Activation of the intrinsic mitochondrial apoptotic pathway in swine influenza virus-mediated cell death

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Abstract

The mitochondrial pathway of swine influenza virus (SIV)-induced apoptosis was investigated using porcine kidney (PK-15) cells, swine testicle (ST) cells, and HeLa cervical carcinoma cells which are known not to support viral replication. As judged by cell morphology, annexin V staining, and DNA fragmentation, PK-15 and ST cells infected with three different subtypes of SIV (H1N1, H3N2, and H1N2) were obviously killed by apoptosis, not necrosis. SIV infection in PK-15 and HeLa cells was shown to decrease the cellular levels of Bcl-2 protein compared to that of mock-infected control cells at 24 h post-infection, whereas expression levels of Bax protein increased in the PK-15 cells, but did not increase in HeLa cells by SIV infection. Cytochrome c upregulation was also observed in cytosolic fractions of the PK-15 and HeLa cells infected with SIV. Apoptosome (a multi-protein complex consisting of cytochrome c, Apaf-1, caspase-9, and ATP) formation was confirmed by immunoprecipitation using cytochrome c antibody. Furthermore, SIV infection increased the cellular levels of TAJ, an activator of the JNK- stressing pathway, and the c-Jun protein in the PK-15 and HeLa cells. Taken together, these results suggest that the mitochondrial pathway should be implicated in the apoptosis of PK-15 cells induced by SIV infection.

Keywords: apoptosis; cytochrome c; influenza virus; mitochondria; proto-oncogene proteins c-Bcl-2; proto-oncogene proteins c-jun

Introduction

Programmed cell death, or apoptosis, is central to many physiological processes, including tissue atrophy, development of the immune system, and tumor biology (Majno and Joris, 1995; Jacobson *et al.,* 1997). Apoptosis also plays an important role in the pathogenesis of many infectious diseases, including those caused by viruses (Young *et al.,* 1997; Ludwig *et al.,* 1999; Roulston *et al.,* 1999). Many viral infections provoke apoptosis in host cells, but several viruses have also been implicated in the inhibition of apoptosis (Teodoro and Branton, 1997). Although there is no obvious advantage for apoptosis induction by a cytopathogenic virus, the influenza virus induces apoptosis in numerous cell types, both *in vivo* (Mori *et al.,* 1995) and *in vitro* (Morris *et al.,* 1999; Schultz-Cherry *et al.,* 2001).

Two primary pathways induce apoptosis in mammalian cells (Hengartner, 2000). First, the death-receptor pathway is trigged by members of the death receptor superfamily (such as CD95 and TNFRI). Binding of CD95 ligand to CD95 induces receptor clustering and formation of a death-inducing signal complex. This complex recruits multiple procaspase-8 molecules into close proximity via the adaptor molecule FADD (fas-associated death domain protein), which results in caspase-8 activation through proximity. Caspase-8 activation can be blocked by recruitment of the degenerated caspase homologue c-FLIP. The second apoptosis pathway is induced by mitochondria in response to extracellular cues

and internal insults, such as DNA damage (Rich *et al.,* 2000). These diverse response pathways converge on mitochondria, often through the activation of a pro-apoptotic member of the Bcl-2 family, such as Bax, Bad, Bim, and Bid. Pro- and anti-apoptotic Bcl-2 family members meet at the surface of mitochondria, where they compete to regulate the exit of cytochrome c by a mechanism that is still disputed. If the pro-apoptotic camp wins, an array of molecules is released from the mitochondrial compartment. Principal among these released molecules is cytochrome c, which associates with Apaf-1 and then casepase-9 to form the apoptosome. Activation of caspase-8 and -9 initiates the activation of a cascade of caspases (aspartate-specific cysteine proteases), including caspase-3 (Kumar, 1999). Caspase-3 activities have been shown to control both the death-receptor and the mitochondrial pathways (Zheng *et al.,* 1998). In the swine population, three predominant subtypes are prevalent; the classical swine H1N1 which is genetically and antigenically similar to the human H1N1 virus, the H3N2 "human-like" virus, and a reassortant H1N2 swine virus containing HA of classical H1N1 and other genes (NA) of "human-like" swine H3N2 virus (Choi *et al.,* 2002).

