

The NS3 protease and helicase domains of Japanese encephalitis virus trigger cell death via caspase-dependent and -independent pathways

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Abstract. Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, causes acute encephalitis and nervous damage. Previous studies have demonstrated that JEV induces apoptosis in infected cells. However, to date the mechanisms of JEV-induced apoptosis are unclear. In order to identify the viral proteins associated with JEV-induced apoptosis, pEGFP-non-structural protein 3 (NS3) 1-619 (expressing the JEV NS3 intact protein, including the protease and helicase domains), pEGFP-NS3 1-180 (expressing the protease domain) and pEGFP-NS3 163-619 (expressing the helicase domain) were transfected into target cells to study cell death. Results demonstrate that the JEV NS3 intact protein and protease and helicase domains induce cell death. In addition, cell death was identified to be significantly higher in cells transfected with the NS3 protease domain compared with the intact protein and helicase domain. Caspase activation was also analyzed in the current study. NS3 intact protein and NS3 protease and helicase domains activated caspase-9/-3-dependent and -independent pathways. However, caspase-8 activity was not found to be significantly different in NS3-transfected cells compared with control. In summary, the present study demonstrates that the NS3 helicase and protease

domains of JEV activate caspase-9/-3-dependent and -independent cascades and trigger cell death.

Introduction

Japanese encephalitis virus (JEV) is a member of the *Flaviviridae* family and infection is associated with nervous damage and encephalitis (1-3). Previous studies have demonstrated that JEV infection causes cell death via the apoptotic pathway (4,5). In addition, infection increases oxidative stress and activates ERK/p38 MAPK signal transduction (6,7). JEV-induced apoptosis via caspase activation has been previously reported (8-10). However, the component of JEV which induces cell death remains unclear.

Similar to JEV, dengue virus type 2 (DENV), hepatitis C virus (HCV), West Nile virus (WNV) and langat flavivirus are all members of the *Flaviviridae* family (11-14). These viruses cause cell death in infected cells, although their clinical presentation varies (15-18). To date, a number of studies have reported that induction of cell death by DENV, HCV, WNV and langat flavivirus may be associated with non-structure protein 3 (NS3) domains present in the viruses (19-22). In addition, previous studies have demonstrated that JEV NS2B-NS3 fragments activate caspase-3 and induce apoptosis in human medulloblastoma cells (23). Based on these observations, we hypothesized that the JEV NS3 protein may be important for JEV-induced apoptosis. Therefore, to examine whether JEV NS3 proteins induce cell apoptosis, pEGFP-NS3 1-619 (expressing the NS3 intact protein) was transfected into cells. Results indicate that the JEV NS3 protein induces apoptotic cell death.

The JEV NS3 protein has important enzyme activities, including helicase, protease and nucleoside triphosphatase activities (24-26). Previous studies have cloned C-terminal residues of JEV NS3 and identified RNA helicase activity (27). In addition, a number of studies have cloned N-terminal residues of NS3 using a PCR method, identifying protease

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activity (28-30). In order to examine which fragments of the JEV NS3 protein induce cell apoptosis, pEGFP-NS3 163-619 or pEGFP-NS3 1-180 (expressing the NS3 helicase and protease domains, respectively), were transfected into cells and cell death percentage was calculated. The current study demonstrates that the NS3 helicase and protease domains induce cell death in a manner comparable with the JEV NS3 intact protein.

Cell death is divided into two pathways, necrosis and apoptosis (31). In the apoptosis pathway, chromatin condensation and DNA fragmentation are observed (32,33) and the process is mediated by caspase cascades (34,35). Two major caspase cascades have been reported, including death receptor and mitochondria-mediated death pathways (36). In the death receptor pathway, initiator caspase-8 and executioner caspase-3 are activated to induce cell apoptosis through death ligands, which bind cell surface death receptors (37). In the mitochondrial death pathway, initiator caspase-9 and executioner caspase-3 are activated to mediate cell apoptosis via mitochondrial dysfunction (38). In this study, caspase-9 and -3 were activated in cells transfected with pEGFP-NS3 1-619, pEGFP-NS3 163-619 and pEGFP-NS3 1-180. However, caspase-8 activity was unchanged in groups compared with the control. Overall, results demonstrate that JEV NS3 and the NS3 protease and helicase domains induce cell death via the caspase-9/-3 cascade pathway.

