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Integrin αvβ3 promotes infection by Japanese encephalitis virus

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ABSTRACT

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that is one of the major causes of viral encephalitis diseases worldwide. The JEV envelope protein facilitates viral entry, and its domain III contains an Arg-Gly-Asp (RGD) motif, that may modulate JEV entry through the RGD-binding integrin. In this study, the roles of integrin αv and β3 on the infection of JEV were evaluated. Reduced expression of integrin αv/β3 by special shRNA confers 2 to 4-fold inhibition of JEV replication in BHK-21 cells. Meanwhile, antibodies specific for integrin αv/β3 displayed ~58% and ~33% inhibition of JEV infectivity and RGD-specific peptides produced ~36% of inhibition. Expression of E protein and JEV RNA loads were clearly increased in CHO cells transfected with cDNA encoding human integrin β3. Moreover, integrin αv mediates JEV infection in viral binding stage of life cycle. Therefore, our study suggested that integrin αv and β3 serve as a host factor associated with JEV entry into the target cells.

1. Introduction

Japanese encephalitis virus (JEV) is a member of the family *Flaviviridae* and the genus *Flavivirus,* which includes yellow fever virus (YFV), dengue virus (DEV), West Nile virus (WNV), tick-borne encephalitis virus (TBEV) and St. Louis encephalitis virus (SLEV). JEV is an arthropod-borne human pathogen that is naturally transmitted in an enzootic cycle among birds, pigs and other vertebrate hosts by mosquitoes, particularly *Culextritaeniorhynchus*. JEV is one of the most common agents of viral encephalitis in humans and animals, with most cases occurring in southern and eastern Asia (Solomon, 2006; Wang and Liang, 2015). Nearly 3 billion people are believed to be at risk for JEV infection, and approximately 30,000 to 50,000 clinical cases, with 10,000 deaths, are reported annually in eastern Asia. The case fatality rate ranges from 20% to 30%, but approximately 30% to 50% of survivors result in permanent neuropsychiatric sequelae (Ghosh and Basu, 2009; Campbell et al., 2011; Impoinvil et al., 2013).

JEV is an enveloped arbovirus with a single strand positive sense RNA genome of approximately 11 kb in length. The genomic RNA contains a single open reading frame (ORF) encoding a polyprotein (with approximately 3400 amino acids), which is subsequently cleaved by both host and viral proteases into three structural proteins (capsid (C), membrane (M) and envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Unni et al., 2011; Pierson and Diamond, 2013).

Flavivirus can replicate in the cytoplasm of various cell types cells including monocytes, macrophages, dendritic cells and neuronal cells mediated by the viral envelope glycoprotein E, indicating that different cell surface molecules are involved in the entry of JEV. In particular, JEV attaches to the cell surface and enters target cell by receptor-mediated endocytosis (Lee et al., 2008; Kaufmann and Rossmann, 2011; Pierson and Kielian, 2013; Lindenbach et al., 2013; Liu et al., 2015; Wang et al., 2016). Several reports showed that HSP70, HSC70 as well as Hsp90β were the putative receptors for JEV infection in different cells (Das et al., 2009; Hung et al., 2011; Zhu et al., 2012; Chuang et al., 2015). Lipid rafts, heparin sulfate, vimentin, GRP78 and 37/67 kDa high-affinity laminin receptor protein were the potential JEV-binding proteins helping for viral infectivity (Zhu et al., 2012a, 2012b; Su et al., 2001; Das et al., 2011; Wu et al., 2011; Thongtan et al., 2012). Recent study showed that DC-SIGN was an attachment factor to facilitate JEV infection of dendritic cells (DCs) (Wang et al., 2016).

