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Short communication

Equine arteritis virus induced cell death is associated with activation of the intrinsic apoptotic signalling pathway

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ABSTRACT

Equine arteritis virus (EAV) causes a respiratory and reproductive disease in horses, equine viral arteritis. Though cell death in infection with EAV is considered to occur by apoptosis, the underlying molecular mechanism has not been extensively elucidated. We investigated the expression of mRNA of pro-apoptotic and caspase genes during EAV infection in BHK21 cells, a well-established cell type for EAV replication. Using a SYBR Green real-time PCR, mRNA of p53, Bax, caspase 3 and caspase 9 were found up-regulated in a time dependent manner in EAV infected cells. Western blot analysis for caspase 3 and caspase 9 showed expression of cleaved forms of these proteins during EAV infection. In addition, a luminescence-based cell assay for caspase 3/7 activation as a hallmark in apoptosis confirmed apoptotic cell death. The findings demonstrate that cell death in EAV infected BHK21 cells results from apoptosis mediated through the intrinsic signalling pathway.

Equine arteritis virus (EAV) causes equine viral arteritis, a respiratory and reproductive tract disease that affects equids such as horses, donkeys and mules. The symptoms of disease include abortions in mares, pneumonia in young fouls, and an influenza-like sickness characterized by fever, oedema and nasal discharge in adults (Huntington et al., 1990). The virus was first isolated in Bucyrus, OH, USA (Bryans et al., 1957) during an outbreak of respiratory disease and abortions. EAV virus belongs to the family *Arteriviridae*, genus *Arterivirus*, in the order *Nidovirales*, which also includes the *Coronaviridae* (Cavanagh, 1997). EAV is an enveloped, spherical virus of 50–60 nm in diameter and has a single-stranded, positive sense RNA genome. The length of the genome is approximately 12.7 kb (Snijder and Meulenberg, 1998), with 5′ and 3′ untranslated regions and nine open reading frames (den Boon et al., 1991).

Apoptosis, or programmed cell death is a process of cell suicide in response to different stimuli, and is necessary in physiological processes like development of the embryo, maintenance of tissue homeostasis and functioning of the immune system. It is triggered by a variety of stimuli, such as UV radiation, oxidative stress, genotoxic chemicals and infectious agents like viruses, which activate intracellular signalling cascades, culminating in cell death. Caspases, which mediate the apoptotic process, belong to the family of cysteine proteases that are cysteine dependent aspartate directed proteases. These caspases are broadly grouped into initiators (caspase 2, 8, 9, 10), effectors or executioners (caspase 3, 6, 7) and inflammatory caspases (caspase 1, 4, 5) (Danial and Korsmeyer, 2004).

Generally, cell death through the apoptotic process occurs via two pathways, the extrinsic and intrinsic pathways. The extrinsic signalling pathway is mediated by the death receptor present on the cell surface, leading to cleavage of procaspase 3 and 7. The intrinsic pathway, on the other hand, is a non-receptor-mediated pathway that is stimulated by intracellular signals like DNA damage, and acts directly on target molecules, where mitochondria play a vital role by releasing pro-apoptotic factors such as cytochrome c. Cytochrome c constitutes an apoptosome by binding with apoptotic protease activating factor Apaf-1 and caspase 9 (Bao and Shi, 2007). Accumulation of procaspase 9 leads to activation of caspase 9, and also the downstream molecules caspase 3/7 resulting in cell death (Jiang and Wang, 2004). Mitochondrial events associated with apoptosis are regulated by the Bcl-2 family of proteins

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including anti-apoptotic proteins, Bcl-2, Bcl-x, Bcl-XL, Bcl-XS and pro-apoptotic proteins such as Bax, Bak, Bid, Bad, Bim, and Bik. These pro-apoptotic proteins regulate the release of cytochrome c by altering mitochondrial membrane permeability (Goldstein et al., 2005). In cross talk between the intrinsic and extrinsic pathway, caspase 8 mediates the cleavage of the pro-apoptotic protein Bid through the Fas death receptor mediated pathway (Li et al., 1998).

Mitogen activated protein kinases (MAPKs) are a specific family of signalling molecules that carry signals from the surface to the target molecule. This super family of kinases includes the extra-cellular signal-related kinase 1 and 2 (ERK 1/2), the c-jun N-terminal kinases 1–3 or stress-activated protein kinases (JNKs or SAPK), p38 families (α, β, γ and δ), the ERK 3 and 4. MAPKs are involved in cell differentiation, survival and cell death, depending on the stimuli received from extra-cellular environment (Kyriakis and Avruch, 2001). Phosphorylated JNK and p38 MAPKs activate their downstream molecules, and may become co-activated, depending on the stimuli from upstream kinases. Substrates for JNK and p38 MAPKs are transcription factors such as c-jun, ATF-2, p53 (Zou et al., 2007) and heat shock factors (Stokoe et al., 1992).