Materials and methods

Plasmids and chemicals. pEGFP (expressing green fluorescent protein), pEGFP-NS31-619 (expressing NS3 intact protein), pEGFP-NS3 1-180 (expressing NS3 protease domain) and pEGFP-NS3 163-619 (expressing NS3 helicase domain) were kindly donated by Dr Jaang-Jiun Wang (Division of Pediatric Infectious Diseases, Emory University School of Medicine, Atlanta, USA). Acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA; caspase-3 substrate), Ac-Leu-Glu-His-Asp (LEHD)-pNA (caspase-9 substrate) and Ac-Ile-Glu-Thr-Asp (IETD)-pNA (caspase-8 substrate) were obtained from Anaspec (Fremont, CA, USA). Gene Jammer® transfection reagent was purchased from Agilent Technologies (Santa Clara, CA, USA). Fetal bovine serum (FBS) was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). DMEM, non-essential amino acid, L-glutamine and penicillin/streptomycin were obtained from Gibco-BRL (Carlsbad, CA, USA).

Cell lines and cell culture. Vero (green monkey kidney epithelial) and HeLa cells (human cervical cancer) were obtained from the Bioresources Collection and Research Center (Hsin Chu, Taiwan). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 0.1 mM non-essential amino acids and maintained at 37°C in a humidified atmosphere containing 5% CO₂. The study was approved by the ethics committee of Graduate Institute of Cancer Biology, China Medical University, Taichung, Taiwan.

Transfection. Vero and HeLa cells were incubated with 2 µg plasmid (pEGFP, pEGFP-NS3 1-619, pEGFP-NS3 1-180 and pEGFP-NS3 163-619) mixed with 6 µl Gene Jammer® transfection reagent and added to 1 ml DMEM, in 6-well plates

(2x10⁵ cells/well) for 3 hours. Following this, the medium was replaced with DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 IU/ml penicillin/streptomycin and 0.1 mM non-essential amino acids and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Following 16 h transfection, the plasmid expressed the gene products.

Cell death percentage assay. Cells (1x10⁵) were grown in a 24-well plate. Following 24 h plasmid transfection, cell number was calculated using the trypan blue exclusion method and a hemocytometer. Percentage of dead cells was calculated using the following formula: (100 - no. of cells in plasmid transfected group/no. of cells in plasmid non-transfected group) x 100.

Nuclear staining. Nuclear staining was performed by Hoechst 33342 as described previously (39). In brief, cells were treated with Hoechst 33342 (10 µg/ml) for 10 min. Following this, DNA condensation and fragmentation were observed under a fluorescence microscope (excitation, 352 nm; emission, 450 nm).

Caspase activity assay. Caspase activity was determined using a substrate cleavage assay as described previously (39,40). Briefly, cells were lysed with lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 1 mM EDTA and 1% NP-40; pH 7.5) in the presence of protease inhibitors. Cell pellets were discarded following centrifugation at 15,000 x g for 30 min at 4°C. The substrate cleavage assay was executed in a working reaction containing 40 µl cell lysate (80 µg total protein), 158 µl reaction buffer (20% glycerol, 0.5 mM EDTA, 5 mM dithiothreitol and 100 mM HEPES; pH 7.5) and 2 µl fluorogenic substrate (either Ac-LEHD-pNA, Ac-IETD-pNA or Ac-DEVD-pNA; 100 µM final concentration). The reaction was incubated at 37°C for 6 h. Cleavage of the fluorogenic substrate released p-NA, which was measured at 405 nm in an ultra-microplate reader (Ceres UV 900, BioTek Instruments, Inc., Winooski, VT, USA). Fold increase in caspase activity was calculated using the following formula: (A405_{sample} - A405_{control})/A405_{control}.

