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Diverse apoptotic pathways in enterovirus 71–infected cells

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Mechanisms related to the neuropathogenesis of enterovirus 71 infection remain unclear. This investigation conducts a comprehensive study of the apoptotic pathways in neural and non-neural cells following enterovirus 71 infection. Infections with enterovirus 71 not only induce classical cytopathic effects in SF268 (human glioblastoma), SK-N-MC (human neuroblastoma), RD, and Vero cells, but also induce classic signs of apoptosis in all cells, including DNA fragmentation and phosphatidylserine translocation. Apoptosis has also been caused by the efflux of cytochrome *c* from mitochondria, and subsequently by cleavage of caspase 9 in all cells. Activation of caspase 8 followed by cleavage of the proapoptotic protein Bid only occurs in non-neural cells. Results of this study demonstrate that a mitochondrial pathway of apoptosis mediated by activation and cleavage of caspase 9 is a main pathway in enterovirus 71–induced apoptosis, especially for enterovirus 71–infected neural cells. *Journal of NeuroVirology* (2004) **10,** 338–349.

Keywords: apoptosis; caspase; cytochrome *c*; enterovirus 71; mitochondria

Introduction

Enterovirus 71 (EV71), a positive-stranded RNA virus in the family of *Picornaviridae*, is also an endemic enterovirus with a global distribution (Alexander *et al*, 1994; Blomberg *et al*, 1974; Chonmaitree *et al*, 1981; Chumakov *et al*, 1979; Gilbert *et al*, 1988; Hayward *et al*, 1989; Kennett *et al*, 1974; Melnick, 1984; Nagy *et al*, 1982). The 1998 EV71 outbreak in Taiwan infected over 120,000 children and caused 78 mortalities (Ho, 2000). During this epidemic, a significant proportion of infected children developed unusual neurological complications such as aseptic meningitis, rhombencephalitis, and polio-like syndrome (Alexander *et al*, 1994; Chang *et al*, 1999; Gilbert *et al*, 1988; Ho *et al*, 1999; Huang *et al*, 1999; Lin *et al*, 2002; Lum *et al*, 1998; McMinn *et al*, 2001). Furthermore, immunofluorescent and molecular studies using autopsied samples during this epidemic demonstrated that EV71 infected the central nervous system (CNS) (Hsueh *et al*, 2000), and also isolated EV71 from the cerebrospinal fluid (CSF), medulla oblongata, and spinal cord (Chang *et al*, 1999; Lin *et al*, 2002; Shih *et al*, 2000). In cell culture and experimental animals, EV71 exhibited the capacity to infect neural cells *in vitro* and *in vivo* (Chen *et al*, 2004; Chumakov *et al*, 1979; Nagata *et al*, 2002; Wen *et al*, 2003). Additionally, EV71 can also infect diverse cultured cell lines, including LLC-MK2, MRC-5, Vero, RD, A549, Hela-229, U373MG, HEp-2, and SK-N-SH cells (Kuo *et al*, 2002; Shih *et al*, 2000; Wen *et al*, 2003). Accordingly, the diverse tissue tropism of EV71 provides an effective means of investigating how neural and non-neural cells differ in virus-cell interaction.

Numerous neurotropic RNA viruses can cause cell damage in CNS involving apoptosis. This has been demonstrated in many RNA neurotropic viruses, including human immunodeficiency virus (Zheng *et al*, 1999), reovirus (Oberhaus *et al*, 1997), Alphavirus (Lewis *et al*, 1996), dengue virus (Despres *et al*,

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1996), West Nile encephalitis virus (Shrestha *et al*, 2003), rabies virus (Theerasurakarn and Ubol, 1998), and Sindbis virus (Ubol *et al*, 1996). Other picornaviruses, poliovirus, and coxsackievirus B3 were also demonstrated to induce apoptosis in cultured cells (Couderc *et al*, 2002; Girard *et al*, 1999; Huber *et al*, 1999; Saraste *et al*, 2003). However, tissue damage resulting from virus infection in vital irreplaceable neurons would have very different consequences to that in non-neural cells. Consequently, the involvement of EV71-infected neural cells in apoptotic cell death must be investigated.

Apoptosis, or programmed cell death, is characterized morphologically by plasma membrane blebbing, cell shrinkage, internucleosomal DNA cleavage, chromatin condensation, nuclear fragmentation, and translocation of phosphatidylserine to the outer leaflet (Adams, 2003; Kaufmann and Hengartner, 2001; Martelli *et al*, 2001). Most morphological changes are associated with a set of cysteine proteases that are activated specifically in apoptotic cells through intrinsic or extrinsic stimuli (Alnemri *et al*, 1996; Buendia *et al*, 1999; Rao *et al*, 1996). The process of cell death is essential for developing or maintaining tissue homeostasis and also for an effective immune system. Furthermore, dysregulation of apoptosis has been linked to the pathogenesis of various human diseases, including cancer, autoimmune syndrome, neurodegenerative diseases, and virus infection (Barber, 2001; Bouillet *et al*, 2002; Friedlander, 2003; Herr and Debatin, 2001).

EV71 has been demonstrated to induce apoptosis in non-neural cell lines, such as Vero and Hela cells, and it has been proposed that cleavage of eIF4GI by viral 2A protease causes cell apoptosis (Kuo *et al*, 2002). Our previous study has demonstrated that transient expression of viral 3C protease can induce apoptosis of human glioblastoma cells. The 3C protease activity triggered the induction of caspase 3 activity, and this apoptotic pathway may play an important role in the pathogenesis of EV71 infection (Li *et al*, 2002). However, the involvement of whole EV71-infected neural cells in the apoptotic process is poorly understood. Identifying the apoptotic pathway of EV71-infected cells was not only critical for understanding the pathogenesis of EV71 infection, but eventually also stimulated the development of new strategies for controlling EV71 infection and other neurological diseases related to apoptosis.

