ARTICLE
Journal of Cellular Biochemistry 114:2809–2822 (2013)

Journal of Cellular Biochemistry

Modulation of Apoptosis by Caprine Herpesvirus 1 Infection in a Neuronal Cell Line

Serena Montagnaro,[1] Roberto Ciarcia,[1] Claudio De Martinis,[1] Carmen Pacilio,[1] Simona Sasso,[1] Maria Valeria Puzio,[1] Morena De Angelis,[1] Ugo Pagnini,[1]* Silvia Boffo,[2] Ivanna Kenez,[3] Giuseppe Iovane,[1] and Antonio Giordano[2,3]

[1]*Department of Veterinary Medicine and Animal Productions, University of Naples "Federico II", Via Delpino no. 180137, Naples, Italy*

[2]*Sbarro Institute for Cancer Research and Molecular Medicine, Center of Biotechnology, College of Science and Technology, Temple University, BioLife Science Bldg. Suite 333, 1900N, 12th StreetPhiladelphia, Pennsylvania, 19122A*

[3]*Department of Medicine, Surgery and Neuroscience, University of Siena, Viale Bracci 1653100, Siena, Italy*

ABSTRACT

Caprine herpesvirus type 1 (CpHV-1), like other members of the alpha subfamily of herpesviruses, establishes latent infections in trigeminal ganglion neurons. Our groups previously demonstrated that CpHV-1 induces apoptosis in goat peripheral blood mononuclear cells and in an epithelial bovine cell line, but the ability of CpHV-1 to induce apoptosis in neuronal cells remains unexplored. In this report, the susceptibility of Neuro 2A cells to infection by CpHV-1 was examined. Following infection of cultured cells with CpHV-1, expression of cell death genes was evaluated using real-time PCR and Western blot assays. Analysis of virus-infected cells revealed activation of caspase-8, a marker for the extrinsic pathway of apoptosis, and caspase-9, a marker for the intrinsic pathway of apoptosis at 12 and 24 h post-infection. Significant increase in the levels of cleaved caspase-3 was also observed at the acme of cytopathic effect at 24 h post-infection. In particular, at 3 and 6 h post-infection, several proapototic genes were under-expressed. At 12 h post-infection several proapototic genes such as caspases, TNF, Cd70, and Traf1 were over expressed while Bcl2a1a, Fadd, and TNF genes were underexpressed. In conclusion, the simultaneous activation of caspase-8 and caspase-9 suggests that CpHV-1 can trigger the death-receptor pathway and the mitochondrial pathway separately and in parallel. Our findings are significant because this is the first published study showing the effect of CpHV-1 infection in neuronal cells in terms of gene expression and apoptosis modulation. J. Cell. Biochem. 114: 2809–2822, 2013.

KEY WORDS: CAPRINE HERPESVIRUS TYPE 1; APOPTOSIS; NEURONS; GENE EXPRESSION

Apoptosis is an highly complex process regulated by a wide range of cell signals and eventually leading to DNA fragmentation and cell demolition. Much insight has been gained in recent years concerning the signalling pathways and the proteins involved, but current models tend to attribute effects to the few known complexes, and many key signalling players remain to be elucidated [Lavrik, 2010].

Two major signalling cascades have been identified, namely the extrinsic and intrinsic pathways [Krammer, 2000]. Both centrally involve the caspase family of proteins as initiators and effectors of apoptosis propagation [Lavrik et al., 2005]. Initiator caspases are, by definition, the first to be activated in the apoptotic pathway. They in turn activate effector caspases, which trigger a series of proteolytic events that eventually lead to cell death [Fuentes-Prior and Salvesen, 2004].

The extrinsic pathway is initiated when certain death receptors (DR) on the cell surface are activated by death ligands (DL). DR mainly comprise the tumour necrosis factor (TNF) receptor family including FAS, TNFR1, and TRAIL (TNF-related apoptosis-inducing ligand) receptor (activated by their respective DL such as FAS ligand, TNF, and TRAIL) [Ashkenazi and Dixit, 1998]. Binding of a DL to a DR results in death-inducing signalling complex formation [Kischkel

Grant sponsor: Human Health Foundation Onlus (www.hhfonlus.org), Sbarro Health Research Organization (www.shro.org)(AG).

*Correspondence to: Prof. Ugo Pagnini, University of Naples "Federico II", Department of Veterinary Medicine and Animal Productions, University of Naples "Federico II", Via Delpino 1, 80137 Napoli, Italy. E-mail: upagnini@unina.it

Manuscript Received: 26 June 2013; Manuscript Accepted: 28 June 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 8 July 2013

DOI 10.1002/jcb.24628 • © 2013 Wiley Periodicals, Inc.

et al., 1995], which brings together the initiator caspases of the extrinsic pathway, that is, procaspase-8 and procaspase-10 [Krammer et al., 2007]. Cleavage of procaspase-8 releases caspase-8 into the cytosol to act on effector caspases.

The intrinsic pathway is triggered by a variety of factors including genotoxic stress, UV irradiation, certain hormones (e.g., glucocorticoids), and cytokine deprivation [Krammer et al., 2007]. Exposure to these factors leads to mitochondrial outer membrane permeabilisation (MOMP) and eventually cytochrome c release from the mitochondria into the cytosol [Green and Kroemer, 2004]. The release of cytochrome *c* from the mitochondria is also regulated by proteins of the B-cell lymphoma 2 (Bcl-2) family. The Bcl-2 family is divided into two classes of molecules that have opposing effects: antiapoptotic members such as Bcl-2 and Bcl-xL that protect the cell against apoptosis, and proapoptotic members such as Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer (Bak) that trigger apoptosis. The apoptosome formed between released cytochrome *c*, apoptosis-activating factor 1 and caspase-9 recruits and activates the initiator caspase of the intrinsic pathway, procaspase-9. Cleavage of procaspase-9 liberates caspase-9, which in turn activates downstream effector caspases [Brenner and Mak, 2009].

A final common pathway for both intrinsic and extrinsic cascades involves activation of the effector caspases (caspase-3, caspase-6, and caspase-7) by caspase-8 in the extrinsic pathway or caspase-9 in the intrinsic pathway. This then triggers cleavage of cellular proteins, nuclear shrinkage, and DNA fragmentation [Krammer et al., 2007; Lo et al., 2011].

Among stimuli that have been associated with apoptosis are infections with mammalian DNA and RNA viruses [Hay and Kannourakis, 2002]. Viruses possess various biochemical and genetic mechanisms to evade and/or induce apoptosis in infected cells through interactions at different stages of the apoptotic pathway and it has been demonstrated that apoptosis modulation through virus-encoded proteins is a key step in herpesviruses pathogenesis [Henderson et al., 1993; Hu et al., 1997; Wang et al., 1997; Cassady et al., 1998; Pagnini et al., 2004; Pagnini et al., 2005; Marfè et al., 2006; Longo et al., 2009].

Apoptotic phenomenon has been observed after infections with herpes simplex virus (HSV) type 2 (in the brain) [Geiger et al., 1995], varicella-zoster virus (in cultured cells) [Sadzot-Delvaux et al., 1995; Brazeau et al., 2010], and CpHV-1 (in blood mononuclear cells) [Pagnini et al., 2005]. Moreover, several herpesvirses such as HSV, VZV, and EHV-1 show a marcked neurotropism and neurovirulence [Watanabe et al., 2000; Pugazhenthi et al., 2011; Mori et al., 2012].

Caprine Herpesvirus 1 (CpHV-1) belongs to the Alphaherpesvirinae subfamily and show genomic similarity to other viruses of this group, such as Bovine Herpesvirus 1 (BoHV-1), the etiological agent of IBR/IPV, Bovine Herpesvirus 5 (BoHV-5), Suid Herpesvirus 1 (SuHV-1), Cervid Herpesvirus 1, and Rangiferine Herpesvirus 1 [Thiry et al., 2006]. Caprine Herpesvirus 1, is associated with two different syndromes in goats, depending on the age of the animals at the time of infection. In young kids, CpHV-1 causes an often generalized disease mainly affecting the digestive tract [Saito et al., 1974; Mettler et al., 1979; Roperto et al., 2000]. Infection in adult goats remains unapparent, or may cause respiratory distress, abortion [Berrios et al., 1975; Waldvogel et al., 1981; Williams et al., 1997], vulvovaginitis or balanoposthitis [Horner et al., 1982; Tisdall et al., 1984]. Moreover, being a member of alphaherpesvirus, CpHV-1 is able to establish latent infection in trigeminal ganglia [Plebani et al., 1983; Buonavoglia et al., 1996] and to cause immunosuppression [Pagnini et al., 2005]. Thus, CpHV-1 infection is responsible for severe economic losses due to abortions, stillbirths, reproductive disorders, and susceptibility of CpHV-1-infected host to secondary bacterial infections.

Like herpes simplex virus 1 (HSV-1), the prototype member of the alpha subfamily of herpesviruses, CpHV-1 establishes life-long, non-productive latent infections in ganglionic sensory neurons (Homan and Easterday, 1980; Jones, 1998; Delhon et al., 2002). During the first days following infection by herpesviruses, some neurons are productively infected (acute phase). However, other neurons are not permissive to virus replication and become latently infected. After the acute phase subsides, these cells continue to harbour viral DNA without producing infectious virus (latent phase). During latency, gene expression is restricted to a small region of the genome known as the latency-related (LR) or latency-associated region [Rock et al., 1987; Stevens et al., 1987].

Our group previously showed that CpHV-1 is able to induce apoptosis in goat peripheral blood mononuclear cells [Pagnini et al., 2005] and we investigated on the pro-apoptotic potential of CpHV-1 in a permissive cell line (Madin Darby bovine kidney cells), evaluating apoptotic profiles like chromatin condensation and DNA laddering [Longo et al., 2009]. In this report, to characterize in more detail the intracellular pathway by which CpHV-1 is able to induce apoptosis, we have analyzed the gene expression response during the apoptotic phase of CapHV-1 infection in a neuronal cell line.

