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Japanese encephalitis virus disrupts blood-brain barrier and modulates apoptosis proteins in THBMEC cells

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ABSTRACT

Japanese encephalitis (JE) is a neurotropic flavivirus that causes inflammation in central nervous system (CNS), neuronal death and also compromises the structural and functional integrity of the blood–brain barrier (BBB). The aim of this study was to evaluate the BBB disruption and apoptotic process in Japanese encephalitis virus (JEV)-infected transfected human brain microvascular endothelial cells (THBMECs). THBMECs were overlaid by JEV with different MOIs (0.5, 1.0, 5.0 and 10.0) and monitored by electrical cell-substrate impedance sensing (ECIS) in a real-time manner in order to observe the barrier function of THBMECs. Additionally, the level of 43 apoptotic proteins was quantified in the virally infected cells with different MOIs at 24 h post infection. Infection of THBMEC with JEV induced an acute reduction in transendothelial electrical resistance (TEER) after viral infection. Also, significant up-regulation of Bax, BID, Fas and Fasl and down-regulation of IGFBP-2, BID, p27 and p53 were observed in JEV infected THBMECs with 0.5 and 10 MOIs compared to uninfected cells. Hence, the permeability of THBMECs is compromised during the JEV infection. In addition high viral load of the virus has the potential to subvert the host cell apoptosis to optimize the course of viral infection through deactivation of pro-apoptotic proteins.

1. Introduction

Japanese encephalitis (JE) is a severe zoonotic disease caused by the mosquito-borne Japanese encephalitis virus (JEV). JEV is a single-stranded, positive-sense RNA belonging to the genus *Flavivirus* of the family *Flaviviridae* (Taylor et al., 1980; Weaver et al., 1999). JEV has become an alarming public health threat due to its recent geographic transition into Pakistan, Western Indonesia, Papua New Guinea and Northern Australia (Mackenzie and Williams, 2009; Van den Hurk et al., 2009). Seventy thousand cases of JE have been recorded annually across Asia; however, the number may be inaccurate due to insufficient laboratory based observation and reporting (Campbell et al., 2011; Solomon, 2006). The mortality rates are approximately 25–30%, while 50% of the infection will result in permanent neurologic sequelae. The highest number of cases was recorded in children under the age of 15 in rural areas (Chen et al., 2002; Solomon et al., 2000).

Neurotropic virus-related neuropathy is categorized by the existence of infectious virus particles, inflammatory mediators, and immune cells, which later results in neuronal dysfunction or

Abbreviations: AJ, adherens junctions; BID, Bcl 2 homology 3-interacting domain death agonist; Bax, Bcl-2 like protein 4; BID, BH3interacting-domain death agonist; BBB, blood–brain barrier; BMEC, brain microvascular endothelial cell; CNS, central nervous system; DAB, 3′-diaminobenzidine; DMEM, Dulbecco's modified eagle medium; ECIS, electrical cell-substrate impedance sensing; ECs, endothelial cells; Fasl, fas ligand; Fas, fas receptor; FBS, fetal bovine serum; FFU, foci forming unit; HSP, heat shock proteins; HRP, horse-radish peroxidase; IGFBP-2, insulin-like growth factor-binding protein 2; ICAM1, intercellular adhesion molecule 1; IL-1β, interleukin 1β; IL-6, interleukin 6; JE, Japanese encephalitis; JEV, Japanese encephalitis virus; JNK, Jun NH2-terminal kinase; JAM, junctional adhesion molecules; MCP-1, monocyte chemoattractant protein-1; MAV-1, mouse adenovirus type 1; MMPs, multiple matrix metalloproteinases; MOI, multiplicity of infection; NF-kappab, nuclear factor kappa B; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TJs, tight junctions; TRADD, TNFR associated death domain; TEER, transendothelial electrical resistance; THBMEC, transfected human brain microvascular endothelial cells; TNF-α, tumor necrosis factor; NFR-1, tumor necrosis factor receptor-1T; VCAM, vascular cellular adhesion molecule; WNV, West Nile virus; ZO, Zona occludens.

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destruction in the parenchymal tissues of the CNS. In several neurotropic viruses, BBB integrity is compromised during the infection, and its disruption leads to brain injury (Afonso et al., 2008; Schäfer et al., 2011; Soilu-Hänninen et al., 1994). During the peripheral amplification of JEV in primary target cells such as dendritic cells and macrophages, JEV gains entry into the CNS through the BBB. BBB is comprised of brain microvascular endothelial cells (BMECs), astrocytes, pericytes, neurons, and basement membrane. Endothelial cells (ECs) play a major role in vascular permeability. Numerous reports showed that during infections with hemorrhagic fever viruses, endothelial cells become infected (DeMaula et al., 2002; Lukashevich et al., 1999; MacLachlan et al., 2000). BMECs are structurally and functionally different from peripheral endothelial cells (Craig et al., 1998; MacLean et al., 2001). Recent studies have suggested the particular role and contribution of endothelial cells in neurotropic virus-associated BBB breakdown (Vandenhaute et al., 2012; Xu et al., 2012a). Therefore, in this study endothelial cells derived from the brain were used to investigate effects induced by this virus.

