Apoptotic and necrotic changes in cultured murine neurons infected with equid herpesvirus 1

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Summary. – Equid herpesvirus 1 (EHV-1), like other members of the Alphaherpesvirinae, is a neurotropic virus, that causes latent infections in the nervous system of the natural host. All alphaherpesviruses have developed sophisticated strategies to interfere with the host cell apoptotic mechanisms, but the ability of EHV-1 to induce apoptosis in neurons has not been determined yet. In this study, apoptotic and necrotic changes in cultured murine neurons were methods identifying key stages of apoptosis. These methods have demonstrated characteristic apoptosis features, like DNA fragmentation, chromatin condensation, membrane blebbing and cell shrinkage in the infected cells. It seems likely that apoptosis was the predominant way of cell death in EHV-1-infected murine neurons. However, we showed also that during acute EHV-1 infection the majority of infected neurons remained unchanged and survived for more than eight weeks in culture, suggesting some protective mechanisms induced by the virus. Furthermore, it was shown that infection of neurons with EHV-1 has no significant influence on the level of the caspase 3, 7, and 8. We speculate that the control of apoptosis may be the key mechanism regulating the balance between productive and latent infection at the site of virus persistence.

Keywords: equid herpesvirus 1; murine neurons; primocultures; apoptosis; necrosis; cytopathic effect

Introduction

Equid herpesvirus 1 (EHV-1) (the subfamily Alphaherpesvirinae) is a major cause of upper respiratory tract infections, neurological disorders and abortion in horses. Similarly to other alphaherpesviruses (e.g. HHV-1, HHV-2, VZV, PRV, BHV-1) (Dehlon et al., 2002; Ch’ng et al., 2005), it is neurotropic and causes latent infection in neurons of natural hosts. In vivo studies in the natural host and mice models showed that EHV-1 replicates in the neurons of trigeminal ganglion and olfactory bulbs, where it also sets the state of latent infection (Awan et al., 1990; Slater et al., 1994; Walker et al., 1999). Other studies showed that in mice experimentally infected with EHV-1 Jan-E strain, isolated from aborted fetus, productive and latent infection occurred not only in neurons of the olfactory bulbs, but also in other parts of rhinencephalon (Banbura et al., 2000). In addition, infection was accompanied by neuronal degeneration, but the mechanism of these changes has not been elucidated yet. One of the possible mechanisms of neurodegenerative effect of the EHV-1 is the induction of programmed cell death – apoptosis of neurons. Apoptosis is utilized by the host organism as a defense mechanism directed against intracellular pathogens which cannot be eliminated in any other way. During apoptosis, degradation of proteins and...
nucleic acids of host and viral origin occurs, thus prevents
the spread of disease. On the other hand, viruses can trigger
anti-apoptotic mechanisms or modulate the pro-apoptotic
response to disrupt the apoptosis (Aubert et al., 2007).
Delhon et al. (2002) suggested that the control of apoptosis
machinery by viruses may be critical for their reproduction
and provision of the adequate yield of progeny virions. Our
previous results demonstrated that infection by two
different strains of EHV-1 (Jan-E and Rac-H) do not induce
caspase-3-mediated apoptosis in equine dermal ED (host)
and monkey kidney Vero (model) cell lines. Our preliminary
data suggested that the primary mechanism of cell death in
both lines was necrosis (Turowska et al., 2010). Those results
are in agreement with Walter and Novotny (1999) observa-
tions in Vero cells. On the other hand, little is known of the
EHV-1 interactions with neurons and the mechanism of the
virus-induced neurodegenerative changes. We suppose
that the virus has ability to affect the apoptotic process in
host cell. It is known that virus replication and transmission
depends on the effective exploitation of the existing cellular
machinery of the infected cell. Additionally, the latent infe-
tions of the host cells also depend on prevention of death of
the infected cells.

In our previous studies we have already showed that EHV-1
was able to replicate in murine neurons in vitro without the
need of adaptation, what was confirmed by the positive
results of nPCR and real-time PCR tests. Furthermore, in
long time infection some neurons degenerated but some
remained unchanged. This is typical for the latent form
of infection (Cymerys et al., 2010). These results led us to
further investigation of mechanisms associated with death
or survival of EHV-1 infected neurons.

