Infection, Genetics and Evolution 51 (2017) 203–210

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Susceptibility and initial immune response of *Tupaia belangeri* cells to dengue virus infection

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ARTICLE INFO

Article history:
Received 24 January 2017
Received in revised form 3 April 2017
Accepted 5 April 2017
Available online 06 April 2017

Keywords:
Dengue virus
Tupaia fibroblast
Innate immunity
TLR

ABSTRACT

Dengue is an emerging disease of great public health significance worldwide. The lack of a suitable infection model has hampered dengue virus (DENV) pathogenesis study, and developing a suitable small animal model has been a long-standing challenge. The aim of this study was to develop a feasible experimental model of DENV infection using *Tupaia belangeri*. The susceptibility of tupaia to DENV infection and characteristics of its innate immune response were examined *in vitro*. We found that tupaia fibroblast cells support replication of DENV serotypes 1–4 with a linear increase in viral load 24–96 h post-infection in both cells and culture supernatants. DENV-2 resulted in the highest viral growth among all serotypes. To characterize the innate immune response in tupaia cells during the early phase of DENV infection, we first evaluated the evolutionary relationship between tupaia Toll-like receptors (TLR1–9) and those of other mammalian species. Phylogenetic analysis showed that tupaia TLRs are evolutionarily much closer to human than they are to rodent. We next established an innate immune response measurement system by assessing the mRNA expression of *TLR1–9* and four cytokines in DENV-infected tupaia cells. All serotypes induced the upregulation of *TLR8* mRNA expression in infected tupaia cells. Silencing of *TLR8* led to an increase in viral replication, indicating the existence of antiviral response through TLR8 on DENV infection. Although upregulation of *IFN-β* and *IL-6* expression was only observed in DENV-1 infected cells and a significant suppression of *TNF-α* was observed in DENV-2 infected cells alone, IL-8 was upregulated in all DENV-1–4. Thus, this study demonstrates for the first time the susceptibility of tupaia cells to DENV infections and the role of TLR8 in the anti-viral response of tupaia cells to DENV. These findings demonstrate the potential utility of tupaia as a model for DENV research in the future.

1. Introduction

Dengue is the most prevalent and rapidly spreading mosquito-borne viral disease affecting humans. It is caused by dengue virus (DENV), which is transmitted through the bites of infected *Aedes aegypti* and *Aedes albopictus* mosquitos. Recent estimates revealed that 3.9 billion people are at risk of acquiring DENV infection, and approximately 390 million DENV infections occur each year worldwide (Bhatt et al., 2013). DENV is a positive-strand RNA virus belonging to the *Flaviviridae* family. It circulates as four distinct but closely related serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), and infection with any of the four serotypes can cause dengue (Simmons et al., 2012). Though DENV infection in humans is often asymptomatic, it may lead to several clinical manifestations ranging from self-limited dengue fever to the potentially life-threatening dengue hemorrhagic fever and dengue shock syndrome (Simmons et al., 2012). The mechanisms underlying dengue-related diseases are still not completely understood. However, it is clear that a complex interplay of virus and host immune responses are likely to determine the outcome of DENV infection (Costa et al., 2013; Yacoub et al., 2013). In addition, evidence suggests a pivotal role of innate immunity during early DENV infection stages in priming both protection and disease induction (Costa et al., 2013).

DENV can infect various cell types, including dendritic cells, peripheral leukocytes, epithelial and endothelial cells, hepatocytes, and

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http://dx.doi.org/10.1016/j.meegid.2017.04.003

fibroblasts (Balsitis et al., 2009; Bonner and O'Sullivan, 1998; Diamond et al., 2000; Kurane et al., 1990; Kurane et al., 1992; Marianneau et al., 1996; Suksanpaisan et al., 2007). Innate immune responses represent the first line of defense and are promptly activated after DENV recognition by specific receptors named pattern-recognition receptors (PRRs), which leads to the activation of intracellular signaling pathways that induce the release of proinflammatory cytokines and type I interferons (IFNs) (Akira et al., 2006). An important class of PRRs, the Toll-like receptors (TLRs), has been shown to play a pivotal role during DENV infection. TLRs are evolutionarily conserved innate receptors expressed in various immune cells including dendritic cells, macrophages, B and T cells, and non-immune cells such as epithelial cells and fibroblasts. To date, 10 functional members of the TLR family have been identified in humans (Blasius and Beutler, 2010). TLR3 has been found to participate actively in early DENV infection stages and initiates strong IL-8 and IFN-α/β responses *in vitro* (Liang et al., 2011; Tsai et al., 2009). TLR7 and TLR8 were also found to promote antiviral mechanisms against DENV, leading to decreased viral replication *in vitro* and *in vivo* (Sariol et al., 2011; Sun et al., 2009).

There are currently no worldwide available vaccines or antiviral treatments for the prevention and control of DENV infection. A major obstacle complicating the progress of therapeutic and preventive interventions, as well as advancements in understanding DENV pathogenesis, is the lack of a suitable animal model that mimics dengue diseases in humans (Chan et al., 2015). Humans and mosquitos are so far the only known natural hosts for circulating DENV serotypes. While many animal models, including different types of mouse models, and non-human primates like rhesus monkeys, chimpanzees, and marmosets have been investigated for use in DENV infection studies, the ability to recapitulate the complete disease remains a challenge (Chan et al., 2015).

