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Bovine herpesvirus type 5 replication and induction of apoptosis *in vitro* and in the trigeminal ganglion of experimentally-infected cattle

Daniel E. Rensetti[a], Maia S. Marin[b,c], Pedro E. Morán[a], Anselmo C. Odeón[c], Andrea E. Verna[b,c], Sandra E. Pérez[d,*]

[a] Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Paraje Arroyo Seco S/N, Tandil, 7000, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Rivadavia 1917, C1033AAJ, Buenos Aires, Argentina
[c] Instituto Nacional de Tecnología Agropecuaria (INTA), Estación Experimental Agropecuaria Balcarce, Ruta 226 Km 73.5, 7620, Balcarce, Buenos Aires, Argentina
[d] Centro de Investigación Veterinaria de Tandil (CIVETAN)-CONICET, Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires, Paraje Arroyo Seco S/N, Tandil, 7000, Argentina

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ABSTRACT

Bovine herpesvirus (BoHV) types 1 and 5 are neuroinvasive. Cases of BoHV-1-induced encephalitis are not as frequent as those caused by BoHV-5. In this study, the capability of BoHV-5 to induce apoptosis in cell cultures and in the trigeminal ganglion during acute infection of experimentally-infected cattle was analyzed. Apoptotic changes in cell cultures agree with the ability of the viral strains to replicate in each cell line. Marked differences were observed between the *in vitro* induction of apoptosis by BoHV-1Cooper and BoHV-5 97/613 strains. Apoptotic neurons were clearly evident in the trigeminal ganglion of BoHV-1-infected calves. For BoHV-5 a fewer number of positive neurons was observed. There is an association between the magnitude of bovine herpesviruses replication and the induction of apoptosis in trigeminal ganglion. These findings suggest that the induction of apoptosis and the innate immune response orchestrate the final outcome of alpha herpesviruses infection of the bovine nervous system.

1. Introduction

Bovine herpesvirus types 1 (BoHV-1) and 5 (BoHV-5) are two closely related alpha-herpesviruses that infect cattle. BoHV-1 causes significant economic losses to the cattle industry worldwide [1] since it is responsible for a variety of clinical syndromes, including respiratory disease, conjunctivitis, abortion and genital infections. Acute infection of the respiratory tract by BoHV-1 causes immunosuppression, leading to secondary bacterial infections, pneumonia and death [2]. BoHV-5 is highly prevalent in South America, and it is the primary etiological agent of non-suppurative meningoencephalitis in calves [3], a condition which is usually fatal [4]. Although both viruses are neuroinvasive, cases of BoHV-1-induced encephalitis are not as frequently reported as those caused by BoHV-5. The reasons why they differ in their ability to cause neurological disease have not been completely elucidated.

Herpesviruses' life cycle is characterized by stages of acute infection, latency and reactivation. Sensory neurons of the trigeminal ganglion (TG) are the main site of latency of alpha-herpesviruses [5]. During latency, viral gene expression is restricted to the latency-related (LR) gene [6]. Sporadic virus reactivation from latency can occur under conditions of natural stress or it can be experimentally induced by glucocorticoids administration. These episodes of reactivation are the main source of virus dissemination [5]. BoHV-1 and BoHV-5 share a high level of amino-acid identity, particularly in those proteins involved in viral DNA replication. However, Delhon et al. [7] reported marked differences in the coding and transcriptional regulatory regions of the LR gene. Previous studies [8,9] have demonstrated that BoHV-1 LR products have anti-apoptotic activity *in vitro* and *in vivo*. The anti-apoptotic activity of the LR gene is required to reach a high number of latently-infected neurons available for reactivation. Recently, Silvestro and Bratanich [10] have shown that components which are essential for the anti-apoptotic functions of BoHV-1 LR gene are not expressed in BoHV-5-infected-cells, suggesting that, at least *in vitro,* BoHV-1 LR gene may have roles different from the homologous BoHV-5 gene.

The purpose of this study was to analyze the capability of BoHV-5 to induce apoptosis in cell cultures and in the trigeminal ganglion of acutely-infected calves, as differences in the apoptotic potential of BoHV-5 and BoHV-1 might be related to the distinct features of their neuropathogenesis.

* Corresponding author.
E-mail address: seperez@vet.unicen.edu.ar (S.E. Pérez).

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2. Material and methods

2.1. Viruses

For *in vitro* assays and animal inoculations, the reference BoHV-1 Cooper strain and the field BoHV-5 isolate 97/613 were used. Furthermore, infection of cell cultures was also performed with the strain A663 of BoHV-5. This strain was considered as a BoHV-5 subtype *b* strain, a subtype only described in Argentina [11]. Recently, it was demonstrated that strain A663 is indeed a natural, field recombinant virus between BoHV-5 and BoHV-1 [12] which was isolated from a case of bovine encephalitis [11]. From here on this strain will be designated as BoHV-5 nrecA663. BoHV-5 strain 97/613 is classified as subtype *a* BoHV-5 strain [10], which was isolated from the brain of a 2 year-old Brangus cow with neurological signs. This strain has been previously characterized [3]. Titers of virus stocks for experimental inoculations and virus titers for the different bovine herpesviruses strains in each cell line at 6 and 24 hpi were determined by the end-point titration method, in 96 well-plates and expressed as $TCID_{50}$/ml, according to Reed and Müench [13]. Determination of virus titers in the different cell cultures was performed by triplicate.