For that reason, in this study we have examined apoptotic
and necrotic changes in cultured murine neurons, infected
with EHV-1, Rac-H, and Jan-E strains, by methods of identi-
ifying the key stages of apoptosis.

Materials and Methods

Virus strains. In this study two strains of EHV-1 from the
virus collection of the Virology Laboratory of the Department
of Preclinical Sciences were used. Jan-E is a field strain isolated
from aborted fetus (mare Ezelda, Janów Podlaski stud, Poland; 12th pas-
sage in ED cells) and identified by PCR using gB-specific primers
(Borchers and Slater, 1993). Rac-H is a reference strain isolated
from mare Heraldia, Racot stud, Poland, which has been passed
through a series of cell cultures, and described as ‘pantropic, non-
pathogenic’ (Nugent et al., 2006).

Cells. Balb/c (H-2d) mice genetically susceptible to EHV-1 infec-
tion (Awan et al., 1990; Gosztowy et al., 2009) were used to establish
primoculture of murine neurons, as described before (Cymerys et
al., 2010). Cells were plated onto poly-L-lysine, or poly-D-lysine
and laminin-coated coverslips at a density of 5x10⁴ to 10⁵ neurons
per well. Primary murine neurons were cultured in B-27 neuron
plating medium (Gibco Life Technologies, UK). Cultures were
maintained at 37°C with 5% CO₂. Primoculture of murine neurons
(10⁷ cells per well) was infected with EHV-1 (MOI 1.0) for 60 min
at 37°C. After adsorption, the inoculum was aspirated and fresh
culture medium was added. Then the cells were incubated for 3,
24, 48, or 72 hrs at 37°C with 5% CO₂.

Immunofluorescence test for viral antigen. Anti-β-tubulin mono-
clonal antibody (Sigma-Aldrich, USA) was used in dilution 1:250 to
detect β-tubulin as a neuronal marker. Secondary goat anti-mouse
antibody conjugated with texas red (Invitrogen Life Technologies,
UK) was used in dilution 1:100. The presence of viral antigen
was detected by indirect immunofluorescence, using anti-equine
rhinopneumonitis virus/equine herpesvirus type 1 (ERV/EHV-1)
polyclonal antiserum conjugated with fluorescein isothiocyanate
(VMRD Inc., USA). Cell nuclei were counter-stained with Bisbenzi-
midine/Hoechst 33258 or DAPI (Sigma-Aldrich, 2μg/ml), according
to the manufacturers protocols. Cells cultured on coverslips
were examined under the BX-60, fluorescence microscope (Olympus
Corp., Germany) and FV-10i, confocal microscope using FluoView
software (Olympus Polska, Poland).

Annexin V and propidium iodiode staining. The Annexin V-FITC
apoptosis detection kit (Sigma-Aldrich) was used to detect early
phases of apoptosis preceding the loss of cell membrane integrity
(Shun et al., 2008). At 3, 24, and 48 hrs post infection (p.i) with
Jan-E and Rac-H strains, suspension of neuron cells (10⁷ cells) was
double stained with FITC-Annexin V/propidium iodid, accord-
ting to the manufacturer’s instructions. In the cells with disturbed
membrane integrity, propidium iodide (PI) was used to discern
necrotic and late apoptotic cells. The samples were analyzed by
FACS flow cytometer equipped with Cell Quest Research Software
(Becton Dickinson, Germany) and by fluorescence microscopy
(Olympus BX60). Proportion of dead cells (Annexin V-FITC+/PI+),
early apoptotic cells (Annexin V-FITC+/PI−), late apoptotic/necrotic
cells (Annexin V-FITC+/PI+) and non-apoptotic cells (Annexin V-
FITC−/PI−) could be distinguished.

