Anti-apoptotic activity of Japanese encephalitis virus NS5 protein in human medulloblastoma cells treated with interferon-β

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Abstract Background: Japanese encephalitis virus (JEV) non-structural protein 5 (NS5) exhibits type I interferon (IFN) antagonists, contributing to immune escape, and even inducing viral anti-apoptosis. This study investigated the anti-apoptotic mechanism of JEV NS5 protein on type I IFN-induced apoptosis of human medulloblastoma cells.

Methods: Vector control and NS5-expressing cells were treated with IFN-β, and then harvested for analyzing apoptotic pathways with flow cytometry, Western blotting, subcellular localization, etc.

Results: Annexin V-FITC/PI staining indicated that IFN-β triggered apoptosis of human medulloblastoma cells, but JEV NS5 protein significantly inhibited IFN-β-induced apoptosis. Phage display technology and co-immunoprecipitation assay identified the anti-apoptotic protein Hsp70 as a NS5-interacting protein. In addition, Western blotting demonstrated that NS5 protein up-regulated the Hsp70 expression, and reduced IFN-β-induced phosphorylation of ERK2, p38 MAPK and STAT1. Hsp70 down-regulation by quercetin significantly recovered IFN-β-
induced apoptosis of NS5-expressing cells, correlating with the increase in the phosphorylation of ERK2, p38 MAPK, and STAT1. Inhibiting the ATPase activity of Hsp70 by VER-155008 resulted in the elevated IFN-β-induced apoptosis in vector control and NS5-expressing cells.

**Conclusions:** The results indicated Hsp70 up-regulation by JEV NS5 not only involved in type I IFN antagonism, but also responded to the anti-apoptotic action of JEV NS5 protein through the blocking IFN-β-induced p38 MAPK/STAT1-mediated apoptosis.

**Introduction**

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus in the Flaviviridae family. Like other mosquito-borne flaviviruses, such as dengue (DEN), yellow fever (YF), St. Louis encephalitis, and West Nile (WNV), JEV is a life-threatening pathogen causing acute flaccid paralysis, meningitis and encephalitis. JEV particle widely appears within the nervous system, including thalamus, basal ganglia, brainstem, cerebellum, cerebral cortex and spinal cord. Especially, JEV infects the basal ganglia and thalamus in 71% patients as well as the brainstem in 43% patients, which are responsible for the movement disorders, acute respiratory failure, and even death.

Japanese encephalitis (JE) with a high fatality rate of 30% occurs in East and Southeast Asia, and Northern Australia. Remarkably, estimated 30,000 to 50,000 JE cases with 10,000–15,000 deaths are reported annually in Asian countries.

Flavivirus contains a plus-sense, single strand RNA genome with one open reading frame encoding a large polyprotein. Viral polyprotein is cleaved by viral and cellular proteases, and then divided into three structural proteins (capsid (C), membrane (prM/M), and envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). Interestingly, several NS proteins like NS2A, NS4A, NS4B and NS5 show type I interferon (IFN) antagonistic activity through blocking JAK-STAT signaling.

In IFN-stimulated JAK-STAT signaling pathways, JEV NS5 suppresses nuclear translocation and tyrosine phosphorylation of STAT1; WNV NS5 protein prevents phosphorylated STAT1 accumulation; DEN NS5 inhibits phosphorylation of STAT2. Interaction of PDZ protein scribble (hScrib) with tick-borne encephalitis virus (TBEV) NS5 is associated with type I IFN antagonism. A recent report indicates type I interferon antagonism by JEV NS5 as the inhibition of Ca²⁺/calreticulin/calcineurin in STAT1-mediated signaling.

JEV-induced neuronal apoptosis and inflammation are responsible for JE pathogenesis. However, JEV could be isolated from the cerebrospinal fluid in JE cases more 3 weeks after occurring encephalitis symptoms. JEV persistence is also detected in peripheral mononuclear cells in infected children several months after acute infection. Type IFN antagonism has been considered as the essential role in the establishment of viral persistence. Since JEV NS5 protein showed type I IFN antagonistic ability, thus this study intends to the anti-apoptotic role of JEV NS5 in IFNβ-induced apoptosis of TE671 human medulloblastoma cells. Heat shock protein 70 (Hsp70) was identified as a JEV NS5-interacting protein using phage display technology; the binding interaction between Hsp70 and NS5 was confirmed by immunoprecipitation assay. In particular, Hsp70 was up-regulated in NS5-expressing cells compared to mock cells, in which involved in anti-apoptotic mechanism of JEV NS5 on IFN-induced apoptosis. Quercetin, reducing the Hsp70 expression, significantly augmented IFNβ-induced apoptosis in human medulloblastoma cells via the activation of p38 MAPK and STAT1.