Inhibition of caspase activity. Z-VAD-FMK is a general caspase inhibitor which inhibits all caspase activity. Inhibition of caspase activity was performed as described previously (39). Briefly, cells were pretreated with 10 mM Z-VAD-FMK prior to transfection. Following this, the percentage of dead cells was determined as described for cell death percentage assay.

Statistical analysis. Data were obtained from four independent triplicate experiments and are presented as the mean values of the representative triplicate experiment (mean ± SD). Statistical differences between 2 groups were determined by the Student's t test.

Results

NS3 intact protein induces cell death through the apoptosis pathway. Plasmid pEGFP expresses a green fluorescent protein and plasmid pEGFP-NS3 1-619 expresses the JEV NS3 intact protein. Following transfection of Vero cells with pEGFP or pEGFP-NS3 1-619 for 24 hours, cell counts were performed and the percentage of dead cells was calculated.

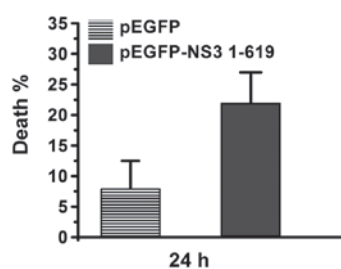


Figure 1. Trypan blue exclusion analysis at 24 h following Vero cell transfection with pEGFP or pEGFP-NS3 1-619. Percentage of dead cells was calculated. Data were obtained from four independent experiments and presented as mean \pm SD. NS3, non-structure protein 3; JEV, Japanese encephalitis virus.

As demonstrated in Fig. 1, percentage of dead cells was \sim 8% in the pEGFP-transfected group. However, the percentage of dead cells was $>20\%$ in the pEGFP-NS3 1-619-transfected group, indicating that JEV NS3 induces cell death. Next, nuclear morphology was analyzed in transfected cells via the Hoechst 33342 staining method. Compared with pEGFP-transfected cells (Fig. 2A), chromatin condensation and DNA fragmentation were found in pEGFP-NS3 1-619-transfected cells (Fig. 2B). Results indicate that the JEV NS3 intact protein induces cell death through the apoptosis pathway.

NS3 helicase and protease domains induce cell death. JEV NS3 intact protein includes two major domains, the protease and helicase domains. pEGFP-NS3 1-180 and pEGFP-NS3 163-619 (expressing the JEV NS3 protease and helicase domains, respectively) were used in this study. The percentage of dead cells was determined 24 h after transfection of Vero cells with pEGFP, pEGFP-NS3 1-619, pEGFP-NS3 1-180 or pEGFP-NS3 163-619 (Fig. 3). In the pEGFP-transfected group, cell death was \sim 6%. In the pEGFP-NS3 1-619 and pEGFP-NS3 163-619-transfected groups, cell death was \sim 20% and in the pEGFP-NS3 1-180-transfected group, cell death was \sim 30%. These results demonstrate that cell death is induced by the JEV NS3 intact protein as well as the the JEV NS3 helicase and protease domains. In addition, the protease domain was observed to exert a higher cytotoxic effect compared with the intact protein and helicase domain.

JEV NS3 intact protein and helicase and protease domains induce the caspase-9/-3 cascade pathway. Caspase activities were also determined in the current study, following 24 h transfection of Vero cells with pEGFP, pEGFP-NS3 1-619, pEGFP-NS3 1-180 or pEGFP-NS3 163-619 (Fig. 4). Compared with the pEGFP-transfected group, caspase-3 and -9 were markedly activated in pEGFP-NS3 1-619, pEGFP NS31-180 and pEGFP-NS3 163-619-transfected groups (Fig. 4A and B). However, compared with pEGFP, caspase-8 was not found to be significantly activated in pEGFP-NS3 1-619, pEGFP-NS3 1-180 and pEGFP-NS3 163-619-transfected groups (Fig. 4C). Results indicate that the JEV NS3 intact protein and protease and helicase domains activate the caspase-9/-3 cascade pathway.