TUNEL labeling. Suspension of neuron cells (10⁶ cells) at 3,
24, 48, and 72 hrs p.i. was stained for TUNEL (Terminal deoxynu-
cleotide transferase dUTP nick end labeling) with an APO-BrdU
kit (Becton Dickinson, Germany) according to the manufacturer’s
instructions. The APO-BrdU kit is a two color TUNEL assay for
labeling DNA breaks in late phases of apoptosis. The percentage
of TUNEL-positive cells was calculated by FACS flow cytometer
equipped with Cell Quest research software (Becton Dickinson,
Germany).

Cell cycle analysis. Primoculture of murine neurons incubated
for 24 and 72 hrs with Jan-E and Rac-H strains was stained with
Hoechst 33258 (dilution 1:1000, 3 min) for nuclei visualization. The
slides were examined using a Scan^R scanning cytometer (Olympus
Polska, Poland), designed for fully automated image acquisition
and data analysis of biological samples. Data were acquired from
225 fields of view distributed all over the sample area. Subsequent
sampling areas were separated by the width of the field of view. Cells were identified on the base of DNA-related fluorescence intensity. Cell cycle was evaluated on the base of nuclei parameters: size, circularity and DNA-related fluorescence intensity. Results were analyzed by using Microsoft Excel 2003 software (Microsoft Corporation, USA) and Prism version 3.00 software (GraphPad Software, USA).

**Caspase assay.** Caspase-3 and 7 activity was evaluated in cell suspension (10⁶ cells) using Vybrant® FAM Caspase-3 and 7 assay kit (Invitrogen Life Technologies) and caspase-8 activity was measured using Vybrant® FAM Caspase-8 assay kit (Invitrogen Life Technologies). Both assays were done according to the manufacturer’s instructions. Samples were then analyzed by FACS (Becton Dickinson, Germany).

**Statistical analysis.** Each analysis was carried out at least in triplicate, and each experiment was repeated at least twice. Differences in mean values were analyzed using Student’s t-test. Statistical differences were interpreted as significant at P < 0.05 and highly significant at P < 0.01. A positive control for apoptotic cells was generated by adding 1 μmol/l staurosporine (Sigma-Aldrich), a potent apoptosis-inducing protein kinase inhibitor with an indirect inhibitory effect on topoisomerase I, to the culture medium (20 hrs incubation), whereas non-infected neurons served as negative control.

**Results**

**Morphology of virus-infected neurons**

Regardless of the EHV-1 strain (Jan-E or Rac-H) a cytopathic effect was observed (Fig. 1c–f). In Jan-E strain infected primoculture of murine neurons, the cytopathic effect was manifested by morphology changes and nuclei degeneration, whereas Rac-H strain caused additional cytoplasm vacuolization and cell lysis, which led to the formation of the plaque. Jan-E strain induced changes that were well visible as early as 24 hrs p.i. (Fig. 1c, d), whereas Rac-H strain yielded a similar result 48 hrs p.i. (Fig. 1e, f). Direct immunofluorescence test confirmed the presence of EHV-1 antigen in the infected cells. The signal was localized mostly within the cytoplasmic region and seldom in the nucleus. Furthermore, infection by Jan-E and Rac-H strains was associated with the reorganisation or destruction of β-tubulin fibres (Fig. 1c–f).

**Apoptotic and necrotic changes in virus-infected neurons**

The annexin V-FITC and PI, FACS study was performed to distinguish and quantitatively determine the percentage of dead, viable and apoptotic (both early and late phases) cells after infection with both EHV-1 strains (Fig. 2). Jan-E strain induced apoptosis of murine neuronal cells within 3 to 24 hrs p.i. The percentage of early apoptotic cells increased from 5.86% in control to 28.07% 3 hrs p.i. and late apoptotic cells from 4.75% in control to 13.64% 3 hrs p.i. After infection with the Rac-H strain the induction of apoptosis was postponed until 24 hrs p.i. – the percentage of early and late apoptotic cells increased from 4.75% and 5.86% in the non-infected control culture to 14.13% and 23.99% in the infected cells, respectively. Additionally, we have shown that regardless of infection, the highest number of cells remained unaffected (57.27%–85.79% neurons).

