Bovine papillomavirus productive infection in cell cultures: First evidences

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Abstract

Bovine papillomavirus (BPV) is the etiological agent of bovine papillomatosis (BP), infectious disease, characterized by the presence of multiples papillomas that can regress spontaneously or progress to malignances. Although recognized as mutagen, BPV action following cancer initiation remains few explored, since studies about cancer progression and metastasis are based on cell cultures. The lack of attention to in vitro models is a reflection of the papillomavirus replication paradigm, which is dependent of epithelium cell differentiation. Since 2008, we have explored the potential of cell lines derived from BPV-infected neoplasms as model to study the oncogenic process. In this study, we described BPV productive infection in cell lines derived from cutaneous papilloma, fibropapilloma and esophageal carcinoma (EC) in which BPV DNA sequences were previously detected by PCR. Considering that the immunodetection of L1 capsid protein is the main evidence of productive infection, we analyzed the expression of this protein by immunofluorescence and flow cytometry. Results showed the immunodetection of L1 protein in cell lines derived from cutaneous papilloma, fibropapilloma and EC, but not in cells derived from BPV-free normal skin. We also observed the presence of spherical and electron-dense particles, with 41.02-61.94 nm diameter in cytoplasmic vesicles of cells in the sixth passage of cutaneous papilloma, fibropapilloma and EC, being compatible with the expected BPV morphology. Cells derived from BPV-free normal skin, in turn, showed membranous particles up to 75.00 nm not compatible with BPV morphology. These results suggest the BPV productive infection in cell lines derived from BPV-infected neoplasm, reinforcing that these cells are useful models to study the viral biology and pathogenesis.

Highlights

- Bovine papillomavirus (BPV) cause multiples papillomas that can regress or progress to malignances;
- BPV action following cancer initiation remains few explored;
- Identification of BPV L1 capsid protein and virion-like particles in cytoplasmic vesicles of cell lines derived from BPV-infected cutaneous papilloma, fibropapilloma and esophageal carcinoma;
- Cell lines derived from BPV-infected neoplasm can be considered useful model to study the viral biology and pathology.

Introduction

Bovine papillomavirus (BPV) is the etiological agent of bovine papillomatosis (BP), infectious disease, characterized by the presence of multiples papillomas that can regress spontaneously or progress to malignance in the presence of co-factors [1-8]. BPV is a cosmopolitan virus, being present in all continents [1,9], leading to important economic losses worldwide [10]. About 60% of Brazilian cattle herd are infected by BPV [2]. However, this percentage can be greater, since the virus can lead to asymptomatic infections [11,12].

Currently there are 15 BPV types described, which are classified in four genres: **Deltapapillomavirus** (BPV-1, 2, 13 and 14), **Epsilonpapillomavirus** (BPV-5 and 8), **Xipapillomavirus** (BPV-3, 4, 6, 9, 10, 11, 12 and 15) and **Dyoxipapillomavirus** (BPV-7) [13–15]. The **Xipapillomavirus** infects epidermis, causing true papillomas, while **Delta** and **Epsilonpapillomavirus** (BPV-5) can infect both epidermis and dermis, resulting in fibropapillomas [16–18]. Although the papillomaviruses (PVs) are recognized as specie-specific, BPV is able to infect felines [19], buffaloes [20–22], giraffe [23,24], tapirs [25], zebra [26], yaks [27,28] and horses [29–31]. In equines, BPV is the causative agent of sarcoid, invasiveness but non-metastatic fibroblastic benign neoplasm [32] that affects 11.5% of horses worldwide [33]. Due to the ability to infect different species and in function of morphological and pathogenic similarities with human papillomavirus (HPV), BPV is considered a useful model to study the HPV-associated oncogenic process [8,34–36].

Although recognized as oncogenic viruses by induce mutation [2,11,37–40] and transformation [41–43], the action of BPV following cancer initiation remains unclear [36]. The reason for this is the absence of in vitro virus replication and, therefore, the lack of models

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Based on cell culture systems [44], which are mandatory to study the BPV action in cancer progression and metastasis [36]. This occurs because, according to the BPV natural history, the viral replication is dependent on cell differentiation [45–47]. Considering the paradigm of PVs replication cycle, the expression of capsid proteins (L1 and L2) and viral assembly are only verified in most differentiated epithelial layers (hypergranulosis) [48–50]. Due to this paradigm, little attention has been given to the primary cultures derived from BPV-infected tissues as model to study the pathogenic mechanism of PVs. However, in last decades, studies have described the presence of BPV DNA sequences, transcripts, proteins and virus-like particles in sites before not recognized as permissive to productive infection due to the absence of cell differentiation, including peripheral blood [51,52] and placenta [53]. Similar results have been also described in humans, in which sequences of HPV were verified in blood and semen [54,55]. In addition, Cerqueira et al. [47] currently demonstrated the cell-free assembly HPV-16 capsid. These results indicate the need to review the PVs natural history, as proposed by Munday [34].

