

Epizootic haemorrhagic disease virus induced apoptosis in bovine carotid artery endothelium is p53 independent

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Summary

Epizootic haemorrhagic disease virus (EHDV) replicates in endothelium and it has been shown that EHDV serotype 2 (Ibaraki) is able to cause cell death by apoptosis in cow pulmonary artery endothelial cells. However, the underlying mechanism has not been established. For some viruses, such as influenza, a p53 dependent mechanism has been demonstrated in viral induced apoptosis. In this study, we investigate the involvement of p53 in the induction of apoptosis in a US isolate of EHDV serotype 2 in cow endothelium. We inoculated cow carotid artery endothelial cell cultures with live and inactivated EHDV-2 isolated from a white-tailed deer (*Odocoileus virginianus*). Using *in situ* nick end-labeling (TUNEL), caspase-3 (cleaved) immunohistochemistry (IHC), flow cytometry and annexin staining we documented the development of apoptosis and its direct relation to viral replication. p53 gene regulation and protein expression were assessed by reverse transcription polymerase chain reaction and IHC, respectively, in infected cells. We show that p53 mRNA was not upregulated and protein expression was not significantly increased. No increase of p53 mRNA or protein expression was observed in cells that stained positive for EHDV antigen. Our results indicate that EHDV induces apoptosis through a p53 independent mechanism.

L'apoptosi indotta dal virus della Malattia emorragica epizootica nelle cellule endoteliali dell'arteria carotidea nei bovini è indipendente dalla proteina p53

Parole chiave

Apoptosi,
Arteria carotidea bovina,
Endotelio,
Malattia emorragica
epizootica,
Proteina tumorale 53,
Virus della Malattia
emorragica epizootica.

Riassunto

Il virus della Malattia emorragica epizootica (EHDV) si replica nell'endotelio vascolare. Si è visto come il sierotipo 2 dell'EHDV, ceppo Ibaraki, è in grado di causare la morte delle cellule endoteliali delle arterie polmonari di bovino per apoptosi. Il meccanismo che sta alla base del fenomeno non è stato del tutto chiarito. Per alcuni virus (ad es. influenza) l'apoptosi è p53 dipendente. Oggetto dello studio è stato il coinvolgimento della proteina p53 nell'induzione dell'apoptosi da parte del sierotipo 2 dell'EHDV nell'endotelio bovino. Pertanto, colture di cellule endoteliali dell'arteria carotidea di bovino sono state inoculate con il ceppo vivo e inattivato del sierotipo 2 dell'EHDV isolato da un cervo della Virginia (*Odocoileus virginianus*). Mediante utilizzo del saggio TUNEL (*in situ* nick end-labeling), dell'immunoistochimica per la caspasi-3 (IHC), della citofluorimetria e della colorazione dell'annexina, è stato possibile documentare il processo apoptotico e la sua relazione diretta con la replicazione virale. Nelle cellule infette, la regolazione del gene p53 e la sua espressione proteica sono state testate mediante reazione a catena della polimerasi trascrittasi inversa (RT-PCR) e IHC. L'indagine non ha rilevato aumenti significativi dei livelli di RNA messaggero (mRNA) della p53 né della sua espressione proteica neanche nelle cellule positive alla colorazione per l'antigene EHDV. I risultati ottenuti indicano che EHDV induce apoptosi attraverso un meccanismo che non è dipendente dalla proteina p53.