To determine whether EHV-1 infection was associated with DNA strand breaks, characteristic for late apoptosis, primary murine neurons were infected with Rac-H and Jan-E strains and processed for TUNEL assay (Fig. 3). The study showed an increase in the DNA strand breaks in neurons infected with Jan-E strain at 3 hrs p.i. (24.82% ± 4.05), compared to uninfected cells (4.76% ± 3.07, P <0.05). The percentage of apoptotic cells in neurons infected with a Jan-E strain remained at a statistically significantly increased value until 48 hrs p.i. (19.12% ± 1.08, P <0.001), and then the percentage of apoptotic cells decreased. Infection of the neurons with EHV-1 Rac-H strain caused a statistically significant increase in TUNEL response only 24 hrs p.i. (12.93% ± 2.66, P <0.05) and 72 hrs p.i. (12.50% ± 1.98, P <0.05), when compared to control.

Scanning cytometry evaluation of the cell cycle showed that in all examined cultures the highest percentage of neurons remained in the G1 phase, both in control (80.01% ± 5.09) and EHV-1-infected cells - from 56.46% ± 15.40 to 64.11% ± 20.77, respectively. The percentage of cells in the SubG1 region of the histogram (characteristic to the late phases of apoptosis) increased from 6.45% ± 2.73 in the control to 11.2% ± 8.78 in Jan-E-infected cells (72 hrs p.i.) and 35.28% ± 20.18 in Rac-H-infected cells (24 hrs p.i.). Increase in the SubG1 region was accompanied by the simultaneous reduction of the cell number in G1 phase in both experimental cultures. Percentage increase of the cells in S and G2M phase of cell cycle was also observed in Jan-E-infected cells after 24 and 72 hrs, whereas in Rac-H-infected cells only after 72 hrs (Fig. 4). Simultaneously with the cytogams, an analysis of the cell morphology and the presence of viral antigens were assessed in uninfected and infected primocultures of murine neurons. The EHV-1-related fluorescence was positively correlated with a SubG1 phase on the cell cycle histograms (data not shown). Furthermore, when analyzed under the confocal microscope, the virus-positive cells exhibited characteristic features of apoptosis such as chromatin condensation, pyknosis and the fragmentation of nuclei (Figs. 5, 6).

**Caspase-3, 7, and 8 in virus infected neurons**

The expression of executor caspase-3 and 7 in neurons infected with both strains of EHV-1 remained at similar or lower level in comparison to the control. In Rac-H-infected
Fig. 1

Morphology of virus-infected cells

Confocal microscopy of β-tubulin fibres (red), viral antigen (green) and nuclei (blue) in non-infected (ctrl) and infected (Jan-E, Rac-H) murine neurons. Arrows indicate areas of destruction of β-tubulin fibres associated with the presence of EHV-1 antigens. Lens magnification: 60x, digital zoom 1.4x (a), 2.2x (b), 1.6x (c).
neurons level of caspase-3 and 7 decreased from 29.22% \pm 9.24 in control to 19.41% \pm 2.32 (24 hrs p.i.) and returned to the initial values (28.16% \pm 11.65) 72 hrs p.i. In Jan-E-infected neurons expression changes of caspase-3 and 7 were similar to Rac-H-infected neurons: 14.49% \pm 11.95 24 hrs p.i. and 26.08% \pm 8.33 72 hrs p.i. (Fig. 7). The expression of regula-

tor caspase-8 (the receptor-mediated form of apoptosis) in the cultured neurons was relatively high, even in the control cultures (22.69% \pm 6.37). Following infection with EHV-1, the caspase-8 expression increased slowly in Rac-H-infected neurons (from 20.70% \pm 3.27 24 hrs p.i. to 27.42% \pm 1.81 72 hrs p.i.) while in Jan-E strain infected neurons it increased from 11.28% \pm 12.85 24 hrs p.i. to 24.27% \pm 2.68 72 hrs p.i. (Fig. 7). As a positive control, neurons were incubated with the chemical inducer of apoptosis – staurosporine (1 μmol/l). In neurons incubated with staurosporine a significantly higher expression of caspase-3, 7, and 8 was observed, when compared to untreated cells. A significant difference was also

ACS flow cytometrical assay of dead, early apoptotic, late apoptotic, and non-apoptotic virus-infected and control murine neurons stained with Annexin V and propidium iodide.