The in vitro models, based on cell culture systems, have been extensively employed to study the oncogenic potential of PVs oncoproteins (E5, E6 and E7) or evaluate the interaction of virus with host cell, as showed in Table 1. Results of these studies demonstrate that these systems mimic several phenotypes observed in vivo (Table 2), reinforcing the potential of these cell cultures as useful models to study the pathogenic mechanism of PVs. In this sense, since 2008, our group has demonstrated the potential of cell lines derived from BPV-infected neoplasm as model to study the cytogenetic [40,56] and biochemical alterations induced by BPV [36,57]. Currently, we reported the maintenance of BPV DNA sequences during six passages of cell lines derived from cutaneous papilloma, fibropapilloma and esophageal carcinoma, suggesting an in vitro productive infection [57]. We also verified that these cell lines present a stem-cell and migratory biomarker phenotype acquisition, suggesting that in vitro systems are useful models to study the metastasis [58]. Despite these data, cell lines derived from BPV-infected cutaneous papilloma, fibropapilloma and esophageal carcinoma remains considered as non-permissive to productive infection. Based on this, we investigated the expression of BPV L1 capsid protein and the presence of virus particle by electron transmission microscopy in order to evaluate a possible productive infection in in vitro systems.

Material and methods

Ethics statement

This study was approved by the Ethic Committee on Animal Use of São Paulo Federal University (UNIFESP, process 1829/09).

Primary cell culture establishment

Three samples of skin papilloma were collected from three adult bovines showing bovine papillomatosis (Bos taurus, Simmental breed). One fragment of normal skin, without morphological alteration, was collected from 8 months aged. Samples of cutaneous papilloma, fibropapilloma and esophageal carcinoma were collected from adults presenting clinical symptoms of esophageal carcinoma. Tissue samples were collected by a veterinarian. Samples were washed in PBS with 2% ammonotericin B and 3% penicillin/streptomycin (Cultilab, Brazil) and transported to Genetics Laboratory of Butantan Institute in Dulbecco medium (DMEM), supplemented with 2% ammonotericin B and 3% penicillin/streptomycin (Cultilab, Brazil).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Results</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Keratinocyte</td>
<td>Viral suspension addition in bovine epithelium cells promotes cell transformation and acidification</td>
<td>[93]</td>
</tr>
<tr>
<td>Papilloma and fibropapilloma</td>
<td>Verified cytogenetic aberration in chromosomes 8 and 14 of BPV1.69 transgenic mouse-derived papilloma</td>
<td>[94]</td>
</tr>
<tr>
<td>Papilloma, fibropapilloma, urinary bladder and esophageal carcinoma</td>
<td>Establishment of cell lines and identification of BPV</td>
<td>[56]</td>
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<tr>
<td>Papilloma</td>
<td>Cytogenetic aberration</td>
<td>[40]</td>
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<td>Primary culture establishment</td>
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Each sample was divided in three fragments, which were destined to: (1) primary cell culture establishment, (2) BPV molecular identification and (3) histopathological analysis. For primary cell culture establishment, the tissue was fragmented mechanically, using a sterile scalpel, which were washed three times PBS with 2% ammonotericin B and 3% penicillin/streptomycin. Tissue fragments were treated with 0.01% collagenase (Sigma, Germany) at 37°C for 15 minutes. The enzymatic product digestion was transferred to three culture flasks of 25 cm², containing 5 mL of DMEM medium supplemented with 10% of fetal bovine serum (FBS) and 1% of penicillin/streptomycin (complete medium) at 37°C. Culture flasks were incubated at 37°C, with 5% CO₂ atmosphere until a confluence of 80%. From this step, cells were treated with trypsin solution and expanded in culture flasks. One culture flask of each cell lineage obtained was cryopreserved in

Table 1. Summary of studies involving cell lines and papillomavirus. Studies based on BPV/HPV gene transfection.

Table 2. Summary of studies involving cell lines and papillomavirus. Studies based on BPV-infected cell lines.

freezing medium (70% DMEM, 10% dymethylsulphoxide and 20% FBS) and stocked at -196°C. These cell lines are part of biological collection of Genetics Laboratory. Cell employed in this study were cultivated until sixth passage (P1-P6) and subjected to morphological analysis by phase contrast using the Nikon Eclipse Ti (Nikon, Japan) inverted microscopy. Images were acquired using the NIS-Elements Br version 3.0 (Nikon, Japan) in total magnification of 100 and 200X. The molecular identification of BPV DNA sequences were performed by PCR using specific primers for BPV-1, 2 and 4, the most frequent virus types verified globally. PCR results are available in Araldi, et al. [57].