**Materials and methods**

**Cells**

Human medulloblastoma TE671 cells were grown in the minimum essential medium (MEM) with 2 mM L-glutamine, 1 mM sodium pyruvate and 10% fetal bovine serum. Stably-transfected TE671 cell lines containing the pCR3.1 vector or JEV NS5 recombinant plasmid were generated in our previous report, and cultured in MEM plus 2 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum (FBS), and 800 μg/ml G418.

**Apoptosis assay with flow cytometry**

Stably-transfected cell lines were treated with 1000 U/ml IFN-β, photographed under microscope 24 and 48 post treatment, and then harvested for apoptosis analysis with the Annexin V-fluorescein isothiocyanate (FITC) apoptosis Detection Kit (BioVision, Milpitas, CA, USA). Stained cells (at least 10,000 cells per sample) were quantitated by flow cytometry with an excitation wavelength of 488 nm and the emission wavelengths at 620 nm for propidium iodide (PI) and 530 nm for FITC, respectively.

**Western blotting analysis of protein expression and phosphorylation**

For testing expression and phosphorylation levels of indicated proteins, stably-transfected cell lines were treated with 0, 250, 500, or 1000 U/ml IFN-β (Merck-Serono, Darmstadt, Germany) for 30 min, 60 min, or 24 h, and then harvested for Western blotting with anti-caspase 3, anti-phospho-STAT1 (Tyr701), STAT1, anti-phospho-ERK1/2, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-Hsp70, and anti-β actin Abs (Cell Signaling, Danvers, MA, USA). The
immune complexes were detected using peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents. The relative intensity ratio was quantified using ImageJ based on triplicate replicates, and then normalized with the band intensity of β-actin in each experiment.

Expression of recombinant JEV NS5 protein in *Escherichia coli* (*E. coli*)

JEV NS5 gene, the nucleotides 7677–10364 of the JEV strain T1P1 genome (GenBank Accession No. AF254453), was amplified by RT-PCR using the primer pair 5'-GTCCGCGATCCGGAAGACCTGGGGGCAGGACG-3' and 5'-CTAGAC TCGAGATGACCCCTGTCTTCCTGGAT-3'. The PCR product was cloned into the BamHI–XhoI site of pET24a plasmid (Merck Millipore, Darmstadt, Germany). *E. coli* BL21 (DE3) cells were transformed with recombinant plasmid pET24a-NS5 NS5, expressing recombinant NS5 proteins after IPTG induction that were purified using Ni²⁺-affinity chromatography as described in our prior reports. Finally, purified NS5 protein was analyzed by SDS-PAGE and Western blotting with anti-His tag mAb and alkaline phosphatase-conjugated goat anti-mouse IgG Abs. The immunoreactive band was developed with nitro blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Thermo Scientific, San Diego, CA, USA) or the enhanced chemiluminescence detection kit (Amer- sham Pharmacia Biotech, Piscataway, USA).

Biopanning of a phage display cDNA library with recombinant NS5 protein

For identifying NS5-interacting host factors, a human brain cDNA library (Merck Millipore) was used to screen high affinity clones for JEV NS5, as previously described. Briefly, six rounds of biopanning the phage display cDNA library with JEV NS5; high affinity phage clones were eluted with soluble recombinant NS5 and picked up from individual plaques using plaque assays. Each phage clone was amplified in *E. coli*, and then determined the relative affinity to NS5 using direct ELISA. The nucleotide sequences of human cDNA fragments displayed on the phage clones were directly sequenced using an ABI PRISM 377 DNA Sequencer (Perkin–Elmer, Waltham, MA, USA). JEV NS5-interacting proteins were identified using the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) according to their deduced amino acid sequences.

Co-immunoprecipitation assay

Stably-transfected cell lines were harvested for co-immunoprecipitation assay with anti-Flag and anti-Hsp70 mAbs, as previously described. Co-immunoprecipitated proteins were detected using Western blotting assay. The resultant blots reacted with the anti-Hsp70 or anti-Flag tag mAb; the immune complexes were detected using horseradish peroxidase-conjugated secondary antibodies. The immunoreactive band was developed by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Subcellular localization of STAT1

60–90% confluency of stably-transfected cell lines were pre-treated with 10 μg/ml quercetin for 1 day, followed by the incubation with 1000 u/ml IFN-β, and then harvested for immunofluorescent staining. The cells were fixed, and stained with primary antibodies anti-STAT1, followed by FITC-conjugated anti-mouse IgG antibodies. After washing, cells were further stained with 4', 6-diamidino-2-phenylindole (DAPI, Sigma, Saint Louis, MO, USA) for 10 min. Images of cells were photographed using a fluorescent microscopy (Olympus, Nagano, Japan).

