Cell death induced by equine arteritis virus: possible roles of JNK and p38 MAPK signaling pathways

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ABSTRACT

Equine Arteritis Virus (EAV) is an enveloped positive single-stranded RNA virus that belongs to the Arteriviridae family. EAV infection causes Equine Viral Arteritis in horses, a disease which affects the economy in the equine industry. Cell infection with EAV ends with death of infected cells, a process that is relevant to the pathogenesis of disease. In a previous study it has been shown that the JNK and p38 MAPK signaling pathways are activated on infection with different strains of EAV in Baby Hamster Kidney 21 (BHK21) cells. Apoptosis or programmed cells death is known to be mediated through the JNK and p38 MAPK signaling pathways. Therefore, the aim of the current study was to investigate if EAV-induced cell death is due to apoptosis, in relation to JNK and p38 MAPK signaling in infected cells. A real-time PCR was optimized and used to analyze the expression of pro-apoptotic molecules like p53, Bax, initiator and executioner caspases (caspase 9, 3, 8) during infection with EAV. Further, activation of caspase 3/7 was evaluated with a fluorescence based assay that also measures cytotoxicity. Up-regulation of p53 and Bax was observed from 12 hr post-infection in BHK21 cells, with differences in kinetics between the two virus strains used. The levels of caspases 9 and 3 by qPCR were not high, however, there was a prominent down-regulation of these caspases in infected cells up to 16 and 20 hr post-infection, respectively, suggesting an inhibition mechanism used by the virus to ensure its replication. Increased levels of activated caspase 3/7 and cytotoxicity were found from 20 hr in infected cells using the caspase 3/7 assay, confirming virus-induced activation. Altogether the results demonstrate that EAV-induced cell death is due to apoptosis through caspase activation, and that the process uses the intrinsic signaling pathway for cell death. The study contributes to an understanding of the interaction of EAV with the host cell and to enlighten on the mechanism of pathogenesis of EAV-induced disease.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma-2</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal related kinase</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas- associated protein with death domain</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>Mitogen activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear-factor kappa- light chain enhancer B</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress activated protein kinase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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1. INTRODUCTION

1.1 Equine Arteritis Virus

Equine arteritis virus belongs to the family Arteriviridae, genus Aterivirus, in the order Nidovirales, which also include 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 & Meulenberg, 1998) with 5’ and 3’ untranslated regions (UTRs) and nine open reading frames (ORFs) (den Boon et al., 1991). ORFs 1a and 1b cover 75% of the genome at the 5’end and encode two replicase proteins, PP1a and PP1ab that are processed into the non-structural proteins of the virus. At the 3’ end, ORFs 2-7 occupy 25% of the genome and encode seven structural proteins, namely E, GP2B, GP3, GP4, GP5, M and a small basic nucleocapsid protein (N) which encapsidates the viral genome (Figure 1) (Snijder et al., 1994).

Figure 1. The genome organization of equine arteritis virus. (Picture by Munir Muhammed.)

1.2 Equine Viral Arteritis

Equine viral arteritis is a contagious respiratory and reproductive tract disease which affects equids like horses, donkeys and mules. EAV was first isolated in Bucyrus, Ohio, USA (Bryans et al., 1957) during outbreaks of respiratory disease and abortions in North America. Later, it was found to be present in countries in Europe and South America (Wood et al., 1995). The symptoms of disease include abortions in pregnant mares, pneumonia in young foul, and
influenza like sickness characterized by fever, edema and nasal discharge (Huntington et al., 1990). EAV persistently infects the reproductive tract of pre-pubertal stallions inducing a carrier state that can be short or long lasting. The virus replicates in macrophages, endothelial cells of small arteries, lungs, intestine, kidney, reproductive tract and may spread to other regions of the body (MacLachlan et al., 1996). The respiratory route is the primary mode of transmission, however, natural and artificial breeding also play an important part (MacLachlan & Balasuriya, 2006).

1.3 Apoptosis

Apoptosis or programmed cell death is a process of cell suicide in response to stimuli. The term apoptosis was coined by Currie and his co-workers in 1972. It is characterized by morphological features and biological changes in the cell, like chromatin condensation, cell shrinkage, membrane blebbing and formation of apoptotic bodies. Apoptosis is triggered by a variety of stimuli which activate intracellular signaling cascades for cell death process. Examples of such stimuli are UV radiation, oxidative stress, genotoxic chemicals and infectious agents like viruses. Apoptosis differs from the necrosis, though they share similar metabolic events in the process. Necrosis is a degradative process and can be fatal to the tissue. Apoptosis is an irreversible, ordered process, which leads to cell death and is necessary in physiological processes like development of the embryo, maintenance of tissue homeostasis and functioning of the immune system.

