Virus Research 145 (2009) 227–235

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Analysis of apoptosis induced by Caprine Herpesvirus 1 *in vitro*

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ARTICLE INFO

Article history:
Received 30 April 2009
Received in revised form 8 July 2009
Accepted 9 July 2009
Available online 18 July 2009

Keywords:
CpHV-1
Apoptosis
Caspases
Cytochrome *c*
Bcl-2 family
PARP

ABSTRACT

It is known that Caprine Herpesvirus 1 (CpHV-1) causes apoptosis in mitogen-stimulated as well as not stimulated caprine peripheral blood mononuclear cells (PBMC). Initial experiments in Madin Darby bovine kidney (MDBK) cells revealed that CpHV-1 infection induced apoptotic features like chromatin condensation and DNA laddering. Thus, to characterize in more detail this apoptotic process, activation of caspase-8, -9 and -3 in MDBK cells CpHV-1 infected was investigated and demonstrated. In addition, CpHV-1 infection resulted in disruption of mitochondrial membrane potential, cytochrome *c* release and alterations in the pro- and anti-apoptotic proteins of Bcl-2 family. Proteolytic cleavage of poly(ADP-ribose) polymerases (PARP), confirming the activation of downstream caspases, was also observed. Our data indicated that a "cross-talk" between the death-receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway occurred in CpHV-1-induced apoptosis *in vitro*.
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1. Introduction

Apoptosis or programmed cell death (PCD) is considered as a physiological form of cell death which occurs during embryonic development, tissue remodeling and tumor regression with no inflammatory response (Schulze-Osthoff et al., 1998). Apoptosis is characterized morphologically by cell shrinkage, apoptotic body formation, and condensation of the chromatin and biochemically by fragmentation of DNA into oligonucleosomal DNA fragments (Shi, 2002). An important regulatory event in the apoptotic process is the activation of caspases, a family of cysteine proteases. Caspases are synthesized as inactive precursors (zymogens) that are processed to large and small subunits to form the active enzymes (Nicholson and Thornberry, 1997). There are two well-known apoptotic pathways involving activation of caspases (Brenner and Kroemer, 2000). The extrinsic pathway is characterized by interaction of a ligand with death receptors. This binding leads to interactions of adaptor molecules and pro-caspases 8, forming the death-inducing signaling complex (DISC), which activates caspase-8 (Lavrik et al., 2005). The intrinsic pathway involves an alteration of the mitochondrial membrane potential ($\Delta\Psi$) leading to mitochondrial membrane permeabilisation (MMP), and followed by a release of cytochrome *c* (Chipuk et al., 2006). Cytosolic cytochrome *c* binds the protein adaptor APAF-1 to trigger the formation of a multimeric protein complex termed the apoptosome that permits recruitment and autocleavage of caspase-9 (Bao and Shi, 2007). MMP is tightly regulated by Bcl-2 family members, including Bcl-2 and Bax, that inhibit or promote MMP respectively (Reed, 2006). Both the extrinsic and intrinsic pathways, converge downstream on the executioner caspase-3, whose activity produces the morphological hallmarks of apoptosis (Porter and Jänicke, 1999). Alternative pathways have also been described, such as a pathway where intrinsic and extrinsic pathways converge (Gaddy and Lyles, 2005). In this pathway, low levels of activated caspase-8 cleave BID, a BH3 domain containing pro-apoptotic Bcl-2 family member. Cleaved BID (tBID) translocates to mitochondria and transduces apoptotic signals from cytoplasmic membrane to mitochondria. tBID induces the release of cytochrome *c* into the cytosol activating caspase-9 (Gaddy and Lyles, 2005).

Among stimuli that have been associated with cell apoptosis are infections with mammalian DNA and RNA viruses (Hay and Kannourakis, 2002). Viruses possess various biochemical and genetic mechanisms to evade and/or induce apoptosis in infected cells through interactions at different stages of the apoptotic pathway and it has been demonstrated that apoptosis modulation through virus-encoded proteins is a key step in herpesviruses

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doi:10.1016/j.virusres.2009.07.008

pathogenesis (Cassady et al., 1998; De Martino et al., 2007; Henderson et al., 1993; Hu et al., 1997; Wang et al., 1997).

Caprine Herpesvirus 1 (CpHV-1) belongs to the *Alphaherpesvirinae* subfamily and show genomic similarity to other viruses of this group, such as Bovine Herpesvirus 1 (BoHV-1), the etiological agent of IBR/IPV, Bovine Herpesvirus 5 (BoHV-5), Suid Herpesvirus 1 (SuHV-1), Cervid Herpesvirus 1, and Rangiferine Herpesvirus 1 (Thiry et al., 2006). Caprine Herpesvirus 1, is associated with two different syndromes in goats, depending on the age of the animals at the time of infection. In young kids, CpHV-1 causes an often-generalized disease mainly affecting the digestive tract (Saito et al., 1974; Mettler et al., 1979; Roperto et al., 2000). Infection in adult goats remains unapparent, or may cause respiratory distress, abortion (Berrios et al., 1975; Waldvogel et al., 1981; Williams et al., 1997), vulvovaginitis or balanoposthitis (Horner et al., 1982; Tisdall et al., 1984). Moreover, being a member of alphaherpesvirus, CpHV-1 is able to establish latent infection in trigeminal ganglia (Buonavoglia et al., 1996; Plebani et al., 1983) and to cause immunosuppression (Pagnini et al., 2005). Thus, CpHV-1 infection is responsible for severe economic losses due to abortions, stillbirths, reproductive disorders and susceptibility of CpHV-1-infected host to secondary bacterial infections.

