

INDUCTION OF APOPTOSIS IN MDCK, RK13, AND NEURO-2A CELLS INFECTED WITH EQUINE INFLUENZA VIRUS

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Abstract

The purpose of the experiment was to compare apoptosis induced by equine influenza virus (EIV A1 and EIV A2) infection in MDCK, RK13, and NEURO-2A cell lines. Flow cytometry was used to observe two symptoms of apoptosis: phosphatidylserine translocation in plasmalemma (annexin V assay) and the fragmentation of DNA generated by endonuclease activity (TUNEL assay-terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling). The differences in the onset of apoptosis in the studied cells was observed. In MDCK cells infected with EIV A1 and A2, a weak signal of the phosphatidylserine translocation was observed but more cells showed the DNA fragmentation. An opposite effect was observed in case of RK 13 cells. NEURO-2A cells displayed a similar number of annexin V and TUNEL positive cells after the infection with EIV A2, while in case of EIV A1 infection, only the early symptoms of apoptosis were noted. Differences between both viral serotypes could originate from functioning of viral proteins responsible for induction or inhibition of apoptosis. The differences between cell types may result from the activation of cellular pro or anti-apoptotic mechanisms.

Key words: equine influenza virus, cell culture, apoptosis, annexin V, TUNEL.

Influenza A viruses appear to be important respiratory pathogens in humans, pigs, and horses (23). Two subtypes: H7N7 (EIV A1) and H3N8 (EIV A2) are involved in equine influenza, but EIV A1 infection has not been confirmed in horses since the early 1980s (24). Influenza viruses replicate in the epithelial cells of the upper respiratory tract but macrophages and other leukocytes are also infected (7, 20). EIV regulates host cell transcriptional and translational systems (17, 18). EIV infection leads to initiation of apoptotic cell death mechanisms in host's cells, manifested by DNA fragmentation or induction of the Fas antigen (12, 13, 21, 22). During early stages of the infection, host cells turn on the apoptotic pathways to limit the virus spread by elimination of the infected cells. Viruses express anti-apoptotic mechanisms to prevent this cellular response. During the late stages of infection, virus can influence apoptosis machinery to facilitate efficient propagation, disabling anti-apoptotic pathways, and subsequently activating the signals of apoptosis initiation. Finally, apoptotic mechanisms may lead to cell death by caspase-dependent mechanism (7, 9, 10).

The purpose of the experiment was to compare apoptosis induced by EIV A1 and EIV A2 infection of cells of three different cell lines: MDCK, RK13, and NEURO-2A. Two symptoms of apoptosis: phosphatidylserine translocation in plasmalemma and fragmentation of DNA generated by endonuclease activity will be detected.

Material and Methods

Viruses. The strains of equine influenza virus A/Equi/1/Prague 56 (H7N7) and A/Equi/2/Kentucky 81 (H3N8) were obtained from the National Veterinary Services Laboratories, Ames, USA. The viruses were propagated in embryonated chicken eggs.

Cell cultures. Madin Darby canine kidney cells (MDCK), rabbit kidney cells (RK13), and mouse neuroblastoma cells (NEURO-2A) were obtained from the European Collection of Animal Cell Cultures (ECACC). Cells were propagated in Eagle's minimal essential medium with Earle's salts, 2 mM L-glutamine, 1% non essential amino acids, and 10% foetal bovine serum in six well plates. The monolayer was obtained within 24 h.

Infection of cells. The same infection dose - 100 EID₅₀ for EIV A1 and A2 was applied to all cells. Incubation was carried out at 37°C for 24 h. Then, the medium was collected and the cells were detached from the plate surface by treatment with 0.25% solution of trypsin-EDTA. The time of the trypsinisation was shortened to protect the integrity of the surface antigens. Afterwards, the cells were suspended in the removed medium, centrifuged (500 g, 5 min, 8°C), and washed in PBS (500 g, 3 min, 8°C).

Detection of infected cells. Cells were fixed with Cytofix/Cytoperm (BD Pharmingen) kit according to the producer's instruction. For labelling the infected

cells, the sera from rabbits immunised with H7N7 (HI titer 8,192) or H3N8 (HI titer 8,192) were used and the secondary anti-rabbit antibody, FITC-labelled (Jackson ImmunoResearch) was applied. The labelling protocol was as follows: 1×10^6 fixed cells were incubated for 1 h at room temperature with 5 μ l of rabbit sera in final volume of 100 μ l. In the next step after washing, the cells were incubated in dark with 5 μ l of secondary antibodies diluted 1:20 in PBS. Flow cytometric analysis was performed with the use of Epics XL cytometer (Beckman Coulter) and histograms were generated with WinMDI 2.8.