In response to virus infection, the host cell can activate defence mechanisms against viral replication including undergoing cell death to eliminate virus-infected cells. One such mechanism used by viruses that subvert infection is apoptosis (Cristina et al., 2001; Jeurissen et al., 1992; Lomonosova et al., 2002). However, viruses have evolved mechanisms that suppress apoptosis through interference with the cell death pathways orchestrated by viral gene products. Other viruses like human adenovirus (Debbas and White, 1993; Han et al., 1996), cytomegalovirus (Utama et al., 2006), Epstein–Barr virus (EBV) (Fries et al., 1996) and hepatitis viruses (Cao et al., 2004) block apoptosis by targeting p53 gene expression. Apoptosis mediated by the activation of JNK and p38 MAPK signalling pathways has been demonstrated previously for viruses such as reovirus (Clarke et al., 2001), hepatitis B virus (Wang et al., 2004), porcine circovirus type 2 (Wei et al., 2009), HIV-1 (Perfettini et al., 2005) and porcine reproductive and respiratory syndrome virus (Yin et al., 2012; Lee and Lee, 2012).

It has been shown that EAV infection induces apoptosis from 24 h of infection in Vero cells (Archambault and St-Laurent, 2000), and that the cell death process is mediated by caspase 8 and caspase 9 activation (St-Louis and Archambault, 2007). In order to further understand the mechanism of cell death induced by EAV, we have investigated the expression of pro-apoptotic and caspase genes in EAV infected cells.

The expression of different apoptotic genes was studied by SYBR Green real-time PCR. Baby Hamster Kidney 21 (BHK21) cells are permissive to EAV and therefore have been used as an infection system in this study. The cells were infected with two strains of EAV, SP3A and Arvac at a moi of 5. SP3A, a low passage strain derived from the Bucyrus "pleural fluid isolate" by plaque purification in a separate study, has been evaluated to cause severe clinical signs of equine viral arteritis in ponies (Wescott et al., unpublished). The Arvac virus is a recognized attenuated vaccine strain of EAV. Infected and mock-infected cells were incubated at 37 °C in a CO_2 incubator. Total RNA was extracted from the cells at different time points using the TRizol® Reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions, and the samples were subjected to DNAse treatment. Complementary DNA was synthesized using Oligo$(dT)_{20}$ to select mRNAs, and with Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (200 U) (Invitrogen, Grand Island, NY) as per manufacturer's protocol. The PCR was performed using Brilliant II SYBR Green QRT-PCR kit (Agilent Technologies, La Jolla, CA) in a RotorGene 3000 instrument (Corbett Research, Australia) with gene specific primers (Table 1). Analysis of gene expression data from real-time PCR was evaluated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Beta actin gene was taken as a reference endogenous control for normalization of mRNA expression values.

As JNK and p38 MAPKs mediate virus-induced apoptosis, we examined the expression of genes that are regulated by the JNK and p38 MAPK pathways, like p53 and Bax. It is known that p53 acts as a tumour suppressor gene and is an important regulator in apoptosis (Amaral et al., 2010). p53 can turn off the cell cycle and turn on the apoptosis mechanism under stress conditions, as it was observed with many viral infections, for example with HIV-1 (Perfettini et al., 2005). The gene expression analysis by real-time PCR showed an increase in p53 mRNA in cells infected with EAV compared to mock-infected cells, with steady levels of two to three fold increase from 8 h and 12 h post-infection with the SP3A (Fig. 1b) and Arvac (Fig. 1a) strains respectively. Induction of p53 provokes activation of genes like Bax, Noxa and PUMA. Therefore investigations on the expression of the Bax gene, which mediates cytochrome c release from the mitochondrial membrane (Granville et al., 1999) were included. The Bax mRNA has shown a pattern similar to that seen in p53 in the course of infection with the SP3A and Arvac strains. A signal for Bax mRNA observed at 4 h in the Arvac infection was followed by a decrease and a subsequent up-regulation from 12 h post-infection (Fig. 1c). Differently, in the SP3A infection, low p53 and Bax mRNA were detected early in infection, followed by an up-regulation in a recurrent manner from 8 h post-infection (Fig. 1d). As mentioned, some viral genes encode protein homologues which have anti-apoptotic properties and can suppress Bax gene expression at initial stages of infection (Cuconati and White, 2002). It is not known whether EAV encodes early proteins with such properties.

We analyzed the expression of initiators of programmed cell death, caspases 8 and 9, as they become activated due to the release

Table 1
Sequence of primers used for amplification of pro-apoptotic genes and endogenous control.

Gene	Product length (bp)	Forward and reverse primer sequences[a] (5′–3′)	T_a [b] (°C)	GenBank accession no.
Caspase 3	72	GTCTAACTGGAAAGCCCAAACTC CTCAATGCCACAGTCCAGTTC	55	FJ940732.1
Caspase 8	85	AACAGCAGCAAGGAGGAGATG GCATGACCCTGTAGGCAGAAA	55	EU527788.1
Caspase 9	98	CTCGAGGCAGGGACTTAGACA AAACTTGACACGGCATCCA	53	EU527789.1
p53	232	AAGGCGATAGTTTGGCTCCT3 CTGGGGTCTTCCAGTGTGAT	53	Y08900
BAX	215	AGCTGCAGAGGATGATTGCT CTCTCGGAGGAAGTCCAGTG	53	AJ582075.1
β-Actin	600	TGGGTCAGAAGGACTCCTATG AGAAGAGCTATGAGCTGCCTG	53	NM001101

[a] The final concentration of each primer was 150 nM.
[b] T_a – PCR annealing temperature.