Statistical analysis

Each assay was performed by three independent experiments, and then its standard error of the mean was calculated. Statistical analysis of data was made using Student’s t-test and Scheffe’s test; p < 0.05 was considered to be a significant result.

Results

**JEV NS5 protein reduced IFN-β-induced apoptosis of human medulloblastoma cells**

Initially, apoptotic activity of IFN-β to human medulloblastoma cells was evaluated (Fig. 1). Microscopic photography indicated IFN-β inducing a markedly cytopathic effect on stably-transfected TE671 cell lines containing the pCR3.1 vector (vector control cells), but a lightly cytopathic effect on stably-transfected TE671 cell lines containing the pCR3.1-JEV NS5 plasmid (NS5-expressing cells) 24 and 48 h post treatment, respectively (Fig. 1A). Annexin-V/PI apoptosis analysis using flow cytometry showed that the fraction of apoptotic cells with annexin-V positive and PI-positive was 11.8% in mock-treated vector control cells, 8.7% in mock-treated NS5-expressing cells, 69.0% in IFN-β-treated vector control cells, and 6.4% in IFN-β-treated NS5-expressing cells, respectively (Fig. 1B and C). Western blotting also showed IFN-β concentration-dependently stimulating the increase of caspase-3 active form in treated vector control cells, but not in treated NS5-expressing cells (Fig. 1D). The result indicated that IFN-β significantly elicited apoptosis in human medulloblastoma cells, but JEV NS5 protein exhibited the anti-apoptotic potency to reduce IFN-β-induced apoptosis in medulloblastoma cells.

Identification of anti-apoptotic proteins binding to JEV NS5 using phage display technology

To identify JEV NS5-interacting host proteins, recombinant NS5 proteins fused with His tag were synthesized in *E. coli*, purified using immobilized-metal affinity chromatography, and then used for biopanning of a phage display human brain cDNA library (Fig. 2). Coomassie blue staining of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel demonstrated the purity of recombinant NS5 proteins; Western blotting analysis with anti-His tag mAb indicated ~105-kDa immunoreactive bands as His tagged
fusion proteins of recombinant JEV NS5 (Fig. 2A). After sixth round of biopanning with recombinant JEV NS5, phage clones with high affinity host proteins to NS5 were eluted, mixed with *E. coli* in top agar, and then poured onto plate for plaque assays. Phage clones from individual plaques at the sixth round of biopanning were randomly selected for determining relative JEV NS5-binding affinities by direct binding ELISA assay (Fig. 2B). NS5-binding protein-encoding sequences fused in-frame with protein III gene of high affinity phage clones were sequenced. BLAST alignment search of NS5-binding protein-encoding sequences indicated heat shock protein 70 (Hsp70), ATP synthase subunit d, and β-tubulin as the most common NS5-binding proteins. Of them, Hsp70 was anti-apoptotic. To test the binding interaction between NS5 and Hsp70, stably-transfected TE671 cell lines containing pCR3.1-Flag or pCR3.1-NS5-Flag were harvested for co-immunoprecipitation (Fig. 2C). After 4-h incubation with anti-Hsp70 antibodies in cool room, the NS5/Hasp70/anti-Hsp70 complex was co-immunoprecipitated using protein A-Sepharose beads, and then examined using Western blotting with the anti-Flag tag or anti-Hsp70 antibodies (Fig. 2C). Western blotting analysis of co-immunoprecipitated proteins revealed the protein—protein interaction between Hsp70 and JEV NS5 protein (Fig. 2C, lane 2). The results demonstrated that Hsp70 specifically interacted with JEV NS5 in cells. Therefore, Hsp70 could involve in anti-apoptotic mechanisms of JEV NS5 on IFN-β-induced apoptosis in medulloblastoma cells.