Detection of phosphatidylserine translocation (annexin V). Cell infected with EIV A1 or EIV A2 were washed with annexin V binding buffer and incubated with annexin V - PE (BD Pharmingen) for 20 min at room temperature (5 μ l per 1×10^6 cells). Uninfected cells and cells treated for 1 h with 9 μ M camptothecin were used as control (2). Flow cytometric analysis was performed with Epics XL Beckman Coulter and histograms were generated with WinMDI 2.8 software.

Detection of DNA fragmentation (TUNEL assay). The same cells as for annexin V assay were used to detect DNA fragmentation. Cells were fixed with Cytofix/Cytoperm (BD Pharmingen) and labelled with FITC-conjugated anti-BrdU antibodies (BD Pharmingen). Flow cytometric analysis was performed with Epics XL Beckman Coulter and histograms were generated with WinMDI 2.8 software.

Microscopic analysis. Observation of cytopathic effect was performed under light microscope.

Results

Flow cytometry was applied to analyse apoptosis induced by EIV in MDCK, RK13, and NEURO-2A cells. The cells were infected with EIV A1 or EIV A2 and analysed 24 h post infection. This time point was selected on the basis of observation of the cytopathic effect in order to maximise post infection changes in RK 13 cells and simultaneously to avoid degradation of MDCK or NEURO-2A cultures (the cytopathic effect was observed in MDCK and NEURO-2A cells inoculated with EIV A2). The total number of infected cells and cells in the early (annexin V positive) and late (TUNEL positive) stages of apoptosis, as well as the cytopathic effect after infection were evaluated (Table 1).

In the first step, the number of cells expressing viral antigens after EIV infection was determined. The similar percentage of positive cells for all three tested cell lines after EIV A2 infection was observed (63.06%, 69.09%, and 60.32% for MDCK, NEURO-2A, and RK13, respectively). The number of cells expressing viral antigens after EIV A1 infection was lower (25.04%, 32.7%, and 37.95% for MDCK, NEURO-2A and RK13, respectively) (Fig. 1).