The BBB is complexed by a network of tight junctions (TJs), adherens junctions (AJ) and gap junctions. These specialized complexes form a paracellular diffusion barrier at the vascular interface with circulating blood. Under physiological circumstances, the BBB preserves CNS homeostasis and confines diffusion of soluble molecules and immune cell migration from the systemic compartments of the body into the CNS. The structural and functional integrity of the BBB is critically dependent on the TJs between BMECs rather than other kinds of cells (Hawkins and Davis, 2005; Persidsky et al., 2006). The TJs play a vital role in sustaining the permeability barrier of epithelial and endothelial cells which control tissue homeostasis. The TJ comprises of a multi-protein complex including tetraspanin claudins, occludin and the cytosolic proteins such as zona occludens (ZO), which joins the cytoskeletal network to the TJ membrane proteins. TJ acts as a functional diffusion barrier for pathogens and various viruses either use components of TJ for establishing infection in epithelial or endothelial cells or modulate the TJ components to gain access to the tissue space for further spread of the infection (Anderson and Van Itallie, 2009; Schneeberger and Lynch, 2004). A previous study has demonstrated that JEV may damage the TJ proteins leading to increase the multiple matrix metalloproteinases (MMPs), contributing to BBB disruption (Roe et al., 2012). Furthermore, it has been shown that disruption of AJ alone is enough to increase the permeability of BBB dramatically (Gao et al., 2000), which contributes to pathogenesis of related complications, such as poisoning (Hossain et al., 2004), stroke (Paul et al., 2001), sepsis (Angelini et al., 2006), and tumor metastasis. To evaluate the transendothelial electrical resistance (TEER) and cell membrane barrier function, an electric cell-substrate impedance-sensing (ECIS) 1600R (Applied BioPhysics, Troy, NY, USA) was applied. Several studies have evaluated cell morphology, cell-substrate interactions, cell layer barrier function, cell motility and wound healing, using ECIS system (Giaever and Keese, 1986; Giaever and Keese, 1991; Giaever and Keese, 1993; Keese et al., 2004; Lo et al., 1993; Mitra et al., 1991; Tiruppathi et al., 1992; Wegener et al., 2000).

On the other hand, apoptosis has been shown to be one of the main mechanisms of cell death (Arends et al., 1990). It is considered as a physiological suicide process in many different cell types. Specifically, some viral genes have been revealed to stimulate or prevent apoptosis (Collins, 1995). JEV may induce cytopathic effect due to neuronal apoptosis and inflammation (Ghoshal et al., 2007; Yang et al., 2010). There are several mechanisms of apoptosis that have been described in JEV infection including initiation of endoplasmic reticulum stress (Su et al., 2002), reactive oxygen species (ROS) generation, activation of nuclear factor kappa B (NF-kappaB) (Lin et al., 2004), activation of caspase 8 in a FADD-independent manner, stimulation of caspase 9 by mitochondrion-dependent pathway (Tsao et al., 2008), as well as the engagement of tumor necrosis factor receptor-1 (TNFR-1) and TNFR associated death domain (TRADD) (Swarup et al., 2008).

Although viruses can be identified in BBB endothelial cells after systemic infection (Liou and Hsu, 1998), our knowledge of cellular mechanisms accompanying with JEV-induced BBB disruption is not clear. In addition, the role of apoptosis in modulating the immune response to JEV infection at varying multiplicity of infection (MOI) levels is vague. Therefore, this study aimed to evaluate the effects of JEV infection on the BBB and the cellular properties of cultured THBMECs using ECIS and to understand the apoptosis process in JE pathogenesis.

2. Materials and methods

2.1. JEV propagation in vero cells

JEV (Nakayama/MY/2009/P578662, Accession Number: HE861351) was obtained from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia. African green monkey kidney Vero cell line (ATCC® CCL-81™), which was purchased from the American Type Culture Collection (ATCC) and used for JEV propagation and titration. Viral propagation was conducted by overlaying JEV at MOI of 0.1 on monolayer of Vero cells growing in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, USA), supplemented with 5% heat-inactivated fetal bovine serum (FBS) in humidified 37 °C, 5% CO_2 incubator for 120 min (Han et al., 2014). Complete DMEM was then added to the cells and further incubated under the same conditions. Five days post-infection, the supernatant containing progeny viruses was collected and after centrifugation in order to eliminate the cell debris aliquoted and kept at −80 °C.

2.2. JEV titration assay

Foci forming unit (FFU) assay was used to titrate JEV as described previously (Fong, 2011). Briefly, Vero cells were seeded in 24 wells cell culture microplate. After cell monolayer formation, growth medium was removed, and the cells were infected by serial dilutions of JEV under the conditions described earlier. Virus adsorption and attachment occurred after incubating the microplate at 37 °C for 1 h. Sterile phosphate-buffered saline (PBS) was used to wash the cells twice and incubated at 37 °C for four days for viral foci formation. To visualize the JEV foci, the cell culture medium was removed and the cells were washed gently thrice with PBS. To fix the cells, 10% paraformaldehyde was added for 30 min at room temperature (RT), followed by three washes with PBS. Tergitol-type NP-40 1% (Sigma, St. Louis, MO, USA) was used to permeabilize the cells for 10 min at RT. Cells were then washed thrice with PBS and blocked with 3% skim milk solution prepared in PBS for 2 h at RT. Following three times of washings with PBS, the cells were then incubated at 37 °C for 1 h with rabbit JEV hyperimmune rserum diluted in 1:500 using 1% skim milk solution. Cells were then washed three times with PBS and incubated with goat anti-rabbit IgG conjugated with horse-radish peroxidase (HRP) at a final concentration of 1:250 in 1% skim milk solution (Sigma, St. Louis, MO, USA). Finally, to stain the virus foci, 3′-diaminobenzidine (DAB) peroxidase substrate (Thermo Scientific Pierce, Rockford, IL, USA) was added to each well. An SMZ 1000 stereomicroscope (Nikon, Tokyo, Japan) was used to count viral foci and the results were expressed as FFU. The titer of the cultured JEV was determined as 1.55×108 FFU/ml. Cytopathic effect was observed during JEV-infected Vero cells at varying MOI, even at the lowest tested MOI which was 0.5.