Influenza viruses induce apoptosis in cells that are permissive of viral replication (Morris *et al.,* 1999), as well as cells which do not support viral replication, such as HeLa cells and lymphocytes. Both cellular and viral factors are involved in this process, which may also depend on the cell type. Although influenza virus-induced apoptosis is inhibited by Bcl-2 (Olsen *et al.,* 1996), v-FLIP, and crmA (Takizawa *et al.,* 1999), the best characterized pathway of influenza-induced apoptosis is that activation of receptor-mediated signaling is associated with the activation of caspase-8 (death receptor pathway) (Takizawa *et al.,* 1995; Fujimoto *et al.,* 1998). However, although the Bcl-2 family is associated with apoptosis in various viral infections (Conti *et al.,* 1998; Duncan *et al.,* 1999; Lewis *et al.,* 1999), there have been few reports regarding the activation of members of the Bcl-2 family by the influenza virus. Hence, we examined expression mitochondrial pathway-associated gene regulation in the porcine kidney cell line (PK-15) and HeLa cells infected with three subtypes of swine influenza viruses (SIV).

Materials and Methods

Cells and viruses

The porcine kidney epithelial (PK-15) cell line and swine testicle (ST) cell line were purchased from American Type Culture Collection (ATCC) and main-

tained in Minimum Essential Medium (MEM) containing 5% fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin/streptomycin at 37°C. HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 5% FBS, 2 mM glutamine, and 1% penicillin/streptomycin at 37°C. All cell culture reagents were purchased from GibcoBRL. Swine influenza viruses [A/Swine/New Jersey/11/76 (H1N1), A/SW/MN-1480/00 (H1N2), and A/Swine/MN/9088-2/98 (H3N2)] were propagated and maintained using the Madin-Darby canine kidney (MDCK) cell line (Meguro *et al.,* 1979).

Viral infection

Cells grown in 10 cm dishes or T-150 flasks were infected with different SIV subtypes. Each of the SIV subtypes (1×10^5 tissue culture infectious dose 50; TCID$_{50}$) were inoculated onto the cell monolayer and incubated for 1 h at 37°C to allow cells to be adsorbed by the viruses. PBS was used as a control inoculum. After washing with PBS, cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 2% FBS for up to 2 d at 37°C. Prior- and post-inoculation, the cell monolayer was rinsed twice with $1\times$ trypsin solution (1:1,000 dilution, GibcoBRL) with SIV.

Analysis of apoptosis

To determine nuclear genomic DNA fragmentation, one of the hallmarks of apoptosis, genomic DNA was extracted from either mock- or SIV-infected cells using the Wizard genomic DNA purification kit (Promega) according to the protocols of the manufacturer. Genomic DNA (5 μg) was subjected to electrophoresis (1.8% agarose gel) and visualized by staining with ethidium bromide. To examine the apoptotic cell population infected with either mock or SIV, annexin V-stained cell populations were analyzed using the Annexin-V-FLUOS staining kit (Roche) and the FACScan flow cytometer (BD BioSciences) after adding propidium iodide (PI) staining to gate out dead cells according to the instructions of the manufacturers.