Previous studies approved that the envelope (E) glycoprotein, especial the domain III (DIII), can induce the production of neutralizing antibodies and inhibit the entry of flavivirus into target host cells (Rey et al., 1995; Batra et al., 2010; Chin et al., 2007; Chu et al., 2005; McAuley et al., 2016). Similar to other flavivirus, JEV E protein is the major factor for binding cell receptors and mediating low-pH triggered membrane fusion (Liu et al., 2015; Zhu et al., 2012a, 2012b). Several studies revealed that the recombinant E DIII of JEV blocked JEV entry into host cells, and the recombinant E DIII of heterologous flavivirus induced cross-

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protection against JEV infection in mice (Luca et al., 2012; Li et al., 2012; Fan et al., 2013; Zu et al., 2014). Meanwhile, there is a conserved RGD integrin-binding motif or an extremely similar sequence in most mosquito-borne flaviviruses. Mutation of RGD motifs in the E proteins of Murray Valley encephalitis virus might alter tropism or virulence, which fueled the speculation that the cell attachment of these viruses might involve integrin, but not to yellow fever virus 17D (YFV-17D) (van der Most et al., 1999; Lee and Lobigs, 2000). Though Medigeshi's studies indicate that integrin αVβ3 is not required for viral entry of WNV, Chu and Ng identified that integrin αvβ3 was putative receptor of WNV without a RGD motif in the E protein (Chu and Ng, 2004; Medigeshi et al., 2008). Consequently, Lee et al. revealed that the interaction between WNV E DIII and integrin αvβ3 results in the phosphorylation of focal adhesion kinase, which mediates the ligand-receptor internalization into cells (Lee et al., 2006). Schmidt et al. also confirmed the important role of integrin in WNV infection but not at the level of binding to target cells (Schmidt et al., 2013). Nevertheless, the role of integrin in the infection of flavivirus, particularly in JEV, is rather superficial.

Integrins are heterodimeric transmembrane proteins that consist of α and β subunits and mediate adhesion to the extracellular matrix (ECM) and cell-cell contact, participating in many cellular processes (Giancotti and Ruoslahti, 1999). In mammals, the combination of at least 18α and 8β subunits engenders 24 distinct heterodimerics that are expressed in large numbers on almost all cell types. Integrins play an important role in regulating endocytosis and recycling of cell migration and invasion (Hynes, 2002; Gahmberg et al., 2009). Integrins binds specific ligands in a divalent-cation-dependent manner, and a subset of integrins bind to specific recognition sequences such as the amino acid Arg-Gly-Asp (RGD) motif (Jolly and Sattentau, 2013). RGD-binding integrins were used as receptors or coreceptors for viruses' infection including hantavirus, human picornaviruses, human metapneumovirus and human papillomaviruses (Gavrilovskaya et al., 1998; Merilahti et al., 2012; Cox et al., 2012; Raff et al., 2013).

In this study, the role of integrin αv or β3 in JEV infection was further investigated. Consequently, the data demonstrated that the reduced expression of integrin αv or β3 on the surface of BHK-21 cells by specific shRNAs obviously diminished the infection efficiency of JEV. In particular, function-blocking αv or β3 integrin-specific antibodies inhibited the infection of JEV, and the introduction of a receptor to competitively bind RGD polypeptides decreased the infectivity of both JEV and FMDV. The divalent cation chelat or EDTA, which reduces integrin-ligand binding, diminished JEV infectivity. Moreover, the findings of this study indicated that the capacity of JEV to bind host cells was inhibited when the expression of integrin αv was decreased. Nevertheless, transfection of β3 cDNA into non-permissive cells CHO conferred JEV infectivity. Collectively, this study verified that both integrin αv and β3 were involved in JEV infection.

2. Material and methods

2.1. Cells and viruses

BHK-21 cells and Hela cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) containing 10% of fetal bovine serum (FBS) (Gibco, USA). Chinese hamster ovary cells (CHO, ATCC) without integrin β3 expression were grown in F12 K medium (Wuhan Boster Biological Technology, LTD) containing 10% FBS. Genotype GI Japanese encephalitis virus (JEV) strain SX09S-01 and type O FMDV strain O/ES/2001(used as positive control) were propagated on BHK-21 cells and used for viral infection (Cao et al., 2011; Fan et al., 2016).