JEV NS3 protein and protease and helicase domains induce cell death through caspase-independent pathways. To determine whether JEV NS3 intact protein- and protease and helicase domain-induced cell death is dependent on caspase activity, Vero cells were treated with Z-VA-FMK (a general

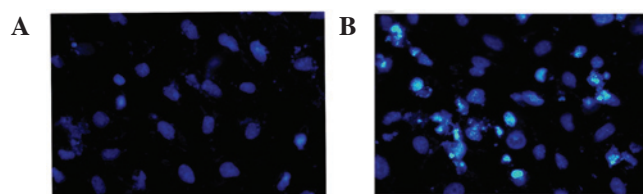


Figure 2. Hoechst 33342 nuclear staining for analysis of nuclear morphology. (A) pEGFP- and (B) pEGFP-NS3 1-619-transfected cells. Chromatin condensation and DNA fragmentation was observed in pEGFP-NS3 1-619-transfected cells, indicating that JEV NS3 intact protein induces apoptosis. NS3, non-structure protein 3; JEV, Japanese encephalitis virus.

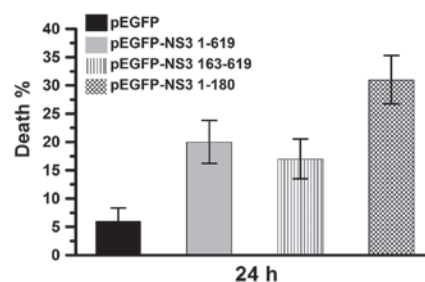


Figure 3. Trypan blue exclusion analysis at 24 h following transfection of Vero cells with pEGFP, pEGFP-NS3 1-619, pEGFP-NS3 163-619 or pEGFP-NS3 1-180. The percentage of dead cells was calculated. Data were obtained from four independent experiments and presented as mean \pm SD. NS3 helicase and protease domains of JEV induced cell death. NS3, non-structure protein 3; JEV, Japanese encephalitis virus.

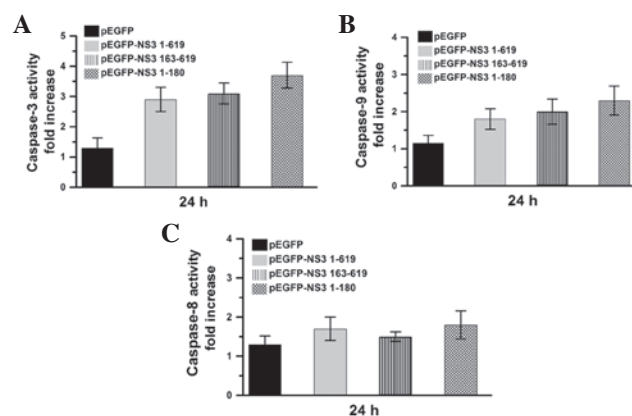


Figure 4. Caspase activity assay. Activities of (A) caspase-3, (B) -9 and (C) -8 were determined in Vero cells transfected with pEGFP, pEGFP-NS3 1-619, pEGFP-NS3 163-619 and pEGFP-NS3 1-180. Caspase-3 and -9 activity significantly increased in the pEGFP-NS3 1-619-, pEGFP-NS3 163-619- and pEGFP-NS3 1-180-transfected groups, indicating that the JEV NS3 intact protein and helicase and protease domains induce cell death via a caspase-9/-3-dependent pathway. Data were obtained from three independent experiments and presented as mean \pm SD. NS3, non-structure protein 3; JEV, Japanese encephalitis virus.

caspase inhibitor) prior to plasmid transfection and cell death percentage was calculated. As demonstrated in Fig. 5, cell death was $<10\%$ in pEGFP-, $>15\%$ in pEGFP-NS3 1-619- and pEGFP-NS3 163-619- and \sim 25% in pEGFP-NS3 1-180-transfected groups. Therefore, the NS3 intact protein and protease and helicase domains may also induce cell death by caspase-independent pathways. In summary, this study revealed that the JEV NS3 intact protein and protease and NS3

