Virus Research 198 (2015) 53–58

Contents lists available at ScienceDirect

Virus Research

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

An embryonic heart cell line is susceptible to dengue virus infection

Antonio H. Angel-Ambrocio[a,1], Rubén Soto-Acosta[a,1], Eshwar R. Tammineni[b], Elba D. Carrillo[b], Patricia Bautista-Carbajal[a], Ascención Hernández[b], Jorge A. Sánchez[b], Rosa M. del Angel[a,*]

[a] Departamento de Infectómica y Patogénesis Molecular, México, D.F., Mexico
[b] Departamento de Farmacología, Centro de Investigación y Estudios Avanzados del IPN, México, D.F., Mexico

ARTICLE INFO

Article history:
Received 2 December 2014
Accepted 7 January 2015
Available online 15 January 2015

Keywords:
Dengue
Cardiomyocytes
H9c2 cells
Infection

ABSTRACT

Dengue virus (DENV) is the causative agent of dengue fever. In recent years, patients with more severe form of the disease with acute heart failure or progression to cardiogenic shock and death have been reported. However, the pathogenesis of myocardial lesions and susceptibility of cardiomyocytes to DENV infection have not been evaluated. Under this perspective, the susceptibility of the myoblast cell line H9c2, obtained from embryonic rat heart, to DENV infection was analyzed. Our findings indicate that H9c2 cells are susceptible to the infection with the four DENV serotypes. Moreover, virus translation/replication and viral production in this cell line is as efficient as in other susceptible cell lines, supporting the idea that DENV may target heart cells as evidenced by infection of H9c2 cells. This cell line may thus represent an excellent model for the study and characterization of cardiac physiopathology in DENV infection.

1. Introduction

Dengue Virus (DENV), a positive-single strain RNA virus of the *Flaviviridae* family is the causative agent of dengue fever. In most cases, the symptoms of dengue fever are self-limited. However in a small number of people the disease progresses to the severe form of the infection called severe dengue, previously called dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Severe dengue disease is characterized by plasma leakage and organ dysfunction. These injuries may be a consequence of viral replication in a specific organ or a result of the exacerbated immune response during infection. Most of the histopathological studies performed on dengue patients are mainly obtained from fatal cases and it is not clear whether the tropism observed in fatal cases or in the mice model is the same that the one detected in patients with a dengue fever infection (Balsitis et al., 2009; Bhoopat et al., 1996; Couvelard et al., 1999). Additionally, some tissues that are positive for viral antigens may not be targets for active viral replication (Balsitis et al., 2009; Jessie et al., 2004). Specifically, it has been described that DENV is able to infect resident cutaneous Langerhans dendritic cells (DCs). Additionally, monocytes and macrophages, as well as B and T cells have been characterized as the primary targets of the viral infection in humans and in mice *in vivo* (Blackley et al., 2007; King et al., 1999; Martins Sde et al., 2012; Mota and Rico-Hesse, 2011). Hematopoietic lineage cells as well as some non-hematopoietic origin cells are permissive to DENV during natural infection such as hepatocytes and Kupffer cells (Huerre et al., 2001; Jessie et al., 2004). Endothelial cells have also been documented as targets for DENV infection (ECs) (Balsitis et al., 2009; Zellweger et al., 2010). However, in recent years it has been described atypical clinical symptoms in dengue patients, involving the lung, kidney, central nervous system and heart. Injuries in these organs have been confirmed by hemorrhage, edema and inflammatory infiltrates (Povoa et al., 2014; Salgado et al., 2010; Setlik et al., 2004).

Interestingly, in patients with the more severe form of the disease, progression to cardiogenic shock and death has been reported (Kularatne et al., 2006; Nagaratnam et al., 1973; Obeyesekere and Hermon, 1973), however, the pathogenesis of myocardial lesions and the infection of cardiac cells have been poorly studied (Miranda et al., 2013; Salgado et al., 2010). Moreover, the susceptibility of cardiomyocytes to DENV infection has not been described. Under this perspective and given the importance of myocardial lesion in severe dengue pathogenesis, the susceptibility of the myoblast cell line H9c2, obtained from embryonic rat heart to DENV infection was analyzed.

* Corresponding author. Tel.: +52 55 57473800; fax: +52 55 57473377.
E-mail addresses: rmangel@cinvestav.mx, rmdelangel@gmail.com (R.M. del Angel).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.virusres.2015.01.004
0168-1702/

2. Materials and methods

2.1. Cell culture

H9c2 cells (passage 17–24, American Type Culture Collection) were cultured in monolayers in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), sodium bicarbonate 1.5 g/l, penicillin (50 IU), and streptomycin(50 μg/ml) under atmospheric conditions of 95% air and 5% CO_2 at 37 °C. Stocks of cell lines were propagated in culture flasks for successive passage. Cell culture medium was replaced with fresh medium every 2 days.

VERO cells (passage 18–23, American Type Culture Collection) were cultured in monolayers in advanced DMEM supplemented with 2 mM glutamine, penicillin (5×10^4 U/ml)–streptomycin (50 mg/ml), 5% fetal calf serum (FCS) and 1 ml/l of amphotericin B (Fungizone) under atmospheric conditions of 95% air and 5% CO_2 at 37 °C. Stocks of cell lines were propagated in culture flasks for successive passage. Cell culture medium was replaced with fresh medium every 2 days.

The human monocytic cell line, constitutively expressing DC-SIGN (U937-DC SIGN) receptor (a kind gift from Dr. Aravinda M. de Silva from the University of North Carolina School of Medicine) was grown in RPMI Advanced medium supplemented with 5% fetal bovine serum, 2 mM glutamine, 2× penicillin/streptomycin and 5 ml/l fungizone at 37 °C under a 5% CO_2 atmosphere.

