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Negative effect of heat shock on feline calicivirus release from infected cells is associated with the control of apoptosis

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ABSTRACT

FCV infection causes rapid cytopathic effects, and its replication results in the induction of apoptosis changes in cultured cells. It is well established that the survival of apoptotic cells can be enhanced by the expression of heat-shock proteins (Hsp) to prevent damage or facilitate recovery. Hsps can act as molecular chaperones, but they can also have anti-apoptotic roles by binding to apoptotic proteins and inhibiting the activation of caspases, the primary mediators of apoptosis. Because apoptosis occurs during FCV infection and heat shock (HS) treatment has a cytoprotective role due to the expression of Hsps, we studied the effect of the HS response to hyperthermia during FCV infection in cultured cells. We found that FCV infection does not inhibit the expression of Hsp70 induced by HS and that non-structural and structural protein synthesis was not modified during HS treatment. However, HS caused a delay in the appearance of a cytopathic effect in infected cells, as well as a reduction in the extracellular but not in the cell-associated viral yield. This antiviral effect of HS correlates with the inhibition of caspase-3 activation. Thus, the HS-induced reduction in virus production appeared to be associated with the control of apoptosis, supporting previous data that indicate that apoptosis is necessary for FCV release.

1. Introduction

The Caliciviridae family is composed of small, nonenveloped, positive-strand RNA viruses, including numerous pathogens that infect a broad range of vertebrate hosts and cause several diseases that affect animals and humans. In particular, calicivirus infections in humans are the leading cause of gastroenteritis worldwide (Koo et al., 2010). Studies investigating the biology and pathogenesis of these viruses are still restricted to viruses that replicate in cell culture or for which appropriate animal models are available (Sosnovtsev et al., 2003). One of the most valuable models for understanding calicivirus biology is feline calicivirus (FCV) (Pesavento et al., 2008; Vashist et al., 2009). Although FCV causes an infection in cats that produces signs of oral ulceration and/or upper respiratory disease, its ability to grow in vitro and the possibility to be genetically manipulated, have contributed to the study of the molecular mechanism of calicivirus translation and genome replication (Sosnovtsev and Green, 1995; Vashist et al., 2009).

As with many other viruses, FCV has evolved sophisticated strategies to exploit the metabolic machinery of the host cell for its own multiplication, such as hijacking the host transcriptional/translational machinery (Kuyumcu-Martinez et al., 2004; Willcocks et al., 2004) and controlling cellular signaling and apoptotic pathways (Al-Molawi et al., 2003; Natoni et al., 2006; Roberts et al., 2003; Sosnovtsev et al., 2003). FCV infection causes rapid cytopathic effects, and its replication results in the induction of apoptosis in cultured cells.

Apoptosis is a highly coordinated process for controlling cell homeostasis in multicellular organisms in response to a wide range of stimuli, including stress and viral infections (Vaux and Strasser, 1996). A number of viruses are known to express proteins that inhibit apoptosis (Garnett and Duerksen-Hughes, 2006; Liu et al., 2012; McNees and Gooding, 2002; O'brien, 1998). However, for some other viruses, and probably for FCV, apoptosis facilitates the

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release and spread of viral progeny (Deng et al., 2008; O'brien, 1998; Tran et al., 2013). The modulation of mitochondrial apoptosis during FCV infection is dependent on viral protein synthesis (Sosnovtsev et al., 2003) and is characterized by changes such as chromatin condensation, DNA fragmentation, mitochondrial membrane potential loss, and cytochrome C release, resulting in the formation of an Apaf-1-caspase 9 apoptosome that induces the apoptotic protease cascade via the activation of pro-caspase 3 (O'brien, 1998; Saleh et al., 2000; Tran et al., 2013). The activation of caspase-8 and -9 has been reported to occur at low levels, in contrast to the significantly higher activation of caspase-3 (Sosnovtsev et al., 2003). Caspase-2 and -7 are also activated during FCV infection (Al-Molawi et al., 2003).