Although it is known that EV71 can induce apoptosis in non-neural cells, little is known about neural cell damage response to EV71 infection and pathways that differ from non-neural cells. Therefore, studying the cellular pathway in EV71-infected neural and non-neural cells is of worthwhile interest. This investigation demonstrates that EV71 infection can produce the hallmarks of apoptosis in neural and non-neural cells, including DNA fragmentation and phosphatidylserine translocation. EV71 can also infect all cell lines when efflux of cytochrome c from mitochondria and subsequent sequential cleavage of caspase 9 and caspase 3 occur. However, activation of caspase 8, followed by cleavage of Bid protein, only occurs in non-neural cells. In addition to indicating the existence of diverse pathways to apoptosis in different EV71-infected cells, the results of this study demonstrate that the mitochondrial pathway is a main pathway in EV71-induced apoptosis in neural cells.

Results

EV71 infection induced cytopathic effects in both neural and non-neural cell lines

This work examined the cytopathic effects (CPEs) of EV71 infections in neural (SF268 and SK-N-MC) and in non-neural (RD and Vero) cells. Cells were infected with EV71 at an multiplicity of infection (m.o.i.) of 1, and the morphological change associated with EV71 infection was examined. Numerous cells that had rounded up and become detached from the bottom of the dish, representing the typical cytopathic effects of EV71, were clearly present, including 12 h post infection (p.i.) in RD, 36 h p.i. in Vero, 48 h p.i. in SF268, and 72 h p.i. in SK-N-MC cells (Figure 1A). In contrast, mock-infected cells retained intact monolayers. These CPE results demonstrate that EV71 has the capacity to induce cell loss or damage in cultured cells, including neural cells.

To examine how the EV71 infection and CPE are related, this investigation performed indirect immunofluorescence in EV71-infected or mock-infected cells. The presence of EV71 in cells displayed specific apple-green fluorescence, and was found in all four cell lines (Figure 1B, right panels). Mock-infected cells exhibited a dull red staining by Evans blue counterstain (Figure 1B, left panels). Taken together, the experimental results suggest that cell damage to both neural and non-neural cells resulted from EV71 infection.

Induction of internucleosomal DNA fragmentation in neural and non-neural cells following EV71 infection

Previous studies have demonstrated that EV71 proteins such as 2A and 3C protease induced apoptosis in cultured cells (Kuo *et al*, 2002; Li *et al*, 2002). However, cell death induced by EV71 whole virus in various culture cells, especially neural cells, was poorly understood. To understand whether EV71 infection could induce apoptosis in these four cell lines, this work examined internucleosomal DNA fragmentation in infected cultures. From Figure 2A, the characteristic nucleosomal ladder appeared in RD cells 1 day following EV71 infection, whereas in Vero, SK-N-MC, and SF268 cells, it appeared 2 and 3 days following infection, respectively.

DNA fragmentation was also examined using the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay, and the fluorescein-labeled DNA was analyzed using fluorescence microscopy. When infected with EV71 (Figure 2B, panels D, H, L, and P), all four cell lines showed prominent green fluorescence, consistent with EV71-induced apoptosis (Figure 2A). The mock-infected cells displayed no such signal (Figure 2B, panels B, F, J, and N). The above results suggest that EV71 infection induces apoptosis in both neural and non-neural cells.