2.2. Experimental inoculation of cattle

All procedures for animal handling and experimentation were performed according to the Animal Welfare Committee of the University of the Center of Buenos Aires Province (Res. 087/02). Five BoHV-1- and BoHV-5-free and seronegative cross-bred, 1 year-old calves were used. Calves were randomly assigned to each group. For the study of primary acute infection, two calves were intranasally inoculated with a high dose ($10^{6.3}$ $TCID_{50}$ in 10 ml) of BoHV-1 Cooper strain and two calves were inoculated with $10^{6.3}$ $TCID_{50}$ (in 10 ml) of BoHV-5 97/613 isolate. One calf was intranasally inoculated with 10 ml of cell culture medium as placebo. Calves were euthanized at 6 days post-infection (dpi). At necropsy, TG samples were collected and properly stored for further processing.

2.3. Cells

Two cell lines of bovine origin (Madin-Darby bovine kidney [MDBK] and bovine turbinate [BT] cells) and a human cell line (HeLa, cervical carcinoma cells) were selected for this study. HeLa cells were chosen to compare the findings in routine substrates for bovine herpesviruses growth with the findings in cells which are susceptible to infection but do not sustain high levels of virus replication. Cells were grown in Minimum Essential Medium (MEM, Sigma-Aldrich) supplemented with 10% fetal calf serum (Natocor SRL, Argentina), L-glutamine (10 µl/ml), penicillin (100 µg/l) and streptomycin (200 µg/l). Cultures were incubated at 37 °C in a 5% CO_2 atmosphere.

2.4. Inoculation of cell cultures

Cell lines were grown in 24 well-plates containing coverslips in each well, for cell attachment. Cells were infected with BoHV-1 or BoHV-5 strains at a low multiplicity of infection (moi = 0.1). Infected monolayers were fixed at 6 or 24 h post-inoculation (hpi). Cells were also treated with deoxynivalenol (DON, Sigma-Aldrich) (2.8 µg/ml in MEM) and 5% dimethyl-sulfoxide (DMSO, Sigma-Aldrich), as controls for the induction of apoptosis and fixed at the same time-points. Evaluation at 6 h was chosen as a starting point to determine initial alterations in nuclear morphology after infection or chemical treatment. DON is a mycotoxin which has been previously demonstrated to induce apoptosis in intestinal and immune cells [14] and in several cell culture lines [15]. Apoptosis induced by treatment with DMSO has been also previously described [16]. Virus strains, DMSO and DON were added to the preformed cell monolayer and incubated for 1 h at 37 °C. Then, cells were washed with PBS and culture medium was added for the remaining incubation time.

2.5. Identification of nuclear changes in cell cultures

Nuclear morphological changes (chromatin condensation, nuclear fragmentation and presence of apoptotic bodies) were evaluated by staining with 4′, 6-diamidino-2-phenylindole (DAPI) after fixing infected or treated cells with 4% paraformaldehyde. Experiments were done by triplicate and cells with nuclear alterations in six microscopic fields per sample were counted. The percentages of cells with altered nuclear morphology were determined.

2.6. Detection of apoptotic cells by TUNEL (Terminal dUTP Nick End-Labeling)

For detection of cells with fragmented DNA, a TUNEL assay (DeadEndColorimetric TUNEL System, Promega) was performed according to the manufacturer's instructions. Cells were seeded on coverslips in 24 well-plates and treated as described for DAPI staining. Cells were fixed with 1% paraformaldehyde. Experiments were done by duplicate.

2.7. Immunohistochemistry for detection of cleaved caspase 3 in bovine TG

Tissue sections were deparaffinized and rehydrated in graded ethanol. Sections were incubated in 0.03% hydrogen peroxide solution to block endogenous peroxidase. For antigen unmasking, sections were treated with proteinase K (20 µg/ml, Sigma-Aldrich) for 20 min at 37 °C. After blocking, tissues were incubated overnight at 4 °C with diluted primary antibody (1:100, cleaved caspase 3, Cell Signaling # 9661). The secondary antibody was applied for 30 min at room temperature. The peroxidase substrate system (DAB, SK-400, Vector Laboratories) was used according to the manufacturer's instructions. Sections were counterstained with hematoxylin, mounted and observed under microscope.

2.8. Statistical analysis

Differences in the percentages of cells with nuclear changes were evaluated for each virus or treatment within a cell line and among the different cell lines. Virus titers for the different bovine herpesvirus strains were also evaluated in the different cell lines. The percentages of cells with nuclear changes and virus titers were analyzed by ANOVA followed by Tukey test. A P value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Nuclear morphological changes in BoHV-1 and BoHV-5-infected cell cultures

Marginated nuclei and chromatin condensation were easily visualized in infected or treated cells, particularly after 24 hpi. Apoptotic bodies, which are membrane bound portions of chromatin [17] were also observed, particularly after infection with strain BoHV-5 nrecA663. On the contrary, nuclei in mock-infected cells remained uniformly stained (Fig. 1).

As expected, the percentages of cells with nuclear morphological changes observed at 6 hpi were lower when compared with the percentages detected at 24 hpi (Fig. 2A and B). At 6 hpi, only significant changes ($P \leq 0.05$) were detected in BT cells infected with the strain BoHV-5 nrecA663. In mock-infected BT, DAPI staining also revealed significantly higher numbers of cells with nuclear changes with respect to the other mock-infected cell lines ($P \leq 0.05$) (Fig. 2A). Differences were not detected after chemical treatment or infection of the other cell