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Canine parvovirus NS1 induced apoptosis involves mitochondria, accumulation of reactive oxygen species and activation of caspases

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ABSTRACT

The non-structural protein (NS1) of parvoviruses plays an important role in viral replication and is thought to be responsible for inducing cell death. However, the detailed mechanism and the pathways involved in canine parvovirus type 2 NS1 (CPV2.NS1) induced apoptosis are not yet known. In the present study, we report that expression of CPV2.NS1 in HeLa cells arrests cells in G1 phase of the cell cycle and the apoptosis is mitochondria mediated as indicated by mitochondrial depolarization, release of cytochrome-c and activation of caspase 9. Treatment of cells with caspase 9 inhibitor Z-LEHD-FMK reduced the induction of apoptosis significantly. We also report that expression of CPV2.NS1 causes accumulation of reactive oxygen species (ROS) and treatment with an antioxidant reduces the ROS levels and the extent of apoptosis. Our results provide an insight into the mechanism of CPV2.NS1 induced apoptosis, which might prove valuable in developing NS1 protein as an oncolytic agent.

1. Introduction

Viruses in the nature have evolved various strategies which facilitate their survival and proliferation in the host cells. While some viruses have evolved to inhibit apoptosis for their extended multiplication, several others have developed ways to induce apoptosis for their survival. Some of these viruses and their gene products specifically target high proliferating cells such as cancer cells, and kill them without affecting the healthy cells. Such viruses are called as oncolytic viruses, while the viral genes with oncolytic properties are termed as anti-cancer genes (Grimm and Noteborn, 2010; Gupta et al., 2014). Parvovirus is the class of viruses with an intrinsic property of killing the malignant tumor cells while sparing the normal cells. These viruses such as, Minute virus of mice (MVM), rat parvovirus (H-1PV) etc., have been extensively studied for their tumor lytic properties (Rommelaere et al., 2010; Geletneky et al., 2012; Nüesch et al., 2012).

Canine parvovirus (CPV), one of the members of Parvoviridae family, has been reported to induce apoptosis in the permissive cell lines (Dolley et al., 2014; Nykky et al., 2010). Its infection causes induction of apoptosis in Norden laboratory feline kidney (NLFK) cells and canine fibroma cells (A72) (Nykky et al., 2010). The results of our laboratory have shown that CPV2 (Canine parvovirus type 2) is able to induce apoptosis in MDCK cells through the activation of caspases and involves both extrinsic and intrinsic pathway of apoptosis (Doley et al., 2014).

Parvovirus genome encodes three nonstructural proteins, including non-structural protein 1 (NS1). NS1 is a multifunctional protein, which is responsible for the cytotoxic activities of the parvoviruses. NS1 plays crucial role in virus replication, regulation of viral promoter, cellular transcription and induction of cell death (Lorson et al., 1998; Krady and Ward, 2007). The ectopic expression of NS1 protein of many parvoviruses such as MVM, H-1PV, B19 (Human parvovirus B19) and CPV2 has been reported to cause cell cycle arrest and initiation of apoptosis in the tumor cells (Mincberg et al., 2011; Hristov et al., 2010; Poole et al., 2006; Saxena et al., 2013). B19 NS1 expression has been shown to cause cell cycle arrest in G1 phase and induction of apoptosis in both permissive and non-permissive cell lines through the activation of caspases (Hsu et al., 2004; Poole et al., 2006; Morita et al., 2003). Parvovirus H-1 and MVM NS1 protein is also cytotoxic and causes cell cycle arrest by generating nicks in chromatin. MVM NS1 is associated with the alteration in the cytoskeleton structures of the transformed and cancer cells and causes cell death (Nüesch et al., 2005; Nuesch and Rommelaere, 2006). H-1PV NS1 causes accumulation of reactive oxygen species (ROS) and leads to cell death through the intrinsic pathway of apoptosis (Hristov et al., 2010).

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In our previous study, we had reported for the first time that expression of NS1 causes apoptosis in the HeLa cells (Saxena et al., 2013). We, further, demonstrated that apoptosis is caspase dependent and p53 independent. However, it is still not known whether apoptosis induced by CPV2.NS1 follows an intrinsic pathway only or it involves an extrinsic pathway as well. Moreover, involvement of mitochondria and oxidative stress also needs to be examined for a clear understanding of the mechanism behind its cytotoxic action. In light of the above facts, we, in the present study, investigated the mechanisms and pathways involved in the CPV2.NS1 induced apoptosis in the HeLa cells, and observed that apoptosis proceeds through the intrinsic pathway which involves mitochondrial depolarization, release of cytochrome-c and activation of caspase 9.

2. Materials and methods

2.1. Cell culture

HeLa cells were obtained from NCCS (National Centre for Cell Science, Pune, India) and maintained in Dulbecco's modified Eagle's medium with high glucose (Gibco, USA) supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin, and 100 μg/ml streptomycin.; Gibco, USA) at 37 °C and 5% CO_2 in a CO_2 incubator.