Many viruses have been shown to cause cell death through induction of apoptosis (Shen and Shenk 1995), thereby limiting (e.g. simian immunodeficiency virus) or enhancing (e.g. bovine herpes virus 1) virus spreading (Devireddy and Jones 1999). Viral encoded proteins may interact with apoptotic pathways to inhibit or initiate apoptosis (Danen-van Oorschot *et al.* 2000, Martin and Berk 1998, Teodoro and Branton 1997). The pathophysiology of Bluetongue virus (BTV), which causes severe haemorrhagic disease in sheep and ovine and bovine cell death *in vitro*, has been linked to caspase-dependent extrinsic and intrinsic pathways of apoptosis (Nagaleekar *et al.* 2007, Stewart and Roy 2010, Mortola and Larsen 2010, Mortola and Roy 2004). It has been proposed that injury to the endothelium of small blood vessels is responsible for the lesions in infected sheep (DeMaula *et al.* 2001) and that BTV outer capsid proteins are sufficient to trigger apoptosis (Mortola and Roy 2004). p53 is an important transcription factor, which is involved in regulating apoptosis. Viruses may induce apoptosis by a p53 dependent (e.g. bovine herpes virus 1 and herpes simplex virus) or independent mechanism (e.g. Chicken anaemia virus and Dengue virus) (Devireddy and Jones 1999, Müller *et al.* 2004, Danen-van Oorschot *et al.* 2000, Teodoro *et al.* 1997, Thongtan *et al.* 2004).

Epizootic haemorrhagic disease viruses (EHDV), which are closely related to BTV, cause disease in white-tailed deer (WTD), which is characterized by endothelial damage, primarily of the microvasculature, resulting in oedema, haemorrhage, tissue necrosis, and disseminated intravascular coagulation (Fletch and Karstad 1971, Howerth *et al.* 1988, Howerth and Tyler 1988). These viruses primarily replicate in endothelial cells resulting in cell death (Howerth and Tyler 1988, Tsai and Karstad 1973). Furthermore, the Ibaraki strain of EHDV-2 has been shown to induce apoptosis in spontaneously immortalised ovine kidney cells and calf pulmonary aortal endothelial cells *in vitro* (Shai *et al.* 2013). However, the relationship between EHDV induced apoptosis and p53 has not been fully established. Our objectives for this study were to determine whether US EHDV-2 causes endothelial cell death via apoptosis, and whether apoptotic cell death is receptor mediated or directly related to virus replication. Furthermore, we evaluate whether apoptotic cell death is p53-dependent.

To demonstrate that EHDV causes apoptotic cell death in endothelial cells, bovine carotid artery endothelial cell (CAE) monolayers grown in 25 cm² flasks or chambered slides were inoculated with EHDV-2 (10^{5.7} TCID₅₀/25µl) and processed for agarose gel electrophoresis, Annexin V-binding assay to detect phosphatidylserine, and *in situ* end-labeling by terminal transferase (TUNEL) assay

at various time points. Tissues from deer naturally infected with EHDV were also assayed by TUNEL. Monolayers inoculated with tumor necrosis factor (100 ng/ml; TNF α , mouse; recombinant, Sigma), or left untreated were used as positive and negative controls, respectively. The cells were incubated for 24 to 48 hours and then either dissociated with trypsin/EDTA, and used for annexin V binding/electrophoretic DNA fragmentation assays, or fixed in 10% buffered formalin and processed for fragment end labeling (FragEL) and TUNEL to detect DNA fragmentation typical of apoptosis.

Agarose gel electrophoresis (Gentra Puregene kit, Qiagen, Valencia, California, USA) showed DNA laddering typical of apoptosis in EHDV-2 infected bovine CAE cell cultures. Laddering was seen as early as 24 hours post inoculation and became more prominent within 36 hours (Figure 1). Annexin V-binding assay was performed with an Annexin V-biotin apoptosis detection kit (Oncogene Research Products, La Jolla, California, USA) and fluorescein-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA); detection was by either fluorescence microscopy or flow cytometry using an Epics-Elite flow counter (Beckman Coulter, Brea, California, USA) using aliquots of cells. Fluorescent microscopy showed that many cells in the EHDV-2 infected and tumor necrosis factor (TNF) treated bovine CAE cultures had green fluorescence on the cell membrane surface indicating annexin binding to phosphatidylserine and thus early apoptosis within 24 hours post inoculation. Few EHDV infected cells stained positive for annexin and propidium iodide (PI), an indication of membrane leakage and late apoptosis or necrosis. Within