Caspases belongs to the family of cysteine proteases that are cysteine dependent aspartate directed proteases that cleave specific motifs at aspartic acid residues. The name “caspase” was given based on its catalytic activity. These are formed in inactive form of zymogens (proenzymes) (Cohen, 1997). Until now, ten caspases have been recognized and broadly grouped into initiators (caspase 2, 8, 9, 10), effectors or executioners (caspase 3, 6, 7) and inflammatory caspases (caspase 1, 4, 5) (Danial & Korsmeyer, 2004).

Generally, cell death through the apoptotic process occurs via two pathways, extrinsic and intrinsic pathways. Extrinsic signaling pathway is mediated by the death receptor present on the cell surface. The death receptors come under the family of tumor necrosis factor (TNF) receptor (Locksley et al., 2001). Cytoplasmic death domain of the receptor mediates the signal from the surface to intra cellular signalling pathways. FasL/FasR, TNF-α/TNFR1,
Apo3L/DR3 are some of the well known ligands and corresponding receptors (Ashkenazi & Dixit, 1998). In the process of apoptosis, the ligand binds to the corresponding receptor, followed by binding of adaptor proteins to the cytoplasmic receptor through the death domain. For example, Fas ligand binds to the Fas receptor leading to binding of the adaptor protein, FADD and subsequent recruitment and activation of procaspase 8 (Wajant, 2002). The result is the cleavage of the executers of apoptosis, caspase 3 and caspase 7. The intrinsic pathway on the other hand, is a non receptor mediated pathway which is stimulated by intracellular signals like DNA damage and directly acts on target molecules. Mitochondria play a vital role by releasing pro-apoptotic factors like cytochrome c. Released cytochrome c forms an apoptosome by binding with apoptotic protease activating factor Apaf-1 and caspase 9 (Chinnaiyan, 1999). Accumulation of procaspase 9 lead to activation of caspase 9 and also the downstream molecules caspase 3/7 which lead to cell death (Jiang & Wang, 2004). Mitochondrial events in apoptosis are regulated by the Bcl-2 family of proteins including anti-apoptotic proteins, Bcl-2, Bcl-x, Bcl-XL, Bcl-XS and pro-apoptotic proteins such as Bax, Bak, Bid, Bad, Bim, Bik. These pro-apototic proteins oversee the release of cytochrome c by altering mitochondrial membrane permeability (Gross et al., 1999). In cross talk between the intrinsic and extrinsic pathway, caspase 8 mediates the cleavage of pro-apoptotic protein Bid in Fas death receptor mediated pathway (Li et al., 1998).

1.4 Intracellular signaling pathways in apoptosis

Cell regulation involves stimulation, activation and expression of different transcription factors and genes in response to the stimuli or signal received from the cell surface. These signals are carried from the surface to the target by a specific kind of signaling molecules called Mitogen Activated Protein Kinases (MAPKs), which regulate different signaling cascades, involved in cell differentiation, survival and cell death, depending on the stimuli received from extra-cellular environment (Kyriakis & Avruch, 2001). Until now, in mammals, five different types of MAPKs have been identified: extra-cellular signal-related kinases 1 and 2 (ERK 1/2), c-Jun N-terminal kinases 1-3 or stress-activated protein kinases (JNKs or SAPK), p38 families (α, β, γ and δ), ERK 3 and 4 and, most recently discovered ERK 5 of big MAP Kinases (Chen et al., 2001).
MAPKs are activated by variety of external stimuli, for example, ERK 1 and ERK 2 are activated due to interaction with growth factors (transforming growth factors), serum, cytokines, osmotic pressure and other stimuli (Raman et al., 2007). JNK and p38MAPKs are activated by stress signals like heat, inflammatory responses, DNA damage, ionizing radiation and other environmental stress (Kyriakis & Avruch, 2001). These signaling cascades carried out by three sequential activations of kinases. Upon receiving signal from surface, the first kinase MAPKKK (MAP3K) is activated by phosphorylation on serine and threonine residues, leading to phosphorylation of MAPKK. This in turn activates MAPKs by phosphorylating threonine and tyrosine residues. The activated MAPKs will then act on downstream substrates based on the received stimuli. MAPKs are involved in different biological functions, for example, ERK activation regulates motility of cell, cell proliferation, differentiation and survival (Lewis et al., 1998). JNK and p38 MAPK are involved in cell proliferation, differentiation and programmed cell death (Chen et al., 2001). The illustration in Figure 2 summarises these events.