2.2. Virus

Propagation of four DENV serotypes: serotype 1 YUC18494 strain (clinical isolate from a patient with DENV, was kindly donated by Dr Isabel Salazar IPN-Mexico); DENV serotype 2 New guinea strain; DENV serotype 3 H87 strain and DENV serotype 4 H241 strain, was carried out in CD1 suckling mice brains and titers were determined by plaque assays in BHK-21 cells as previously described (Mosso et al., 2008). CD1 suckling mice brains from mock infected mice were used as control.

2.3. Infection of H9c2 cells with DENV

Before infection, H9c2 cells were washed three times with Hanks medium, and infected with DENV (serotype 1, 2, 3 or 4) at different MOIs in serum-free medium and infection was allowed for 2 h at 37 °C. Thereafter, cells were washed with acid glycine (pH3) to inactivate non-internalized virus, washed 3 times with PBS and fresh DMEM supplemented with serum was added. Infection was permitted for different times at 37 °C.

2.4. Western blot analysis

For Western blot analysis H9c2 cells were grown in p100 plates, infected with DENV1, DENV2, DENV3 and DENV4 at MOI of 1. Twenty four hours after infection cells were lysed with lysis buffer containing protease inhibitors. Thirty microgram of protein were separated by 10% SDS–PAGE and transferred to a nitrocellulose membrane (BIO-RAD). Membranes were blocked with 5% of non-fat milk in PBS. Actin protein was detected by using an anti-actin mouse antibody (1:2000, SIGMA A3853), while NS3 was detected by an anti NS3 antibody (GENETEX 124252) diluted 1:1000. An anti-rabbit HRP antibody and an anti-mouse HRP (1:50,000) were used as secondary antibodies. Proteins were visualized by Immobilon Western Chemiluminescent HRP Substrate (MILLIPORE).

2.5. Flow cytometry and immunofluorescence

Infected H9C2 cells were grown in slide and 6-wells plates, for confocal microscopy and FACS assays, respectively, cells were infected as indicated above. After 24 h of infection, slides or harvested cells were fixed with 1% formaldehyde, incubated for 20 min with permeabilized solution (PBS 1%, saponin 0.1% and FBS 1%), and incubated for 2 h at room temperature with anti-E (4G2 monoclonal antibody 1:100), detected with donkey anti mouse-Alexa 488 and anti-NS3 (GENETEX 124252 1:1000) or anti-Capsid (GENETEX 103343) and detected with goat anti rabbit-Alexa 555. After each step, cells were washed once with cold permeabilized solution by centrifugation at 2500 rpm for 6 min. Slides were observed in a Zeiss LSM700 laser confocal microscopy and flow cytometry was performed in a BD LSR Fortessa quantifying 30,000 events. Cell viability of H9C2 cells was assessed by flow cytometry through the propidium iodide (PI) uptake.

2.6. Real time RT-PCR analysis

Conventional RT-PCR performed from isolated RNA, using DV2C-L 5′-CAATATGCTGAAACGCGAGA-3′ and DV2C-R: 5′-TGCTGTTGGTGGGATTGTTA-3′ primers (Prada-Arismendy et al., 2012) amplified a 151-pb fragment of the dengue virus capsid gene. This PCR product was cloned in the plasmid pJet1.2 Vector System (Thermo). The recombinant plasmid was purified and quantified through spectrophotometry at 260 nm to prepare a dilution containing 10^{10} copies of plasmid/ml using the formula:

$$\text{Number of copies} = \frac{6 \times 1023\,\text{copies/mol} \times \text{concentration (g/μl)}}{\text{Plasmid molecular weight}} + \text{insert (g/μl)}$$

Serial dilutions of the plasmid (10^9 to 10^2 copies/ml) were prepared.

Reverse transcription used 1 μg of total RNA from each experimental condition, and was performed by using random primers (Promega) at a concentration of 0.025 μg/μl and the reverse transcriptase enzyme ImpromII (Promega) at 25 °C for 5 min, 42 °C for 60 min, 70 °C for 15 min. For real-time PCR amplification, SYBR Fast universal (Kapa) was used in the Eco Illumina System apparatus. The reaction mix contained 1 μl of cDNA and 5 μl of Master Mix 2×. The amplification protocol included 2 min at 50 °C, 2 min at 95 °C, and 40 cycles at 95 °C for 5 s and 30 cycles at 55 °C. Finally, a dissociation curve was generated heating the PCR products from 55 °C to 95 °C to confirm primer dimers absence. A standard curve was generated.

2.7. NS1 secretion analysis

Supernatants of infected cells were analyzed for NS1 secretion by ELISA (Platelia, Biorad) (Ludert et al., 2008).

3. Results

3.1. The four DENV serotypes are able to infect H9c2 cells

The myoblast cells line H9c2 was selected to evaluate the susceptibility of the cardiac cells to DENV infection. This cell line was obtained from embryonic rat heart and represents a useful experimental model for cardiac cells because of genetic homogeneity, ease of availability and genetic manipulation (Mejia-Alvarez et al., 1994).

To initially address the susceptibility of H9c2 cells to DENV, the cells were infected with DENV serotype 1 YUC18110 strain (clinical isolate from a patient with DENV in Yucatan, Mexico), DENV serotype 2 New Guinea strain, DENV serotype 3 H87 strain and DENV serotype 4 H241 strain at a MOI of 1 for 24 h and the