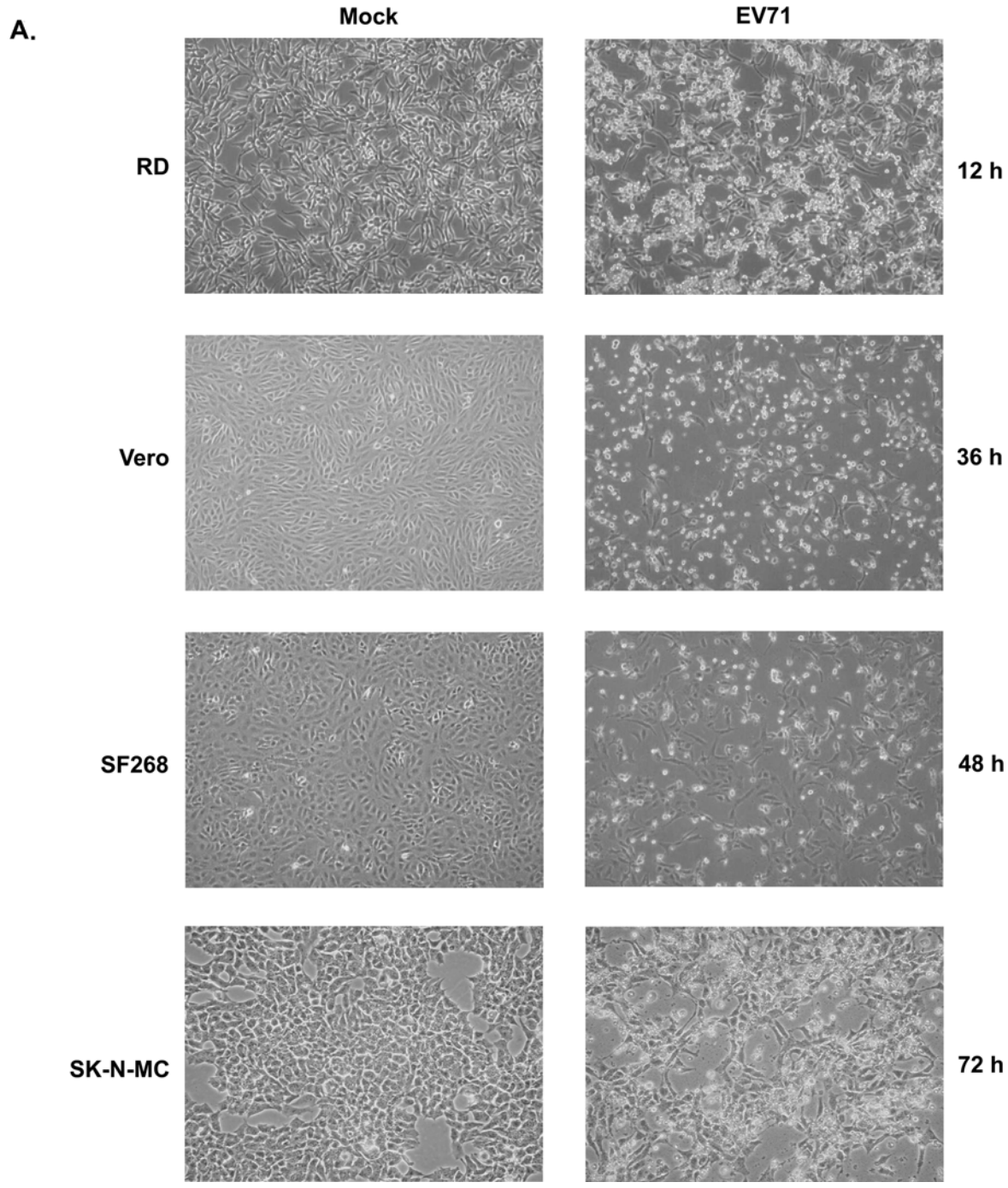

Figure 1 EV71 can infect both neural and non-neural cells. RD, Vero, SF268, or SK-N-MC cells were mock-infected (left panels) or infected with EV71 (right panels) at an m.o.i. of 1. Following the infection at 12 h p.i. in RD cells, 36 h p.i. in Vero cells, 48 h p.i. in SF268 cells or 72 h p.i. in SK-N-MC cells, cell morphology was observed and photographed under light microscopy (A). EV71-infected (right panels) and mock-infected cells (left panels) were further fixed, permeabilized and immunostained with and anti-EV71 mAb followed by a FITC-conjugated secondary antibody and Evans blue counterstain. Cells were visualized and photographed by fluorescence microscopy (B). *(Continued)*

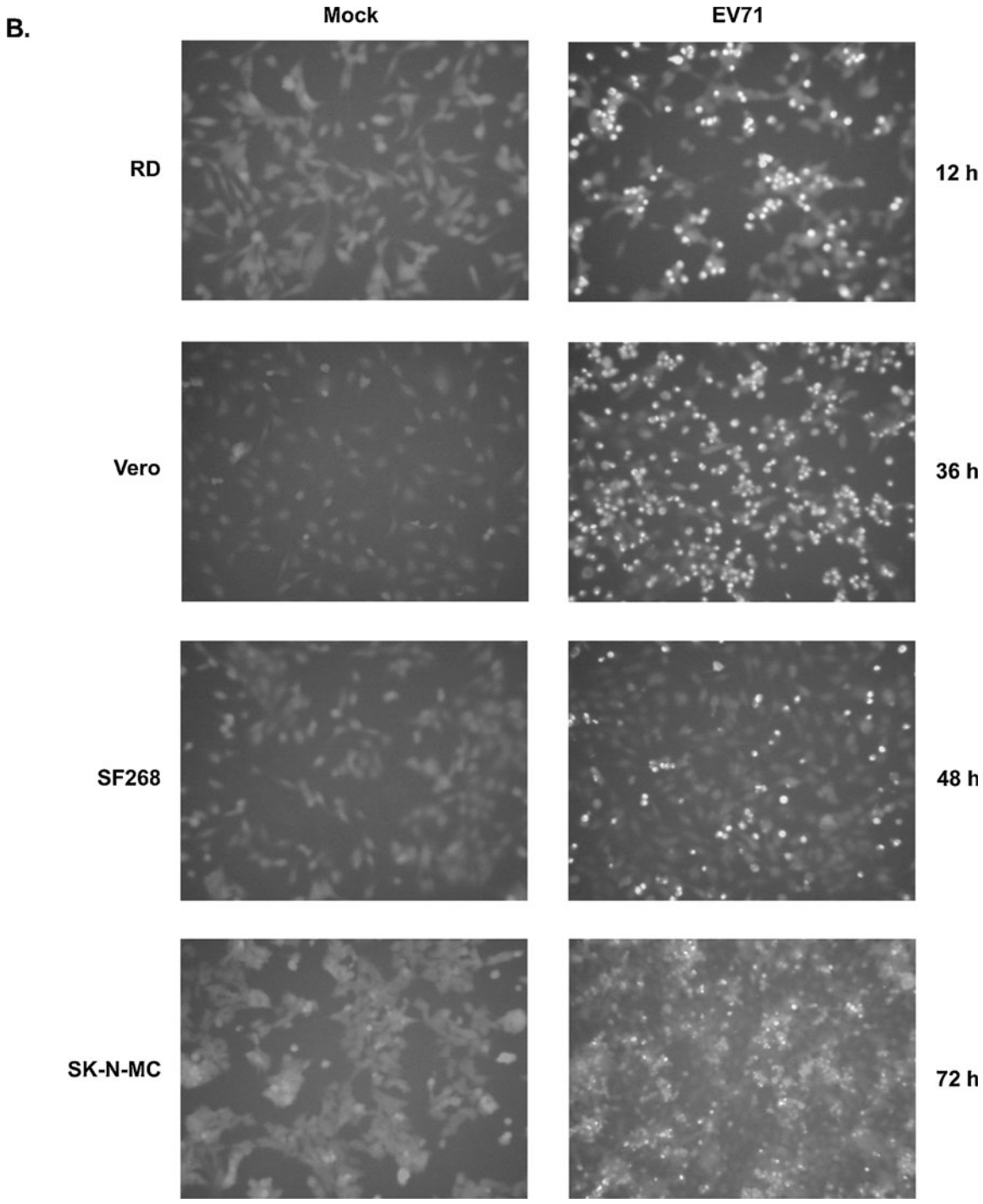

Figure 1 (Continued)

Translocation of phosphatidylserine (PS) in EV71-infected cells

Phosphatidylserine (PS) translocated from the inner to the outer leaflet of the plasma membrane, enabling early detection of apoptosis. This study next examined whether EV71 infection induced translocation of PS to the outer leaflet of the plasma membrane during apoptosis in these four cell lines. Annexin V, a Ca^{2+}-dependent phospholipid-binding protein with a high affinity for PS was used for detection, and the binding of fluorescein isothiocyanate (FITC)-conjugated annexin V was measured via fluorescence microscopy. All four cell lines displayed a green fluorescence, as shown in panels D, H, L, and P of Figure 3. However, no signal was found in the four mock-infected cells (Figure 3, panels B, F, J, and N).