MATERIALS AND METHODS

CELLS AND VIRUS

Mouse Albino Neuroblastoma (Neuro 2A) cells (CCL-131, American-Type Culture Collection) were grown in Dulbecco's modified minimal essential medium (DMEM), supplemented with 100 IU/ml of penicillin, 100 mg/ml of streptomycin, and 5% pestivirus free foetal calf serum. This cell line was maintained free of mycoplasma and of bovine viral diarrhoea virus.

The reference Swiss strain E/CH [Mettler et al., 1979] of CpHV-1 was used. It was multiplied on MDBK, and cell extracts, obtained by three cycles of freezing and thawing, were pooled, collected, and stored in aliquots at −80°C. Infectivity titers were expressed as median tissue culture infectious doses (TCID50)/ml [Reed and Muench, 1938].

APOTOX-GLO™TRIPLEX ASSAY

The ApoTox-Glo™Triplex assay was used in order to assess viability, cytotoxicity, and caspase activation events within a single assay well. In the first part of the assay, it measures two protease activities simultaneously; one being a marker of cell

viability and the other being a marker of cytotoxicity. Peptide substrate (glycylphenylalanyl-aminofluoro-coumarin; GF-AFC) enters intact cells where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. Peptide substrate (bis-alany-lalanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity. Bis-AAF-R110 is not cell-permeable, so no signal from this substrate is generated by intact, viable cells. The live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected simultaneously. In the second part of the assay, the Caspase-Glo® 3/7 Reagent, added in an "add-mix-measure" format, results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal produced by luciferase.

Neuro 2A cells of approximately 500/well were seeded in a flat 96-well micro-plate (Becton Dickinson Labware, USA) as triplicates. Four different types of controls, namely: positive, infected, negative, and background controls were used throughout the study. Positive control had cells with culture medium treated with Staurosporine of 10 μM final concentration for 3, 6, 12, 24, and 48 h to induce apoptosis. Infected cell cultures contained cells treated with CapHV-1 at MOI 1 for 3, 6, 12, 24, and 48 h. Negative control consisted of mock infected Neuro 2A cells and no-cell control (background) containing only culture medium without cells [Pelzl et al., 2009].

After 3, 6, 12, 24, and 48 h post-infection 20 μl of viability/cytotoxicity reagent containing both GF-AFC and bis-AAF-R110 substrates was added to each well and briefly mixed by orbital shaking at 300–500 rpm for 30 s and then incubated at 37°C for 30–180 min. Fluorescence was measured at 400Ex/505Em (Viability) and 485Ex/520Em (Cytotoxicity) by using Glomax Multi Detection System multiwell plate reader (Promega Corporation, USA). After that 100 μl of Caspase-Glo 3/7 reagent was added to each well, and briefly mixed by orbital shaking at 300–500 rpm for 30 s and then incubated at room temperature for 30–180 min. Luminescence was measured using a Glomax Multi Detection System multiwell plate reader (Promega Corporation, USA) by Luminiscence protocol which is proportional to the amount of caspase activity present.

VERIFICATION OF APOPTOTIC GENE EXPRESSION PROFILING OF CPHV-1-INFECTED NEURO 2A CELLS BY QUANTITATIVE REAL-TIME PCR

The RT2 qPCR profiler Mouse Signal Transduction Pathway array (catalog number PAMM-012, SABiosciences, Frederick, MD), representing 84 genes involved in signal transduction pathways, plus five housekeeping genes and three controls, was used to analyze the effect of CpHV-1 infection on signaling-related gene expression in Neuro 2A cell line. The total RNA was isolated from the infected (MOI 1) and mock infected cells cultures using the Rneasy Mini Kit (Qiagen), at 3, 6, 12, 24, and 48 h post-infection. cDNA was generated from 1 μg total RNA using the RT2qPCR Array First Strand Kit in accordance with manufacturer's instructions. The template was combined with RT2 SYBR Green/Fluorescein PCR master mix. Equal amounts of this mixture (25 μl) were added to each well of the RT2qPCR profiler plate containing the predispensed gene-specific primer sets, and the reaction was performed using StepOnePlus (Applied Biosystems) according to the manufacturer's protocols.

Data analysis was based on the $\Delta\Delta C_t$ method with the aid of an Excel (Microsoft Excel; Microsoft, Redmond, WA) spreadsheet containing algorithms provided by the manufacturer (RT2ProfilerPCR Array data Analysis software http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

The expression levels of the mRNA of each gene were normalized using the expression of the housekeeping gene GAPDH. A positive value indicates that the gene was upregulated and a negative value indicates that the gene was downregulated.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot analysis were performed on CpHV-1 infected Neuro 2A cells and on mock infected Neuro 2A cells.

Neuro 2A cells in 75 cm^2 flask, at confluency, were infected with CpHV-1 at MOI 1. At 3, 6, 12, 24, and 48 h post-infection, adherent cells were washed twice with PBS and scraped.

Cells were then mixed with cells previously collected by centrifugation from supernatant of the same flask and resuspended in PBS. The pellets, obtained by centrifugation, were stored at −20°C and then processed by Western blot analysis as previously described [Montagnaro et al., 2013]. To measure caspases activation at least 1×10^6 cells were used. Cells were homogenized directly into lysis buffer 50 mM Tris pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.25% deoxycholic acid, 1% Triton X-100) with 20 mM sodium pyrophosphate, 0.1 mg/mL aprotinin, 2 mM phenylmethylsulphony fluoride (PMSF), 10 mM sodium orthovanadate (Na_2VO_3), and 50 mM sodium fluoride (NaF). Protein concentrations were determined by use of a protein assay kit (Bio-Rad Laboratories).

Equal amounts of lysate samples were boiled and loaded on bis/acrylamide gels, separated by electrophoresis and proteins were blotted from the gel onto nitrocellulose membranes. The membranes were blocked with 5% bovine serum albumin (BSA) in tris buffered saline (TBS: 12.5 mM Tris–HCl pH 7.4; 125 mM NaCl) at room temperature, washed with TBS-0.1% Tween and incubated with primary antibody.

The primary antibodies used were rabbit anti-cleaved caspase 3 (Cell Signaling Technologies catalog no. 9662; 1:1,000 diluition), or rabbit anti-caspase 8 p18 (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc-7890; 1:200 dilution), rabbit anti-caspase 9 (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc-8355; 1:200 dilution), rabbit anti-Bcl2 (Abcam plc, Cambridge, UK; catalog no. ab7973; 1:100 diluition); rabbit anti-phospho-Bcl2 (Cell Signaling Technologies catalog no. 2827; 1:1,000 diluition), mouse anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA; catalog no. sc-99; 1:200 dilution), rabbit anti-phospho-p53 (Cell Signaling Technologies, catalog no. 2528; 1:1,000 diluition).

After appropriate washing steps, peroxidase-conjugated anti-rabbit IgG (GE Healtcare, UK, catalog no. NA934) or anti-mouse IgG (GE Healtcare, UK, catalog no. NA931) was applied for 1 h at

a 1:1,000 dilution. The blots were stripped and reprobed against mouse anti-actin antibody (Calbiochem, San Diego, CA; catalog no. CP10) at 1:5,000 dilution to confirm equal loading of proteins in each lane. Protein expression levels were quantitatively estimated by densitometry using a Gel Doc scanner (BioRad) equipped with a densitometric workstation. The protein concentrations were normalized to the actin level and expressed as relative band density (arbitrary units).

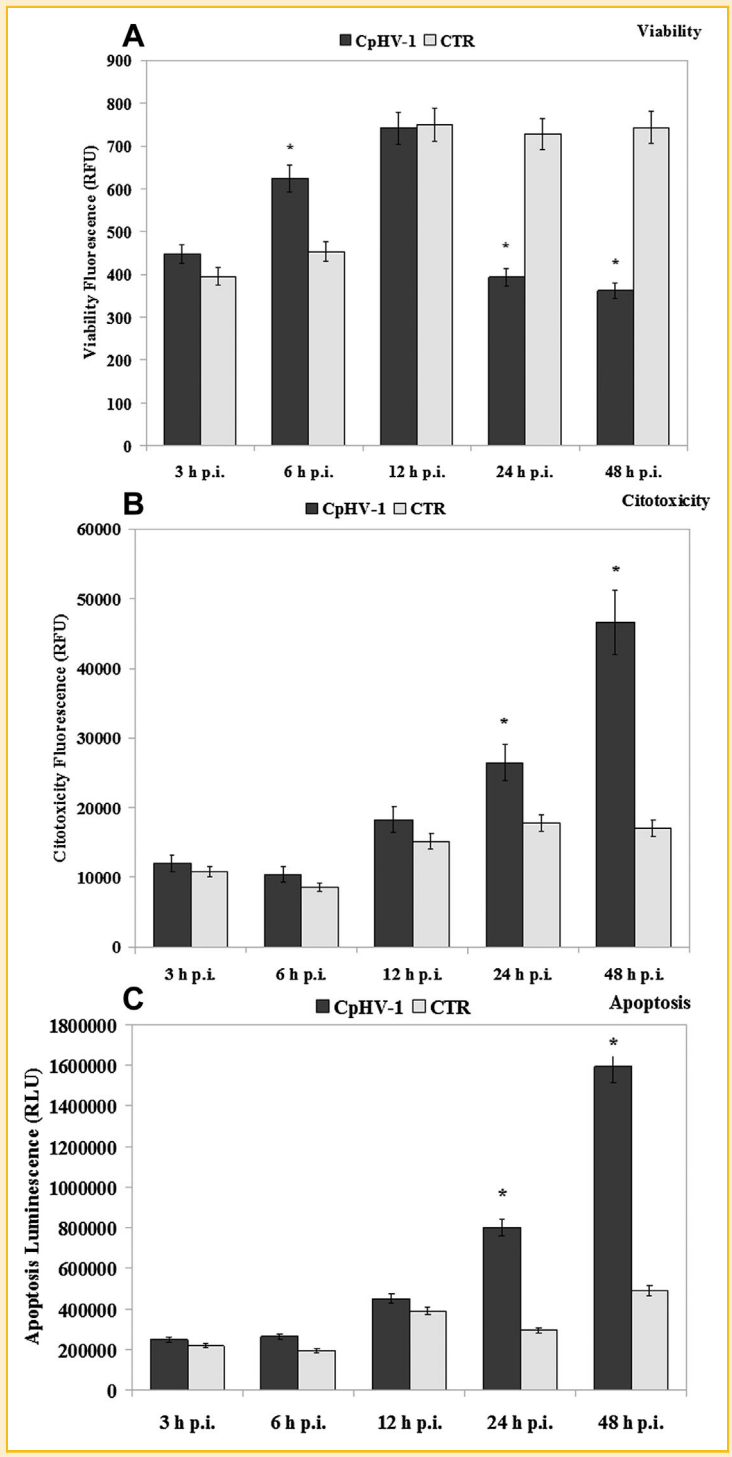

Fig. 1. CpHV-1 induce apoptosis in Neuro 2A cells. Replicate cultures of Neuro 2A cells were mock infected or infected with inoculum consisting of criolysated infected Neuro 2A cells. Inoculum had been titrated in advance, and the multiplicity of infection was approximately 1. At different time points after infection, the viability, cytotoxicity, and apoptosis were assayed using ApoTox-Glo™Triplex assay. Results are expressed as Relative Fluorescence Units (RFU) and Relative Luminescence Units (RLU) and reported as the mean ± SD of three separate experiments. A *P*-value <0.05 was selected as significant.