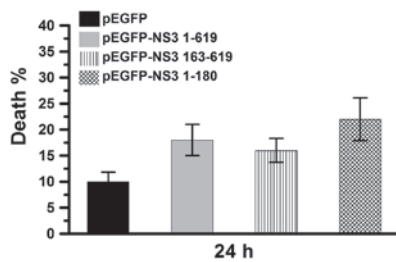


Figure 5. Trypan blue exclusion analysis. Vero cells were treated with caspase inhibitor, Z-VAD-FMK, for 30 min, followed by transfection with pEGFP, pEGFP-NS3 1-619, pEGFP-NS3 163-619 or pEGFP-NS3 1-180. The percentage of dead cells was calculated. Data were obtained from four independent experiments and presented as mean \pm SD. JEV NS3 intact protein and helicase and protease domains induced cell death via a caspase-independent pathway. NS3, non-structure protein 3; JEV, Japanese encephalitis virus.

helicase domains induce cell death via caspase-9/-3 cascade and caspase-independent pathways.

Discussion

A number of previous studies have demonstrated that JEV infection induces cell death via a caspase-dependent cell death pathway (4,8-10,41). However, the involvement of various JEV proteins with cell death remains unclear. A previous study indicated that JEV NS2-NS3 fragments induce cell death (23). Consistent with these observations, the current study demonstrates that the JEV NS3 protein alone is sufficient to trigger cell death (Fig. 1). JEV NS3 protein has been previously identified to exhibit protease and helicase enzyme activities at the N- and C- terminals, respectively (25,42,43). JEV NS3 protein is known to induce cell death; however, the enzyme activities associated with this process are not known and were analyzed in the present study. Results demonstrate that the protease and helicase domains alone trigger cell death (Fig. 3), indicating that these activities induce cell death following JEV infection.

Caspase-dependent death pathways include the caspase-8/-3 and -9/-3 cascade pathways (36-38). The caspase-8/-3 death pathway is induced by ligands acting on death receptors located in the cell membrane (37,44). However, the caspase-9/-3 death pathway is activated by mitochondrial dysfunction (38,44). Previous studies have reported that JEV infection triggers activation of caspase-3, -8 and -9 (10,41), indicating that JEV infection induces cell death through the death receptor and mitochondrial pathways. However, the present results indicate that the JEV NS3 protein and protease and helicase domains activate caspase-9 and -3 activity only and do not activate caspase-8 (Fig. 4). Therefore, these fragments induce the caspase-9/-3 mitochondrial death pathway only. To understand why the death receptor pathway is activated in JEV-infected cells but not in JEV NS3-, protease- and helicase-expressed cells, we hypothesized that the virus enters the cell across the membrane and the viral capsid may interact with death receptors leading to activation of the caspase-8/-3 death pathway during JEV infection. The caspase-9/-3 pathway is not activated until later in the infection process when NS3 protein is expressed in cells. Therefore, results indicate that the JEV NS3 protein and protease and helicase domains markedly induce the caspase-9/-3 but not the caspase-8/-3 cascades.

Numerous studies have demonstrated that cell death is induced through the caspase-dependent and -independent pathways (45-48). JEV infection is known to induce cell death through a caspase-dependent pathway (10,23); however, studies have not determined whether JEV-induced cell death is also associated with caspase-independent pathways. The present results demonstrate that inhibition of caspase activity, using the general caspase inhibitor Z-VAD-FMK (39,49), did not prevent cell death in pEGFP-NS3 1-619-, pEGFP-NS3 1-180- and pEGFP-NS3 613-619-transfected cells (Fig. 5), indicating that these fragments induce cell death through a caspase-independent pathway. Recent studies have demonstrated that a number of compounds, including isoegomaketone, transglutaminase 2 and blazeispirol A, induce cell death through caspase-dependent and -independent pathways (50-52). Consistent with these observations, the current study indicates that the JEV NS3 protein, as well as the JEV NS3 protease and helicase domains induce cell death through caspase-dependent (caspase-9/-3 cascade) and -independent pathways (Figs. 4 and 5).

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