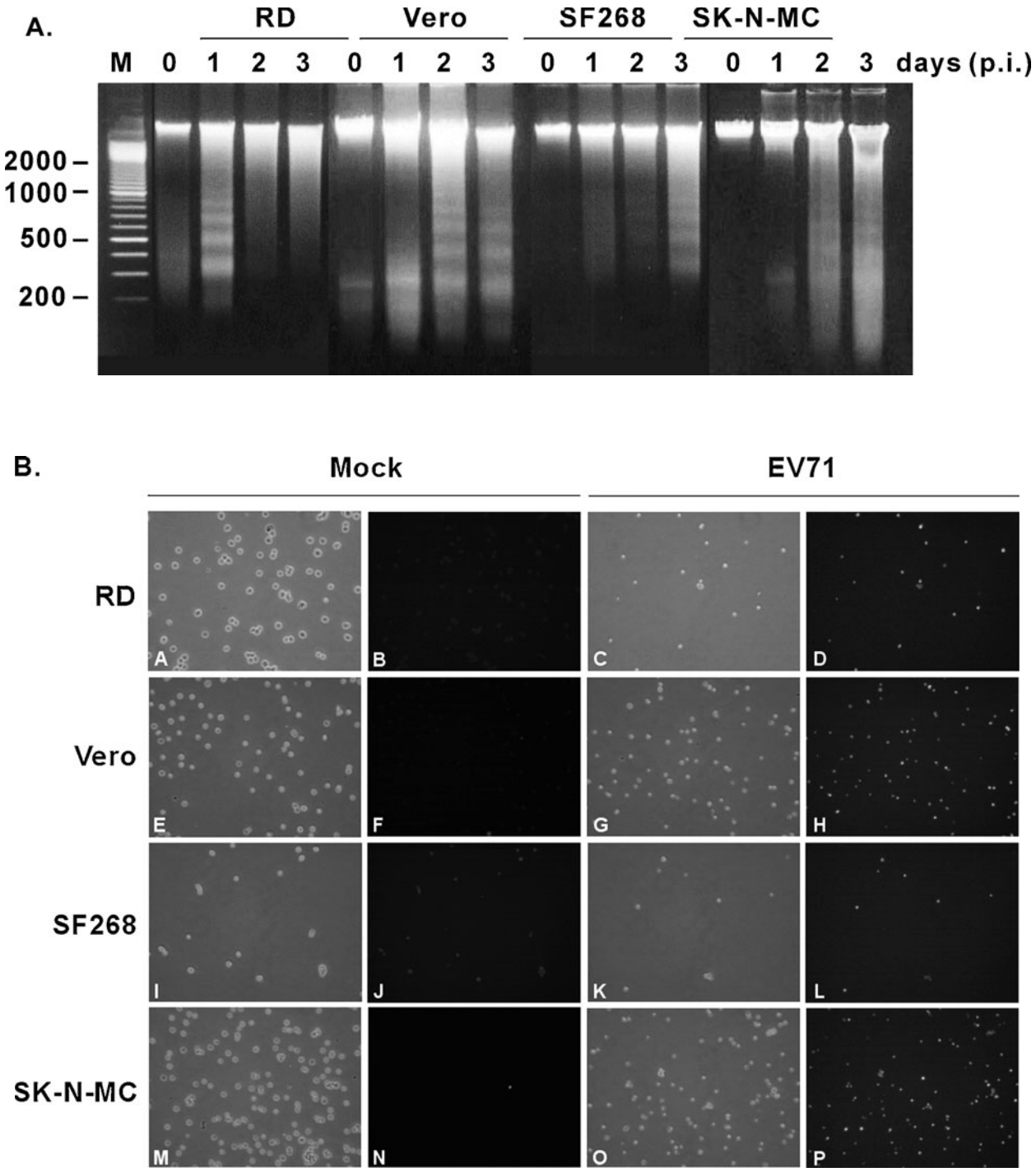

Figure 2 Fragmentation of chromosomal DNA in EV71-infected cells. Cells were infected with EV71 at an m.o.i. of 1 and incubated at 37°C in DMEM containing 2% FCS. At the indicated time, cells were harvested and DNA extracted as described. The oligonucleosomal ladder was detected in a 1% agarose gel (**A**). (**B**) Cells for TUNEL assay were harvested from mock-infected (B, F, J, and N) or EV71-infected (D, H, L, and P) cells and stained on cells with clear CPE. Moreover, TUNEL-positive cells were observed and photographed under fluorescence microscopy. EV71-infected (C, G, K, and O) and mock-infected (A, E, I, and M) cells are shown and photographed under light microscopy.

Overall, the DNA fragmentation and PS translocation results indicate that cell damage caused by EV71 infection in both neural and non-neural cells induces apoptosis.

Activation of caspases 9, 8, and 3 in neural and non-neural cell lines following EV71 infection

After verifying that EV71 infection induces apoptosis in both neural and non-neural cells, the next

