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JNK1/2 inhibitor reduces dengue virus-induced liver injury

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ABSTRACT

High viral load with liver injury is exhibited in severe dengue virus (DENV) infection. Mitogen activated protein kinases (MAPKs) including ERK1/2 and p38 MAPK were previously found to be involved in the animal models of DENV-induced liver injury. However, the role of JNK1/2 signaling in DENV-induced liver injury has never been investigated. JNK1/2 inhibitor, SP600125, was used to investigate the role of JNK1/2 signaling in the BALB/c mouse model of DENV-induced liver injury. SP600125-treated DENV-infected mice ameliorated leucopenia, thrombocytopenia, hemoconcentration, liver transaminases and liver histopathology. DENV-induced liver injury exhibited induced phosphorylation of JNK1/2, whereas SP600125 reduced this phosphorylation. An apoptotic real-time PCR array profiler was used to screen how SP600125 affects the expression of 84 cell death-associated genes to minimize DENV-induced liver injury. Modulation of caspase-3, caspase-8 and caspase-9 expressions by SP600125 in DENV-infected mice suggests its efficiency in restricting apoptosis via both extrinsic and intrinsic pathways. Reduced expressions of TNF-α and TRAIL are suggestive to modulate the extrinsic apoptotic signals, where reduced p53 phosphorylation and induced anti-apoptotic Bcl-2 expression indicate the involvement of the intrinsic apoptotic pathway. This study thus demonstrates the pivotal role of JNK1/2 signaling in DENV-induced liver injury and how SP600125 modulates this pathogenesis.

1. Introduction

Dengue virus (DENV) infection is one of the most important arbo-viral diseases of the 21st century, most prevalent in tropical and sub-tropical countries (Gubler, 2002). DENV-infected patients show different levels of disease severity; dengue fever, dengue hemorrhagic fever, or the most severe dengue shock syndrome. The hemorrhage signs of DENV infection are represented by plasma leakage and hematologic disorders. Severe infection may lead to hypovolemic shock in DENV infection (Halstead, 2007) with multiple organ injuries (Ghosh et al., 2011; Schmitz et al., 2011).

Liver injury is reported in severe DENV-infected patients (Trung et al., 2010) where apoptosis is evident (Limonta et al., 2007). Elevated alanine transaminase (ALT) and aspartate transaminase (AST) are observed in the patients (Arora et al., 2015; Nguyen et al., 1997; Treeprasertsuk and Kittitrakul, 2015) and animal models (Franca et al., 2010; Paes et al., 2005, 2009; Sreekanth et al., 2016; Sreekanth et al., 2014) of DENV infection. Histopathology correlated with transaminase level is used to understand the severity of liver injury (de Macedo et al., 2006; Huerre et al., 2001). Histopathology of DENV-induced liver injury in Balb/C mice was thoroughly studied (Sakinah et al., 2016). DENV infection induces apoptosis in HepG2 cells (Morchang et al., 2011; Thepparit et al., 2013; Thongtan et al., 2004). DENV infection undergoes apoptosis via activated caspase-8 (Liao et al., 2010), and overexpression of pro-inflammatory cytokines and chemokines including TNF-α, IL-8 and RANTES leads to vascular permeability in DENV-infected patients (Chareonsirisuthigul et al., 2007; Pang et al., 2007). DENV-induced TNF-α mediates apoptosis (Cardier et al., 2005), and TNF-related apoptosis-inducing ligand (TRAIL) modulates type I and

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type II interferon responses (Warke et al., 2008). The role of p53 in cellular functions including apoptosis is studied (Yan et al., 2016). The anti-apoptotic protein Bcl-2 depletion promotes death signals in DENV-infected HepG2 cells via the mitochondrial pathway of apoptosis (Catteau et al., 2003).

Mitogen-activated protein kinases (MAPKs) including p38 MAPK, JNK (c-Jun N-terminal kinase) and ERK1/2 (Extracellular-signal Regulated Kinase) are involved in apoptosis and cytokine responses. Phosphorylation of ERK1/2 and p38 induces liver injury in DENV-infected mice and treatments with ERK1/2 and p38 inhibitors minimized the liver injury (Sreekanth et al., 2014, 2016). JNK, the stress-activated protein kinase, is phosphorylated during inflammatory responses and an anthrapyrazolone inhibitor of JNK1/2, SP600125 was reported to inhibit this phosphorylation (Bennett et al., 2001). JNK phosphorylation is vital for DENV infection in human monocyte-derived macrophages (Ceballos-Olvera et al., 2010). However, the role of JNK1/2 in animal models of DENV-induced liver injury has never been investigated. We therefore investigated the effect of SP600125 on the inhibition of JNK1/2 and its molecular signaling in DENV-induced liver injury.