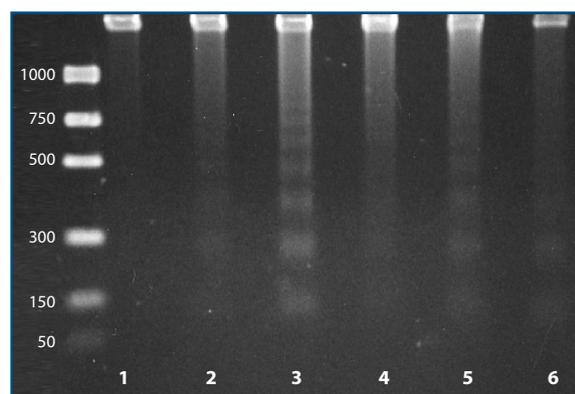


Figure 1. Gel electrophoresis of genomic DNA from bovine endothelium. Lane 1: 24 hour, untreated. Lane 2: 24 hour, infected with serotype-2 of Epizootic haemorrhagic disease virus (EHDV-2). Lane 3: 24 hour tumor necrosis factor (TNF) treated. Lane 4: 36 hour, untreated. Lane 5: 36 hour, EHDV-2 infected. Lane 6: 36 hour, TNF treated. Slight laddering consistent with apoptosis is seen in the 24 hour, EHDV-2 infected cells and is more obvious by 36 hours.

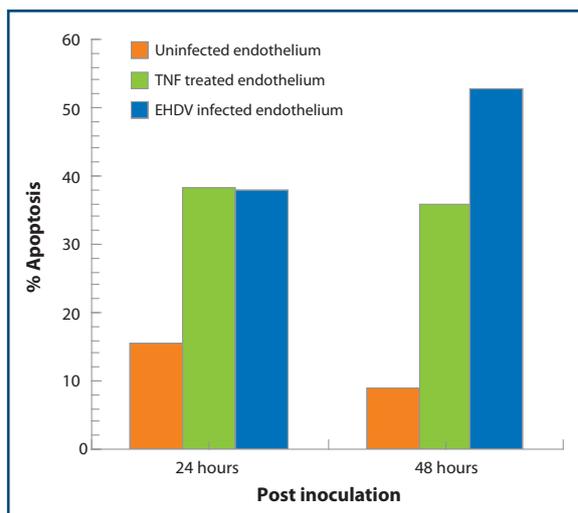


Figure 2. Annexin V-binding in bovine carotid artery endothelium at 24 and 48 hours post inoculation either with serotype 2 of the Epizootic haemorrhagic disease virus (EHDV-2) or with tumor necrosis factor (TNF) analysed by flow cytometry and expressed as % apoptosis; EHDV-2 inoculated cells had increased apoptosis as compared to controls at 24 hours, and apoptosis increased over time.

48 hours of inoculation, an increased number of annexin-positive cells showed nuclear staining with PI, indicating progression to late apoptosis. These findings were confirmed by flow cytometry (Figure 2). By 24 hours post inoculation, more cells stained for annexin in the EHDV-2 infected cultures than the control cultures, while a small number of the EHDV-2 infected cells stained with both annexin and PI. Over time, most cells in EHDV-2 infected cultures shifted from staining single positive for annexin to co-staining with PI.

FragEL DNA fragmentation detection assays were performed with a Klenow-FragEL kit (Oncogene Research Products, La Jolla, California, USA). The TUNEL assay was performed using Apotag Plus In situ Apoptosis Detection kit (Chemicon International, Inc., Temecula, California, USA) according to manufacturer's instructions. These assays labelled numerous cells with morphologic

features of apoptosis in EHDV infected and TNF treated endothelial cell cultures as early as 24 hours post inoculation. The numbers of stained cells increased over time in the EHDV infected cultures but not in negative control cultures. Most cells staining positive by TUNEL also showed nuclear condensation and pyknosis typical of apoptosis. A few cells with normal morphology were also labelled by this method. Mitotic cells were not labelled.