STATISTICAL ANALYSIS

The results are presented as mean ± SD of three experiments. One-way ANOVA with Turkey's post-test was performed using GraphPad InStat Version 3.00 forWindows 95 (GraphPad Software, San Diego, CA). *P*-value <0.05 was considered statistically significant.

RESULTS

APOTOX-GLO™TRIPLEX ASSAY

In a first series of experiment we have analysed the effect of CpHV-1 infection on cytotoxicity, viability, and apoptosis within a single assay well, by using the ApoTox-Glo™Triplex assay.

The graphs in Figure 1 depicts the results of an ApoTox-Glo™Triplex assay showing the cytotoxicity, viability, and apoptosis of Neuro 2A cells infected with CpHV-1 at MOI 1.

After 24 and 48 h post-infection, the cytotoxicity of CpHV-1 infected cells, compared to the mock infected cells, increased significantly in a time dependent manner (Fig. 1B).

The graph in Figure 1A depicts the results of an Apo-Tox-Glo™ Triplex assay showing the viability of Neuro 2A cells infected with CpHV-1 at MOI 1. After 24 and 48 h post-infection, the viability CpHV-1 infected cells significantly decreased respect to untreated control cells.

The Figure 1C show the results of an Apo-Tox-Glo™ Triplex assay showing the activation of caspase 3 in Neuro 2A cells infected with CpHV-1 at MOI 1 at 3, 6, 12, 24, and 48 h post-infection. CpHV-1 infected cells showed high level of caspase 3 at 24 and 48 h post-infection respect to mock infected control cells.

VERIFICATION OF APOPTOTIC GENE EXPRESSION PROFILING OF CPHV-1-INFECTED NEURO 2A CELLS BY QUANTITATIVE REAL-TIME PCR

To detect the apoptotic genes involved in CpHV-1-induced apoptosis, we scanned 84 apoptosis genes by RT2 Profiler PCR microarray (Tables I–V). A wide spectrum of apoptosis-relavant genes was upregulated and downregulated during CpHV-1 infection.

TABLE I. Genes Over- and Under-Expressed at 3 h Post-Infection by CpHV-1

Symbol	Description	Fold change	Symbol	Description	Fold change
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	5.5644	Xiap	X-linked inhibitor of apoptosis	4.0695
Abl1	C-abl oncogene 1, non-receptor tyrosine kinase	−3.8019	Cd40	CD40 antigen	−5.5257
Aifm1	Apoptosis-inducing factor, mitochondrion-associated 1	−7.3549	Cidea	CD40 ligand	−2.2402
Akt1	Thymoma viral proto-oncogene 1	−6.3038	Dad1	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	−2.6346
Apaf1	Apoptotic peptidase activating factor 1	−9.9738	Dapk1	Death associated protein kinase 1	−7.7399
Api5	Apoptosis inhibitor 5	−4.2568	Dffb	DNA fragmentation factor, beta subunit	−3.18
Atf5	Activating transcription factor 5	−2.6639	Diablo	Diablo homolog (Drosophila)	−7.7399
Bag1	Bcl2-associated athanogene 1	−3.3355	Fadd	Fas (TNFRSF6)-associated via death domain	−3.6342
Bag3	Bcl2-associated athanogene 3	−4.1664	Fas	Fas (TNF receptor superfamily member 6)	−2.9981
Bak1	BCL2-antagonist/killer 1	−8.8519	Fasl	Fas ligand (TNF superfamily, member 6)	−17.0168
Bax	Bcl2-associated X protein	−3.2272	Lhx4	LIM homeobox protein 4	−7.6796
Bcl10	B-cell leukemia/lymphoma 10	−4.7136	Mapk1	Mitogen-activated protein kinase 1	−7.6803
Bcl2	B-cell leukemia/lymphoma 2	−17.6559	Mcl1	Myeloid cell leukemia sequence 1	−7.205
Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a	−2.6827	Naip2	NLR family, apoptosis inhibitory protein 2	−8.527
Bcl2l1	Bcl2-like 1	−3.1334	Nme5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	−4.5562
Bcl2l10	Bcl2-like 10	−5.7487	Nod1	Nucleotide-binding oligomerization domain containing 1	−4.8126
Birc3	Baculoviral IAP repeat-containing 3	−4.8778	Nol3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	−2.3031
Birc5	Baculoviral IAP repeat-containing 5	−5.4074	Polb	Polymerase (DNA directed), beta	−7.7399
Bnip2	BCL2/adenovirus E1B interacting protein 2	−192.3201	Prdx2	Peroxiredoxin 2	−2.2155
Bnip3	BCL2/adenovirus E1B interacting protein 3	−7.7399	Pycard	PYD and CARD domain containing	−7.0338
Bnip3l	BCL2/adenovirus E1B interacting protein 3-like	−23.202	Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	−4.3588
Bok	BCL2-related ovarian killer protein	−9.2989	Tnf	Tumor necrosis factor	−3.1233
Card10	Caspase recruitment domain family, member 10	−2.0503	Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	−3.6758
Casp1	Caspase 1	−7.8312	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	−25104851.5815
Casp12	Caspase 12	−3.2708	Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	−3.538
Casp14	Caspase 14	−2.1739	Tnfsf12	Tumor necrosis factor (ligand) superfamily, member 12	−3.1993
Casp2	Caspase 2	−2.3658	Traf1	Tnf receptor-associated factor 1	−7.7399
Casp3	Caspase 3	−7.7399	Traf2	Tnf receptor-associated factor 2	−9.2416
Casp4	Caspase 4	−3.247	Traf3	Tnf receptor-associated factor 3	−3.3477
Casp7	Caspase 7	−7.7399	Trp53bp2	Transformation related protein 53 binding protein 2	−13.3396
Casp8	Caspase 8	−9.3871	Trp73	Transformation related protein 73	−3.1029
Casp9	Caspase 9	−2.2536			

Regulated genes with fold change ≥2.0 (either up or down) and *P*-values of ≤0.05 are presented, expressed in Neuro 2A cell line infected with CpHV-1 (MOI 1) at 3 h post-infection are presented.

In particular, at 3 h post-infection, several proapototic genes such as Fas, Fadd, TNF, and caspase were under-expressed (Table I). Starting from 6 h post-infection Fas and TNF genes resulted to be over-expressed (Table II). At 12 h post-infection several proapototic genes like caspase (1, 3, 4, 6, 7, 8, 10, 12, 14,) TNF, Cd70, and Traf1 were over expressed. Conversely, at 12 h post-infection Bcl2a1a, Fadd, and TNF genes were underexpressed (see Table III). After 24 and 48 h post-infection many genes were up and down regulated probably due to the massive citopatyc effect (see Tables IV and V).

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

To investigate the modulation of apoptotic pathway in Neuro 2A infected with CpHV-1, we also measured the apoptotic protein expression by Western blot analysis.

Activation of the DNase responsible for oligonucleosomal degradation of nuclear chromatin is dependent on cleavage of an inhibitor complexed with the DNase molecule [Sakahira et al., 1998]. The cleavage is mediated by caspase-3, which in turn is activated by either initiator caspase-8 or caspase-9. Caspase-8 and caspase-9 represent the extrinsic and intrinsic pathways of apoptosis cascade triggering, respectively.

In order to characterize the pathway that triggers the apoptotic cascade during CpHV-1 infection, we examined cleavage of caspase-8 and caspase-9 in samples of mock- and CpHV-1-infected Neuro 2A cells collected at 3, 6, 12, 24, and 48 h post-infection by the use of Western blotting and caspase-specific antibodies. Western blot analysis showed significant changes in the amounts of caspase 8 at 24 h post-infection (Fig. 2A,B). In fact, we have observed, at the same time, a 18-kDa protein band corresponding to the processed form of caspase-8 that is detected by the same antibody. Similarly, the appearance of a 37-kDa protein corresponding to the cleaved form of caspase-9 was observed starting at 24 h p.i. (Fig. 2A,C). Once processed, caspase-9 further cleaves procaspase-3, activating caspase-3. A cleaved caspase-3 fragment corresponding to the 17-kDa protein band was detected starting at 12 h post-infection (Fig. 2A, D), whit an acme at 24 h post-infections.

In CpHV-1-infected Neuro 2A cells the expression of Bcl2 and phospho-Bcl2 was significantly increased at 12 and 24 in time dependent manner (Fig. 3). We also tested p53 and phospho-p53 expression in CpHV-1-infected and mock infected Neuro 2A cells. At 24 h post-infection p53 was significantly increased, while phospho-p53 increased starting from 3 h post-infection with an acme at 12 h post-infection (Fig. 4).