Isolation of cytoplasmic fractions

Cytosolic and mitochondrial fractionations were prepared using the Cytochrome c Release Apoptosis Assay Kit (Oncogene) according to the instructions of the manufacturer. In brief, after incubation in $1 \times$ cytosol extraction buffer containing protease inhibitor, cells were subjected to homogenization with tissue grinder. The efficiency of homogenization was checked by microscopy. When the nuclei (70-80%) were observed to have lost their tiny rings, homo-
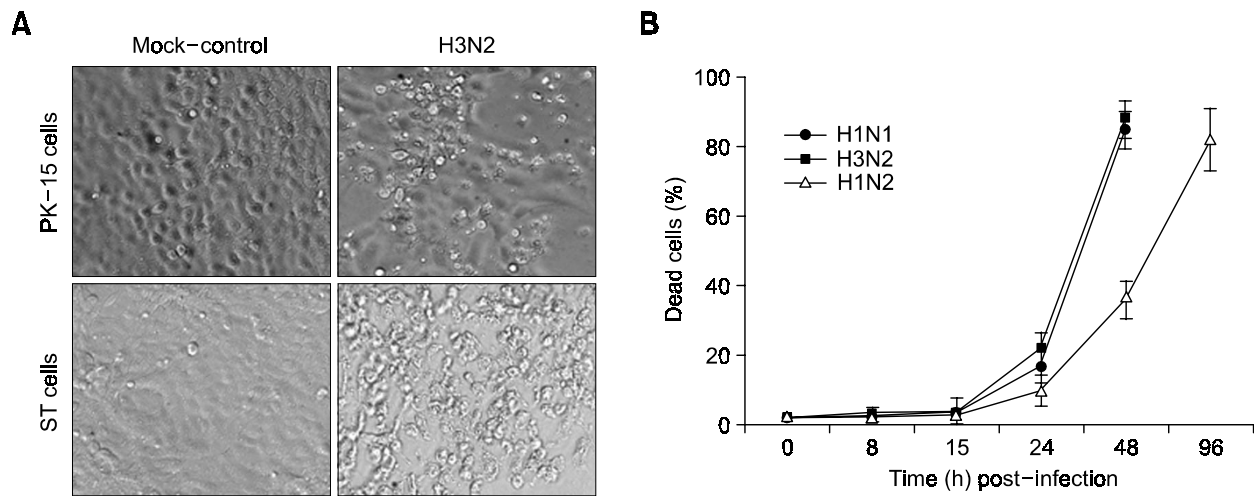
Figure 1. Infection of swine influenza virus (SIV) in porcine cell lines. (A) Representative photographs showing porcine kidney (PK-15) and swine testicle (ST) cell lines infected with either mock-control or H3N2 subtype of swine influenza virus (SIV). (B) Cell death rate (%) of PK-15 cells infected with the H1N1, H3N2, and H1N2 subtypes of SIV at different time points post-infection. The similar results were obtained from three identical experiments that were independently carried out.

genates were transferred to a microcentrifuge tube (1.5 ml) and centrifuged at 700 × *g* for 10 min at 4°C. The supernatants were then transferred to a fresh microcentrifuge tube and centrifuged at 10,000 × *g* for 30 min at 4°C. In this step, supernatants were collected as cytosolic fractions; pellets were resuspended in 0.1 ml mitochondrial extraction buffer and were then saved as mitochondrial fractions.

Immunoprecipitation and Western blot analysis

Immunoprecipitation assay was conducted using whole cell lysate (200 μg), anti-cytochrome c antibody (Oncogene), and Protein A PLUS-Agarose (Santa Cruz Biotechnology). The whole cell extracts, cytosolic fractions, and immunoprecipitates were mixed with sample buffer (0.5M Tris-Cl, pH6.8, 10% SDS, 2-mercaptoethanol, 0.05% bromphenol blue). The protein samples were boiled at 100°C for 4 min and were then cooled on ice. Fifteen microgram of each of the denatured protein samples was separated by 8 or 10% SDS-polyacrylamide gel and then transferred to a PVDF membrane (Millipore). The membranes were blocked with 5% nonfat milk and incubated with anti-Bcl-2 (Santa Cruz Biotechnology), anti-Bax (Santa Cruz Biotechnology), anti-cytochrome c (Santa Cruz Biotechnology), anti-Apaf-1 (Santa Cruz Biotechnology), anti-caspase-9 (Santa Cruz Biotechnology), anti-TAJ (Oncogene), anti-c-Jun (Oncogene), and anti-β-actin (Santa Cruz Biotechnology) antibodies. Membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG antibodies (Pierce) and visualized using SuperSignal West Pico Che-

miluminescent Substrate (Pierce).