To study whether EHDV-2 induced apoptosis is directly related to viral replication and whether p53 plays a role, bovine CAE cell monolayers grown in 25 cm² flasks were inoculated with partially purified EHDV-2 ($10^{6.78}$ TCID₅₀/ml)/unpurified EHDV-2 ($10^{7.3}$ TCID₅₀/ml) or ultraviolet-inactivated unpurified and partially purified EHDV-2 inoculums. Mock inoculated or TNF (0.1 µg/ml) inoculated bovine CAE cells were used as negative and positive controls, respectively. At 0, 18, 24 and 42 hours post inoculation the monolayers in the flasks were scraped and cells and supernatants harvested for virus titer assessment, p53-specific semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR), TUNEL, and immunohistochemistry for caspase-3 (cleaved), p53 and EHDV-2.

Table I shows the viral titers, cytopathic effect, and cells staining positive for caspase 3 (cleaved), TUNEL, and p53 in bovine endothelium at 18, 24, and 48 hours post treatment. TUNEL was done as previously described. Only cells inoculated with live EHDV, both purified and unpurified inoculums, had appreciable numbers of cells staining positive by TUNEL (Table I). The number of TUNEL labelled cells increased with time and correlated with increasing viral titers and percent CPE.

Immunohistochemistry for caspase-3 was performed by using rabbit anti-caspase-3 (cleaved) antibody (Biocare Medical, Concord, California, USA; 1:100). The reaction was visualized with Vulcan fast Red chromogen (Biocare Medical, Concord, California, USA). Caspase 3 was detected in controls and all treatment groups (Table I). However, only in cultures inoculated with live virus or TNF (positive

Table I. Virus titers, % CPE, and EHDV, caspase 3 (cleaved), TUNEL and p53 staining cells in bovine endothelium at 18, 24, and 48 hours post treatment.

Treatment	Virus titer [*]			% CPE ⁺			EHDV [#]			Caspase ^{&}			TUNEL [^]			P53 ^{>}		
	18	24	42	18	24	42	18	24	48	18	24	48	18	24	48	18	24	48
Control ¹	0	0	0	0	0	0	0	0	0	0	25	5	0	0	1	0	0	0
Killed partial purified ²	0	0	0	0	0	0	0	1	0	16	8	3	0	0	1	6	5	2
Live Partial Purified ³	4.1	4.6	5.1	10	30	60	29	35	44	75	8	7	7	4	19	0	2	0
Killed unpurified ⁴	0	0	0	0	0	0	0	1	0	5	2	5	1	1	1	2	3	0
Live Unpurified ⁵	2.6	2.6	3.1	1	4	10	11	70	86	30	2	42	1	7	30	0	2	0
TNF ⁶	0	0	0	4	10	15	0	0	0	60	12	4	2	2	1	13	2	1

^{*}Virus titer log₁₀ TCID₅₀; ⁺% cytopathic effect; [#]Number of EHDV staining cells/100 cells; [&]Number of caspase 3 (cleaved) staining cells/100 cells; [^]Number of TUNEL staining cells/100 cells; [>]Number of p53 staining cells/100 cells. Treatments included: ¹mock inoculated control; ²Inactivated partially purified EHDV-2 at similar dose as 3; ³Partially purified EHDV-2 at $10^{5.8}$ TCID₅₀/ml; ⁴Inactivated unpurified EHDV-2 at similar dose as 5; ⁵Unpurified EHDV-2 at $10^{5.8}$ TCID₅₀/ml; ⁶TNF at 0.1 µg/ml.

control) were appreciable numbers of caspase positive cells detected. At 18 hours post inoculation, the number of caspase positive cells in the live partially purified virus and TNF treatment groups was 3 and 2.4 time greater than the maximum number in the sham inoculated control (24 hours post inoculation; 25 cells/100 cells). Nevertheless, the number of caspase positive cells in these groups decreased rapidly with time. Caspase expression in cells inoculated with live unpurified virus was biphasic and increased over the maximum control count at 18 and 42 hours post inoculation. There was no apparent correlation of caspase expression with virus titer, CPE, TUNEL staining or p53 expression.

