitted light; (d) corresponds to the overlap of the images except the channel of transmitted light.
With the confirmation of the presence of viable stem cells in the amniotic fluid, we can consider about the use of these cells as a new therapeutic strategy, as has occurred in Brazil and all over the world. This new tool for stem cell directed to differentiate into specific types offers the possibility of a renewable source of cells and tissues in order to spare those for the treatment of various diseases [7].

The possibility of the HPV16 to infect stem cells from hematopoietic precursor lineage could give support to the hematological way as possibility of infection, not considered in public health nowadays, mainly in the mother-fetal
transmission hypothesis of HPV DNA sequences. In addition, it stimulates a reflection about a potential application of these cells for fetal therapy and tissue enginery.

4. Conclusion

In this study, CD34+/CD117+/Oct4+/SSEA4+ stem cells lineage of the amniotic fluid interacted with HPV16 L1L2 VLP in vitro. New experiments are being conducted for further confirmation of these cells interaction with HPV16, as an alternative pathway of infection, in the mother-fetal transmission. Also, the relevance of this study was clearly demonstrated, going against the prospect of new therapeutic strategies using stem cells in cell therapy, without considering the possibility of the HPV presence.

The virus-cell interaction represents the initial step of viral infection; therefore the prevention of virus attachment would prevent the entry process. Thus, the study of interaction and inhibition of interaction is important for a better understanding of this pathology, as well as to the development of a strategy to combat the papillomatosis and HPV associated cancers.

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