**Figure 2.** MAPK signaling pathways mediate cellular responses upon extra- or intra-cellular stimuli.

### 1.5 JNK MAPK signaling pathway in apoptosis

Apoptosis depends on the stimuli received at cell surface and also on the intra-cellular signaling pathways like c-Jun amino terminal Kinase (JNK) and p38 signaling pathways. As mentioned before, JNK MAPKs are also called stress-activated protein kinases as they get activated under stress conditions. JNK has 3 isoforms, 1-3, with JNK1 and JNK2 isoforms being present in all types of tissues while JNK3 is mainly present in cardiac and brain tissues.
JNK activation involves phosphorylation on Thr183 and Tyr185 in upstream sequential events, and both phosphorylations are required for its complete activation. Different phosphorylations of JNK serve different functions. Phosphorylation at Tyr185 can be observed in TNF-α induced JNK Activation (Tournier et al., 2001). The activated JNK MAPKs mainly control the activity of c-jun transcription factor, a member of activator protein 1(AP-1) family, by binding and phosphorylating it (Barr & Bogoyevitch, 2001). c-jun activated by JNK plays a major role in neuronal apoptosis, NGF withdrawal in PC12 cells (Ham et al., 1995), and induces apoptosis in NIH3T3 fibroblasts (Bossy-Wetzel et al., 1997). c-jun activation is also required for the cytochrome c release from mitochondria (Tournier et al., 2000). Pro-apoptotic family members promote cytochrome c release (Martinou, 1999).

It has also been reported that cells exposed to UV light can cause apoptosis mediated through c-jun and p53 (Shaulian et al., 2000). JNK signaling pathway is involved in TNF-α induced apoptosis when anti-apoptotic action of NF-kB is inhibited (Tang et al., 2001). Chemical substances like Cisplatin, generally used in cancer therapy, activate JNK and p38 MAPK pathways differentially, and persistent activation of JNK leads to increased production of phosphorylated c-jun transcription factor, and subsequently apoptosis mediated by death ligand/receptor(FasL/Fas) (Mansouri et al., 2003).

1.6 p38 MAPK signaling pathway in apoptosis

p38 MAPK was first identified as a 38 kDa protein produced in response to lipopolysaccharide induction (Han et al., 1993). To date, there are four isoforms of the p38 family identified as: p38α, p38β, p38γ (also called ERK 6 or SAPK3) and p38δ (Jiang et al., 1996; Lechner et al., 1996; Li et al., 1996). The p38 MAPK signaling pathway is activated by numerous extra-cellular signals, growth factors, oxidative stress, osmotic shock, UV light, heat, cytokines etc. and depends on the stimulus and type of cell (Raingeaud et al., 1995; Rouse et al., 1994). This pathway is activated upon dual phosphorylation at the Thr-Gly-Tyr activation loop (Hanks & Hunter, 1995) by upstream kinases, MKK3 and MKK6. Some times MKK4, an activator of JNK, can also be involved in the p38 activation depending on the cell type (Zou et al., 2007), JNK and p38 MAPKs are co-activated depending the stimuli from upstream kinases. The substrates for p38 downstream, along with its family member MK3, activates heatshock proteins (Stokoe et al., 1992), cAMP response element-binding protein
(CREB) (Nemeth et al., 2003) and many transcription factors such as activating transcription factors 1, 2 & 6 (ATF 1/2/6), p53, CHOP (growth arrest and DNA damage inducible gene 153), and also regulate AP-1 which has a binding site for the jun family of transcription factors (TFs) (Zhao et al., 1999). Apart from TFs, p38 MAPKs also regulate genes encoding cytokines, cell surface receptors and inflammatory proteins (Hunt et al., 2002). Activation of p38 has different biological responses including cell growth and differentiation, tumour suppression, inflammation and programed cell death. Apoptosis mediated by p38 signaling pathway can be induced by various stimuli, such as NGF withdrawal and Fas receptor that, for example, lead to activation of JNK and p38 MAPKs in rat PC-12 cell (Sarker et al., 2003); Fas ligand binding to the Fas receptor lead to the activation of p38 MAPK which mediates mitochondrial cell death in CD8\(^+\) T cells (Farley et al., 2006). Induction of apoptosis was observed in cardiac muscle cells infected with upstream activators of p38 Kinases with the help of adenoviral vectors (Wang et al., 1998).