DISCUSSION

Apoptosis or programmed cell death is an encoded suicide program that allows the elimination of cells that have been produced in excess, developed improperly, or sustained genetic damage.

Several viruses trigger apoptosis in infected cells at an early stage of infection when virus particles interact with receptors on the cell surface or at the time of fusion with cell membrane and disassembly

TABLE II. Genes Over- and Under-Expressed at 6 h Post-Infection by CpHV-1

Symbol	Description	Fold change	Symbol	Description	Fold change
Fasl	Fas (TNF receptor superfamily member 6)	6.4839	Nol3	Nucleotide-binding oligomerization domain containing 1	2.1385
Tnf	Tumor necrosis factor	2.233			
Abl1	C-abl oncogene 1, non-receptor tyrosine kinase	−2.9488	Dad1	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	−10.4774
Aifm1	Apoptosis-inducing factor, mitochondrion-associated 1	−5.7699	Dffa	DNA fragmentation factor, alpha subunit	−19.7952
Akt1	Thymoma viral proto-oncogene 1	−6.4817	Fadd	Fas (TNFRSF6)-associated via death domain	−5.6866
Api5	Apoptosis inhibitor 5	−2.4396	Fas	Fas (TNF receptor superfamily member 6)	−11.8323
Atf5	Activating transcription factor 5	−4.4826	Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	−2.009
Bag1	Bcl2-associated athanogene 1	−2.3089	Il10	Interleukin 10	−3.9316
Bak1	BCL2-antagonist/killer 1	−2.6708	Lhx4	LIM homeobox protein 4	−5.7746
Bcl2	B-cell leukemia/lymphoma 2	−3.1533	Ltbr	Lymphotoxin B receptor	−2.4825
Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a	−3.4393	Mapk1	Mitogen-activated protein kinase 1	−11.6602
Bcl2l10	Bcl2-like 10	−6.9293	Naip2	NLR family, apoptosis inhibitory protein 2	−2.2603
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	−6.5965	Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105	−4.5239
Bcl2l2	Bcl2-like 2	−5.9661	Nod1	Nucleotide-binding oligomerization domain containing 1	−6.7005
Bid	Bcl2-like 1	−2.5608	Prdx2	Peroxiredoxin 2	−3.2921
Birc2	Baculoviral IAP repeat-containing 2	−66.5446	Pycard	PYD and CARD domain containing	−13.4001
Birc3	Baculoviral IAP repeat-containing 3	−16.7838	Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	−2.798
Birc5	Baculoviral IAP repeat-containing 5	−20.4182	Tnfrsf10b	Tumor necrosis factor receptor superfamily, member 10b	−38.9134
Bnip2	BCL2/adenovirus E1B interacting protein 3	−5916.5031	Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	−3.8718
Bnip3l	BCL2/adenovirus E1B interacting protein 3-like	−20.6852	Tnfsf12	Tumor necrosis factor (ligand) superfamily, member 12	−2.5211
Bok	BCL2-related ovarian killer protein	−3.7808	Traf3	Tnf receptor-associated factor 3	−2.2858
Card10	Caspase recruitment domain family, member 10	−2.5367	Trp53	Transformation related protein 53	−24.684
Casp6	Caspase 6	−5.4272	Trp63	Transformation related protein 63	−3.9655
Casp8	Caspase 8	−16.4795	Trp73	Transformation related protein 73	−49.0112
Cidea	CD40 ligand	−3.1964	Xiap	X-linked inhibitor of apoptosis	−6.1029

Regulated genes with fold change $\geq$2.0 (either up or down) and *P*-values of $\leq$0.05 are presented, expressed in Neuro 2A cell line infected with CpHV-1 (MOI 1) at 6 h post-infection are presented.

TABLE III. Genes Over- and Under-Expressed at 12 h Post-Infection by CpHV-1

Symbol	Description	Fold change	Symbol	Description	Fold change
Abl1	C-abl oncogene 1, non-receptor tyrosine kinase	2.5434	Cd70	CD70 antigen	10.4642
Anxa5	Annexin A5	11.706	Cideb	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector B	3.1213
Apaf1	Apoptotic peptidase activating factor 1	2.1478	Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	6.7399
Bad	BCL2-associated agonist of cell death	2.0824	Dapk1	Death associated protein kinase 1	13.4782
Bag3	Bcl2-associated athanogene 3	3.2227	Dffa	DNA fragmentation factor, alpha subunit	2.1903
Bax	Bcl2-associated X protein	5.2575	Dffb	DNA fragmentation factor, beta subunit	5.8786
Bcl10	B-cell leukemia/lymphoma 10	13.4782	Diablo	Diablo homolog (Drosophila)	13.4782
Bcl2l1	Bcl2-like 1	4.2583	Fasl	Fas ligand (TNF superfamily, member 6)	3.5359
Bcl2l2	Bcl2-like 1Bcl2-like 2	9.147	Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	3.4116
Bnip2	BCL2/adenovirus E1B interacting protein 2	13.4782	Igf1r	Insulin-like growth factor I receptor	10.4614
Bnip3	BCL2/adenovirus E1B interacting protein 2	13.4782	Il10	Interleukin 10	2.7443
Bnip3l	BCL2/adenovirus E1B interacting protein 3-like	4.2965	Mcl1	Myeloid cell leukemia sequence 1	8.6676
Bok	BCL2-related ovarian killer protein	8.6406	Naip2	NLR family. apoptosis inhibitory protein 2	5.6412
Card10	Caspase recruitment domain family, member 10	3.5986	Polb	Polymerase (DNA directed), beta	13.4782
Casp1	Caspase 1	13.4782	Tnfrsf10b	Tumor necrosis factor receptor superfamily, member 10b	2.9891
Casp12	Caspase 12	4.7559	Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	4.381
Casp14	Caspase 14	3.596	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	8.3783
Casp3	Caspase 3	13.4782	Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	2.2295
Casp4	Caspase 4	13.4782	Tnfsf12	Tumor necrosis factor (ligand) superfamily, member 12	2.1111
Casp6	Caspase 6	5.7523	Traf1	Tnf receptor-associated factor 1	13.4782
Casp7	Caspase 7	13.4782	Traf2	Tnf receptor-associated factor 2	2.4416
Casp8	Caspase 8	4.0298	Trp63	Transformation related protein 63	2.2063
Cd40	CD40 antigen	8.4609			
Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a	−2.3584	Fadd	Fas (TNFRSF6)-associated via death domain	−3.5719
Tnf	Tumor necrosis factor	−3.5503			

Regulated genes with fold change ≥2.0 (either up or down) and *P*-values of ≤0.05 are presented, expressed in Neuro 2A cell line infected with CpHV-1 (MOI 1) at 12 h post-infection are presented.

TABLE IV. Genes Over- and Under-Expressed at 24 h Post-Infection by CpHV-1

Symbol	Description	Fold change	Symbol	Description	Fold change
Xiap	X-linked inhibitor of apoptosis	4.0695			
Aifm1	Apoptosis-inducing factor, mitochondrion-associated 1	−15.9044	Dad1	Defender against cell death 1	−3.2977
Akt1	Thymoma viral proto-oncogene 1	−3.1839	Dffb	DNA fragmentation factor, beta subunit	−2.6608
Api5	Apoptosis inhibitor 5	−14.4781	Fadd	Fas (TNFRSF6)-associated via death domain	−10.5391
Atf5	Activating transcription factor 5	−2.1774	Fas	Fas (TNF receptor superfamily member 6)	−17.8168
Bad	BCL2-associated agonist of cell death	−3.938	Fasl	Fas ligand (TNF superfamily, member 6)	−60.2972
Bag1	Bcl2-associated athanogene 1	−6.3526	Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	−4.0851
Bag3	Bcl2-associated athanogene 3	−6.6082	Igf1r	Insulin-like growth factor I receptor	−10.0671
Bak1	BCL2-antagonist/killer 1	−37.8345	Il10	Interleukin 10	−11.7901
Bcl2	B-cell leukemia/lymphoma 2	−19.0913	Lhx4	LIM homeobox protein 4	−5.8171
Bcl2l10	Bcl2-like 10	−32.9371	Ltbr	Lymphotoxin B receptor	−7.3538
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	−6.8497	Mapk1	Mitogen-activated protein kinase 1	−5.0789
Bcl2l2	Bcl2-like 2	−2.4005	Naip1	NLR family, apoptosis inhibitory protein 1	−13.7733
Bid	BH3 interacting domain death agonist	−7.3919	Naip2	NLR family, apoptosis inhibitory protein 2	−4.0633
Birc2	Baculoviral IAP repeat-containing 2	−3.1317	Nod1	Nucleotide-binding oligomerization domain containing 1	−107.9962
Birc3	Baculoviral IAP repeat-containing 3	−19.8521	Polb	Polymerase (DNA directed), beta	−2.209
Birc5	Baculoviral IAP repeat-containing 5	−2.0833	Prdx2	Peroxiredoxin 2	−11.77
Bnip3l	BCL2/adenovirus E1B interacting protein 3-like	−9.7815	Pycard	PYD and CARD domain containing	−39.7069
Bok	BCL2-related ovarian killer protein	−101.9957	Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	−12.1399
Card10	Caspase recruitment domain family, member 10	−2.3448	Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	−3.0839
Casp2	Caspase 2	−4.3444	Tnfsf12	Tumor necrosis factor (ligand) superfamily, member 12	−7.3892
Casp6	Caspase 6	−2.7112	Traf2	Tnf receptor-associated factor 2	−4.7817
Casp8	Caspase 8	−6.6039	Traf3	Tnf receptor-associated factor 3	−2.9862
Casp9	Caspase 9	−4.2025	Trp53	Transformation related protein 53	−2.0039
Cd40lg	CD40 ligand	−5.9645	Trp53bp2	Transformation related protein 53 binding protein 2	−30.856
Cideb	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector B	−3.5427	Trp63	Transformation related protein 63	−9.5018
Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	−2.7054			

Regulated genes with fold change ≥2.0 (either up or down) and *P*-values of ≤0.05 are presented, expressed in Neuro 2A cell line infected with CpHV-1 (MOI 1) at 24 h post-infection are presented.