2.2. shRNA, antibodies and peptides

The plasmids containing the short hairpin RNA (shRNA) corresponding to the mRNA sequence of integrin β3 (sc-29375-SH, consisting of pools of three to five target-specific 19–25 nt siRNAs, shRNA-β3) and integrin αV (sc-29373-SH, shRNA-αV), which used to knockdown the endogenous αV and β3 gene expression, negative shRNA (sh-NC, sc-108060) and integrin β3 rabbit polyclonal antibody (H-96, sc-14009, 200 μg/ml, referred to as ITGβ3) were purchased from Santa Cruz Biotechnology Inc. Integrin αV rabbit polyclonal antibody (BA0957, 200 μg/ml, referred to as ITGαV) and labeled second antibodies in this study were purchased from Boster Bioengineering Ltd. Linear RGD peptides GRGDSP (SCP0157) and control peptides SDGRG (F4215-98C) were purchased from Sigma and US Biological, respectively. The monoclonal antibody (mAb) against JEV E protein was kindly provided by professor Cao shengbo (Huazhong Agriculture University).

2.3. RNA interference

BHK-21 cells or Hela cells were seeded at a density of 4.0×10^5 cells in 6-well plates. When cell density reached about 70– 80%, cells were transfected with 100 nM of each shRNA plasmid for integrin αV and β3 with lipofectamine 2000. Cells were harvested and the knockdown efficacy was validated by western blotting analysis at indicated time post-transfection. Meanwhile, 45 h after transfection, cells were infected with JEV at a MOI of 1.0 for 1 h and maintained with free-serum DMEM containing for 24 h before the cells and supernatants were harvested. The virus titers of harvested supernatants were measured by plaque assay in BHK-21 cells. Meanwhile, the JEV RNA loads were analyzed using real-time RT-PCR.

2.4. Antibody blocking assay

To confirm whether integrin αvβ3 is involved in JEV infection or not, a functional antibody blocking experiment was performed on BHK-21 cells based on previous studies (Cseke et al., 2009). Briefly, BHK-21 cells were incubated with antibodies specific for either ITGβ3 rabbit polyclonal antibody or ITGαV at 25 μg/ml (according to the manufacturer's instructions), or with normal rabbit sera as control for 1 h at 37 °C. BHK-21 cells were then inoculated with 1.0 MOI of JEV and scored for infection by plaque assay 4–5 days later.

2.5. Expression of integrin β3 in CHO cells change the infection of JEV

CHO cells were seeded in 6-well plates and transfected with pCA-β3 (containing the full length cDNA of integrin β3) or pCAGGS (kindly provided by Prof. Jin meilin, Huazhong Agricultural University) with Lipofectamine 2000 according to manufacturer's instruction. Cells were infected with JEV at a high MOI of 5.0 at 24 h post-transfection. The cultured supernatants were harvested at 48 h post JEV infection and JEV RNA loads were analyzed using real-time RT-PCR. Meantime, cells were harvested at 60 h post-infection and subjected to Western blot assay with JEV E protein monoclonal antibody.

2.6. The influence of RGD peptides on JEV infection

The confluent monolayers of BHK-21 cells cultivated in 24-well plates were washed twice with PBS. RGD peptides GRGDSP (100 μg/mL) and control peptides SDGRG (100 μg/mL) and different dilutions of EDTA (0 mM, 1.25 mM and 2.5 mM) were prepared in serum-free DMEM. 100 μl of medium were separately added to the wells in triplicate. The cells were incubated at 37 °C for 90 min and at 4 °C for 10 min. Subsequently, the cells were adsorbed with JEV at MOI of 1.0 and FMDV at MOI of 0.01 (BHK-21 cells were highly permissive for FMDV infection) per well and were incubated at room temperature (RT) for 60 min (adsorption) or 37 °C for 60 min (internalization), with occasional rocking. Then the cells were washed three times with PBS and overlaid with DMEM containing 2% FBS and 2% carboxymethyl cellulose (CMC, Sigma-Aldrich) for plaque assay.