### 1.7 Viruses and Apoptosis

Viruses are the common infectious agents causing diseases in animals and humans. They use host cell mechanisms for their replication and survival. Some virus infections lead to the host cell’s death after producing enough progeny. In response to the virus infection, the host cell can also activate defence mechanisms against viral propagation including undergoing cell death, perhaps by apoptosis, to eliminate virus-infected cell. Cell death, be it initiated by virus as means to invade surrounding cells or by the cell defence as means to limit spread of infection often involve apoptosis. However, viruses have evolved mechanisms that suppress apoptosis through interference with the cell death pathways orchestrated by viral gene products. Human adeno virus blocks apoptosis through the E1B 19k (Han et al., 1996) and E1B 55K (Debbas & White, 1993) proteins which are mediated by p53 expression. Some viral genes encode proteins which are similar to the Bcl-2 family proteins and suppress apoptosis. For example, african swine fever virus encodes a LMW5-HL/A179L protein homologue to Bcl-2 that inhibits apoptosis (Afonso et al., 1996). Reports have shown that many viruses express products targeting host p53 expression to inhibit the cell death, like cytomegalo virus, epstein-Barr Virus (EBV), Hepatitis viruses, Pailloma viruses etc. Other viruses, like Baculovirus, have established a mechanism that can target and inhibit caspase activity (Wang
et al., 2003). These findings suggest that viral proteins target Bcl-2 family proteins and p53 in blocking apoptosis.

Many viruses induce apoptosis using the host cell intracellular machinery by activating many signaling pathways. As discussed before, JNK and p38 MAPK pathways play a major role in apoptosis and, these pathways are activated to initiate a death signal, when the cell is exposed to viral stress. The mechanism behind the activation of these pathways is still unclear. Reovirus infection in L929 cells activates the JNK pathway, transcription factors c-jun (Clarke et al., 2001), NF-kB (Connolly et al., 2000), and tumour necrosis factor related apoptosis inducing ligand (TRAIL) (Clarke et al., 2000) to induce apoptosis through the extrinsic signalling pathway. In the same way hepatitis B virus protein X, induces apoptosis mediated by JNK and p38 MAPKs in AML12 derived cell lines (Wang et al., 2004). Porcine circovirus Type 2 infection in PK15 cell leads to apoptosis mediated by p38 and JNK MAPK (Wei et al., 2009). In the downstream events p38 MAPK phosphorylates p53 and initiates cell death induced by HIV-1 envelope (Perfettini et al., 2005).

Infection with equine arteritis virus results in cytopathic effects, however the mechanism of cell death has not been elucidated. Infection of Vero cells with EAV was shown to induce apoptosis after 24 hr (Archambault & St-Laurent, 2000), and that the process was mediated by caspase-8 and caspase 9 activation (St-Louis & Archambault, 2007). Our recent studies on EAV shown that JNK and p38 MAPKs are activated by different strains of EAV during infection in BHK21 cells (Paidikondala et al., submitted). As signaling pathways might mediate EAV-induced cell death, understanding the EAV-host interactions would expand the knowledge in mechanism of pathogenesis of equine arthritis virus.

1.8 Aims of this study

- To identify apoptotic molecules induced by infection with Equine Arteritis Virus (EAV).
- To confirm that EAV-induced cell death occurs by apoptosis.
- To relate this to activation of JNK and p38 MAPK signaling pathways in infected cells.
2. MATERIALS AND METHODS

2.1 Cell cultures

Baby Hamster Kidney 21 (BHK21) cell line was propagated in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% of fetal bovine serum, 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml of streptomycin and incubated at 37 °C in a 5% CO2 incubator. The cells were infected when they were 80% confluent.

