Programmed Cell Death Induced by Japanese Encephalitis Virus YL Vaccine Strain or Its Recombinant Envelope Protein in Varied Cultured Cells

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Abstract

Objective: The Japanese encephalitis virus YL vaccine strain (JEV-YL) was investigated as regards its organ tropism and the role of recombinant envelope glycoprotein in the induction of apoptosis was explored. Methods: Three varied cell lines (HepG2, Vero and C6) were infected with JEV-YL or transfected with eukaryotic expression plasmids (pcE, pcF1R2, pcF1R1 and pcF2R2) which contain different parts of the envelope gene and phenotypic properties were examined by flow cytometry and DNA fragmentation analysis. Results: After JEV-YL infection, smaller plaque was produced on HepG2 cells than on Vero cells, whereas no cytopathic effect was observed on C6 cells; moreover, by apoptosis and DNA fragmentation assays, the hallmark cytopathic effects were detected in HepG2 and Vero cells but not in C6 cells. Furthermore, cells used in our study transfected with recombinant core plasmid, pcE, which include full-length E gene but the deleted forms (pcF1R2, pcF1R1 and pcF2R2) did not have similar results as JEV-YLs. Conclusions: The JEV-YL vaccine strain had changed cell tropism to liver cells different from other virulent strains which have neural tropism, and in this study we proved that the transient-expressed entire E protein of JEV-YL could induce apoptosis and the mutations of E protein may change the organ tropism of JEV-YL.

Introduction

Japanese encephalitis is one of the important mosquito-borne encephalitis in Asia, and causes severe disease of the central nervous system in humans. The clinical features manifest as impaired consciousness and paralysis of the extremities and the fatality rate is 5–40%. In a zoonotic cycle, the virus is maintained in the transmission cycle between amplificer swine and vector mosquitoes. A national JE vaccination program has successfully controlled the disease and swine vaccination can prevent disease in swine and help to reduce JE infection in humans [1–3]. Recently, molecular biology studies of this virus have provided a better way for the development of several viral vaccines [1–9]. The Japanese encephalitis virus (JEV) is a member of the genus flavivirus in the family Flaviviridae. The JEV genome is a single-stranded (+) RNA, approximately 11 kb in length, and it contains three structural proteins (C, prM, E) and seven nonstruc-
Materials and Methods

Virus and Cells

The vaccine strain JEV-YL was a kind gift from the Formosa Biomedical Inc., Taiwan. The virus was cultured in three different cell lines including Vero cells (kidney cell line), HepG2 cells (liver cell line) and C6 cells (neural glial cell line) in this study. Vero and HepG2 cell lines were grown in Dulbecco’s modified Eagle medium (HyClone, USA) and the C6 cell line was maintained in Ham’s F12 (HyClone, USA) both supplemented with 100 units penicillin/glutamine and 10% fetal bovine serum (HyClone, USA).

Plaque Assay

In order to study the cell tropism of JEV-YL, cells were seeded into twelve-well plates and grown for at least 24 h. Then they were infected with a multiplicity of infection of 0.01 with JEV-YL, which was carried out at 37°C for 60 min. After viral infection, the cells were overlaid with Eagle’s minimal essential medium containing 2% fetal bovine serum and 1% methylcellulose. The plates were incubated at 37°C for 4–7 days until plaque formation. Then cells were stained with 1% crystal violet for 30–60 min and washed with tap water. After the plates were dried, plaques were visualized by phase contrast microscopy at 100–200× magnifications.

Plasmid Constructions

The eukaryotic expression vector, pcDNA 3.1 (Invitrogen), was used for the expression of envelope E gene. The entire E gene that contains 1,500-bp cDNA was cut from pJE-struct, which was reported in our previous study [5, 10] by restriction enzyme (EcoRI and EcoRV) digestion and subcloned into pcDNA3.1, named pcE (fig. 1). The other three deleted fragments of E gene (1,018, 525 and 319 bp) generated from prokaryotic plasmids (pRSET/F1R2, pRSET/F1R1 and pRSET/F2R2, respectively) were also subcloned into vector pcDNA3.1 by restriction enzyme (BamHI, BglII, EcoRI, Hind III or XhoI) digestion and named pcF1R2, pcF1R1 and pcF2R2, respectively (fig. 1). These above prokaryotic plasmids containing different parts of gene and their related locations to the genome of JEV-YL were described in our previous study, too [5, 10]. All the resulting plasmids were verified by DNA sequencing and further for DNA transfection.

DNA Transfection

Cells (1 × 10^5) were seeded in 35-mm diameter culture dishes for 24 h and then DNA was transfected with Qiagen Effectene™ (BioLab) according to the manufacturer’s instructions. The brief procedure is as follows: cDNA plasmid (1 μg) is added with EC buffer (BioLab) to 75 μl and mixed with 2.4 μl Enhancer (BioLab) at room temperature for 5 min. Then, 6 μl Effectene reagent (BioLab) was added to the mixture and 400 μl culture medium to cells for 10 min at room temperature. Following this, cells were washed with 1× PBS and maintained in culture medium at 37°C, 5% CO2 for further analysis.

Flow Cytometry

After infection or transfection described above, cells were detached with 0.25% trypsin and washed with 1× PBS at 1,000 rpm for 5 min. Then, the pellets were fixed with 75% alcohol and labeled in a PI reaction solution (12.5 ml PBS, 200 μl Triton X, 200 μl propidium iodine, 50 μl RNase A) for 30 min. Finally, these treated cells were examined by FACS Calibur flow cytometry (Becton Dickinson). The percentage of cells in the G1, S and G2/M phases of the cell cycle was determined using the CellQuest software program (Becton Dickinson). The recorded values of subdiploid DNA content represented the percentage of apoptotic cells relative to the total number of cells counted.