TABLE V. Genes Over- and Under-Expressed at 48 h Post-Infection by CpHV-1

Symbol	Description	Fold change	Symbol	Description	Fold change
Aifm1	Apoptosis-inducing factor, mitochondrion-associated 1	2.0531	Casp9	Caspase 9	2.013
Akt1	Thymoma viral proto-oncogene 1	2.0509	Cd40	CD40 antigen	4.9025
Anxa5	Annexin A5	4.3591	Cd70	CD70 antigen	4.4661
Bax	Bcl2-associated X protein	5.5601	Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	4.8141
Bcl10	B-cell leukemia/lymphoma 10	4.9081	Cradd	CASP2 and RIPK1 domain containing adaptor with death domain	58.7839
Bcl2	B-cell leukemia/lymphoma 2	16.0066	Dapk1	Death associated protein kinase 1	3.7831
Bcl2l1	BCL2-like 11 (apoptosis facilitator)	6.1211	Diablo	Diablo homolog (Drosophila)	6.1211
Birc5	Baculoviral IAP repeat-containing 5	3.0134	Mapk1	Mitogen-activated protein kinase 1	7.4227
Bnip2	BCL2/adenovirus E1B interacting protein 2	6.1211	Mcl1	Myeloid cell leukemia sequence 1	10.4372
Bnip3	BCL2/adenovirus E1B interacting protein 2	6.1211	Nme5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	2.4022
Bnip3l	BCL2/adenovirus E1B interacting protein 3-like	3.7832	Polb	Polymerase (DNA directed), beta	2.2492
Casp14	Caspase 14	2.8301	Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a	2.7313
Casp2	Caspase 2	4.5538	Tnfsf10	Tumor necrosis factor (ligand) superfamily, member 10	2.4444
Casp3	Caspase 3	4.7171	Traf2	Tnf receptor-associated factor 2	3.3169
Casp4	Caspase 4	6.1211	Trp53bp2	Transformation related protein 53 binding protein 2	5.0888
Casp7	Caspase 7	6.1211			
Api5	Apoptosis inhibitor 5	−2.7489	Dad1	Defender against cell death 1	−2.2515
Atf5	Activating transcription factor 5	−4.8525	Dffb	DNA fragmentation factor, beta subunit	−2.6478
Bcl2l2	Bcl2-like 1Bcl2-like 2	−2.6165	Fas	Fas (TNF receptor superfamily member 6)	−2.8243
Bid	BH3 interacting domain death agonist	−2.6999	Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	−2.3895
Birc2	Baculoviral IAP repeat-containing 2	−3.6729	Lhx4	LIM homeobox protein 4	−3.7444
Birc3	Baculoviral IAP repeat-containing 3	−2.3721	Ltbr	Lymphotoxin B receptor	−2.3913
Bok	BCL2-related ovarian killer protein	−2.3496	Pycard	PYD and CARD domain containing	−3.2115
Casp6	Caspase 6	−2.0995	Ripk1	Receptor (TNFRSF)-interacting serine-threonine kinase 1	−2.5729
Casp8	Caspase 8	−2.068	Tnfsf12	Tumor necrosis factor (ligand) superfamily, member 12	−2.3227
Cideb	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector B	−2.9094	Xiap	X-linked inhibitor of apoptosis	−3.8246

Regulated genes with fold change $\geq$2.0 (either up or down) and *P*-values of $\leq$0.05 are presented, expressed in Neuro 2A cell line infected with CpHV-1 (MOI 1) at 48 h post-infection are presented.

[Ramsey-Ewing and Moss, 1998; Jan et al., 2000; Barton et al., 2001; Connolly and Dermody, 2002; Bok et al., 2009]. In addition, certain viruses induce apoptosis at late stages of replication, providing a mechanism for dissemination of progeny virus.

Apoptotic changes in Herpesvirus-infected cells had been documented for several herpesviruses [Delhon et al., 2002; Pagnini et al., 2005; Gardell et al., 2006; Odeberg et al., 2006; Longo et al., 2009; Brazeau et al., 2010], in the present study we described the mechanism of cell death in a neuroblastoma cell line in order to investigate on the apoptotic process induced by CpHV-1 during infection.

In a first series of experiments, we evaluated the ability of CpHV-1 to induce apoptosis using ApoTox-Glo™Triplex assay. These experiment, allowed us to determine the viability, the citotoxicity and the activation of caspase 3 in Neuro 2A cell line. The cytotoxicity of CpHV-1 infected cells increased significantly in a time dependent manner and the viability of infected cells significantly decreased respect to untreated control cells inducing apoptosis at 24 and 48 h p. i. (Fig. 1).

Thus, we clarified the apoptotic pathway in Neuro 2A cells by RT2 Profiler PCR microarray and by Western blot analysis providing evidence that CpHV-1 induces apoptotic cell death in vitro, involving both initiators and effectors caspases.

Our data indicate that CpHV-1 triggers apoptosis in neuronlike cells and that this process occurs rapidly after infection. Indeed, in these cells an over expression of pro-apoptotic genes like Cradd, Fas, and TNF occurred starting from 6 h post-infection.

Cradd is a death domain (DD)-containing adaptor/signaling molecule that promotes apoptosis. It has a dual-domain structure similar to that of FADD, a NH2-terminal caspase homology domain that interacts with caspase-2 and a C-terminal DD that interacts with receptor-interacting protein RIP. Cradd recruits caspases and induces the activation of the apoptosis cascade [Ahmad et al., 1997].

At 12 and 24 h post-infection we have found that CpHV-1 infection triggered activation of caspases genes, in particolar caspase 8, which confirm the involvement of death receptor. Furthermore, at 24 h post-infection, Western blot analysis, showed the presence of caspase 9, 8, and 3.

Triggering of apoptosis and downstream caspases activation can be initiated through two main pathways. The "intrinsic or mitochondrial pathway" involves changes in mitochondrial membrane permeability that are regulated by a family of Bcl-2-related proteins. Activated proapoptotic members of this group (Bax, Bad, Bok) form pores in the mitochondrial membrane and promote leakage of apoptotic factors sequestered by the mitochondria, such as cytochrome *c* and Smac/DIABLO, which was overexpressed at 12 h p.i. (Table III), and is directly linked to permeability of the organelle outer membrane [Epand et al., 2002; Annis et al., 2005]. Loss of the mitochondrial membrane integrity and cytochrome c release result in apoptosome formation and processing of procaspase-9 followed by downstream activation of executioner caspase-3 [Jiang and Wang, 2004].

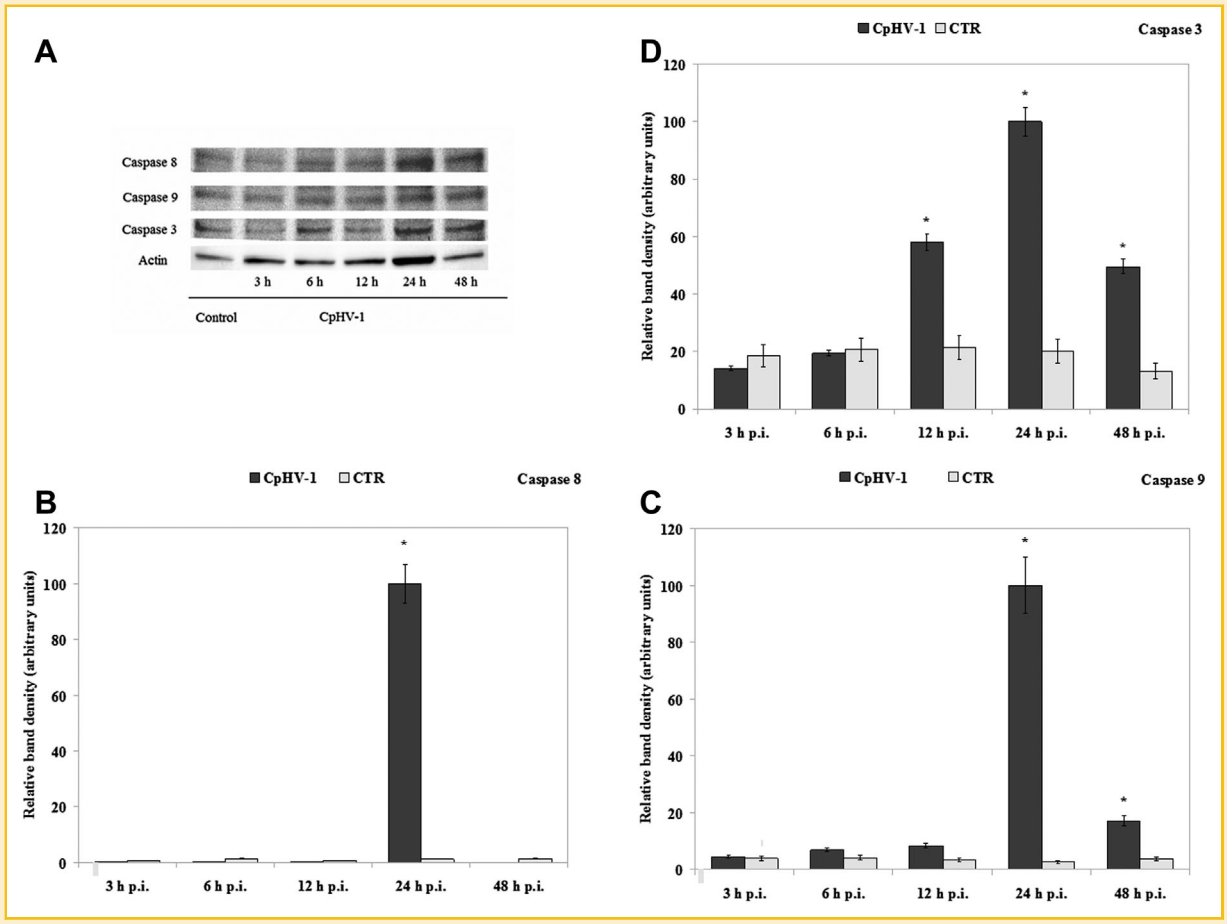

Fig. 2. Caspases activation is induced in Neuro 2A cells infected with CpHV-1. Replicate cultures of Neuro 2A cells were mock infected or infected with inoculum consisting of criolysated infected Neuro 2A cells. Inoculum had been titrated in advance, and the multiplicity of infection was approximately 1. At different time points after infection, cells were scraped, harvested, lysate, and subjected to Western blot analysis with antibodies against Caspase 8, 9, and 3. Actin protein levels were detected to ensure equal protein loading (A). Densitometric analysis of blots relative to Caspase 8, 9, and 3 (B, C, and D). Results are expressed as the mean ± SD of three separate experiments, a *P*-value <0.05 was selected as significant.