Fig. 2

Membrane integrity damage in apoptotic virus-infected murine neurons

![ACS flow cytometrical assay of dead, early apoptotic, late apoptotic, and non-apoptotic virus-infected and control murine neurons stained with Annexin V and propidium iodide.](image-url)
observed between staurosporine-treated neurons and cells infected with EHV-1 (P <0.05) (Fig. 7).

Discussion

Mechanisms of virus-induced apoptosis play an important role in understanding pathogenesis of virus infection. In this report, we demonstrated that the infection of murine primary neurons with two strains of EHV-1 (Rac-H and Jan-E) induces changes associated with apoptotic cell death such as DNA fragmentation, chromatin condensation, membrane blebbing and cell shrinkage. In support of these observations, we have recently found that infection of neurons with EHV-1 induces loss of plasma membrane, which is an early marker of apoptosis. The use of annexin V-FITC assay with PI to distinguish and quantitatively determine the percentage of viable apoptotic and necrotic cells after infection with each EHV-1 strain showed

![Graph showing DNA fragmentation in apoptotic virus-infected murine neurons](image1)

**Fig. 3**

DNA fragmentation in apoptotic virus-infected murine neurons

FACS flow cytometrical assay of non-infected, non-treated cells (control (-)), non-infected, staurosporine-treated cells (control (+)), virus-infected cells (3, 24, 48, and 72 hrs p.i.). Asterisk (*) indicates statistically significant difference in comparison with control (P <0.05) and (P <0.001).

![Graph showing cell cycle phase analysis](image2)

**Fig. 4**

Cell cycle phase analysis of virus-infected murine neurons

Scanning cytometry of non-infected (control) and virus-infected (Jan-E, Rac-H) neurons in SubG1, G1, S, and G2M phases 24 and 72 hrs p.i.
that the predominant type of death is apoptosis (both early and late phases were detected), rather than necrosis. When infected cultures were analysed at different time points, the differences in the susceptibility to Jan-E and Rac-H strains infection have been observed. We speculate that observed differences may depend on the virulence of various EHV-1 strains. It is known that due to the repeated passage of the Rac-H strain in vitro, some parts of the viral genome have been deleted, significantly reducing its virulence. In addition, we have shown that the highest percentage of infected cells remained viable. Similar results were obtained in our previous studies (Cymerys et al., 2010). After infection, some neurons changed their shape and degenerated, although some remained unchanged and as such survived for more than eight weeks. It is worth noting, that microscopic observations confirmed presence of the EHV-1 antigens in the nucleus and cytoplasm of the majority of the neurons to constant, 'limited' replication, followed by the release of viral progeny, as suggested by the observations that trigeminal ganglion, and other neurons may survive virus replication during primary infection or reactivation, and enter or resume latency afterwards (Geiger et al., 1999; Perng et al., 2000; Aleman et al., 2001).

Fig. 5
Virus-infected murine neurons with apoptotic morphological changes
Confocal microscopy of β-tubulin fibres (red), viral antigen (green) and nuclei (blue) in non-infected (ctrl) and infected (Jan-E, Rac-H) murine neurons. Lens magnification: 60x.

Fig. 6
Apoptotic morphological changes in nuclei of virus-infected murine neurons
Nuclear staining (blue) of non-infected (ctrl) and infected (Jan-E, Rac-H) murine neurons. Note the clusters of cells with chromatin condensation, pyknosis and the fragmentation of nuclei (b, c). Lens magnification: 60x.
For further investigation of the EHV-1-induced apoptosis, we performed DNA fragmentation TUNEL assay. In the Jan-E-infected cell cultures the significant increase of TUNEL-positive neurons were observed as early as 3 hrs p.i., and remained elevated until 72 hrs p.i., similarly to the results of annexin tests. In contrary, in the Rac-H-infected neurons a significant increase in the percentage of TUNEL-positive cells occurred only 24 hrs p.i. and 72 hrs p.i. In control cell cultures less than 5% of positive cells have been observed. The extent of the apoptosis was strongly strain dependent. Use of Jan-E strain, presumably more virulent, resulted in higher positive results in both tests. In addition, the scanning cytometry analysis of the cell cycle confirmed the results obtained from the apoptosis-specific tests. Infection with both strains resulted in the increase of the number of cells in SubG1 phase of cell cycle, which is commonly associated with apoptosis (consists predominantly of pyknotic and fragmented nuclei). The increase of the SubG1 phase was accompanied by the proportional decrease of the cell number in G1 phase, suggesting that the majority of the dying cells came from this, non-dividing cell population. What is interesting, the number of cells in S and G2M phases increased notably upon infection, regardless of the status of infection.