DNA Fragmentation Assay

For DNA fragmentation assay, viral-infected or DNA-transfected cells were detached with a mixture of trypsin (Hyclone) and centrifuged at 5,000 g for 2 min. The pellet was incubated in 1× PBS, 10 mg/ml RNase (Promega), and 0.1 mg/ml proteinase K (Promega) for 30 min at 37°C. Total DNA was extracted with phenol chloroform and then precipitated with 2 vol of ethanol and 0.1 vol of 5 M NaCl at –70°C for 30 min. The mixture was centrifuged at 12,000 g for 15 min at 4°C and the pellet of nucleic acid was allowed to dry. Finally, the DNA was dissolved in 50 μl TE (pH 8.0) and analyzed by 1.2% agarose gel electrophoresis.

Results

To study the cell tropism of JEV-YL, we selected three different cell lines including Vero cells (kidney cell line), HepG2 cells (liver cell line) and C6 cells (neural glial cell line) for plaque assay and flow cytometry analysis. The cells were infected at a multiplicity of infection of 0.01 with JEV-YL and cultured for 4–7 days until the infected
cells showed obvious cytopathic effects (CPE). The JEV-YL strain caused apparent varied phenotypic properties on those cell lines (fig. 2). In Vero cells, the CPE was produced on the 5th day after viral infection and pieces of cells were detached from the bottom (large-plaque variant) on the 6th day. However, in HepG2 cells, the CPE was produced on the 4th day after viral infection and formed smaller plaques (<1.0 mm in diameter) on the 6th day, but no CPE was observed on C6 cells (fig. 2).

By flow cytometry analysis, the JEV-YL strain caused varied apoptosis in cell lines and showed that approximately 34.6% cells were apoptotic at 72 h postinfection.
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in JEV-YL-infected HepG2 cells; in Vero cells, the apoptotic responses after JEV-YL infection were initiated (22.1%) and nearly total apoptosis (99.8%) at 120 and 168 h, respectively, whereas no obviously apoptotic responses were observed in JEV-YL-infected C6 cells (fig. 3a). To determine whether the apoptosis was induced by E protein, cells were transfected with plasmids (pcE, pcF1R1, pcF2R2 or pcF1R2) and examined by the flow cytometry analysis. After transfection with plasmid (pcE), approximately 30% HepG2 cells were apoptotic at 48 h, and in Vero cells the apoptotic response (74%) was observed at 72 h but not in C6 cells after transfection (fig. 4a). Besides, mammalian cells transfected with other plasmids (pcF1R1, pcF2R2 or pcF1R2) had no obvious apoptotic response in our study (data not shown). These results showed only host cells transfected with plasmid (pcE) which by encoding the entire E protein could cause apoptosis in both HepG2 and Vero cells but not in C6 cells, the same as JEV-YL (fig. 3a, 4a).

Furthermore, we used the DNA fragmentation assay to confirm apoptotic responses in mammalian cultured cells. After JEV-YL infection, apoptotic DNA fragments could be detected from 72 and 120 h postinfection in HepG2 cells and Vero cells, respectively, but were not observed in C6 cells (fig. 3b). The results of pcE transfection showed that apoptotic DNA fragments could be detected in HepG2 cells at 48 h (fig. 4b, pcE/HepG2, lane 2), whereas in Vero cells the DNA fragmentation was determined until 72 h (fig. 4b, pcE/Vero, lane 2) and no apoptosis was observed in C6 cells at 72 h yet (fig. 4b, pcE/C6, lane 2).

Discussion

In this study, JEV-YL has hepatotropism, whereas other virulent strains were reported to be able to infect neural cells but not liver cells [12]. The E-glycoprotein of
JEV plays a crucial role in viral pathogenesis by determining the cellular susceptibility and organ tropism of the virus [2, 12, 14] and it was reported that a single amino acid change of E (138) to K (138) on viral envelope protein would cause a loss of viral neuroinvasiveness and change its organ tropism, of which the main target organ was the liver [8, 12]. In our previous study, we compared E-glycoprotein sequences of JEV-YL with other virulent JEVs, revealing two amino acid mutations in the E protein of JEV-YL; one is K(138) and the other is G(389) [18, 19] and as a result of these mutations there may be a tropism change from neural cells to liver cells. Therefore, this strain was used as a live-attenuated vaccine.

In addition, the envelope proteins of several RNA viruses, such as Sindbis virus, Langat virus and human immunodeficiency virus, may trigger apoptosis in transfected cells [16, 17, 20], whether the E protein of JEV will induce the effect or not. In this study, we further investigated inducing programmed cell death by recombinant E protein of JEV-YL. The results revealed that only a transient expression containing full-length E protein (pcE) could induce apoptosis in HepG2 cells and Vero cells but not in C6 (fig. 3), and another transient expression of plasmids (pcF2R2 or pcF1R2) merely caused HepG2 cells to stay at the G0/G1 phase (data not shown).

In conclusion, in this study we proved that the transiently expressed entire E protein of JEV-YL could induce apoptosis and the mutations of E protein may change JEV-YL organ tropism. Further experiments are necessary to better delineate the mechanisms by which E protein induces apoptosis, and the consequence of host immune responses to JEV-YL is also in need of investigation in the future.

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References