2.2 Virus inoculums

Two strains of EAV were used for infections, ARVAC and SP3A, which have distinct behaviour in the natural host. The ARVAC virus is avirulent while the SP3A is virulent causing severe disease in ponies. The cell monolayers were washed with PBS and infections performed at a multiplicity of infection (MOI) of 10 PFU/cell. Infected cells were incubated at 39.5 °C in CO2 incubator. Mock-infected cells were also included as control. The cells were lysed for RNA extraction at different time points in the course of infection.

2.3 RNA extraction and cDNA synthesis

Total RNA was extracted from infected and mock-infected cells cells using the TRizol method as per the manufacturer’s instructions (Invitrogen). Concentration of the RNA was determined in a Nanodrop. cDNA was synthesized by incubating 200 ng of RNA with 1 µM of Oligo(dT)$_{20}$ (Invitrogen) in a 20 µl reaction at 65 °C for 10 min. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (200U), RNasin (32U), 0.1 M DTT and 5 X first strand buffer were used in the reaction mixture. The synthesis was carried out at 22 °C for 5 min, 37 °C for 90 min and 95 °C for 5 min.

2.4 Quantification of gene expression by real-time PCR

Five pro-apoptotic and Bcl-2 family genes were selected for evaluation of their mRNA expression during EAV infection. Primer pairs for the real-time PCR were designed based on sequences of Syrian hamster (Golden hamster; *Mesocricetus auratus*) available in GenBank, and are listed in Table 1. The PCR conditions were optimized for annealing temperature, template and primer concentrations for each gene. The optimized real-time PCR was then used
for each target and run with cDNA prepared from the time-course RNA extracts. The reaction mix, 2x Brilliant SYBR Green qPCR Master Mix (Agilent Technologies) was used with 1 µl of cDNA template. The cycling profile was initial denaturation at 95 °C 10 min, followed by denaturation 95 °C for 20 sec, annealing at 55 °C for 30 sec, extension 72 °C for 30 sec for 40 cycles. Corbett Rotor-Gene 3000 PCR machine (Qiagen) was used for the experiment.

Table 1: Primer pairs for amplification of pro-apoptotic genes and endogenous control

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product length (bp)</th>
<th>Primer sequences (forward and reverse)</th>
<th>GenBank accession no.</th>
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<td>Caspase 3</td>
<td>72</td>
<td>5'GTCTAACTGGAAGGCCAAACTC3',</td>
<td>FJ940732.1</td>
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<tr>
<td></td>
<td></td>
<td>5'CTCAATGCCACAGTCCAGTTC3'</td>
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<tr>
<td>Caspase 8</td>
<td>85</td>
<td>5'AACAGCAGCAAGGAGGAGATG3',</td>
<td>EU527788.1</td>
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<tr>
<td></td>
<td></td>
<td>5'GCATGACCTGTAGGCAGAAGA3'</td>
<td></td>
</tr>
<tr>
<td>Caspase 9</td>
<td>98</td>
<td>5'CTCGAGGCGAGGACTTAGACA3',</td>
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<tr>
<td></td>
<td></td>
<td>3'AAACTTGACACGGCATCCA3'</td>
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<tr>
<td>p53</td>
<td>232</td>
<td>5'AAGGCGATAGTTTGGCTCCT3',</td>
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<td></td>
<td></td>
<td>5'CTCTCGGGAAGGATGATCCAGTG3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>600</td>
<td>5' TGGGTCAGAAGGACTCTATG3',</td>
<td>NM001101</td>
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<tr>
<td></td>
<td></td>
<td>5'AGAAGAGCTATGAGCTGCTG3'</td>
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</table>

2.5 Statistical evaluation of the data

Analysis of gene expression data from Real-Time Q-PCR were evaluated by the $2^{\Delta \Delta Ct}$ method (Livak & Schmittgen, 2001), which gives the changes in expression of the target gene relative to the endogenous reference control such as β-actin used in the experiment. -ΔΔCt can be calculated by the normalization of the target gene with the control gene at each time point and at zero time.

\[-\Delta \Delta Ct = (C_{T, Target} - C_{T, Actin})_{Time x} - (C_{T, Target} - C_{T, Actin})_{Time 0}\]