Analysis of viral proteins

Immunofluorescence

A total of 1 x 10^6 cells were seeded per well, employing a six-well plate, containing 2 mL of complete DMEM medium and using a 24 x 24 mm sterile cover slip. Cells were incubated at 37°C, with 5% CO2 atmosphere, until the confluency of 80% (about 24 hours). Medium was removed and cells were washed three times with sterile PBS for 5 minutes. Cells were fixated with 4.0% formalin, diluted in PBS, at 4°C for 30 minutes and then washed three times with PBS for 5 minutes. Cells were permeabilized with 0.01% Triton X-100 (Sigma, Germany), diluted in PBS, at 4°C for 10 minutes. Cells were washed once with PBS and incubated overnight at 4°C with anti-L1 [BPV-1/H8 + CAMVIR] (Abcam, Cambridge, UK) at a 1:100 dilution in 1% of BSA. Cells were washed three times with PBS under described conditions and then incubated at 4°C for 3 hours with anti-mouse IgG-FITC (Sigma, Germany), at dilution of 1:100 in 1.0% of BSA. A cutaneous papilloma 01 cell line, incubated only with secondary antibody, was used as negative control. Cells were washed three times with PBS and cover slips were mounted on slides, using 20 μL of ProLong Gold (Invitrogen, Carlsbad, USA) with DAPI. Slides were analyzed in Axio Scope A1 fluorescent microscope (Carl Zeiss, Germany) under total magnification of 400X. This analysis was performed in third passage (P3).

Flow cytometry

Cell lines were seeded in culture flasks of 25 cm2 with 5.0 mL of complete DMEM medium. Cells were subjected to monolayer disaggregation with 2 mL of Trypsin solution, centrifuged at 400 x g for 5 minutes. Cells were transferred to 1.5 mL polypropylene tubes with PBS, fixed in 1.0 mL of 1.0% formalin solution at 4°C for 2 hours. The material was centrifuged under described conditions, and washed twice with 1.0 mL of PBS at 4°C to remove the formalin residues. Cells were incubated with 1.0% BSA at 4°C for 20 minutes, washed once with PBS, and incubated overnight at 4°C with primary antibodies showed in table 3. Cells were centrifuged under described conditions and washed twice with PBS at 4°C. The material was incubated at 4°C for 2 hours with anti-mouse IgG1 conjugated with Alexa Fluor 488 secondary antibody (Invitrogen, Carlsbad, USA) at 1:200 dilution. Next, cells were washed with PBS, centrifuged under described conditions, and resuspended in 100 μL of PBS. The material was analyzed in FACS Calibur (BD Bioscience, USA), employing the CellQuest software (BD Bioscience, USA). A total of 10,000 events were analyzed using the FlowJo software (TreeStar, Oregon, USA). Analysis were based on the percentage of immunostained cells. Cutaneous papilloma 01 cell line incubated with only secondary antibody was used as control. Analysis were performed in third passage (P3).

Electron microscopy (EM)

Cell lines were seeded in a six-well plate, containing a sterile cover slip/well, with 2 mL of complete medium until forming a confluence of 80–90%. Medium was removed by aspiration and cells were fixated with 2.5% glutaraldehyde buffer for one hour at 4°C. The material was washed five times with cacodylate buffer for two minutes/wash. Cells were incubated with 1% osmium tetroxide (OsO4), diluted in 0.1 M cacodylate buffer, containing 3 mM CaCl2 and 0.8% potassium ferrocyanide, for 30 minutes at 4°C. The material was washed five times with ultrapure water and, later, contrasted with 2% uranyl acetate for 1 hour at room temperature. Cells were washed with ultrapure water and dehydrated with increasing concentrations of alcohol (20%, 50%, 70%, 80%, 90%, and 100%) at 4°C for three minutes. The material was subjected to additional dehydration with absolute ethanol at room temperature for three minutes. Cells were embedded in 1:1 solution of Epon-ethanol, under agitator, for 30 minutes at room temperature. We performed four complete exchanges of Epon resin with intervals of one hour except for an overnight interval, which was the last one. The material was transferred to 60ºC for 72 hours, to polymerize the resin. Blocks were subjected to ultrafine cuts, which were analyzed in JEM-2100 transmission electron microscopy (JEOL Solution, USA). Morphometric analysis was performed using the AxioVision 4.9.1 software (Carl Zeiss, Germany). This analysis was performed in sixth passage (P6) to confirm the presence of virus particles. Morphometric analysis was performed using the AxioVision version 4.1.9.0 software (Carl Zeiss, Germany). Statistical analysis was performed based on medium diameter of electron-dense spherical particles detected by EM.