Apoptotic cells can enhance survival in response to adverse stress through the expression of so-called heat-shock proteins (Hsp), or stress response proteins, which prevent damage or facilitate recovery to maintain cell survival (Beere, 2001; Beere et al., 2000). Hsps are associated with their role as molecular chaperones, to prevent misfolding or aiding protein folding and promoting the correct cellular localization of their respective substrates (Parsell and Lindquist, 1993). Recently, numerous studies have attributed an anti-apoptotic role to Hsps in response to a wide range of stresses, such as heat shock (HS) (Hahn and Li, 1982), nitric oxide (Beere et al., 2000), and viral infections (Beere, 2004; Parcellier et al., 2003; Young, 2012), implicating negative regulation at different points within the multiple signaling cascades of apoptosis (Beere, 2004). This anti-apoptotic role can but does not always depend upon chaperone activity (Beere and Green, 2001). One of the anti-apoptotic-specific response mechanisms occurs through the expression of the stress-induced Hsp27, Hsp90, and major inducible Hsp70 proteins; the regulatory roles of these proteins depend on their ability to interact with protein or polypeptide substrates (Beere, 2004; Beere et al., 2000; Lanneau et al., 2007). Hsp27 essentially blocks caspase-dependent apoptotic pathways by directly associating with cytosolic cytochrome C and inhibiting the formation of the caspase-3 activation complex (Lanneau et al., 2007; Paul et al., 2002). Moreover, Hsp27 binds to procaspase-3 to prevent its cleavage and activation by caspase-9 (Pandey et al., 2000a). The quintessential inhibitor of apoptosis, Hsp70, has been shown to inhibit apoptotic pathways at different levels. At the pre-mitochondrial level, Hsp70 binds to and blocks c-Jun kinase activity (Park et al., 2001) and also has been shown to bind to and stabilize non-phosphorylated protein kinase (C) and Akt (Gao and Newton, 2002). At the mitochondrial level, Hsp70 inhibits Bax translocation and insertion into the outer mitochondrial membrane, preventing membrane permeabilization and the release of cytochrome C and apoptosis-inducing factor (AIF) (Stankiewicz et al., 2005). At the post-mitochondrial level, Hsp70 directly associates with apoptosis-protease activating factor (APAF-1), thereby preventing apoptosome formation and the recruitment of caspase 9 and thus inhibiting the activation of effector caspase-3 (Beere et al., 2000; Saleh et al., 2000). Thus, the modulation of caspase activity might represent a mechanism for preventing apoptosis.

Because apoptosis occurs during FCV infection and HS treatment has a cytoprotective role due to the induction of Hsps, we studied the effect of the HS response induced by hyperthermia during FCV infection in cultured cells. FCV infection did not inhibit the expression of Hsp70 induced by HS. Although HS did not modify the synthesis of non-structural (NS) and structural proteins, it caused a delay in the cytopathic effect as well as a reduction in the extracellular but not in the cell-associated viral yield, that correlated with the inhibition of caspase-3 activation. These data support previous results that indicate that apoptosis is necessary for FCV and that its blockage by HS inhibits virus release.

2. Materials and methods

2.1. Cells and virus infection

CrFK cells obtained from American Type Culture Collection (ATCC) (Rockville, MD) were grown in Eagle's minimal essential medium with Earle's balanced salt solution (BSS) and 2 mM L-glutamine (EMEM) modified by ATCC to contain 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1.5 g/l sodium bicarbonate. The medium was supplemented with 10% fetal serum, 5000 U of penicillin, and 5 g/ml of streptomycin. The cells were grown in a 5% CO_2 incubator at 37 °C, and CrFK infection with the FCV F9 strain was performed as previously described (Vashist et al., 2009). The virus titer was determined by the plaque assay (Escobar-Herrera et al., 2007).

2.2. Heat shock, staurosporine, and caspase inhibitor treatments

For HS treatment, CrFK cell monolayers were washed, and medium heated to 45 °C was added. The cells were maintained at 45 °C for 20 min and then at 37 °C for 1 h; the cells were then processed or infected as required. When indicated, cells were first infected and after 1 h adsorption, medium heated to 45 °C was added, cells were maintained at 45 °C for 20 min and then at 37 °C for the indicated times. The cytopathic effect was monitored by evaluating cell morphology using a phase-contrast microscope. As a positive control for apoptosis, CrFK cell monolayers were incubated with 1 μM staurosporine at 37 °C for 4 h. For caspase activity detection, the cells were infected with FCV at a multiplicity of infection (MOI) of 5 at the indicated times in the absence or presence of 200 μM Z-VAD-FMK irreversible caspase inhibitor (Promega).

2.3. Cell extracts and Western blot analysis

Non-treated or treated CrFK cells were washed with PBS, lysed in Laemmli sample buffer, and boiled for 7 min. The proteins were analyzed by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 10% skimmed milk for 2 h and incubated overnight at 4 °C with the following: monoclonal anti-Hsp70 (Santa Cruz Biotechnology), anti-actin (kindly donated by Manuel Hernández, Cinvestav, Mexico), rabbit polyclonal anti-Hsc70 (kindly donated by Susana López, IBT UNAM, Mexico), anti-caspase-3, and anti-PARP (Cell signal), or anti-N6/7 and NS3 (kindly donated by Ian Goodfellow, University of Cambridge, UK) antibodies. The blots were washed extensively with 0.5% PBS–Tween and incubated for 2 h with the appropriate secondary antibodies (Santa Cruz Biotechnology); the blots were developed using chemiluminescence (PIERCE). Quantification of the protein levels was achieved by the band intensities in the scanned images using ImageJ software (http://rsb.info.nih.gov/ij) and is expressed as arbitrary units.

2.4. Proliferation assay

To determine the viability of non-infected cells after HS treatment, the nucleic acid stain Sytox-green assay (Molecular Probes Inc.) was performed according to the manufacturer's direction. Briefly, CrFK cells were seeded in a 96-well plate for 24 h. The medium was replaced with a medium preheated at 45 °C, and the cells were incubated at the same temperature for 20 min and then at 37 °C for 14 h. Cell viability was determined using an AlphaScan fluorescence spectrophotometer. Each experiment was repeated three times.