Immunohistochemistry for p53 was performed with mouse monoclonal anti-human p53 antibody (PharMingen, San Jose, California, USA; 1:300) and the reaction was visualized with the avidin biotin complex (ABC) peroxidase method followed by DAB chromogen. A bovine squamous cell carcinoma with p53 expression was used as a positive control. No p53 expression was detected in controls. Virtually no staining was observed in cells treated with either live virus inoculums (Table I). Surprisingly, p53 expression was detected in cultures inoculated with both killed virus inoculums, however, the number of cells expressing p53 was never as high as the maximum number in the TNF treated group (18 hours post inoculation; 13 cells/100 cells). p53 and EHDV-2 co-staining cells were not observed in any of the investigated slides, though cells with individual positive staining for p53 (nuclear staining) or EHDV-2 (cytoplasmic staining) were detected during the analysis of tissue sections.

EHDV-2 was detected with rabbit anti-EHDV-2 antibody (1:300) and visualized using the streptavidin-biotin alkaline phosphatase method followed by fast red chromogen (Dako, Via Real, California). The specimens were double-labelled for p53 and EHDV-2 by sequential immunohistochemistry, as mentioned above. Negative control samples were incubated with isotype controls (Biogenex, Fremont, California, USA). The slides were then counterstained with Mayer's hematoxylin. The number of EHD virus immunopositive cells increased with time, viral titer and percent CPE for both live viruses (Table I). However, the number of positive cells did not correlate with the virus titer or percent CPE. Rare EHD virus immunopositive cells were seen in cultures inoculated with both killed viruses.

p53 targeting RT-PCR was performed using 0.1 µg RNA extracted from the harvested bovine CAE cells (RNA-Bee, Amsbio, Cambridge, MA, USA) and the Titan One Tube RT-PCR System (Roche, Indianapolis, Indiana, USA). Primers that amplified bovine p53 were designed from sequences in GenBank (p53-forward

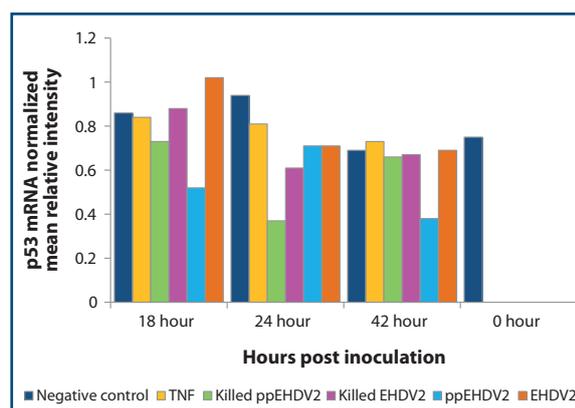


Figure 3. Normalized mean relative intensities for rt-PCR of p53 mRNA from bovine carotid endothelium that was inoculated with tumor necrosis factor (TNF), killed partially purified serotype 2 of the Epizootic haemorrhagic disease virus (EHDV-2) (Killed ppEHDV2), killed EHDV-2 (killed EHDV2), or live partially purified EHDV-2 (ppEHDV2), and live EHDV-2 (EHDV2). Cell cultures were sampled at 0, 18, 24, and 42 hours post inoculation. There was no appreciable upregulation of p53 mRNA in viral inoculated cells.