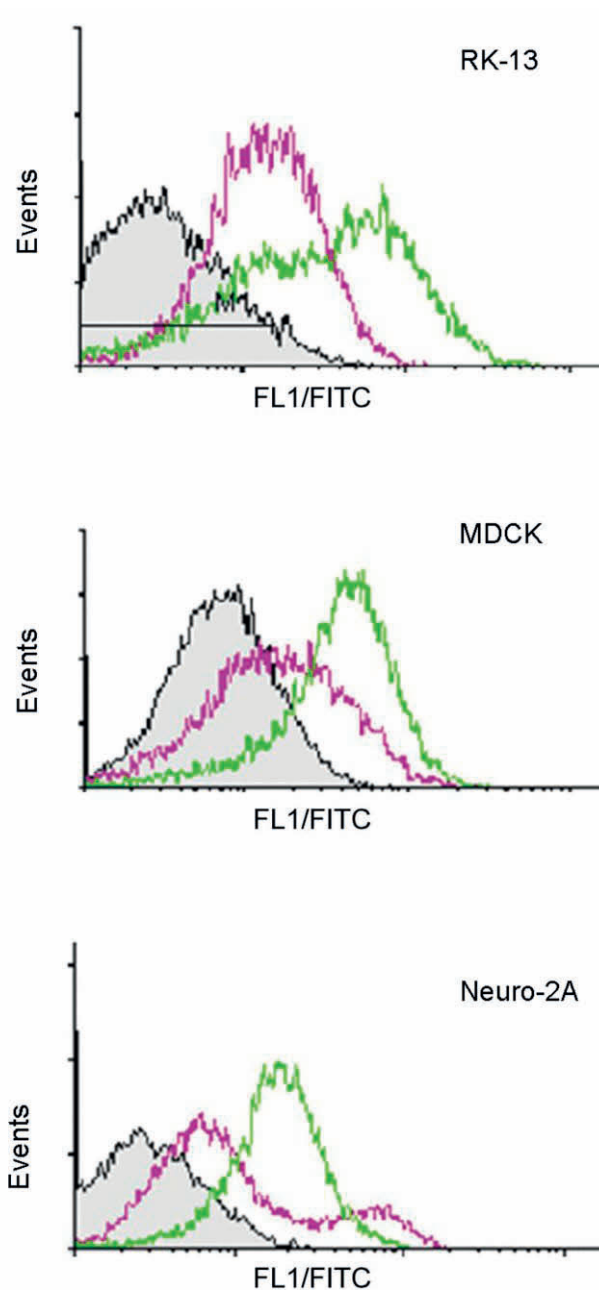


Fig. 1. Flow cytometry analysis of cells expressing EIV A1 and EIV A2 antigens (grey - uninfected control cells; purple - cells infected with EIV A1; green - cells infected with EIV A2)

In the experiment, the low number of MDCK cells infected with EIV A1 showed early stages of apoptosis (0.76%), but 9.81% of the cells were found in the advanced stage of apoptosis (TUNEL positive). In case of EIV A2 infection, 4.44% of cells were in the early phase of apoptosis, 13.58% of cells - in the late phase, and strong cytopathic effect was observed (Fig. 2, Table 1).

After the infection with EIV A1 and EIV A2, 0.87% and 3.88% of RK 13 cells were found to be positive for annexin V, respectively. No cells positive for TUNEL test were detected (Table 1).

Table 1
Comparison of infection, cytopathic effect, and results of annexin V and TUNEL assays
for cells from MDCK, RK 13, and NEURO-2A cell lines

	Infection		Cytopathic effect		Annexin V		TUNEL	
	EIV A1	EIV A2	EIV A1	EIV A2	EIV A1	EIV A2	EIV A1	EIV A2
MDCK	25.04 %	63.06%	-	+++	0.76%	4.44%	9.81%	13.58%
NEURO-2A	32.7 %	69.09%	-	+	2.84%	15.62%	-	11.54%
RK13	37.95 %	60.32%	-	-	0.87%	3.88%	-	-