Results

Apoptosis of the various cells infected with SIV

Porcine kidney epithelial (PK-15) cells and swine testicle (ST) cells were infected with one of the three different subtypes (H1N1, H1N2, and H3N2) of SIV. Cytopathic effects (CPE) were not observed until 15 h post-infection and were less than 30% at 24 h post-infection with H1N1 and H3N2 subtypes. H1N2- infected cells, however, did not show CPE until 36 h post-infection. Maximum CPEs in the PK-15 and ST cells were observed at 48 h post-infection with H1N1 and H3N2, and at 96 h post-infection with the H1N2 SIV subtype (Figure 1A and B).

We performed annexin-V and propidium iodide (PI) staining to determine whether CPE occur as a result of apoptosis by SIV replication in the PK-15, ST and HeLa cells. As shown in Figure 2A, high levels of apoptotic cell death were observed in live virus-infected PK-15, ST and HeLa cells, but were not seen in mock-infected and UV-irradiated (dead) virus-infected counterpart cells. Genomic DNA fragmentations were also observed in the PK-15 and ST cells infected with live viruses, but not observed in mock-infected and UV-irradiated virus-infected counterpart cells (Figure 2B). These results suggest that SIV-induced apoptosis in PK-15 and ST cells might be accompanied by replication of live virus, not by just attachment of the virus to cells, as judged by the lack of CPE and apoptotic effects in the cells inoculated with UV-irradiated (infection-com-
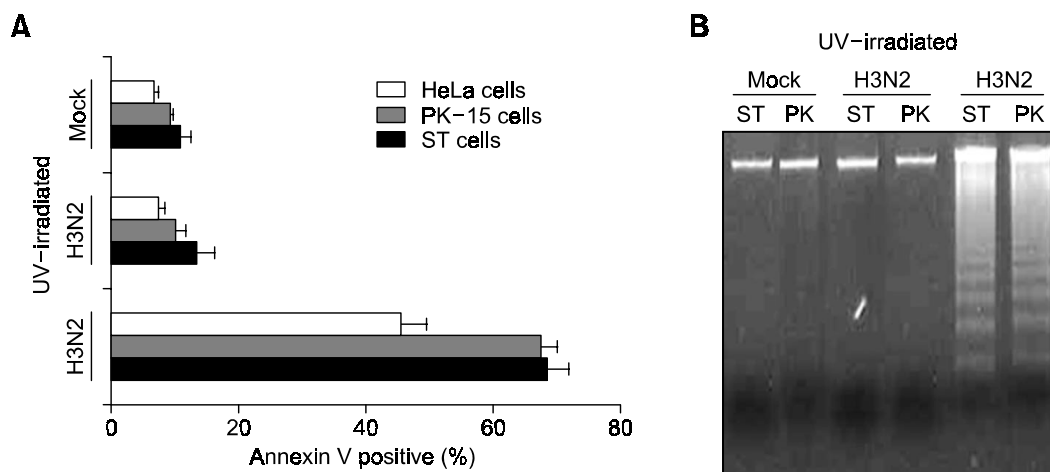
A

B

Figure 2. Apoptosis analysis of cells infected with the H3N2 subtype of SIV. (A) Annexin V-positive cells (%) of HeLa, swine testicle (ST), and PK-15 cells infected with mock-control, UV-irradiated H3N2 subtype (dead virus) or non-treated H3N2 subtype (live virus) of SIV. (B) DNA fragmentation analysis of PK-15 and ST cells infected with mock-control, UV-irradiated H3N2 subtype (dead virus) and non-treated H3N2 subtype (live virus) of SIV at 36 h post-infection.
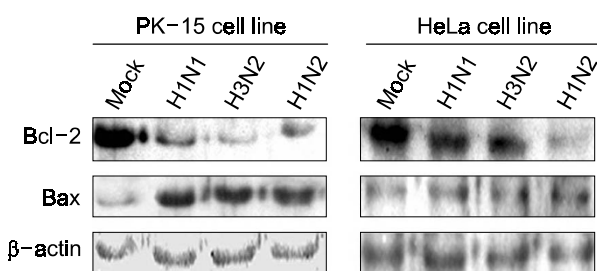