primer-5'-CTCACCATCATCACAAGTGG; p53 reverse primer-5'-ACCCACGGATCTGAAGAGTG). Beta actin was used as a housekeeping gene (Keefe et al. 1997). Polymerase chain reaction (PCR) products (p53) were extracted from 2% agarose gel using QIAquick Gel Extraction Kit (Qiagen, Valencia, California, USA) and sequenced for confirmation. The extracted p53 product matched 100% with bovine sequences for p53 in GenBank (Accession number: X81704.1). p53 mRNA expression was detected in bovine endothelial cells at 0 hours post infection, in controls and all treatment groups and at all subsequent time points. However p53 mRNA expression was not upregulated upon viral infection. The normalized relative intensity for each treatment group is shown in Figure 3. At 18 hours post inoculation, cells treated with unpurified virus showed a slight increase in normalized mean relative intensity compared to the negative control. Overall, the normalized mean relative intensities were lowest, reaching below the negative control, for the partially purified virus (killed and live) groups. No correlation of p53 gene expression with number of cells staining positive for p53 by immunohistochemistry was observed.

Increased TUNEL and caspase 3 (cleaved) staining in endothelium infected with partially purified virus confirmed that apoptosis can be virally induced and was not dependent on cellular proteins or mediators released into the media from the cells used to produce the inoculum. Additionally, TUNEL staining in live virus inoculated cultures increased over time as did the CPE and viral titer. These results suggest that apoptosis is an important mechanism for viral release. Apoptosis appeared to be dependent on the presence of live virus as the number of TUNEL

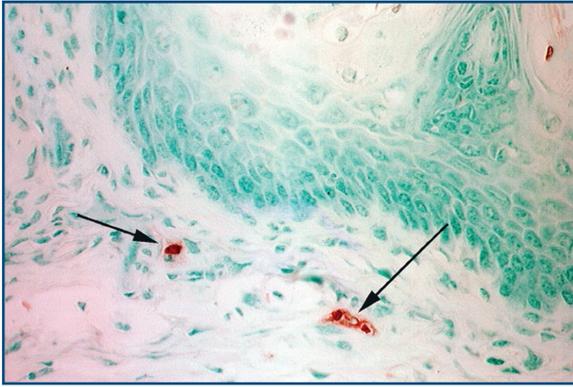


Figure 4. Buccal mucosa from a deer naturally infected with serotype 2 of the Epizootic haemorrhagic disease virus (EHDV-2). Endothelium lining small vessels in the submucosa (arrows) has positive staining indicative of apoptosis. Fast green counterstain. 200X original magnification.

staining cells in cultures inoculated with inactivated virus was negligible compared to those inoculated with live virus. In addition, even though uninfected cultures did show rare cells with morphologic features of apoptosis, the cultures inoculated with live virus had a remarkably increased number of cells showing an apoptotic phenotype. Caspase 3

was clearly activated in the live virus inoculated cells as the number of caspase 3 (cleaved) positive cells was higher than in the control cultures or cultures receiving inactivated virus. As no significant increase in p53 mRNA or protein expression was found in any of the virus treated groups, it appears that EHDV-induced endothelial apoptosis is not p53 dependent.

Our study confirms EHDV can induce apoptosis. By applying several methods including end labeling of fragmented DNA via TUNEL, annexin binding, and gel electrophoresis of genomic DNA, we demonstrated that EHDV induces apoptosis in bovine endothelial cells *in vitro*. These results indicate that, *in vitro* EHDV-2 induced apoptosis is directly related to viral replication and most likely independent of p53. In our preliminary work, we have demonstrated apoptosis via TUNEL staining in buccal mucosal endothelium of WTD with EHDV-2 infection, suggesting that apoptosis might play a role *in vivo* (Figure 4). Future studies are warranted to determine the exact mechanism of EHDV-induced endothelial cell apoptosis, the identity of involved viral proteins, and the biological significance to both host and virus.

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