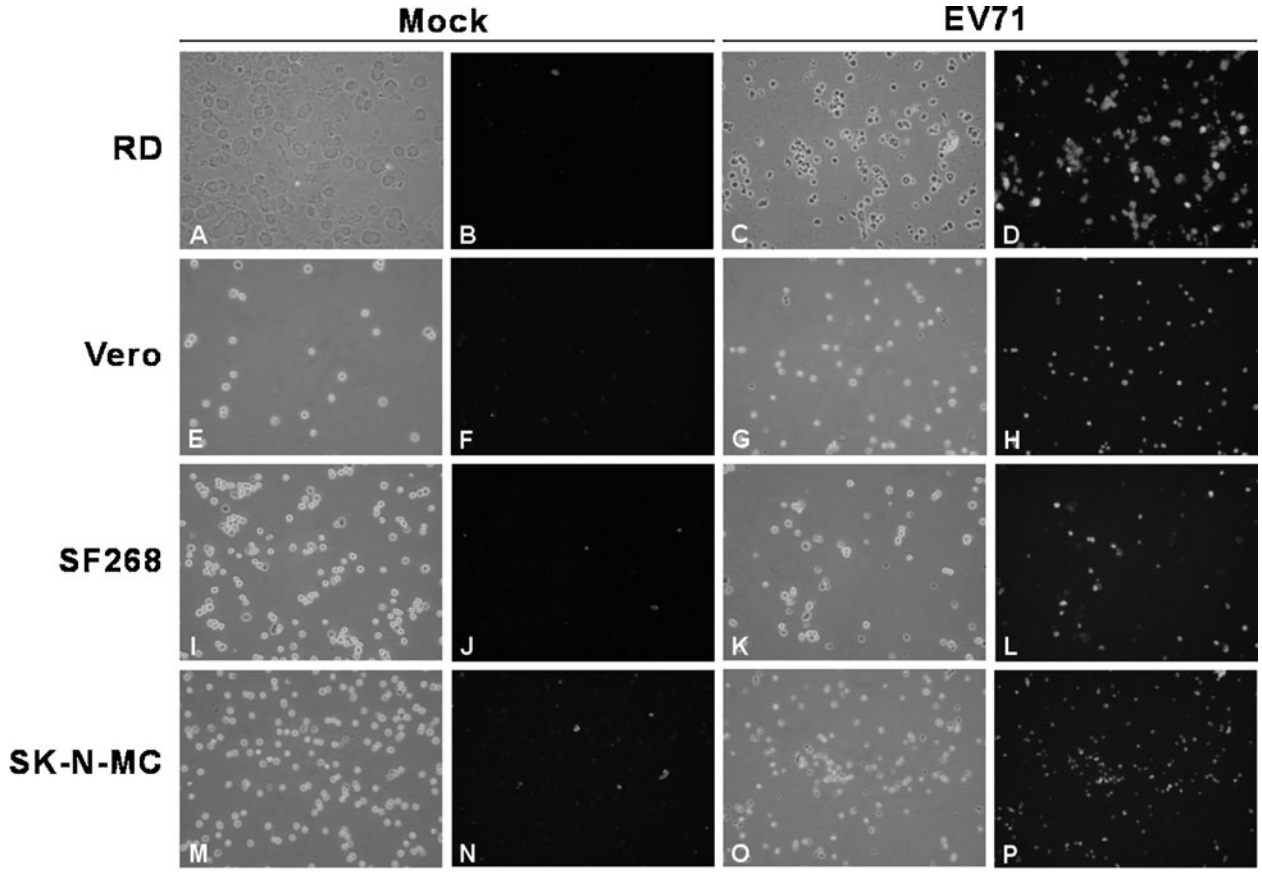

Figure 3 Immunofluorescence analysis was applied to membrane PS exposure associated with apoptosis by annexin V staining in EV71-infected cells. Cells infected (D, H, L, and P) or mock-infected (B, F, J, and N) with EV71 at an m.o.i. of 1 upon clear CPE were incubated with annexin V–FITC and observed using fluorescence microscopy. EV71-infected (C, G, K, and O) and mock-infected (A, E, I, and M) cells are shown and photographed under light microscopy.

step is to identify the specific apoptotic pathways in these cells. Caspases 9 and 8 are involved in two major pathways, the mitochondria pathway and the death receptor pathway, respectively. Additionally, caspase 3 is one of the executioner caspases. This work examined the activation of these caspases by Western blotting using specific antibodies against each of the precursor and cleaved caspases. In EV71-infected RD cells, cleavage of caspase 9 (p37), caspase 8 (p43/p41), and caspase 3 (p19/p17) were all assessed as 9 h p.i. (Figure 4A), indicating the activation of these three caspases. Similar results were observed in EV71-infected Vero cells, with caspases 9 and 8 being activated after 12 h p.i. (Figure 4B). In contrast, caspase 8 was not activated in either SF268 or SK-N-MC cells, suggesting the death receptor pathway might not considerably contribute to EV71-infected neural cell apoptosis (Figure 4C, D). Cleavage of caspase 9 was detected 12 h p.i. in SF268 cells and 9 h p.i. in SK-N-MC cells. Additionally, cleavage of caspase 3 was detected 12 h p.i. and 9 h p.i. in both SF268 and SK-N-MC lines, respectively (Figure 4C, D). The experimental results demonstrated that EV71 induced-apoptosis in non-neural cells was followed by activation and cleavage of initiator caspases 8 and 9, but no caspase 8 activation was observed in EV71-infected neural cells.

Release of cytochrome c *into cytoplasm in EV71-infected cells*

All four cell lines displayed activation of caspase 9 (Figure 4), indicating the mitochondria pathway is involved in the EV71-induced apoptosis. Because mitochondrial pathway of apoptosis was initiated by caspase 9 activation when a proapoptotic protein, cytochrome *c*, was released into cytoplasm from the space between inner and outer mitochondrial membranes, levels of cytochrome *c* were measured in the cytoplasmic and mitochindrial fractions in EV71-infected cells. The cytoplasmic and mitochondrial fractions were separated following the protocol described in Materials and Methods. Additionally, Western blot was performed for monitoring the level of cytochrome *c* in the cells via an antibody against cytochrome *c*. From Figure 5, the accumulation of cytochrome *c* in the cytosol increased significantly either 9 h or 12 h p.i. in all cell lines. These observation results suggest that EV71-infected neural and