Results

Histopathological analysis of tissue fragments

Histopathological analysis of BPV-free normal skin, used as control, showed the absence of morphological alterations, presenting both epidermis and dermis preserved (Figure 1). Cutaneous papilloma and fibropapilloma samples showed morphological alterations in epidermis compatible to those verified in BPV infection: hyperkeratosis, koilocytosis, acanthosis and hypergranulosis (Figure 1). Fibropapillomas showed a fibroelastic dermis, with extensive fibroblastic proliferation (Figure 1), which was not verified in cutaneous papilloma (Figure 1). Esophageal carcinoma sample showed a tissue disorganization, with the presence of mitotic and pleomorphic cells, presenting hydropic degeneration in spinous layer (Figure 1). It was also observed the presence of transformed cell islands into dermis (Figure 1), characterizing an esophageal epidermoid carcinoma.

Morphological analysis of primary cell cultures

Results showed the presence of both epithelioid and fibroblastoid cells in primary cultures of BPV-free normal skin, skin papilloma,
fibropapillomas and esophageal carcinoma (Figure 1). However, we verified the prevalence of fibroblastoid cells in primary culture of BPV-infected tissues, but not in normal skin (Figure 1).

Analysis of BPV L1 protein expression

We verified the expression of L1 proteins in primary cell cultures of cutaneous papilloma (papilloma 01), fibropapilloma (papilloma 02 and 03) and esophageal carcinoma, but not in BPV-free normal skin cells (Figure 2). Results of immunofluorescence showed the nuclear and cytoplasmic labelling of BPV L1 capsid protein (Figure 2). These results were confirmed by flow cytometry, which showed high levels of L1 expression specially in cell lines derived from cutaneous papilloma (papilloma 01), fibropapilloma (papilloma 02 and 03) and esophageal carcinoma (Figure 2). Negative controls of both immunofluorescence and flow cytometry showed the absence of labelling (Figure 2).

Identification of BPV-like particles in primary cell cultures

Results of electron microscopy showed the presence of electron-dense spherical structures with 41.02-61.94 nm and icosahedral morphology, present in cytoplasmic vesicles of cutaneous papilloma, fibropapillomas and esophageal carcinoma cells (Figures 3), but not in BPV-free normal skin cells (Figures 4 and 5). These structures are compatible with the expected morphology of BPV particles [51,59].

Discussion

Since 2008, our group have explored the potential of both primary cell cultures and cell lines derived from primary cell cultures of BPV-infected benign and malignant neoplasms as model to study both natural history and pathogenic mechanism of BPVs [56–58]. In 2013, we described the presence of cytogenetic aberrations in primary cell cultures derived from BPV-infected cutaneous, urinary bladder and esophageal papilloma [40] similar to those verified in lymphocyte short-term culture of BPV-infected animals [2,39]. Cytogenetic damages was also detected in primary cell cultures derived from BPV-infected bovine, equine and canine papillomas [60]. Using cell lines derived from primary cultures of BPV-infected cutaneous papilloma, fibropapilloma and esophageal carcinoma, the same cell lines employed in this study, we showed that virus infection promotes metabolic deregulation, leading to oxidative stress as a consequence of pro-oxidant action of BPV-1 E6 oncoprotein [57]. Currently, we also verified that cell lines derived from BPV-infected neoplasms acquire a stem-cell-like and migratory biomarker phenotype acquisition [58]. Despite these data show the potential of BPV-infected cell lines and/or primary cultures as model to study the viral biology and pathology, especially on oncogenesis, up to date the in vitro systems remains considered as not able to develop a productive infection. This is a reflection of viral replication paradigm, which states that the BPV replication is dependent of epithelial cell differentiation, since the expression of capsid proteins (L1 and L2) is restrict to the most differentiated epithelium layers [8,61].

However, in last years it was verified that presence of BPV DNA sequences in different sites not passive of cell differentiation, including urine, spermatozoa [62], blood [2,11,39,63–65] and placenta [53]. Considering that the L1 protein expression is verified in sites of viral assembly, once this protein is able to self-organize in pentameric structures that composes the BPV capsid [66], L1 immunodetection is pointed out as the main evidence of productive infection [51,67]. Based on BPV DNA sequences identification and L1 immunodetection, Roperto et al. [52] and Melo et al. [51] described the productive infection in lymphocytes and, later in placenta [53].