**Up-regulation of Hsp70 in human medulloblastoma cells by JEV NS5**

Stress protein response like Hsp70 up-regulation could inhibit STAT1 signaling17,18, STAT1 also is a proapoptotic factor that involves in p38 MAPK-mediated apoptosis.19–21 Subsequently, the relative protein and phosphorylation levels of Hsp70, STAT1, ERK1/2, and p38 MAPK were determined in vector control and NS5-expressing cells in the presence or absence of IFN-β using Western blotting (Fig. 3A). Hsp70 was significantly up-regulated by JEV NS5 (Fig. 3A, Lane 4 vs. Lane 1). IFNβ treatment significantly increased the phosphorylation of STAT1, ERK2, and p38 MAPK in vector controls (Fig. 3A, Lanes 2–3); relative phosphorylated levels of STAT1, ERK2, and p38 MAPK in NS5-expressing cells was lower than those in vector controls 30- and 60-min post IFNβ treatment (Fig. 3A, Lanes 5–6 vs. Lanes 2–3). To determine the association of Hsp70 up-regulation with the decrease of STAT1, ERK2, and p38 MAPK phosphorylated levels, vector control and NS5-expressing cells were treated with quercetin (an inhibitor of Hsp70 synthesis), and then analyzed with Western blotting (Fig. 3B). Quercetin (10 μg/ml) significantly reduced the expression of Hsp70 in vector control and NS5-expressing cells (Fig. 3B vs. 3A), but Hsp70 was still observed in NS5-expressing cells. Quercetin, down-regulating Hsp70, significantly elevated IFNβ-induced apoptosis on IFN-β-induced apoptosis in medulloblastoma cells.
phosphorylation of STAT1, ERK2, and p38 MAPK in NS5-expressing cells (Fig. 3B, Lanes 5–6 vs. Fig. 3A Lanes 5–6). Particularly, quantitative analysis of immunoreactive bands using imageJ software indicated that quercetin caused a greater than 2-fold increase of STAT1, ERK2, and p38 MAPK phosphorylation in IFNβ-treated NS5-expressing cells. Moreover, confocal imaging analysis indicated IFNβ treatment activating STAT1 nuclear translocation in vector control cells, but not in NS5-expressing cells (Fig. 4). Hsp70 down-regulation by quercetin significantly enhanced IFNβ-induced STAT1 nuclear translocation in both types of cell lines (Fig. 4), supporting elevation of IFNβ-induced STAT1 phosphorylation at Tyr701 in NS5-expressing cells by quercetin. To investigate the effect of Hsp70 down-regulation by quercetin on IFNβ-induced apoptosis, the relative levels of pro and active forms of caspase 3 in IFNβ-treated cells were examined using Western blotting (Fig. 3B). Hsp70 down-regulation by quercetin correlated with the rise of active caspase 3 in IFNβ-treated NS5-expressing cells compared to the cells without quercetin treatment (Fig. 3B, Lanes 5–6 vs. Fig. 1D, Lanes 6–8). The results indicated that Hsp70 down-regulation in NS5-expressing cells by quercetin elevated the phosphorylation of STAT1, ERK2 and p38 MAPK, raising the protein levels of active caspase 3 in response to IFNβ. A novel HSP70 inhibitor, VER-155008, blocking the ATPase binding domain of HSP70,22 was further used to confirm the involvement of Hsp70 overexpression in the anti-apoptosis of JEV NS5 protein in IFNβ-treated human medulloblastoma cells (Fig. 5). VER-155008 alone slightly raised the apoptotic (sub-G1) phase, but co-treatment of IFNβ with VER-155008 synergistically induced a 3-fold increase of the apoptotic fraction in both type cells compared to IFNβ alone. The results demonstrated the Hsp70 up-regulation in NS5-expressing cells was responsible for the anti-apoptotic action of JEV NS5 protein via the repression of ERK2, p38 MAPK and STAT1 signaling in IFNβ-induced apoptosis of human medulloblastoma cells.

Discussion

This study showed the anti-apoptotic activity of JEV NS5 protein in type I IFN-induced apoptosis of human medulloblastoma cells (Fig. 1). Phage display technology identified Hsp70 as one of the three most common NS5-interacting proteins (Fig. 2). Hsp70 has demonstrated a specific binding to NS5 in cell using immunoprecipitation assays
Anti-apoptotic mechanism of JEV NS5

Fig. 3. Western blotting analysis of Hsp70 protein, and phosphorylation of STAT1 and p38 MAPK in vector control and NS5-expressing cells. Both types of transfected cells were treated with IFNβ (A) or in combination with quercetin (B). After 0, 30 and 60-min incubation, the lysate was analyzed by Western blotting with anti-Hsp70, anti-phospho-STAT1 (Tyr701), anti-phospho-p38 MAPK, anti-apoptotic and IFN antagonistic abilities of JEV NS5 protein (Figs. 3–4). Hsp70 overexpression correlated with the inhibitory effect of JEV NS5 on phosphorylation and nuclear translocation of STAT1, as well as the activation of EKK2- and p38 MAPK-mediated apoptosis (Figs. 3–4). Moreover, inhibiting the ATPase activity of Hsp70 by VER-155008 increased the apoptosis of IFNβ-treated NS5-expressing medulloblastoma cells (Fig. 5). The results let us to propose the role of interaction between JEV NS5 and Hsp70 in anti-apoptotic action via inhibiting EKK2-, p38 MAPK-, and STAT1-mediated signals. Since JEV persistence was identified in the peripheral mononuclear cells and the cerebrospinal fluid of infected patients, the anti-apoptotic and IFN antagonistic abilities of JEV NS5 might be involved in the establishment of viral persistence.