2.6 Infection of cells and assay for caspase 3/7 activation

Freshly passaged cells were seeded in opaque 96 well plate in 100 µl of F-DMEM medium per well. The next day, cells were infected with ARVAC and SP3A strains of EAV at MOI of 10 PFU/cell and incubated at 39.5 °C with 5% CO₂. Reagents for cytotoxicity and caspase 3/7
activation tests were prepared according to manufacturer’s protocol (ApoTox-Glo™ Triplex Assay, Promega, Madison, USA) and stored at 4 °C. To check cytotoxicity, 20 μl cytotoxicity reagent was added and briefly mixed by shaking for 30 sec and incubated at 37 °C for 30 min. Fluorescence was measured with a Luminometer (Promega, Madison, USA) at wavelength 485Ex/520Em. Later, 100 μl of Caspase-Glo® 3/7 reagent was added and mixed by shaking for 30 sec and incubated at room temperature for 30 min. Luminescence was measured to determine caspase 3/7 activation. Readings were taken at different time points after infection of cells (18 hr, 20 hr, 24 hr and 28 hr) based on the results for pro caspases by Q-PCR. Mock infected cell were taken as a control at same time points.

2.7 Preparation of lysates and western blotting

Cell lysates were prepared for western blot analysis from infected and control cells at 0 hr, 8 hr, 12 hr, 14 hr, 16 hr, 18 hr, 20 hr, 24 hr and 28 hr. Cell lysates for protein analysis were prepared using lysis buffer (50 mM Hepes, 1 mM NaCl, 1mM EDTA and IGEPAL (1%)). Infected cells were incubated on ice with PBS (without Ca and Mg) for 5 min. The cells were scrapped and centrifuged at 1400 x g for 4 min in a refrigerated centrifuge and the supernatant was removed. The cells were resuspended with PBS and centrifuged as before. To the cell pellet, 500 μl cell lysis buffer (with protease and phosphatase inhibitors) was added, incubated on ice for 30 min and thereafter centrifuged at 9,300 x g for 20 min at 4 °C. The supernatant was collected and stored at -70 °C until use. Cell lysates were mixed with SDS sample buffer and separated on 12% SDS-PAGE gels. Then, proteins were transferred on to the PVDF membrane (GE Healthcare, Uppsala). The membrane were incubated with blocking solution (TBS-T with 3% Bovine Serum Albumin) (Sigma Life Sciences, St.Louis, USA), for 1 hr, later incubated with p-JNK and p-p38 MAPK antibodies raised in rabbit (Santa Cruz Biotechnology, Inc) overnight at 4 °C. Then, membranes were washed with TBS-T for 10 min for three times. After, the membranes were incubated with HRP-conjugated polyclonal antibodies raised in mouse against rabbit antibodies (DakoCytomation, Denmark) for 1 hr at room temperature and washed with TBS-T as before. The membranes were developed with ECL advanced kit (GE Healthcare Limited, UK) in Chemi Doc instrument (Bio-Rad laboratories Inc, CA).
3. RESULTS

3.1 Optimization of real-time PCRs for analysis of expression of pro-apoptotic molecules

First, six real-time PCRs were optimized to quantify expression of pro-apoptotic molecules and control gene, β-actin in BHK21 cells infected with EAV. The optimization was performed with different template and gene specific primer concentrations using Brilliant SYBR Green qPCR master Mix. Figure 3 shows the optimized real-time PCR amplification curves for each pro-apoptotic gene. The amplification of the gene was detected by measuring the fluorescence of the dye at each cycle.
3.2 Analysis of pro-apoptotic gene expression by qPCR

BHK21 cells infected with a virulent and an avirulent strain of EAV and non-infected control cells were used to extract RNA at different time points after infection. cDNA was synthesized from the RNA using oligo d(T)20 to select for mRNA. Optimized PCRs were used to determine mRNA for five genes that are indicators of apoptosis: p53, Bax, and caspases 9, 3, and 8. The results were as follows:

3.2.1 Expression of p53 and Bax genes

Up to 10 hr post-infection, the levels of p53 remained either close to the values in control cells (0, 4 and 8 hrs) or lower than in control cells (8 hr in ARVAC infection, 4hr and 10 hr in SP3A infection). There was not a clear pattern of expression during this period. Remarkably, from 12 hr post-infection the expression of p53 increased significantly in the infected cells when compared to the non-infected controls at the same. The highest mRNA levels were observed at 12 hr for the SP3A virus and at 14 hr in the ARVAC infected cells (Figure 4, A). Thereafter, the levels declined for both viruses, but SP3A infection showed comparatively higher expression.