Considering that cell lines derived from BPV-infected neoplasm are able to mimetic the in vivo viral pathology, verified by the cytogenetic aberrations [40], DNA damages, metabolic deregulation
Figure 2. Immunodetection of L1 capsid protein by immunofluorescence and flow cytometry. A) Immunofluorescence analysis showing: absence of unspecific labeling in cells derived from cutaneous papilloma only incubated with the secondary antibody conjugated with FITC (control); absence of labeling and, therefore, L1 expression in BPV-free normal skin cells; cytoplasmic and nuclear immunodetection of L1 protein in cells derived from cutaneous papilloma (papilloma 01), fibropapilloma (papilloma 02 and 03) and esophageal carcinoma. Flow cytometry analysis showing absence of unspecific labeling in cells derived from cutaneous papilloma only incubated with the secondary antibody conjugated with Alexa Fluor 488 (control), absence of L1 expression in normal skin cells; immunodetection of L1 protein in cells derived from cutaneous papilloma (papilloma 01), fibropapilloma (papilloma 02 and 03) and esophageal carcinoma. B) Cell percentage expressing L1 protein. C) Median of fluorescent intensity (MFI) of L1 expressing cells. Total of 10,000 events analyzed. Cells in third passage (P3).
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Figure 3. Electron micrographs showing spherical and electron-dense particles observed in: A) cytoplasm of cutaneous papilloma cells (papilloma 01), cytoplasmic vesicles of fibropapillomas (B – papilloma 02 and C – papilloma 03) and D) esophageal carcinoma. Analysis performed in the sixth passage (P6).

Figure 4. Electron micrographs of BPV-free normal skin cells showing the absence of virion-like particles into the nucleus and the presence of membranous vesicles with diameter up to 75.00 nm not compatible with BPV virion morphology. Analysis performed in the sixth passage (P6).

[57] and migratory phenotype acquisition [58] in this study we investigated the L1 expression and the presence of BPV particles in cell lines derived from BPV-infected cutaneous papilloma, fibropapilloma and esophageal carcinoma, in which we previous described the maintenance of DNA sequences of BPV-1, 2 and 4 [57]. We detected the nuclear and cytoplasmic labelling of L1 protein in cutaneous papilloma, fibropapilloma and esophageal carcinoma cells, but not in BPV-free normal skin cells (Figure 2). These results were confirmed by flow cytometry (Figure 2), suggesting the in vitro viral assembly. This because, considering the nature obtaining maximum effect for lower energy cost, it will not expect the L1 expression in primary cell cultures if there was not viral assembly.

Once the viruses are smaller than their host, the electron microscopy (EM) is recognized as the best method to identify BPV particles [59], since this technique allows to analyze the ultrastructure with high resolution [68]. However, with the advent of molecular biology, the PCR became the most used method to viral identification. But, the PCR does not allow to identify viral particles, making the EM a mandatory method to demonstrate the productive infection. Based on these data, the different cell lines, in the sixth passage (P6), were subjected to EM analysis. Under EM, BPV virions are visualized as non-enveloped isometric particles, with electron-dense spherical morphology and a diameter of 45-60 nm [51,59,69].
Figure 5. Morphometric analysis of particles detected by electron microscopy of different cell lines. Results show the presence of particles with a diameter of 41.02-61.94 nm in all cell lines derived from BPV-infected neoplasms. Cells from normal skin show the presence of particles with diameter up to 75.00 nm, not compatible with the BPV virion morphology.

EM results showed the presence of electron-dense spherical structures, with isometric morphology and a diameter among 41.02 to 60.94 nm, compatible with BPV particles (Figure 3). These structures were observed in cytoplasmic vesicles of primary cell cultures derived from cutaneous papilloma, fibropapillom and esophageal carcinoma (Figure 3). BPV-free normal skin cells showed the presence of spherical structures, dispersed in cytoplasm, with envelope and diameter higher to 75.0 nm (Figures 4 and 5), being non-compatible with BPV particles. These results are strong evidences of productive infection in primary cells cultures.

BPV viral assembly in cultures cells are plausible, once different of HPV, BPV does not integrate with host genome, being verified in epistol form. Thus, the virus remains its complete genome, being able to codify all proteins, including L proteins, responsible for the viral assembly. In conclusion, our data suggest for the first time the BPV productive infection in in vitro. These results indicate that primary cell cultures derived from BPV-infected lesion can be useful models to study the oncogenic mechanism of these viruses.

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