Our group previously showed that CpHV-1 is able to induce apoptosis in goat peripheral blood mononuclear cells (Pagnini et al., 2005). In the present study, we investigated on the pro-apoptotic potential of CpHV-1 in a permissive cell line (Madin Darby bovine kidney (MDBK) cells), evaluating apoptotic profiles like chromatin condensation and DNA laddering. Moreover, to characterize in more detail the intracellular pathway by which CpHV-1 is able to induce apoptosis, in this report we have analyzed bid cleavage, the cytochrome *c* release and the ratio of bax:bcl-2. Using FLICA method and Western blot analysis, we have described caspase-8, -9, -6 and -3 activation. Since caspase-3 induces proteolytic cleavage of various substrates including poly(ADP-ribose) polymerases (PARP), cytoskeletal reorganization and disintegration of the cell into apoptotic bodies (Elmore, 2007), we also have evaluated PARP cleavage. These results have important implications for the understanding of CpHV-1-induced apoptosis.

2. Materials and methods

2.1. Cells and virus

Madin Darby bovine kidney (MDBK) cells (CCL22, American Type Culture Collection) were grown in Dulbecco's modified minimal essential medium (DMEM), supplemented with 100 IU/ml of penicillin, 100 mg/ml of streptomycin and 5% pestivirus free foetal calf serum. This cell line was maintained free of mycoplasma and of bovine viral diarrhoea virus.

The reference Swiss strain E/CH (Mettler et al., 1979) was used. It was multiplied on MDBK, and cell extracts, obtained by three cycles of freezing and thawing, were pooled, collected, and stored in aliquots at −80 °C. Infectivity titres were expressed as median tissue culture infectious doses ($TCID_{50}$)/ml (Reed and Muench, 1938).

2.2. Morphological analysis by staining with acridine orange

MDBK cells were grown on slides in 24-well plates and were infected with CpHV-1 at multiplicity of infection (MOI) of 10. At 12, 24, 48, 72 h post infection (p.i.), MDBK cells were stained with acridine orange stain (0.2 mg/ml) and were washed with PBS to remove background staining; after then, still wet, slides were covered with coverslips and evaluated with an fluorescence microscope. The identification of apoptotic cells was based on the presence of uniformly stained nuclei showing chromatin condensation and nuclear fragmentation.

2.3. DNA fragmentation assay

Monolayers of MDBK cells were grown in 25 cm^2 flask and, after 24 h were infected with CpHV-1. At indicated time post infection, DNA from infected and mock-infected cells was extracted using a commercial Qiagen DNeasy tissue kit (Qiagen S.p.A., Italy), according to the manufacturer's instructions for cultured cells. Five micrograms of each DNA sample was electrophoresed on a 1.5% agarose gel containing 0.1 mg of ethidium bromide per ml. The DNA was visualized under UV light, and the sizes of the fragments were estimated by comparing the mobilities with a 100-bp ladder (Invitrogen, Milan, Italy).

2.4. Propidium iodide (PI) staining and DNA content assay

Flow cytometric analysis of apoptosis was performed by analyzing the reduced fluorescence of the DNA binding dye propidium iodide (PI) in the apoptotic nuclei as previously described (Pagnini et al., 2005). Briefly, cells were collected by centrifugation at 250 × *g*, washed twice in PBS and the cell pellet (1 × 10^6 cells) was immediately resuspended at room temperature in 1 ml of hypotonic solution consisting of 0.1% sodium citrate pH 6.5, 1% Triton X-100 and 50 mg/ml propidium iodide (PI) in distilled water. Nuclei were analyzed after a 30-min incubation at 4–8 °C in the dark with a flow cytometer (Partek). A correct threshold value was experimentally selected to exclude the majority of cell debris. At least 5000 events were acquired for each sample.

2.5. Protein extraction and Western blot analysis

MDBK cells in 75 cm^2 flask, at confluency, were infected with CpHV-1 at MOI 1. At 4, 8, 12, 24, 36 and 48 h post infection, adherent cells were washed twice with PBS and removed from the flask by treatment with trypsin–EDTA solution. Cells were then mixed with cells previously collected by centrifugation from supernatant of the same flask and resuspended in PBS. The pellets, obtained by centrifugation, were stored at −20 °C. To measure caspases activation at least 1 × 10^6 cells were used. Cells were homogenized directly into lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 0.5 mM sodium orthovanadate, and 20 mM sodium pyrophosphate). The lysates were clarified by centrifugation at 14,000 × 10 min. Protein concentrations were estimated by an assay (Bio-Rad) and boiled in Laemmli buffer [0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue] for 5 min before electrophoresis. Proteins were subjected to SDS-PAGE (12.5% polyacrylamide). After electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon, Millipore Corp., Bedford, MA); complete transfer was assessed using pre-stained protein standards (Bio-Rad, Hercules, CA). After blocking with Tris-buffered saline–BSA [25 mM Tris (pH 7.4), 200 mM NaCl, and 5% BSA], the membrane was incubated with the primary antibodies. The following antibodies, dissolved in 5% bovine serum albumin-TBST, were used: anti-caspase-3 PAb (dilution 1:1000) (Cell Signaling), anti-caspase-8 PAb (dilution 1:2000) (Abcam), anti-caspase-9 PAb (dilution 1:2000) (Stressgen), anti-poly(ADP-ribose) polymerases (PARP) (1:5000) (Biomol), anti-bax PAb (1:1000) (Abcam), anti-bcl-2 PAb (1:1000) (Abcam), anti-bid PAb (1:500) (Santa Cruz), anti-cytochrome *c* (1:1000) (Abcam) and anti-b-MAb (1:7500) (Cell Signaling). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:10,000) (at room temperature), and the reaction was detected with an enhanced chemiluminescence system (Amersham Life Science, Buckinghamshire, UK).

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