Induction of the p53 gene in infected cells provokes the activation of genes like Bax, Noxa and PUMA. Therefore, investigations were done on the expression of the Bax gene, which mediates cytochrome c release from the mitochondrial membrane. The Bax gene has shown a similar expression pattern as seen in p53 in the course of infection with the SP3A and Arvac strains. Increased levels of Bax were observed from 12 hr post-infection. In the ARVAC infection, the peak was at 14 hr post-infection, followed by a drop thereafter (Figure 4, B).
However, in the SP3A infection, recurrent expression of Bax was observed throughout the infection period.

Figure 4: A. p53 and B. Bax, gene expression during time course infection with ARVAC and SP3A strains of EAV, determined by real-time PCR analysis. Mock-infected cells were taken as a control.

3.2.2 Expression of caspase 9 and caspase 3

Caspase 9 and caspase 3 molecules are the executioners of programmed cell death, and are activated by cytochrome c release from mitochondria. Up to 16 hr post-infection, the levels of caspase 9 in infected cells remained below the levels observed in control cells, indicating not only lack of virus-induced caspase but also a down-regulation of basal caspase 9 in infected cells. From 12-14 hr this down-regulation seemed to recede gradually as levels of caspase 9 went up without being significantly different between infected and non-infected cells. Thus,
virus infection does not increase the production of caspase 9 but down-regulates normal caspase 9 at initial 12-14 hr of infection (Figure 5, A). On the other hand, the data seems to indicate that availability and not high level of caspase 9 is needed to engage apoptosis, as later shown from results on analysis of caspase 3.

The activated caspase 9 induces activation of procaspase 3 to form an apoptosome with Apf-1. Therefore, expression of caspase 3 was tested in infected cells in relation to control cells. In general a basal level of expression of caspase 3 was seen in normal cells. The down-regulating effect of infection on caspase 3 was also seen in infected cells, stronger in the SP3A infection (except at 8 hr) (Figure 5, B). Down-regulation receded from 16 hr in both infections, however the levels in the SP3A infected cells did not go above those in non-infected cells. In contrast, in cells infected with the ARVAC strain, caspase 3 levels were higher at 24 and 28 hr post-infection compared to normal cells.
3.2.3 Expression of caspase 8

The results on caspase 8 showed erratic peaks of expression in non-infected cells at 12, 20, 24 and 28 hr, and at 4 hr in cells infected with SP3A. Considering the scale, there appear to be an increased expression in infected cells at 14 and 16 hr (Figure 5, C). However, the pattern in the remaining infection period is indicative of down-regulation in infected cells.

3.3 Analysis of caspase 3/7 activation in an “in vitro” assay

In order to confirm the caspase 3 results obtained by qPCR, activation of caspase 3/7 as unequivocal event in apoptosis was measured using a caspase 3/7 assay that also measures cytotoxicity. Caspase 3/7 levels increased slowly from 16 hr p.i with both the ARVAC and the SP3A infections. Considerable higher activation levels were observed at 24 hr, and a dramatic increase was seen at 28 hr with higher activation being induced by the ARVAC virus (Figure 6, A). This high increase in caspase 3/7 activation correlated with increase in cell toxicity from 20 hr post infection. There were no significant differences between the viruses in inducing cytotoxicity (Figure 6, B).

Figure 5. Gene expression analysis of Caspase 9(A), Capase 3(B), and Caspase 8(C) after infection with ARVAC and SP3A strains of EAV. Mock-infected cells were taken as a control.
Figure 6. Activation of caspase 3/7 (A) and cytotoxicity assay (B).
4. DISCUSSION

Cellular signaling pathways are activated in response to a variety of stimuli like stress, heat, viral infections etc. Importantly, the mitogen activated protein kinase pathways (MAPK pathways) play a major role in biological responses to stimuli, particularly virus infections. Several viruses have been shown to activate MAPK pathways that lead to apoptosis and contribute to cytopathic effect of host cells (Andrade et al., 2004).

Prior research on EAV has shown that apoptosis was induced upon infection of Vero cells with a Canadian strain of EAV and that it was initiated by the activation of caspase-8 and mitochondrion dependent caspase 9 (St-Louis & Archambault, 2007). The activation of intracellular signaling cascades by EAV infection and the crosstalk among these pathways was hypothesized to play a role in the fate of infected cells, presumably by apoptosis (Paidikondala et al., submitted). Since the death of infected cells is responsible for tissue damage, we have investigated cell death induced by EAV as means to shed light on pathogenesis mechanisms.