Figure 3. Expression of Bcl-2 and Bax proteins in the SIV-infected cells. PK-15 and HeLa cells were infected with mock-control and different SIV subtypes (H1N1, H3N2 and H1N2). Expression of Bcl-2 and Bax protein was determined by Western blot analysis using anti-Bcl-2, anti-Bax, and anti-β-actin (loading control) antibodies.

promised) virus.

Activation of the mitochondrial apoptosis pathway

Since the Bcl-2 family is associated with apoptosis in a variety of viral infections (Conti *et al.,* 1998; Duncan *et al.,* 1999; Lewis *et al.,* 1999; Mastrangelo *et al.,* 2000; Hong and Wu, 2002), and because expression of Bcl-2, an anti-apoptosis gene, in MDCK cells blocks influenza virus-induced apoptosis and DNA fragmentation (Hinshaw *et al.,* 1994), we determined the expression of Bcl-2 and Bax proteins to investigate whether members of the Bcl-2 family are implicated in SIV-mediated apoptosis of PK-15 and HeLa cells. As shown in Figure 3, the cellular level of Bcl-2 protein was remarkably downregulated following SIV infection in both cell lines compared to mock-infected control cells at 24 h post-infection,

whereas expression of Bax protein, a pro-apoptosis gene, was upregulated in SIV-infected PK-15 cells, but was not upregulated in SIV-infected HeLa cells. We also determined subcellular localization of Bax protein by Western blot analysis using both cytosolic and mitochondrial fractions of cell lysates. Consistent with expression levels of Bax protein from total cell lysates of mock-infected control and SIV-infected PK-15 cells (Figure 3), Bax protein was found to be increased in the mitochondrial fraction of the SIV-infected PK-15 cells as compared to that of the mock-infected control cells (data not shown). Taken together, these findings indicate that SIV infection in PK-15 cells might provoke cell death by both suppression of anti-apoptosis gene expression and promotion of pro-apoptosis gene expression.

A number of Bcl-2 pro-apoptotic members, such as Bax and Bad, are known to exert their mitochondria-relative pro-apoptotic effects either by interacting with and inhibiting Bcl-2 and Bcl-XL or by direct interaction with the mitochondrial membrane, after which they induce the release of cytochrome c from the mitochondria to the cytosol. Hence, we examined whether cytochrome c is released from mitochondria to cytosol in the SIV-infected cells at 24 h post-infection. As shown in Figure 4A, the cytosolic release of cytochrome c was shown to be dramatically increased in the SIV-infected PK-15 cells and slightly increased in the SIV-infected HeLa cells as compared to mock-infected counterpart cells. To further confirm the formation of apoptosomes (multi-protein complexes consisting of cytochrome c, Apaf-1, pro-caspase-9, and ATP), we performed immunoprecipitations using anti-cytochrome c anti-
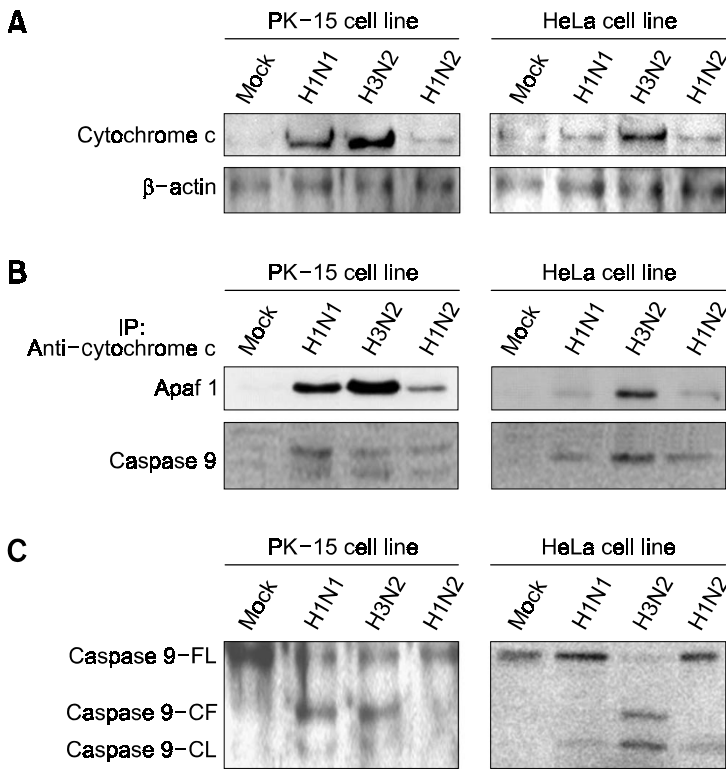
A