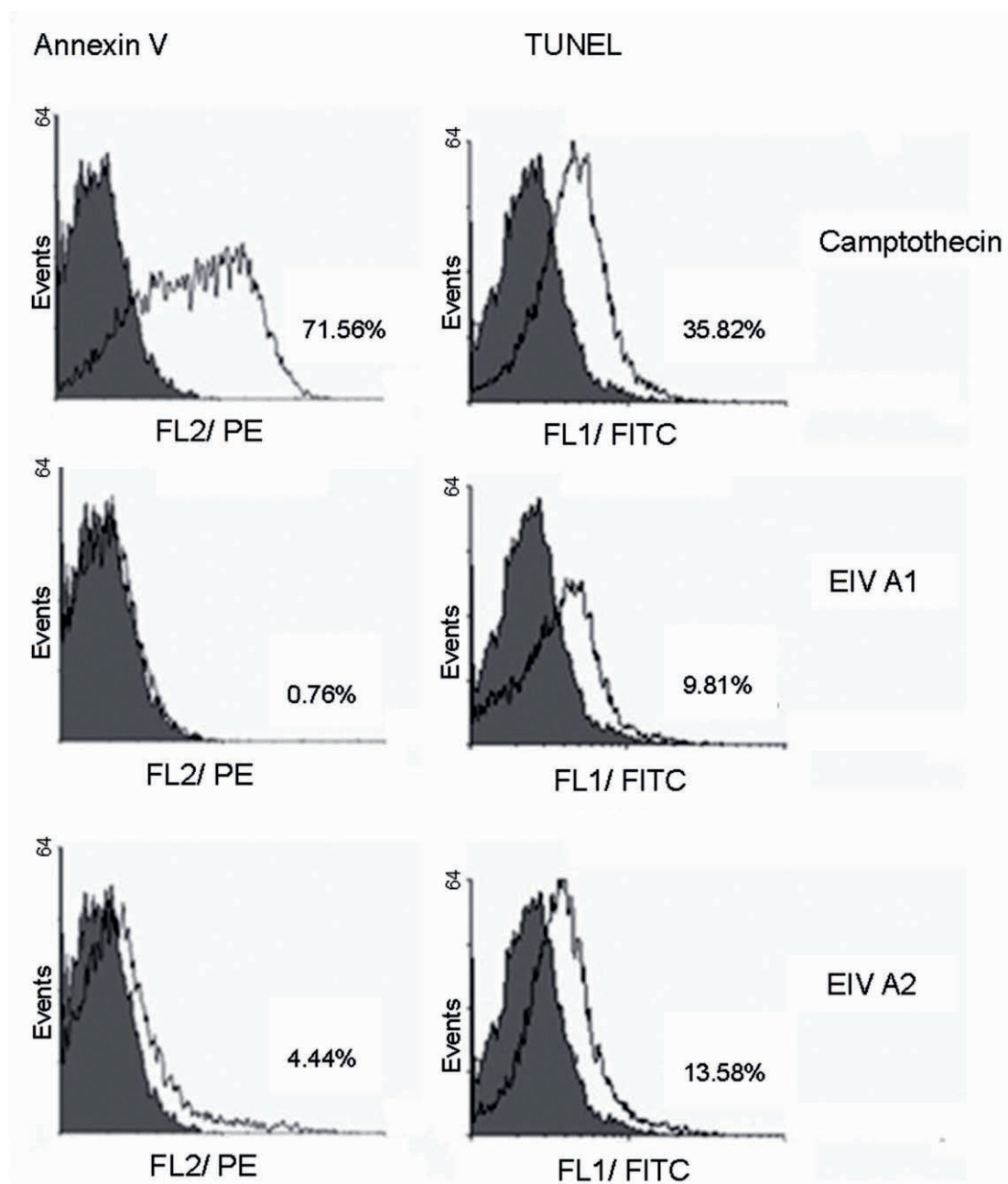


Fig. 2. Flow cytometry analysis of apoptosis induced by EIV in MDCK cells. Uninfected cells shadowed

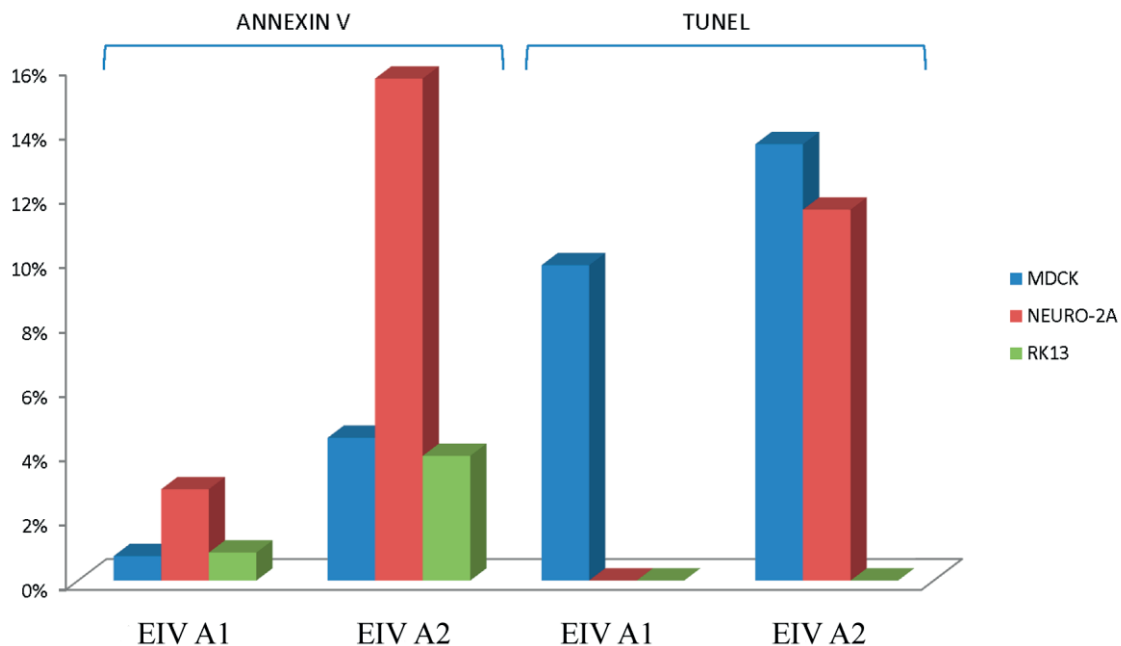


Fig. 3. Results of annexin V and TUNEL assays for MDCK, NEURO-2A and RK13 cells infected with EIV

The infection of NEURO-2A cells with EIV A1 induced early symptoms of apoptosis in 2.84% of cells. There were neither cells in the late stage of apoptosis nor cytopathic effect was observed. The progress of infection of NEURO-2A with EIV A1 seems to be similar to the course of infection in RK13. Infection of the cells with EIV A2 resulted in 15.62% of annexin V positive cells and 11.54% of TUNEL positive cells. Viral infection resulted in induction of the advanced stage of apoptosis and a weak cytopathic effect (Table 1). The results of the annexin V and TUNEL assays are summarised on Fig. 3.