Stress proteins, most notably Hsp70, have been upregulated in neuronal cell injuries, exerting a neuroprotective activity. Overexpression of HSP70 significantly inhibited mitochondria-mediated apoptosis through reducing the release of Smac and Bax from mitochondria, and increasing the stability of Bcl-2. Interestingly, overexpression of Bcl-2 in JEV-infected cells was demonstrated to relate with the inhibition of apoptosis and JEV persistence. In addition, Hsp70 up-regulated MAP kinase phosphatase-1 (MKP-1) expression that caused the inactivation of MAP kinases (ERK, JUN, and p38 MAPK). However, ERK and p38 MAPK were required for type I and II IFN-induced phosphorylation of STAT1 at Ser727. STAT1 inhibitor significantly reduced the apoptosis of damaged spinal code tissues. Therefore, reduction of type I IFN-induced apoptosis by Hsp70 up-regulation in NS5-expressing cells might involve in JE pathogenesis such as neuronal apoptosis and viral persistence.

STAT1-mediated Bim protein activation was identified as the key response for TNF-/IFN-γ-induced apoptosis of pancreatic β-cells and high glucose-induced apoptosis of retinal pericytes. A chimeric cyclic interferon-alpha2b peptide has been demonstrated the antitumor and apoptotic response to...
Moreover, p38 MAPK-mediated STAT1 phosphorylation was required for EGF-induced apoptosis of epidermoid carcinoma cells. Hsp70 up-regulation showed anti-apoptosis effects on hyperthermic injury- and heat-induced cardiomyocyte apoptosis, promoting cell survival via decreasing apoptosis-inducing factor nuclear translocation. Up-regulating HSP70 significantly reduced the p38 MAPK phosphorylation in TLR4/MyD88 signaling pathway. Down-regulation of Hsp70 expression by quercetin reduced NS5-mediated inhibition of IFN-β-induced p38 MAPK activation and STAT1 phosphorylation and nuclear translocation (Figs. 3 and 4).

Since ERK/STAT1 signaling pathway induced by type I IFN was associated with antiviral activity, thus inhibiting p38 MAPK/STAT1 signaling by Hsp70 up-regulation was suggested to play the critical role in the anti-apoptotic action of JEV NS5 against type I IFN-induced apoptosis of human medulloblastoma cells.

Beside Hsp70 overexpression, we previously identified up-regulation of heat shock proteins Hsp60 and Hsp27-1 in NS5-expressing cells compared to vector control cells. The finding suggests that JEV NS5 activated the stress protein response in human medulloblastoma cells. Heat shock proteins regulate protein quality control and contribute to protein folding and complex assembly. Quercetin-mediated HSP70 down-regulation suppresses the activity of the phosphatases PP2a and SHP-2, correlating with the increase of caspase 3 activation in HeLa cells.

Hsp70 directly interacts with calcineurin, and enhanced the calcineurin activity. Since JEV NS5 have been demonstrated its type I IFN antagonistic activities with inhibiting Jak/Stat signaling pathway through the activation of protein tyrosine phosphatases and calreticulin-Ca²⁺-calcineurin signals, Hsp70 overexpression might be responsible for the type I IFN antagonisms of JEV NS5 protein.

In conclusion, we demonstrated the anti-apoptotic effect of JEV NS5 protein on IFN-β-induced apoptosis of human medulloblastoma cells. NS5-induced Hsp70 overexpression in human medulloblastoma cells correlated with inhibiting the activation of p38 MAPK/STAT1 signaling pathway, and diminishing Type I IFN-induced apoptosis. A lowered Hsp70 expression by quercetin markedly recovered IFNβ-induced activation of p38 MAPK and STAT1. VER-155008, an Hsp70 inhibitor, synergistically enhanced IFNβ-induced apoptosis of JEV NS5-expressing medulloblastoma cells. NS5-induced Hsp70 overexpression, linking with blocking type I IFN-induced activation of p38 MAPK and STAT1, elucidates its role in the anti-apoptotic mechanism of JEV NS5 protein during type I IFN treatment.
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