With a view to developing *Tupaia belangeri* (or tree shrew) as an animal model for DENV infection, the present study aimed to investigate the susceptibility of *tupaia* to DENV infection *in vitro*, using a tupaia fibroblast cell (T-238). Tupaia is a small mammal belonging to the *Tupaiidae* family, similar to a squirrel in appearance. Genomic analysis has revealed that tupaia is much more closely related to humans than it is to rodents (Fan et al., 2013; Tsukiyama-Kohara and Kohara, 2014). In addition, tupaia has been reported to be susceptible to several pathogenic viruses infecting humans, including hepatitis B virus (Sanada et al., 2016; Yang et al., 2015), hepatitis C virus (Amako et al., 2010; Xu et al., 2007), hepatitis E virus (Yu et al., 2016a), herpes simplex virus (Li et al., 2016), Newcastle disease virus and Sendai virus (Xu et al., 2015, Xu et al., 2016), until now there is no report of dengue virus susceptibility of tupaia cells. Also, the major limitation of using the tupaia as an animal model was the lack of tools for its characterization previously, therefore we have performed genome analysis to develop specific antibodies and cDNAs (Tsukiyama-Kohara and Kohara, 2014).

In the present study, we characterized the genomic sequence and transcript expression of the tupaia TLR genes and evaluated the susceptibility of tupaia cells to all DENV serotypes. We also characterize the innate immune response in tupaia cells upon DENV infection by assessing TLRs and cytokines expression.

2. Materials and methods

2.1. Cell cultures

Tupaia fibroblast cell line T-23 clone 8 (T-238) was established from a lung of a new-born male Tupaia as described previously (Taketomi et al., 1986) and provided from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Osaka, Japan). Human hepatoma cells (HuH-7), highly susceptible to DENV infection, were used as positive control. T-238 and HuH-7 cells were cultured at 37 °C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM) with high glucose and low glucose, respectively, supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 0.2% sodium bicarbonate.

2.2. Virus stocks and titration

The low-passage DENV strains used in this study, including DENV-1 strain Hue-525, DENV-2 strain Hue-397, DENV-3 strain Hue-453, and DENV-4 strain Hue-420, were isolated during a dengue outbreak that occurred in Hue, Vietnam in 2013. To generate working stocks, the viruses were propagated in C6/36 (E2) cells, as described previously (Aoki et al., 2006). The virus titer was determined by fluorescent focus assay (FFA) using C6/36 (E2) cells, as described previously (Payne et al., 2006), and expressed as fluorescent focus units (FFU) per ml.

2.3. DENV infection of cells

T-238 and HuH-7 cells were seeded into 60 mm cell culture plates at a density of 5×10^5 cells and maintained at 37 °C in a 5% CO_2 incubator. After 24 h, growth medium was removed, and cells were infected with DENV-1 strain Hue-525 at an multiplicity of infection (MOI) = 1. Cells were incubated for 1 h at 37 °C with gentle agitation every 10 min. Next, the inoculum was removed, and fresh culture medium was added. All cells were incubated at 37 °C with 5% CO_2. At 24, 48, 72, and 96 h post-infection, cells and culture supernatants were collected for DENV RNA quantification. T-238 cells were similarly infected with DENV-1 strain Hue-525, DENV-2 strain Hue-397, DENV-3 strain Hue-453, or DENV-4 strain Hue-420 at an MOI = 0.1 and incubated at 37 °C with 5% CO_2. At 24, 48, 72, and 96 h post-infection, cells and culture supernatants were collected for DENV RNA quantification.

2.4. DENV RNA quantification by quantitative reverse transcription PCR (qRT-PCR)

Viral RNA was extracted from culture supernatants using an ISOGEN-LS Kit (Nippon Gene, Japan) according to the manufacturer's instructions and eluted in 20 μl nuclease-free water. Total RNA was extracted from infected tupaia cells and HuH-7 cells using an ISOGEN Kit (Nippon Gene) according to the manufacturer's instructions and eluted in 50 μl nuclease-free water. The concentration and purity of each extracted RNA sample were determined using a Nanodrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., USA). All RNA samples were stored at −80 °C for further use.

Viral RNA copy numbers were quantified in culture supernatants and cells using Brilliant III Ultra-Fast SYBR® Green qRT-PCR Master Mix (Agilent Technologies, USA). Primers targeted a region of the non-structural protein 1 (*NS1*) gene that was highly conserved among the four DENV serotypes and consisted of a forward primer (5′-GTB CAC ACH TGG ACA GA-3′) as previously reported (Suwanwong et al., 2010) and a reverse primer (5′-KGH TAT TTG YTT CCA CA-3′). All reactions were performed in 96 micro-well plates using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA). Each 20-μl reaction was performed in triplicate. The cycling conditions comprised reverse transcription at 50 °C for 10 min, initial denaturation at 95 °C for 3 min, and 35 cycles at 95 °C for 5 s and 45 °C for 10 s. The standard curve was generated using serial 10-fold dilutions of the full-length DENV serotype 1 *NS1* gene (strain Hue-525). Tupaia and human *GAPDH* were used as endogenous controls for normalization of DENV quantification. Primers used for quantification of tupaia *GAPDH* mRNA are provided in Table S2. To quantify human *GAPDH* mRNA, TaqMan Human GAPDH Control Reagents including primer sets (Applied Biosystems) were used.

2.5. Immunofluorescence assay

T-238 cells were seeded in 24-well culture plates containing glass coverslips at 5×10^4 cells per well and maintained at 37 °C in a 5%