The overexpression of caspases, FasL, and TNF receptor family genes at 6 and 12 h post-infection suggest that the induction of apoptosis by CpHV-1 infection also involves the extrinsic pathway.

The over expression of FasL gene indicates that caspase-8 activation in CpHV-1-infected neuronal cells can be mediated by Fas/FasL signal payhway. However, just how FasL expression is regulated by CpHV-1 remains unclear, and further studies are under way to elucidate the mechanism of FasL regulation in CpHV-1-infected MDBK cells.

These data are confirmed by Western blot assay, in fact we have found at 12 and 24 h post-infection a significant increase of caspase 8, 9, and 3.

Our data agree with results of Gautier et al. [2003] who described induction of apoptosis in neuronlike cells infected with HSV-1 at early stage of infections, with the results of Xu et al. [2012] which reported similar findings for BHV-1 infection in MDBK cell line and with several studies that reported concomitant caspase-8 and caspase-9 activation in various apoptotic systems [Aubert et al., 2007; St-Louis and Archambault, 2007; Fiorito et al., 2008; Longo et al., 2009].

In fact, at 12 h post-infection the gene profile showed an over expression of genes that play an important role in intrinsic and extrinsic pathways of apoptosis like caspase 8 gene, Apaf1 that activates caspase 9, Bax, Bad, Bok, Bcl10 that encode for a protein caspase recruitment domain (CARD) and have been shown to induce apoptosis and to activate NF-kappaB, Card10, Dapk1, Polb, Diablo, and the other caspases genes.

In our study the levels of p53 protein and p53 phosphorylation increased after infection, suggesting that p53 plays a role in CpHV-1-induced apoptosis. Genes that are regulated by p53 (like Bax, e. g.) were also induced in a p53-dependent fashion. It has been demonstrated that the cleavage of p53 occurs in response to DNA damage, generating p50 or p40 [Molinari et al., 1996], and consequently alters the transcriptional regulatory activity of p53 [Hupp et al., 1992; Horikoshi et al., 1995; Chen et al., 1996]. Several other herpesviruses such as HSV-1, HSV-2, and BHV-1

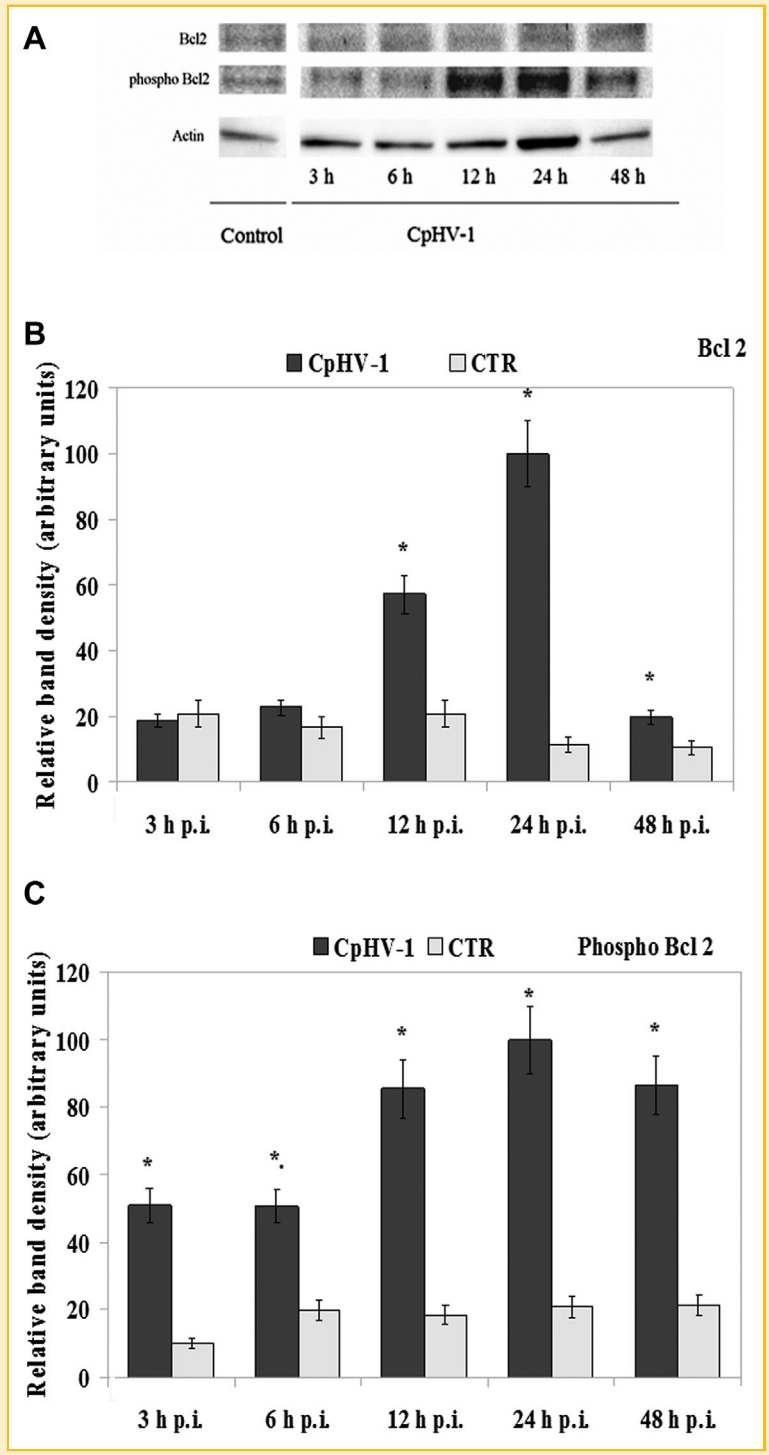

Fig. 3. CpHV-1 upregulates Bcl-2 and phospho-Bcl-2. Replicate cultures of Neuro 2A cells were mock infected or infected with inoculum consisting of criolysated infected Neuro 2A cells. Inoculum had been titrated in advance, and the multiplicity of infection was approximately 1. At different time points after infection, cells were scraped, harvested, lysate, and subjected to Western blot analysis with antibodies against actin, Bcl-2 and phospho-Bcl-2. Actin protein levels were detected to ensure equal protein loading (A). Densitometric analysis of blots relative to Bcl-2 and phospho-Bcl-2 (B and C). Results are expressed as the mean ± SD of three separate experiments, a *P*-value <0.05 was selected as significant.

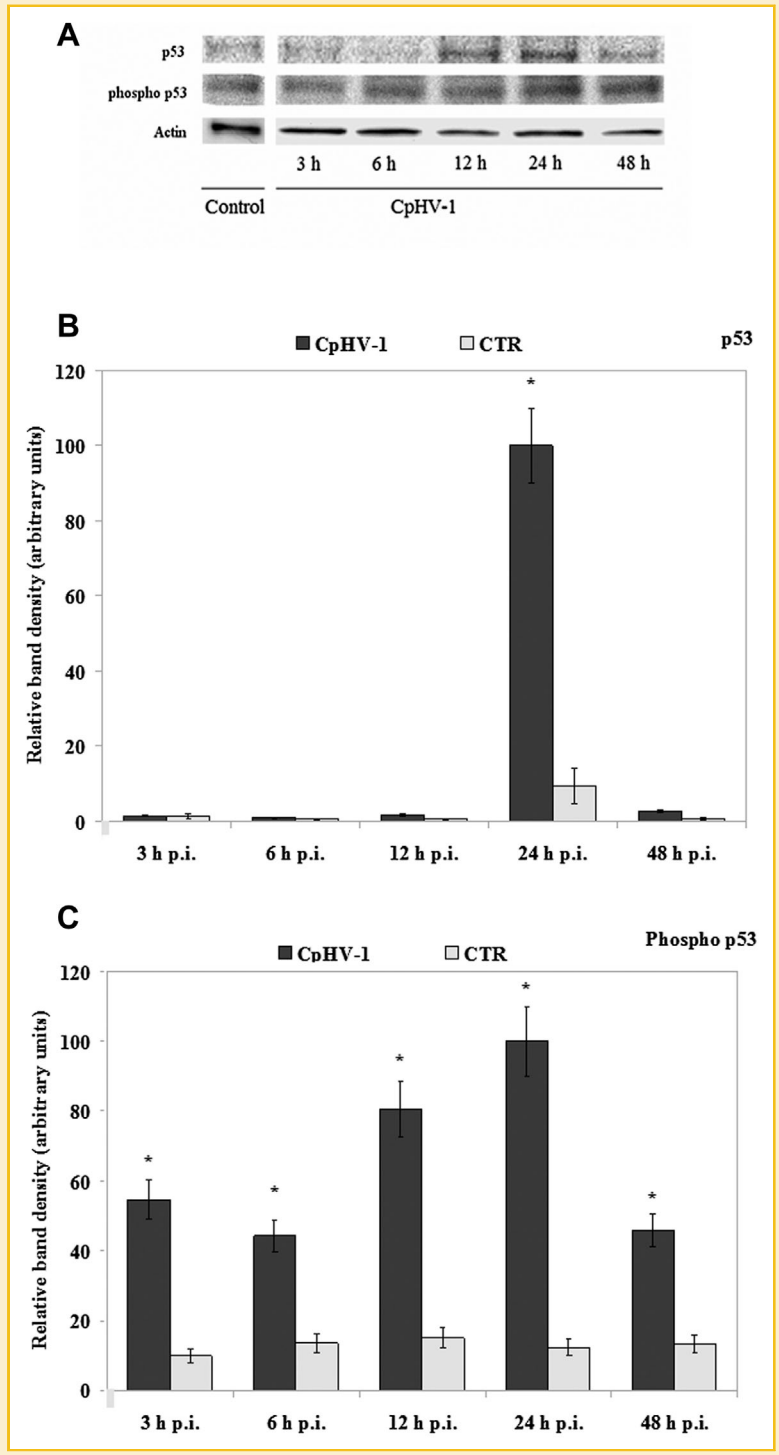

Fig. 4. CpHV-1 upregulate p53 and phospho-p53. Replicate cultures of Neuro 2A cells were mock infected or infected with inoculum consisting of criolysated infected Neuro 2A cells. Inoculum had been titrated in advance, and the multiplicity of infection was approximately 1. At different time points after infection, cells were scraped, harvested, lysate, and subjected to Western blot analysis with antibodies against actin, p53, and phospho-p53. Actin protein levels were detected to ensure equal protein loading (A). Densitometric analysis of blots relative to p53 and phospho-p53 (B and C). Results are expressed as the mean ± SD of three separate experiments, a *P*-value <0.05 was selected as significant.

induce DNA damage after infection [Schlehofer and Hausen, 1982; Pilon et al., 1986; Devireddy and Jones, 1999], suggesting that also infection by CpHV-1 can leads to DNA damage and p53 induction.