Other members of the subfamily Alphaherpesvirinae, including VZV, HSV-1, BHV-1, and PRV have been shown to induce and/or suppress apoptosis in a cell-type-specific manner (Hood et al., 2003). HSV-1 can induce apoptosis in many cell types but it has been reported to suppress apoptosis in neuronal cell lines in vitro and neurons in vivo (Perng et al., 2000). BHV-1 induces apoptosis in activated CD4 T cells, epithelial cell lines and dissociated rabbit sensory neurons but not in rabbit trigeminal ganglionic neurons in vivo (Devireddy et al., 1999; Delhon et al., 2002; Geiser et al., 2008). Furthermore, PRV can induce apoptosis in inflammatory cells, such as monocytes/macrophages and lymphocytes, however, it is unable to induce apoptosis in swine trigeminal ganglionic neurons in vivo (Aleman et al., 2001). Thus, among the alphaherpesviruses studied to date, all appear to have evolved mechanisms to suppress apoptosis in neurons. Relying on those data and results presented in this study, it can be assumed that there is a reason why most neurons survive the primary infection of EHV-1, therefore we suggest that EHV-11 is responsible for suppression of apoptosis in these cells. An ability to inhibit apoptosis during infection of neurons may represent a key mechanism involved in the establishment, maintenance of EHV-1 latency or reactivation. Other reports show, that during latency period, the
viral genome is transcriptionally silent, except for a single region encoding the latency-associated transcript (LAT). LAT can suppress apoptosis (either in vivo or in vitro) and this may explain the importance of LAT in alphaherpesvirus (for example HSV-1, BHV-1, PRV) latency and reactivation (Geiger et al., 1999; Perng et al., 2000; Ahmed et al., 2002; Geenen et al., 2005).

To verify whether the apoptosis induced by EHV-1 infection is dependent on caspase activity, we performed activity and expression studies with a set of key caspases involved in the regulatory and executor phases. The activity of executor caspases-3 and 7 in neurons infected with both strains of EHV-1 remained at a similar or lower level in comparison to the control. The activity of caspase-8 (regulatory caspase on the extrinsic pathway) in the cultured neurons was relatively high. Upon infection no significant changes have been observed. In neurons incubated with staurosporine (positive control) we observed a significant increase of the activity of caspase-3, 7, and 8, as compared to uninfected cells (negative control). In addition to that, the activity of these caspases was higher than in the infected cells. These results suggest that infection of the cultured neurons with EHV-1 does not affect the activity and expression of the studied caspases and it can be assumed that apoptosis in murine neurons is not related to extrinsic (receptor) pathways.

In conclusion, this study showed that two different strains of EHV-1 (Jan-E and Rac-H strains) were able to induce apoptosis in murine neurons. The extent of the apoptosis depended strongly on the strain, with Jan-E strain, presumably more virulent, resulted in higher positive results in all tests. In addition, infection of murine neurons with both EHV-1 strains had no significant influence on the level of the caspase-3, 7, and 8. We observed also that apoptotic neurons represented only a small percentage of total cell number, suggesting that regardless of the infection, majority of the murine neurons were not only resistant to the EHV-1-induced cell death, but also re-entered the active proliferation cycle (increase in the S and G2M phases of cell cycle). Further work will elucidate the mechanisms of control of the apoptosis in EHV-1-infected murine neurons. We speculate that the control of apoptosis may be the key mechanism regulating the balance between productive and latent infection at the site of virus persistence.

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**References**