PK-15 cell line   HeLa cell line

Mock H1N1 H3N2 H1N2   Mock H1N1 H3N2 H1N2

Cytochrome c

β-actin

B

PK-15 cell line   HeLa cell line

IP:
Anti-cytochrome c

Mock H1N1 H3N2 H1N2   Mock H1N1 H3N2 H1N2

Apaf 1

Caspase 9

C

PK-15 cell line   HeLa cell line

Mock H1N1 H3N2 H1N2   Mock H1N1 H3N2 H1N2

Caspase 9-FL

Caspase 9-CF

Caspase 9-CL

Figure 4. Cytosolic release of cytochrome c, formation of apoptosomes, and activation of caspase-9 in the SIV-infected cells. (A) Cytochrome c released from the mitochondria to the cytosol in the PK-15 and HeLa cells infected with mock-control, H1N1, H3N2, and H1N2 was detected by Western blot analysis. The β-actin was used as loading control. (B) Apaf-1 and caspase-9 immunoprecipitated by anti-cytochrome c antibody in the PK-15 and HeLa cells infected with mock-control and different SIV subtypes (H1N1, H3N2, and H1N2). (C) Expression levels of the active caspase-9 in the PK-15 and HeLa cells infected with mock-control and different SIV subtypes (H1N1, H3N2, and H1N2).

body. The results revealed that more Apaf-1 and caspase-9 proteins in the SIV-infected PK-15 and HeLa cells were coprecipitated with anti-cytochrome c antibody than were coprecipitated in mock-infected counterpart cells (Figure 4B). Furthermore, activated caspase-9 (proteolytic fragmented caspase-9) was found to increase in the cytosolic fractions of the SIV-infected PK-15 and HeLa cells, but this was not the case with mock-infected counterpart cells (Figure 4C). Taken together, these results suggest that SIV-mediated apoptosis in the PK-15 and HeLa cells should occur through the intrinsic mitochondria-mediated apoptosis pathway.

Activation of stress-associated proteins

The c-Jun/AP-1 transcriptional factor has been of great interest to researchers because its activity is influenced by oxidative stress and can induce apoptosis (Sawai *et al.,* 1995; Gong *et al.,* 2004; Kim *et al.,* 2004). TAJ, also known as TROY, is a -46 kDa membrane protein possessing characteristic cysteine-rich motifs in the extracellular domain and a TNF receptor-associated factor (TRAF)-2 binding sequence in the cytoplasmic domain (Eby *et al.,* 2000). TAJ binds a number of different TARF family members and enables the activation of the JNK pathway, most likely via interaction with a TRAF homologue. Since infection of the equine influenza
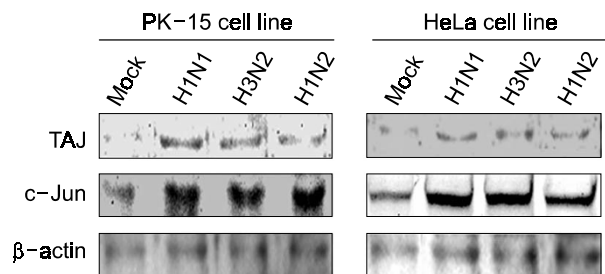
PK-15 cell line   HeLa cell line