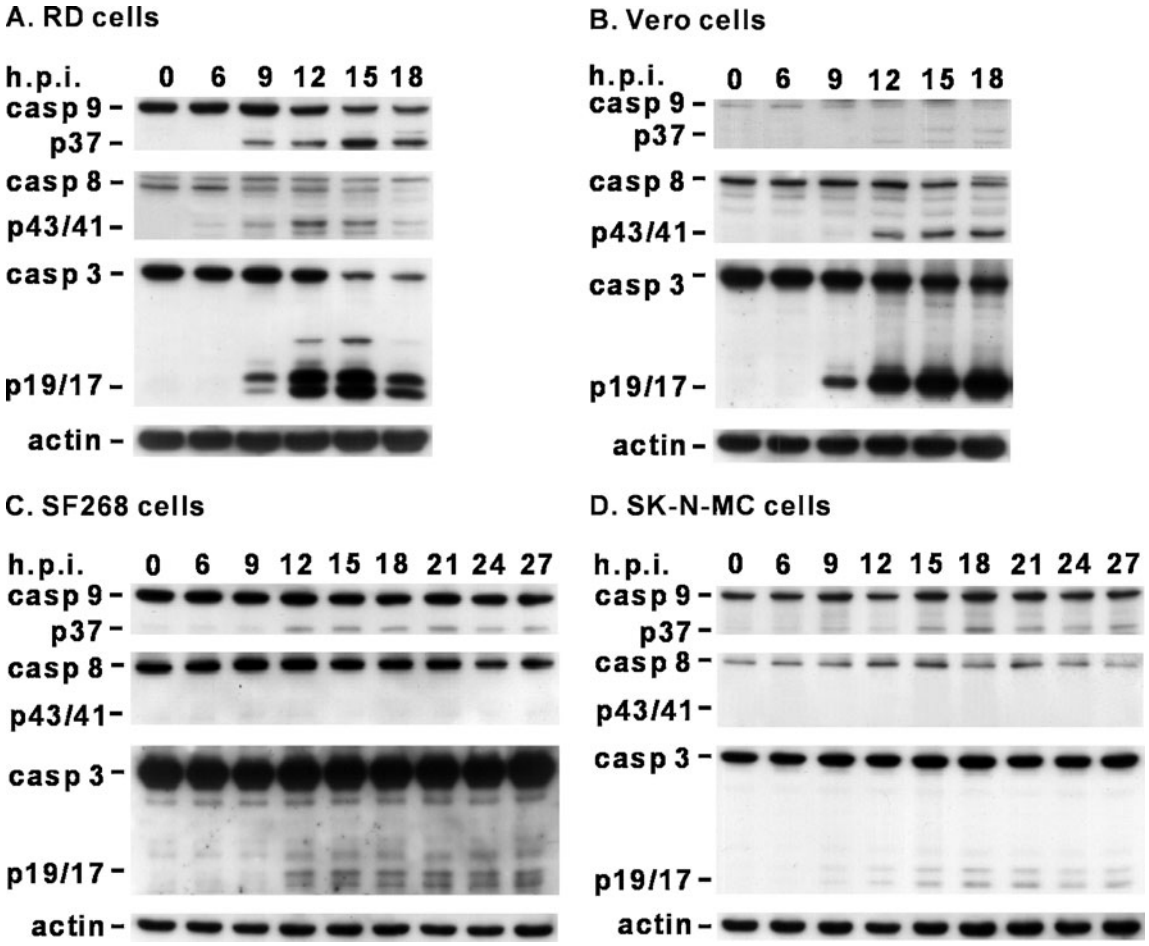

Figure 4 EV71 infection induced activation of caspase in cell lines. Cell lysates (40 μg protein per lane) were prepared from EV71-infected RD cells (**A**), Vero cells (**B**), SF268 cells (**C**) or SK-N-MC cells (**D**) at the indicated time and resolved with 12% SDS-PAGE. Western blot analysis for caspase 9, 8, 3, or β-actin was conducted as described in Materials and Methods. The active fragment of caspase 9 was detected at 37 kDa (p37), the processed fragments of caspase 8 were detected at 43 and 41 kDa (p43/41), and the active fragment of caspase 3 was detected at 19 and 17 kDa (p19/17). Expression of β-actin was used to control equal protein loading.

non-neural cells undergo apoptosis via the mitochondria pathway.

EV71 infection leads to cleavage of full length Bid only in non-neural cells

Bid, a BH3 domain–containing protein from the Bcl-2/Bcl-xL family, was activated on proteolytic cleavage by caspase 8 in the Fas signaling pathway. Cleaved Bid can translocate into mitochondria, and causes the release of cytochrome *c* into the cytoplasm (Bossy-Wetzel and Green, 1999; Chou *et al*, 1999; Esposti, 2002). To clarify whether Bid acts as an apoptotic signal link between the death receptor and mitochondrial pathway, this investigation evaluated Bid activation after EV71 infection. Western blot was conducted to detect Bid by using an antibody against both its precursor and cleaved forms. From Figure 6A and B, the cleaved Bid was detected 12 h p.i. in RD and Vero cells, indicating Bid activation (Figure 6A, B). These observations suggest a possible link between death receptor and mitochondrial pathway via cleavage of Bid in non-neural cell apoptosis. Furthermore, caspase 8 was activated in RD and Vero cells (Figure 4A, B), suggesting the EV71-infected non-neural cells undergo apoptosis via the death receptor pathway. However, no such activation was observed in EV71-infected SF268 and SK-N-MC cells (Figure 6C, D). This study thus concluded that the death receptor pathway is not significantly associated with EV71-infected non-neural cell apoptosis.

Discussion

The mechanism of CNS injury and cell damage caused by EV71 infection is poorly characterized. Neurovirulence of EV71 has been demonstrated in cynomolgus monkey system (Nagata *et al*, 2002), and reports also exist of neonatal mice orally infected by mouse-adapted EV71 strains with involvement of CNS pathogenesis (Chen *et al*, 2004). However, mice