As JNK and p38 MAPKs mediate virus-induced apoptosis, we examined the expression of genes which are regulated by the JNK and p38 MAPK pathways like, p53 and Bax. p53 gene is a tumour suppressor gene and an important regulating gene in apoptosis (Amaral et al., 2010). p53 gene can turn off the cell cycle and turn on the apoptosis mechanism under stress conditions as was observed with many viral infections, for example with HIV-1 (Perfettini et al., 2005). From gene expression analysis by real-time PCR, we have shown that expression of p53 gene is up-regulated from 10 hr post-infection, reaching maximum levels at 24 hr after infection with the ARVAC strain, and from 14 hr upon infection with the SP3A strain. This indicates that the up-regulation of p53 gene may inhibit cell cycle progression and activates response genes like Bax that was also found up-regulated. The reason for the suppression of p53 gene in the early stages of infection may be due to that some viral product(s) inhibit(s) the p53 gene, to enable production of enough viral progeny in the host cells. Similar low levels of Bax expression was detected at initial stages of infection with up-regulation observed from 16 hr p.i. Viral genes encoding proteins homologues to Bcl-2 family proteins, which have anti apoptotic properties (Cuconati & White, 2002) suppress the Bax gene expression at initial stages of infection. Further examinations were done on Bax gene expression, which facilitates cytochrome c release by permeabilizing the mitochondrial membrane (Granville et al., 1999). p53 dependent Bax transcription induces cytochrome c release from mitochondria resulting is
caspase 9 activation (Chipuk et al., 2004). Released cytochrome c initiates caspase 9 activation to form the apoptosome, followed by subsequent activation of caspase 3. Activated caspase 3 was observed from 24 hr and correlated with upstream caspase 9 expression, confirming the involvement of caspase activation in EAV induced cell death.

The pattern of expression of caspase 8 was not conclusive, however in the overall it appear to indicate inhibition. It might be that the viral proteins were suppressing caspase 8 formation from procaspase 8 or the adapter protein, FADD, was inhibited to associate with procaspase 8. This indicates that receptor mediated signaling pathway in apoptosis is not at play in EAV infections in BHK21 cells. In contrast, the results from screening the other apoptosis marker show that EAV-induced apoptosis occur through the intrinsic signaling pathway. For better understanding about the suppression of apoptosis at initial stages of infection, we can further study the role of early proteins EAV, i.e. non-structural proteins in inhibition of cell death.

ApoTox assay was performed to detect activation of caspase 3/7 in cells infected with EAV. Activation levels of caspase 3/7 were increased from 16 hr and reached maximum levels at 28 hr after infection, varying with ARVAC and SP3A strains of EAV. This confirmed that caspase 3/7 is involved in cell death induced by EAV in BHK21 cells. The cytotoxicity assay detects proteases in the cell medium that come from dead cells. The levels of cytotoxicity increased with the caspase 3/7 levels, showing that cell death was induced by EAV infection. Differences in kinetics of p53/Bax and caspase 9 and caspase 3 in cells infected with the ARVAC and SP3A suggest that these strains that differ in biological properties probably have distinct mechanisms to engage apoptosis. Collectively, the results from this study expand the findings by Archambault & St-Laurent (2007) that infection with EAV induces apoptosis in Vero cells mediated through caspase 8 and caspase 9. Differences in cell type and in EAV strains could be the reason for the discrepancy of results on caspase 8 activation between this study and that of Archambault & St-Laurent (2007).

The activation of JNK MAPK signaling pathway influences apoptosis by various mechanisms, including cytochrome c release (Tournier et al., 2000) and requires pro-apoptotic Bcl-2 associated proteins. In addition various transcription factors (TFs) including c-Jun, p53 and activation transcription factor 2 (ATF2) are activated by JNK, and these TFs may affect apoptosis by up-regulating apoptosis-related genes.
The results from the present studies have shown that previously reported activation of JNK and p38MAPK in BHK21 cells infected with EAV lie behind cell death by apoptosis, and also that EAV induces apoptosis through the intrinsic signaling pathway mediated by mitochondria. The study contributes to an understanding of the interaction of EAV with the host cell and to enlighten on the mechanism of pathogenesis of EAV-induced disease.
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