Discussion

EIV A1 and EIV A2 infection of MDCK, RK13, and NEURO-2A cells was analysed. The cytopathic effect and the number of infected and apoptotic cells were estimated. The main part of the experiment was based on detection of phosphatidylserine (PS) translocation in plasmalemma and the DNA fragmentation as the early and late symptoms of apoptosis. Annexin V has high affinity to PS and allows to detect cells with exposed PS (1, 11). TUNEL assay allows to identify the DNA fragmentation characteristic for the late stages of apoptosis. Endonucleases degrade chromatin to 300 kbp fragments and next to 50 bp fragments. Exogenous form of TdT (terminal deoxynucleotidyl transferase) catalyses a template-independent addition of brominated deoxyuridine triphosphates (Br-dUTP) to the 3'-hydroxyl (OH) termini of double and single stranded

DNA. DNA fragments are identified by staining the cells with a fluorochrome labelled anti-BrdU (8).

Replication of EIV induces apoptotic symptoms in many susceptible cells and differences in the onset of apoptosis during infection with EIV A1 and EIV A2 were noticed. The differences in apoptosis induction between EIV A1 and EIV A2 could cause variation in viral proteins activities, especially in case of the surface glycoproteins: HA and NA (14, 16). The interaction of the HAs and their receptors is the major factor influencing infectivity and apoptosis. The viral NA possibly plays an indirect role, by removing sialic acid from the HA, thereby increasing its receptor binding (12). Other viral proteins, like NS1, M1, and PB1-F2 also play an important role in apoptotic mechanisms (5, 10, 12).

MDCK cells are widely used for the primary isolation of influenza viruses because of their high susceptibility to infection with various strains of the viruses (3, 4, 19). For MDCK cells infected with EIV A1 and A2, a weak signal of the PS translocation was observed, while more cells indicated the DNA fragmentation. The published data clearly demonstrate that all of the mammalian and avian influenza viruses induce apoptosis in MDCK cells (6). Hindshaw *et al.* (6) reported that the cytopathic effect in MDCK cells is always accompanied by DNA fragmentation, typically within 3 to 6 h after infection. This indicates that the cellular damage is coupled with DNA fragmentation and occurs quickly after infection. In the experiment, no cytopathic effect was observed after EIV A1 infection, despite that 9.81% cells showed DNA fragmentation.

Low number of annexin positive and no TUNEL positive cells were observed after EIV infection of RK13 cells. Probably the EIV infection induces

apoptosis in RK13 cells but the process is withheld at the early phase. In the experiment neither significant apoptotic symptoms nor cytopathic effect were induced by EIV infection of RK 13 cells. As it is known, the induction of apoptosis in RK13 cells by EIV infection was not previously described. Other viruses like RHDV or Rubella caused fragmentation of DNA in these cells (15).

A similar level of annexin V and TUNEL positive cells was observed after EIV A2 infection of NEURO-2A (Fig. 3, Table 1). For EIV A1 infection only the early symptoms of apoptosis were noted, and neither cells in the late stage of apoptosis nor cytopathic effect were observed. The course of infection with EIV A1 in NEURO-2A cells seems to be similar to the infection in RK13. It seems that 24 h post infection apoptosis did not reach the advanced phase. EIV A2 infection resulted in induction of advanced stage of apoptosis and in a weak cytopathic effect (Table 1). The low level of apoptotic cells in general *versus* relatively high level of infected cells may be influenced by the time of incubation post infection. Therefore, the experiment is intended to be repeated for extended time after inoculation.

To summarise, the differences in induction of apoptosis by two EIV serotypes in cells of different cell lines were observed. The differences between serotypes could originate from functioning of the viral proteins responsible for induction or inhibition of apoptosis; whereas, the differences between cell types may result from the activity of cellular pro- and anti-apoptotic mechanisms.

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