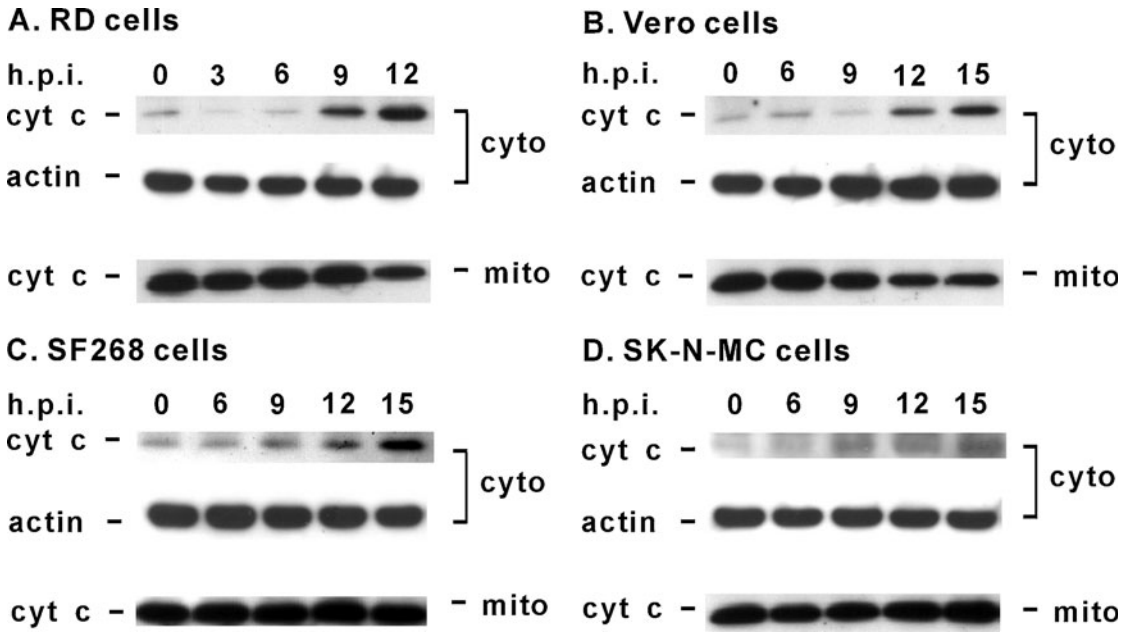

Figure 5 EV71 infection induced release of cytochrome *c*. RD cells (**A**), Vero cells (**B**), SF268 cells (**C**), or SK-N-MC cells (**D**) were infected with EV71 at an m.o.i. of 1 as indicated. Mitochondrial (mito) or cytosolic (cyto) extract (40 μg protein per lane) were prepared as described in Materials and Methods. Western blot analysis for cytochrome *c* (cyt c) was performed using mouse anti-cytochrome *c* monoclonal antibody. Expression of β-actin was used to control for equal gel loading.

older than 6 days of age were less susceptible to wild EV71 strains and displayed no disease symptoms (Yu *et al*, 2000). This study used cultured neural and non-neural cells to investigate the pathogenesis of EV71 infection. Here we demonstrate that either neural (SF-268 and SK-N-MC) cells or non-neural (RD and Vero) cells permitted EV71 infection and displayed the typical cytopathic effect (Figure 1). The cellular damage that results from EV71 infection is the outcome of cell death. These results show that EV71 kills the target cells, including irreplaceable neural cells.

Apoptosis is an active, noninflammatory, but energy-dependent process of cell death in response to various stimuli, including viral infection (Holtzman *et al*, 2000; Martin *et al*, 1994; Melcher *et al*, 1999; O'Brien, 1998). Numerous DNA and RNA viruses have been shown to elicit apoptosis either directly or indirectly upon infection (Jan *et al*, 2000; Seet *et al*, 2003; Teodoro and Branton, 1997). In some studies, specific viral proteins themselves have also been identified as potent apoptosis inducers, for example the viral proteases 2A and 3C of poliovirus and of EV71 (Barco *et al*, 2000; Goldstaub *et al*, 2000;

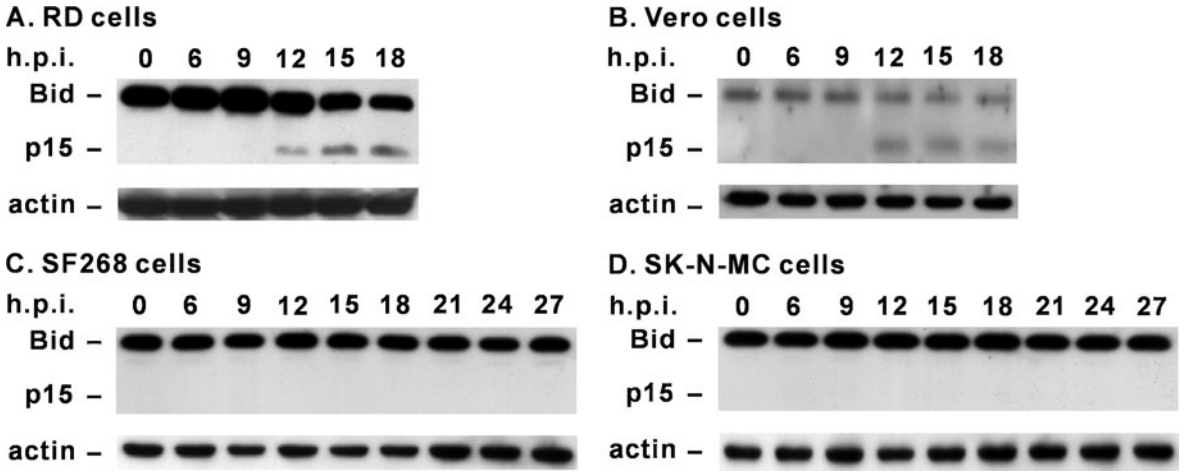

Figure 6 EV71 infection induced cleavage of Bid. Cell lysates were prepared from RD cells (**A**), Vero cells (**B**), SF268 cells (**C**), or SK-N-MC cells (**D**) (40 μg protein per lane) infected with EV71 at an m.o.i. of 1 as indicated, and were analyzed by Western blotting using rabbit anti-Bid polyclonal antibody and anti-β-actin antibodies. Cleaved products of Bid were detected at 15 kDa (p 15).

Kuo *et al*, 2002; Li *et al*, 2002). Accordingly, it is important to understand the mechanism of cell death resulting from EV71 infection. However, the study of apoptosis during whole EV71 infection has been done only on non-neural cells (Kuo *et al*, 2002). This study showed that multiple tissue tropisms of EV71 infection resulting in apoptosis occurred in both neural and non-neural cells. EV71-infected cells were demonstrated to exhibit fragmented DNA analyzed by agarose gel (Figure 2A), nicked DNA by TUNEL assay (Figure 2B), and translocation of phosphatidylserine from the inner to outer plasma membrane (Figure 3), all characteristic of apoptosis.