2. Materials and methods

2.1. Mouse infection, SP600125 treatment and sample collection

Male BALB/c mice were purchased from National Laboratory Animal Center (NLAC), Mahidol University, Thailand, and experiments were performed in compliance with ethics principles and institutional policies, with the protocol approved by the Siriraj Animal Care and Use Committee, Mahidol University (SI-ACUP 004/2556) and Siriraj Biosafety Risk Management Taskforce, Mahidol University (SI-2013-11). Eight week-old mice were infected with 4×10^5 FFU of DENV-2 (Strain 16881) intravenously via lateral tail vein. An un-infected group of mice is maintained with 2%-dimethyl sulfoxide (DMSO) treatment via the same route. DENV-infected mice were treated with 2%-DMSO (v/v) or SP600125 (dose of 20 mg/kg dissolved in 2%-DMSO). The volume of all injections was 0.4 ml and the SP600125 treatment was given 1 h before and 1 h and 24 h after DENV-infection. Blood samples were collected on both day 3 and 7 for serum preparations. At day 7 post-infection, the mice were euthanized with an intraperitoneal injection of sodium pentobarbital and liver tissues were collected and stored. For a total of three groups, six mice per group were challenged. Two independent experiments were conducted with a total of 36 mice.

2.2. DENV-NS1 viral RNA quantification and focus forming unit (FFU) assay

RNA was extracted from the serum and livers of uninfected, DENV-infected, and DENV-infected and SP600125-treated mice. DENV-NS1 viral RNA was quantified by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) using specific primers as previously described (Sreekanth et al., 2016). For FFU assay, liver tissues were homogenized in RPMI medium and centrifuged at 6000×*g* for 5 min repeatedly till clear supernatants were obtained. Supernatants were filter-sterilized for standard FFU assay (Jirakanjanakit et al., 1997).

Table 1
The specific primer set for each individual gene of interest.

Primer	Gene description
TNF-α F	5′ CCC CCA GTC TGT ATC CTT CT 3′
TNF-α R	5′ TTT GAG TCC TTG ATG GTG GT 3′
TRAIL F	5′ GAT GTT GGT GCC TGG AGT TT 3′
TRAIL R	5′ AAG CAA AGG GCA GAA AGT CA 3′
GAPDH F	5′ TGA ATA CGG CTA CAG CAA CA 3′
GAPDH R	5′ AGG CCC CTC CTG TTA TTA TG 3′

2.3. Histopathology and immunohistochemistry

Liver tissues were fixed in 10% formalin and paraffin embedded. Hematoxylin and eosin (H&E) staining was conducted for histopathology. The paraffin embedded liver tissues were allowed for standard immunohistochemistry staining with DENV-E antigen as previously mentioned (Aye et al., 2014).

2.4. Apoptotic mRNA expression profiler

RNA samples extracted from the liver tissues were converted to cDNA using SuperScript® III First-Strand Synthesis System (Invitrogen). The cDNA was further mixed with SYBR Green RT^2 qPCR Mastermix (Qiagen), and were aliquoted into the Mouse Apoptosis RT^2 Profiler™ PCR Array (Qiagen) containing 84 apoptosis related genes. The PCR amplification steps were performed in a Roche LightCycler 480 instrument and Ct values were copied and uploaded to the web program http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php for $2^{-\Delta\Delta Ct}$ analysis using β-actin as housekeeping gene control. The results were presented as fold increased or decreased compared to those of the uninfected group of mice.

2.5. Expression of pro-inflammatory cytokines by real-time RT-PCR

RNA samples were prepared from the liver tissues and reverse transcribed to cDNA, allowed to mix with LightCycler® 480 SYBR Green Mastermix (Invitrogen) and the specific primer set for each individual gene of interest (Table 1). The reactions were allowed to run in a Roche LightCycler 480 instrument for the Ct values. GAPDH (house-keeping gene control) was used to normalize the Ct values. The results were further analyzed by $2^{-\Delta\Delta Ct}$ analysis and the expressions were represented as fold increased or decreased.

2.6. Western blot analysis

Proteins from the livers were extracted in protease inhibitor premixed RIPA buffer and a cocktail of phosphatase inhibitor was added to detect phosphorylated proteins. Bradford assay (Bio-Rad Laboratories) was used to estimate the protein concentration. Proteins were separated with SDS-PAGE, blotted onto nitrocellulose membrane and further blocked with 5%-BSA or 5%-skim milk. Membranes were incubated overnight with rabbit anti-total JNK1/2 or mouse anti-phosphorylated JNK1/2 or rabbit anti-total p53 or rabbit anti-phosphorylated p53 (ser15) or rabbit anti-total p38 MAPK or mouse anti-phosphorylated p38 MAPK or mouse anti-total ERK1/2 or rabbit anti-phosphorylated ERK1/2 or goat anti-TNF-α or rabbit anti-caspase-8 or mouse anti-caspase-9 or goat anti-pro caspase-3 or rabbit anti-cleaved caspase-3 or rabbit anti-