In our study the levels of Bcl-2 protein and its phosphorylated form increased after infection which is surprising due to the antiapoptotic role of Bcl-2 protein [Gross et al., 1999]. Simultaneous increase of Bcl-2 and phospho Bcl-2 at 12 h post-infection, preceded by an increase of

phospho-p53 at 6 h post-infection suggest that the induction of apoptosis by CpHV-1 infection involves also the extrinsic pathway.

Bcl-2 protein is normally expressed in a wide range of tissues and is required for normal development and maintenance of the immune system [Veis et al., 1993].

The function of cellular Bcl-2 family members is regulated in part by caspases. Bellows and others have reported that caspase-3 cleaves Bcl-2 at Asp-34 and Bcl-xL at Asp-61 and Asp-76 to produce N-terminally truncated proteins that have lost their antiapoptotic activities [Cheng et al., 1997; Clem et al., 1998; Fujita et al., 1998; Grandgirard et al., 1998; Kirsch et al., 1999; Bellows et al., 2000]. These cleavages are likely to be physiologically significant, as mutation of the cleavage sites in Bcl-2 and Bcl-xL enhances their antiapoptotic activities [Cheng et al., 1997; Clem et al., 1998]. The caspase cleavage products of Bcl-2 and Bcl-xL are potently proapoptotic, based on transfection studies expressing protein fragments that are equivalent to caspase cleavage products [Cheng et al., 1997; Clem et al., 1998]. Thus, the generation of these fragments inside cells may accelerate cell death by amplifying the caspase cascade and in support of this hypothesis, it has been demonstrated that N-terminally truncated Bcl-2 triggers the release of cytochrome c from mitochondria, similar to Bax [Jurgensmeier et al., 1998; Kirsch et al., 1999].

In conclusion, the simultaneous activation of caspase-8 and caspase-9 suggests that CpHV-1 can trigger the death-receptor pathway and the mitochondrial pathway separately and in parallel. It will be interesting to reveal the upstream signals that trigger these two pathways following CpHV-1 infection.

Our findings are significant because this is the first published study showing the effect of CpHV-1 infection in neuronal cells in terms of gene expression and apoptosis modulation.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the careful reading of the manuscript by Dr. Fabiana Di Pascale and the technical contribution of Dr. Gennaro Altamura and Annunziata Corteggio. Human Health Foundation Onlus (www.hhfonlus.org), Sbarro Health Research Organization (www.shro.org) (A.G.).

REFERENCES

Ahmad M, Srinivasula SM, Wang L, Talanian RV, Litwack G, Fernandes-Alnemri T, Alnemri ES. 1997. CRADD, a novel human apoptotic adaptor molecule for caspase-2, and FasL/tumor necrosis factor receptor-interacting protein RIP. Cancer Res 57(4):615–619.

Annis MG, Soucie EL, Dlugosz PJ, Cruz-Aguado AJ, Penn LZ, Leber B, Andrews DW. 2005. Bax forms multispanning monomers that oligomerize to permeabilize membranes during apoptosis. EMBO J 24:2096–2103.

Ashkenazi A, Dixit VM. 1998. Death receptors: Signaling and modulation. Science 281:305–1308.

Aubert M, Pomeranz LE, Blaho JA. 2007. Herpes simplex virus blocks apoptosis by precluding mitochondrial cytochrome c release independent of caspase activation in infected human epithelial cells. Apoptosis 12(1):19–35.

Barton E.S, Chappell JD, Connolly JL, Forrest JC, Dermody TS. 2001. Reovirus receptors and apoptosis. Virology 290:173–180.

Bellows DS, Chau BN, Lee P, Lazebnik Y, Burns WH, Hardwick JM. 2000. Antiapoptotic herpesvirus Bcl-2 homologs escape caspase-mediated conversion to proapoptotic proteins. J Virol 74(11):5024–5031.

Berrios PE, McKercher DG, Knight HD. 1975. Pathogenicity of a caprine herpesvirus. Am. J Vet Res 36:1763–1769.

Bok K, Prikhodko VG, Green KY, Sosnovtsev SV. 2009. Apoptosis in murine norovirus-infected RAW264.7 cells is associated with downregulation of survivin. J Virol 83(8):3647–3656.

Brazeau E, Mahalingam R, Gilden D, Wellish M, Kaufer BB, Osterrieder N, Pugazhenthi S. 2010. Varicella-zoster virus-induced apoptosis in MeWo cells is accompanied by down-regulation of Bcl-2 expression. J Neurovirol 16 (2):133–140.

Brenner D, Mak TW. 2009. Mitochondrial cell death effectors. Curr Opin Cell Biol 21:871–877.

Buonavoglia C, Tempesta M, Cavalli A, Voigt V, Buonavoglia D, Conserva A, Corrente M. 1996. Reactivation of caprine herpesvirus 1 in latently infected goats. Comp Immunol Microbiol Infect Dis 19:275–281.

Cassady KA, Gross M, Roizman, B. 1998. The second-site mutation in the herpes simplex virus recombinants lacking the $\gamma_1$34.5 genes precludes shuto□ of protein synthesis by blocking the phosphorylation of eIF-2α. J Virol 72:7005–7011.

Chen J, Wu X, Lin J, Levine AJ. 1996. mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. Mol Cell Biol 16:2445–2452.

Cheng EHY, Kirsch DG, Clem RJ, Ravi R, Kastan MB, Bedi A, Ueno K, Hardwick JM. 1997. Conversion of Bcl-2 to a Bax-like death effector by caspases. Science 278:1966–1968.

Clem RJ, Cheng EHY, Karp CL, Kirsch DG, Ueno K, Takahashi A, Kastan MB, Griffin DE, Earnshaw WC, Veliuona MA, Hardwick JM. 1998. Modulation of cell death by Bcl-xL through caspase interaction. Proc Natl Acad Sci USA 95:554–559.

Connolly JL, Dermody TS. 2002. Virion disassembly is required for apoptosis induced by reovirus. J Virol 76:1632–1641.

Delhon GA, González MJ, Murcia PR. 2002. Susceptibility of sensory neurons to apoptosis following infection by bovine herpesvirus type 1. J Gen Virol 83 (Pt9):2257–2267.

Devireddy LR, Jones CJ. 1999. Activation of caspases and p53 by bovine herpesvirus 1 infection results in programmed cell death and efficient virus release. J Virol 73(5):3778–3788.

Epand RF, Martinou JC, Montessuit S, Epand RM, Yip CM. 2002. Direct evidence for membrane pore formation by the apoptotic protein Bax. Biochem Biophys Res Commun 298:744–749.

Fiorito F, Marfè G, De Blasio E, Granato GE, Tafani M, De Martino L, Montagnaro S, Florio S, Pagnini U. 2008 2,3,7,8-tetrachlorodibenzo-*p*-dioxin regulates bovine herpesvirus type 1 induced apoptosis by modulating Bcl-2 family members. Apoptosis 13(10):1243–1252.

Fuentes-Prior P, Salvesen GS. 2004. The protein structures that shape caspase activity, specificity, activation and inhibition. Biochem J 384:201–232.

Fujita N, Nagahashi A, Nagashima K, Rokudai S, Tsuruo T. 1998. Acceleration of apoptotic cell death after the cleavage of Bcl-xL protein by caspase-3-like proteases. Oncogene 17:1295–1304.

Gardell JL, Dazin P, Islar J, Menge T, Genain CP, Lalive PH. 2006. Apoptotic effects of human herpesvirus-6A on glia and neurons as potential triggers for central nervous system autoimmunity. J Clin Virol 37(Suppl1):S11–S16.

Gautier I, Coppey J, Durieux C. 2003. Early apoptosis-related changes triggered by HSV-1 in individual neuronlike cells. Exp Cell Res 289(1):174–183.

Geiger KD, Gurushanthaiah D, Howes EL, Lewandowski GA, Reed JC, Bloom FE, Sarvetnick NE. 1995. Cytokine-mediated survival from lethal herpes simplex virus infection: role of programmed neuronal death. Proc Natl Acad Sci USA. 92(8):3411–3415.

Grandgirard D, Studer E, Monney L, Belser T, Fellay I, Borner C, Michel MR. 1998. Alphaviruses induce apoptosis in Bcl-2-overexpressing cells: Evidence

for a caspase-mediated, proteolytic inactivation of Bcl-2. EMBO J 17(5):1268–1278.

Green DR, Kroemer G. 2004. The pathophysiology of mitochondrial cell death. Science 305:626–629.

Gross A, McDonnell JM, Korsmeyer SJ. 1999. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 13:1899–1911.