2.2. Plasmid, gene constructs and transfection

The CPV2 NS1 gene was available as a pcDNA.cpv.ns1 (Saxena et al., 2011) which was further amplified with the primers having Bsp HI and Nhe I restriction sites and cloned into MCS1 of pVIVO1 vector (InvivoGen, USA). Transfection of HeLa cells with pVIVO1.NS1 and empty vectors was carried out as per the manufacturer's protocol with Lipofectamine® reagent (Invitrogen, USA) and as described in our previous study (Saxena et al., 2013; Singh et al., 2015). The untransfected (mock control) and vector pVIVO1 transfected HeLa cells were used as mock and vector controls, respectively.

2.3. Fluorescence microscopy

Transfected HeLa cells were analyzed for the expression of NS1 protein by Immunofluorescence. In brief, after 48 h of transfection, cells were washed twice with PBS and fixed with 3% PFA followed by permeabilization with 0.2% Triton-X. Cells were then incubated with CPV2 NS1 specific polyclonal sera raised in rabbit (Saxena et al., 2011) for overnight. After PBS washings, cells were incubated with anti-rabbit FITC labelled secondary antibody (Sigma, USA) for 1 h followed by DAPI staining to highlight the nucleus. Cells were analyzed under a fluorescent microscope (Nikon, USA).

2.4. RT-PCR

The whole procedure was carried out in a designated PCR-clean area. After 48 h of transfection, RNA was extracted from the HeLa cells using Trizol reagent (Invitrogen, Carlsbad, California, USA) strictly following manufacturer's protocol. Extracted RNA was resuspended in diethyl pyrocarbonate (DEPC) -treated water and stored in −80 °C until further use. The purity of the sample was determined spectrophotometrically by calculating the ratio of optical density at wavelengths of 260 and 280 nm. The cDNA synthesis of total RNA was carried out using high capacity cDNA reverse transcription kit (ABI Biosystem, USA). The cDNA encoding for NS1 gene was amplified through PCR (Polymerase chain reaction) using the primers as mentioned in the previous section. The amplification was carried out in a 40 μl reaction volume following manufacturer's instructions. Following PCR, samples were subjected to electrophoresis on a 1% agarose gel.

2.5. Western blotting

For Western blot analysis, transfection was carried out in a T-25 flask with Lipofectamine® reagent following manufacturer's protocol. After 48 h of transfection, cells were washed twice with cold phosphate-buffered saline (PBS) and harvested by scraping. After centrifugation, cells lysis was done by using cell lysis buffer (Invitrogen, USA) and protease inhibitor cocktail (Promega, USA) was added to avoid protein degradation. After vortexing briefly, samples were centrifuged at high speed for 5 min and the supernatant was collected and stored at −80 °C. Protein concentration was estimated by Bradford assay and about 30 to 40 μg of protein was loaded per well in SDA-PAGE. Transfer to methanol activated PVDF membranes (Hybond-P membrane, GE Healthcare) was done using a semi-dry apparatus (SCIE-PLAS, UK). The membranes were blocked with 3% BSA for 1 h followed by overnight incubation at 4 °C with the primary antibodies specific for the following: CPV2.NS1 polyclonal antibodies raised in rabbit (Saxena et al., 2011; Kumar et al., 2012) PARP (poly(ADP-ribose) polymerase), caspase 8, 9, and 3, and β-actin antibodies (Santa Cruz Biotechnology, USA). After overnight incubation, membranes were washed thrice with PBS-T and incubated with an appropriate secondary antibody either goat anti-rabbit-HRP conjugated or goat anti-mouse HRP conjugated antibody (Sigma, USA) for 1 h at RT. After briefly washing the membranes, desired proteins were visualized by incubating the membranes with 3,3′-Diaminobenzidine (DAB) substrate kit (Invitrogen, USA).

2.6. Cell viability assay

2.6.1. Trypan blue assay

Following transfection, cells were harvested at different intervals (24, 48 h) to determine the cell viability by Trypan blue dye exclusion method. After PBS washing, cells were resuspended in PBS and equal volumes of cell suspension and 0.4% trypan blue (Invitrogen, USA) solution were mixed. The percentage of live and dead cells were counted using Countess Automated Cell Counter (Invitrogen, USA). The percentage viability was determined by the software in the Countess™ automated cell counter (Invitrogen, USA).

2.6.2. LDH assay

Cell lysis in pVIVO1.NS1 and pVIVO1 empty vector was determined by measuring the release of lactate dehydrogenase in the medium using the LDH Cytotoxicity Assay kit (Cayman Chemical, USA) and following the manufacturer's instructions. Briefly, cells were seeded in a 96 well plate at a density of 10^4–10^5 cells/well in 120 μl of culture medium. Cells were transfected after 24 h of incubation. Each treatment was performed in triplicate.

2.7. Assessment of apoptosis

2.7.1. DNA fragmentation

The DNA fragmentation assay was performed by the method of Herrmann et al. (1994). Briefly, cells were harvested at 48 h of transfection, washed with PBS and the pellet was treated with lysis buffer (1% NP-40 in 20 mM EDTA, 50 nM Tris–HCL, pH 7.5). After centrifugation at 3000 rpm for 5 min, the supernatant was collected and treated with 10% SDS (final 1% SDS) for 2 h at 37 °C followed by digestion with proteinase K (final 2.5 μg/μl) at 56 °C for 3 h. After the addition of 0.5 volume of 10 M ammonium acetate, the DNA was precipitated with 2.5 volume of ethanol. The pellets were washed with 70% ethanol, air dried and suspended in Tris–EDTA