Apoptosis has been demonstrated to significantly influence the pathogenesis of several different CNS viral infections (Belov *et al*, 2003; Johnston *et al*, 2001; Labrada *et al*, 2002), it has been shown that many CNS injury followed by neurotropic virus infection involved apoptosis. However, different cell types employed different pathways in response to apoptotic stimuli (Duncan *et al*, 1999; Fulda *et al*, 2001; Richardson-Burns *et al*, 2002). Therefore, to clarifying the mechanism of apoptotic pathways in EV71-infected neural cells is crucial to future antiviral therapy development, and also may help in identifying neurological diseases associated with apoptosis. Our group previously found that caspase 3 was activated in human glioblastoma cells (SF268) that transiently expressed 3C protease. However, the precise pathways producing whole EV71-induced apoptosis in association with pathogenesis in neural and non-neural cells remain imperfectly understood. This study thus compared the specific apoptotic pathways induced by EV71 infection in neural and non-neural cells.

This work found that caspase 8 was activated at 9 h and 12 h of EV71 infection in RD and Vero cells, respectively. Following activation, caspase 8 cleaved Bid, a proapoptotic member of the Bcl-2 family, at 12 h p.i. in either RD or Vero cells. It suggests that Bid cleavage is closely associated with the caspase 8 activation in non-neural cells. Cleaved Bid can further promote mitochondrial permeabilization and hence caspase 9 activation, providing a potential link between EV71-induced death receptor–mediated caspase 8 activation and mitochondrial pathway activation. In contrast, cleavage of caspase 8 and truncated Bid were not detected in neural cells post infection, but can be activated in both neural cell lines following treatment with anti-Fas antibody (data not shown), suggesting that the death receptor pathway does not markedly contribute to apoptosis in neural cells. The neural cells thus may have evolved a distinctive mechanism if EV71 infection induced apoptosis.

Certain caspases may be activated at other sites in the cell, with other studies having demonstrated that caspases can trigger mitochondrial breach (Adrain and Martin, 2001; Gao *et al*, 2001; Lassus *et al*, 2002). This work found that the next specific proteolytic cleavage of caspases following EV71 infection was closely associated with a mitochondrial initiator caspase, namely caspase 9. Caspase 9 was activated in both neural and non-neural cells, and was accompanied by the efflux of cytochrome *c* from mitochondria.

In summary, in addition to demonstrating that apoptotic pathways induced by EV71 infection varied significantly between neural and non-neural cells, this study provided a pattern of apoptotic pathways related to CNS infection. The absence of caspase 8 in neural cells suggests that caspase 8 may play only a slight or no role in EV71-induced apoptosis in neural cells; that is, the death receptor pathway of apoptosis is not activated in neural cells following EV71 infection. It seems especially crucial for relaying and amplifying cell death signals for apoptosis via mitochondria in neural cells following EV71 infection. Identifying the pathway of apoptosis may help in developing a new strategy against EV71 infection, and also may help prevent neurological complications associated with EV71.

Materials and methods

Cell lines and virus

Green monkey kidney epithelial cells (Vero), human embryonic rhabdomyosarcoma cells (RD), human glioblastoma cells (SF268), and human neuroblastoma cells (SK-N-MC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Enterovirus 71 (EV71 TW/2231/98) was propagated as described previously (Shih *et al*, 2000). In all experiments, cells were infected at an m.o.i. of 1 and maintained in medium containing 2% fetal calf serum following 1 h adsorption at 37°C.

Cytopathic effect

For observing the cytopathic effect, cells were grown on a culture dish and infected by EV71. The morphological changes displayed by individual cell lines then were observed and photographed under microscopy.

Immunofluorescence staining

For immunofluorescence, cells were grown on glass coverslips. Until over 50% cells displayed the cytopathic effect, the cells were washed with phosphate-buffered saline (PBS) and fixed in 3.7% formaldehyde for 30 min. The cells were stained with EV71 monoclonal antibody (3324; Chemicon) for 30 min at 37°C, washed with PBS, and incubated with 1% secondary FITC-conjugated antibody and 0.005% Evans blue counterstain. The cells were viewed using a fluorescence microscope.

DNA fragmentation

Monolayered cells were harvested with trypsin as indicated, and fragmented DNA was extracted using a Blood and Cell Culture DNA Mini Kit (Qiagen,

Hilden, Germany) and analyzed for oligonucleosomal DNA ladders by electrophoresis on a 1% agarose gel.

TUNEL staining and annexin V staining
An ApoAlert DNA Fragmentation Assay kit (Clontech, CA, USA) was employed to perform the TUNEL assay using the manufacturer protocol. The translocation of PS to the outer leaflet of the plasma membrane during apoptosis was detected using an annexin V staining kit according to the instructions of the manufacturer (Clontech, Palo Alto, CA).

Western blot analysis
EV71-infected cells were harvested as indicated. The pellet was washed and lysed with a lysis buffer (2% sodium dodecyl sulfate, 35 mM β-mercaptoethanol, 50 mM Tris-HCl [pH 6.8], 1 mM phenylmethylsulfonyl fluoride). Lysates were cleared by centrifugation and stored at -70°C. The mitochondrial-free extracts were prepared as described previously (Heibein *et al*, 1999). For the mitochondrial-free extracts, cells were pelleted, washed, and incubated at room temperature for 10 min in digitonin lysis buffer (70 mM Tris, 250 mM sucrose, 0.2% digitonin). Lysates were centrifuged at $600 \times g$ for 15 min at 4°C. The supernatant was gathered as cytoplasmic fraction. Subsequently, the pellet containing mitochondria was resuspended at 4°C for 15 min with Triton lysis buffer (50 mM HEPES, 4 mM EDTA, 2 mM EGTA, 1% Triton X-100) and then spun at $1700 \times g$ for 5 min. The supernatant then was collected in the form of mitochondrial fraction. Western blot analysis for protein expression was performed with rabbit anti-caspase 3 polyclonal antibody, rabbit anti-caspase 9 polyclonal antibody, rabbit anti-BID polyclonal antibody, and mouse anti-caspase 8 monoclonal antibody (1:1000; Cell Signaling Technology, Beverly, MA, USA), and horseradish peroxidase–conjugated goat anti-rabbit or anti-mouse immunoglobulin G (IgG) (1:1000; Amersham biosciences) was used as the secondary antibody. The horseradish peroxidase was detected by an enhanced chemiluminescence detection system (ECL; Amersham Pharmacia, Freiburg, Germany).

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