Mock H1N1 H3N2 H1N2   Mock H1N1 H3N2 H1N2

TAJ

c-Jun

β-actin

Figure 5. Upregulation of TAJ and c-Jun proteins in the SIV-infected cells. PK-15 and HeLa cells were infected with mock-control and different SIV subtypes (H1N1, H3N2, and H1N2). Expression of TAJ and c-Jun proteins in the PK-15 and HeLa cells infected with mock-control, H1N1, H3N2, and H1N2 was determined by Western blot analysis using anti-TAJ, anti-c-Jun, and anti-β-actin (loading control) antibodies.

virus increased cellular levels of c-Jun/AP-1 protein (Lin *et al.,* 2001), we wondered whether SIV infection activated expression of c-Jun and TAJ. As demonstrated by Western blot, SIV infection was shown to significantly increase cellular levels of c-Jun and TAJ proteins in PK-15 and HeLa cells (Figure 5), suggesting that TAJ and c-Jun upregulated by SIV infection might play a crucial role in SIV-induced stress and apoptosis-signaling pathways.

Discussion

Although the death receptor-mediated apoptosis pathway induced by influenza infection has been extensively studied (Takizawa *et al.,* 1995; Wada *et al.,* 1995; Fujimoto *et al.,* 1998), little is known about the potential participation of the mitochondria-mediated apoptosis pathway during influenza-induced cell death. Although PK-15 cells are not commonly used for swine influenza virus replication, our results shown in Figure 1 demonstrated that PK-15 cell was shown to fully support SIV replications. Furthermore, we used PK-15 to demonstrate the apoptotic pathway in swine cells because MDCK cells commonly used for SIV culture were originally derived from canine kidney cells. In the present study, we report that SIV infection in the PK-15 and HeLa cells should induce cell death through the mitochondria-mediated apoptosis pathway, as judged by 1) downregulation of anti-apoptotic Bcl-2; 2) upregulation of pro-apoptotic Bax; 3) cytosolic release of cytochrome c from mitochondria; and 4) activation of caspase-9, all of which are known to be implicated in mitochondria-mediated apoptosis. We used three different swine influenza virus subtypes (H1N1, H1N2, and H3N2) to determine whether they exert similar cellular effects in terms of apoptosis. The H1N2 subtype showed relatively delayed CPE in the infected cells as compared to H1N1 and H3N2 subtypes. The delayed CPE in the H1N2 infected cells might result from decrease of cell death rate, cytochrome c release and apoptosome formation as compared to those of H1N1 and H3N2 infected cells. Interestingly, H1N1 and H1N2 subtypes did not show obvious CPE in the HeLa cells (data not shown) and failed to induce cytosolic release of cytochrome c and formation of apoptosomes (Figure 4), whereas H3N2 subtype did. These results suggest that virus-induced cell death might be differentially regulated depending upon cell type and viral factors.

Although upregulation of c-Jun and TAJ in the SIV-infected cells indicates that SIV-induced apoptosis might be partially activated by the stress-signaling pathway, the stress-signaling pathway appears not to be sufficient for inducing SIV-mediated apoptosis, because TAJ and c-Jun were shown to be markedly increased in the H1N1 and H1N2-infected HeLa cells which did not show apoptotic cell death. It is plausible that SIV-mediated apoptosis might be regulated by both receptor- and mitochondrial-mediated cell death.

Acknowledgement

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