Hay S, Kannourakis G. 2002. A time to kill: Viral manipulation of the cell death program. J Gen Virol 83(Pt7):1547–1564.

Henderson S, Huen D, Rowe M, Dawson C, Johnson G, Rickinson A. 1993. Epstein–Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. Proc Natl Acad Sci USA 90:8479–8483.

Homan EJ, Easterday BC. 1980. Isolation of bovine herpesvirus-1 from trigeminal ganglia of clinically normal cattle. Am J Vet Res 41(8):1212–1213.

Horikoshi N, Usheva A, Chen J, Levine AJ, Weinmann R, Shenk T. 1995. Two domains of p53 interact with the TATA-binding protein, and the adenovirus 13S E1A protein disrupts the association, relieving p53-mediated transcriptional repression. Mol Cell Biol 15:227–234.

Horner GW, Hunter R, Day AM. 1982. An outbreak of vulvovaginitis in goats caused by a caprine herpesvirus. NZ Vet J 30:152.

Hu S, Vincenz C, Buller M, Dixit VM. 1997. A novel family of viral death effector domain-containing molecules that inhibit both CD-95- and tumor necrosis factor receptor-1-induced apoptosis. J Biol Chem 272:9621–9624.

Hupp TR, Meek DW, Midgley CA, Lane DP. 1992. Regulation of the specific DNA binding function of the p53. Cell 71:875–886.

Jan JT, Chatterjee S, Griffin DE. 2000. Sindbis virus entry into cells triggers apoptosis by activating sphingomyelinase, leading to the release of ceramide. J Virol 6425–6432.

Jiang X, Wang X. 2004. Cytochrome C-mediated apoptosis. Annu Rev Biochem 73:87–106.

Jones C. 1998. Alphaherpesvirus latency: Its role in disease and survival of the virus in nature. Adv Virus Res 51:81–133.

Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC. 1998. Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci USA 95:4997–5002.

Kirsch DG, Doseff A, Chau BN, Lin DS, de Souza-Pinto NC, Hansford R, Kastan MB, Lazebnik YA, Hardwick JM. 1999. Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. J Biol Chem 274:21155–21161.

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME. 1995. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-in-ducing signaling complex (DISC) with the receptor. EMBO J 14:5579–5588.

Krammer PH. 2000. CD95′s deadly mission in the immune system. Nature 407:789–795.

Krammer PH, Arnold R, Lavrik IN. 2007. Life and death in peripheral T cells. Nat Rev Immunol 7:532–542.

Lavrik IN. 2010. Systems biology of apoptosis signaling networks. Curr Opin Biotechnol 21:551–555.

Lavrik IN, Golks A, Krammer PH. 2005. Caspases: Pharmacological manipulation of cell death. J Clin Invest 115:2665–2672.

Lo AC, Woo TT, Wong RL, Wong D. 2011. Apoptosis and other cell death mechanisms after retinal detachment: Implications for photoreceptor rescue. Ophthalmologica Suppl 1:10–17.

Longo M, Fiorito F, Marfè G, Montagnaro S, Pisanelli G, De Martino L, Iovane G, Pagnini U. 2009. Analysis of apoptosis induced by Caprine Herpesvirus 1 in vitro. Virus Res 145(2):227–235.

Marfè G, De Martino L, Filomeni G, Di Stefano C, Giganti MG, Pagnini U, Napolitano F, Iovane G, Ciriolo MR, Salimei PS. 2006. Degenerate PCR method for identification of an antiapoptotic gene in BHV-1. J Cell Biochem 97 (4):813–823.

Mettler F, Engels M, Wild P, Bivetti A. 1979. Herpesvirus infection in kids in Switzerland. Schweiz Arch Tierheilkd 121:655–662.

Molinari M, Okorokov AL, Milner J. 1996. Interaction with damaged DNA induces selective proteolytic cleavage of p53 to yield 40 kDa and 35 kDa fragments competent for sequence-specific DNA binding. Oncogene 13 (10):2077–2086.

Montagnaro S, Ciarcia R, Pagnini F, De Martino L, Puzio MV, Granato GE, Avino F, Pagnini U, Iovane G, Giordano A. 2013. Bovine herpesvirus type 4 infection modulates autophagy in a permissive cell line. J Cell Biochem. 114 (7):1529–1535.

Mori CM, Mori E, Favaro LL, Santos CR, Lara MC, Villalobos EM, Cunha EM, Brandao PE, Richtzenhain LJ, Maiorka PC. 2012. Equid herpesvirus type-1 exhibits neurotropism and neurovirulence in a mouse model. J Comp Pathol 146(2–3):202–210.

Odeberg J, Wolmer N, Falci S, Westgren M, Seiger A, Söderberg-Nauclér CJ. 2006. Human cytomegalovirus inhibits neuronal differentiation and induces apoptosis in human neural precursor cells. Virol 80(18):8929–8939.

Pagnini U, Montagnaro S, Pacelli F, De Martino L, Florio S, Rocco D, Iovane G, Pacilio M, Gabellini C, Marsili S, Giordano A. 2004. The involvement of oxidative stress in bovine herpesvirus type 4-mediated apoptosis. Front Biosci 1(9):2106–2114.

Pagnini U, Montagnaro S, Sanfelice di Monteforte E, Pacelli F, De Martino L, Roperto S, Florio S, Iovane G. 2005. Caprine herpesvirus-1 (CapHV-1) induces apoptosis in goat peripheral blood mononuclear cells. Vet Immunol Immunopathol 103(3–4):283–293.

Pelzl C, Arcizet D, Piontek G, Schlegel J, Heinrich D. 2009. Axonal guidance by surface microstructuring for intracellular transport investigations. Chemphyschem 10(16):2884–2890.

Pilon L, Langelier Y, Royal A. 1986. Herpes simplex virus type 2 mutagenesis: Characterization of mutants induced at the hprt locus of non-permissive XC cells. Mol Cell Biol 6:2977–2983.

Plebani GF, Engels M, Metzler AE, Wyler R. 1983. Caprine herpesvirus in Switzerland: Distribution, incidence and latency of infection. Schweiz Arch Tierheilkd 125:395–411.

Pugazhenthi S, Nair S, Velmurugan K, Liang Q, Mahalingam R, Cohrs RJ, Nagel MA, Gilden D. 2011. Varicella-zoster virus infection of differentiated human neural stem cells. J Virol 85(13):6678–6686.

Ramsey-Ewing A, Moss B. 1998. Apoptosis induced by a postbinding step of vaccinia virus entry into Chinese hamster ovary cells. Virology 242:138–149.

Reed LJ, Muench H. 1938. A simple method of estimating 50% end points. Am J Hyg 27:493–497.

Rock DL, Nesburn AB, Ghiasi H, Ong J, Lewis TL, Lokensgard JR, Wechsler SL. 1987. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. J Virol 61(12):3820–3826.

Roperto F, Pratelli A, Guarino G, Ambrosio V, Tempesta M, Galati P, Iovane G, Buonavoglia C. 2000. Natural caprine herpesvirus 1 (CpHV-1) infection in kids. J Comp Pathol 122:298–302.

Sadzot-Delvaux C, Thonard P, Schoonbroodt S, Piette J, Rentier B. 1995. Varicella-zoster virus induces apoptosis in cell culture. J Gen Virol 76:2875–2879.

Saito JK, Gribble DH, Berrios PE, Knight HD, Mc Kercher DG. 1974. A new herpesvirus isolate from goats: Preliminary report. Am J Vet Res 35:847–848.

Sakahira H, Enari M, Nagata S. 1998. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature 391:96–99.

Schlehofer JR, Hausen H. 1982. Induction of mutations within the host cell genome by partially inactivated HSV-1. Virology 122:471–475.

Stevens JG, Wagner EK, Devi-Rao GB, Cook ML, Feldman LT. 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science 235(4792):1056–1059.

St-Louis MC, Archambault D. 2007. The equine arteritis virus induces apoptosis via caspase-8 and mitochondria-dependent caspase-9 activation. Virology 367(1):147–155.

Thiry J, Keuser V, Muylkens B, Meurens F, Gogev S, Vanderplasschen A, Thiry E. 2006. Ruminant alphaherpesviruses related to bovine herpesvirus 1. Vet Res 37(2):169–190.

Tisdall DJ, Bentley CB, Collins DM. 1984. New Zealand caprine herpes virus: Comparison with an Australian isolate and with bovine herpesvirus type 1 by restriction endonuclease analysis. NZ Vet J 32:99–100.

Veis DJ, Sorenson CM, Shutter SR, Korsmeyer SJ. 1993. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypo-pigmented hair. Cell 75:229–240.

Waldvogel A, Engels M, Wild P, Stunzi H, Wyler R. 1981. Caprine herpesvirus infection in Switzerland: some aspects of its pathogenicity. Zentralblatt fur Veterinarmedizm Reihe B 28:612–623.

Wang GH, Bertin J, Wang Y, Martin DA, Wang J, Tomaselli KJ, Armstrong RC, Cohen JI. 1997. Bovine herpesvirus 4 BORFE2 protein inhibits Fas- and tumor necrosis factor receptor 1-induced apoptosis and contains death effector domains shared with other gamma-2 herpesviruses. J Virol 71(11):8928–8932.

Watanabe D, Honda T, Nishio K, Tomita Y, Sugiura Y, Nishiyama Y. 2000. Corneal infection of herpes simplex virus type 2–induced neuronal apoptosis in the brain stem of mice with expression of tumor suppressor gene (p53) and transcription factors. Acta Neuropathol 100(6):647–653.

Williams NM, Vickers ML, Tramontin RR, Petrites-Murphy MB, Allen GP. 1997. Multiple abortions associated with caprine herpesvirus infection in a goat herd. J Am Vet Med Assoc 211:89–91.

Xu X, Zhang K, Huang Y, Ding L, Chen G, Zhang H, Tong D. 2012. Bovine herpes virus type 1 induces apoptosis through Fas-dependent and mitoc hondria-controlled manner in Madin-Darby bovine kidney cells. Virol J 9: 202.