Dengue Virus Infection of Primary Human Smooth Muscle Cells

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Abstract. Dengue virus (DENV) infection of humans is presently the most important arthropod-borne viral global threat, for which no suitable or reliable animal model exists. Reports addressing the effect of DENV on vascular components other than endothelial cells are lacking. Dengue virus infection of vascular smooth muscle cells, which play a physiological compensatory response to hypotension in arteries and arterioles, has not been characterized, thus precluding our understanding of the role of these vascular components in dengue pathogenesis. Therefore, we studied the permissiveness of primary human umbilical artery smooth muscle cells (HUASMC) to DENV 1–4 infection and compared with the infection in the previously reported primary human umbilical vein endothelial cells (HUVEC) and the classically used, non-transformed, and highly permissive Lilly Laboratories Cell-Monkey Kidney 2 cells. Our results show that HUASMC are susceptible and productive to infection with the four DENV serotypes, although to a lesser extent when compared with the other cell lines. This is the first report of DENV permissiveness in human smooth muscle cells, which might represent an unexplored pathophysiological contributor to the vascular collapse observed in severe human dengue infection.

INTRODUCTION

Dengue virus (DENV) is a member of the genus *Flavivirus* within the *Flaviviridae* family, for which five serotypes have been described (DENV 1–5).¹ The virion comprises an enveloped spherical particle that harbors a positive single-stranded RNA genome.² Dengue virus is transmitted by mosquito vectors, mainly *Aedes aegypti*, and is considered the most important arthropod-borne viral disease worldwide.³

Dengue virus represents a global threat, for which there is no specific treatment available. Although a tetravalent, live-attenuated, dengue vaccine was recently approved,⁴ safety concerns⁵ have highlighted the urgent need for antiviral drugs to treat DENV infections. However, the development of an antiviral drug targeting viral factors of all DENV serotypes has been problematic. New promising approaches rely on the targeting of host factors to achieve antiviral activity.⁶ Nevertheless, this strategy requires an in-depth understanding of the pathogenesis of dengue disease, including the identification of the key cellular targets involved in severe infections.

The determinants of dengue disease severity are complex and multifactorial, and although several models have been developed over the years to bridge translation from in vitro to human observational studies, no laboratory animals (wild-type or genetically modified) develop all of the clinical manifestations of severe dengue disease in humans.⁷ Dengue virus infects many animal cell lines such as the highly permissive baby hamster kidney cells (BHK-21), C6/36, Vero and LLC-MK2 (macaque kidney cells), and human cell lines such as HepG2, U937, and HEK-293, with varying degrees of permissiveness.^{8–10} However, the question remains as to whether those cell types represent relevant targets of DENV infection in vivo. Therefore, most conclusions regarding the in vivo situation in humans rely on postmortem studies or on the in vitro permissiveness of primary cells such as human umbilical vein endothelial cells (HUVEC) and peripheral blood mononuclear cells to DENV.^{8,11}

Postmortem studies depend on the detection of DENV antigens in tissues. The most specific marker of DENV infection in vivo is the nonstructural protein 3 (NS3) because it does not enter the secretory pathway, and thus demonstrating exclusively intracellular localization.^{11,12} However, localization of NS3 protein in tissues varies depending on which host species is analyzed. In mice, NS3 was detected in phagocytes of the spleen and lymph nodes, as well as in hepatocytes and myeloid cells in the bone marrow.¹¹ In human postmortem tissues, the NS3 protein was detected in phagocytes of spleen and lymph nodes, hepatocytes and endothelial cells in spleen, perivascular cells in brain, and alveolar macrophages in lungs of DENV severe cases.¹¹ Others reported that skeletal and cardiac muscle cells are also infected in vivo.^{13,14} In addition, it has been shown that myotubes can also be infected in vitro.¹⁴

Endothelial cells have long been implicated in the pathophysiology of DENV infection. Microvascular and endothelial dysfunctions are associated with the severity of dengue, and this occurs before the appearance of severe clinical manifestations.¹⁵ Indeed, there are tests and treatments to identify and handle various forms of vascular dysfunction that could be applied for the clinical management of patients with severe dengue.³ It has been reported that endothelial cells are permissive to DENV infection in vitro although they produce low viral titers.¹⁶ Nevertheless, DENV infection of ECV304 human endothelial cells leads to chemokine production and complement activation, suggesting an important role in microvascular dysfunction during DENV pathophysiology.¹⁷ Moreover, others have shown the effect of DENV infection on gene expression in HUVEC cells and identified potentially novel mechanisms involved in dengue disease manifestations such as hemostatic disturbances.¹⁸ However, only a small percentage of endothelial cells were productively infected in vitro using the DENV-2 16681 strain.¹⁹ Despite these observations, the importance of endothelial cells as targets of DENV infection in vivo remains a subject of debate.

Reports addressing the effect of DENV on other vascular components such as smooth muscle cells, which play a physiologically relevant role in arteries and arterioles, are lacking. Dengue virus infection of vascular smooth muscle cells has not been characterized, thus precluding our understanding of the role of these vascular components in

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dengue pathogenesis. To address this issue, here we worked with human umbilical artery smooth muscle cells (HUASMC), which are primary smooth muscle cells isolated from normal healthy human umbilical arteries. Human umbilical artery smooth muscle cells have been used along HUVEC to study the dynamics, maturation, and effects of toxic stimulus on blood vessels, and constitutes a suitable and well-validated model that could be applied on DENV research.^{20,21} This work describes for the first time a DENV-permissive infection of primary arterial smooth muscle cells in vitro, which might represent an unexplored pathophysiological contributor to the reduced vascular reactivity to hypotension observed during dengue shock syndrome and dengue hemorrhagic fever.

MATERIALS AND METHODS

Viruses. Dengue virus-1 Angola (D1/AO/XX/1988) and DENV-4 Dominica (D4/DM/814669/1981) strains were supplied by the Instituto de Medicina Tropical Pedro Kourí, Havana, Cuba. The clinical isolates from Costa Rican patients DENV-2 10066 (D2/CR/10066/2007) and DENV-3 14531 (D3/CR/14531/2007) were provided by the Instituto Costarricense de Investigación y Enseñanza en Nutrición y Salud, Cartago, Costa Rica.²² Viruses were produced in C6/36 cells from *Aedes albopictus* (ATCC, Manassas, VA) by inoculating cellular monolayers with DENV at a multiplicity of infection (MOI) of 0.01 and incubating for 3 days with Roswell Park Memorial Institute-1640 medium supplemented with 2% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD) at 33°C in an atmosphere of 5% CO₂. Then, culture supernatant was collected and centrifuged at 3,000 × *g* for 10 minutes. Before storage at -80°C, 23% newborn calf serum (Gibco) was added.⁹ Culture supernatant from uninfected C6/36 cells was collected and used as negative control (mock control). Viruses were titrated by plaque assay in BHK-21 cells (ATCC) as previously described.²³ Briefly, 10-fold serial dilutions of viruses were added to BHK-21 confluent monolayers. After 2 hours of adsorption, cells were incubated at 37°C in an atmosphere of 5% CO₂ for 5 days with minimum essential medium (MEM) supplemented with 2% FBS (Gibco) and 1% carboxymethylcellulose (Sigma, St. Louis, MO). Plaque numbers were counted after staining with crystal violet.

Cell lines and virus infections. Human umbilical artery smooth muscle cells and HUVEC were purchased and maintained in smooth muscle cell growth medium and endothelial cell growth medium, respectively, according to the manufacturer's instructions (Cell Applications, San Diego, CA). LLC-MK2 cells (ATCC) were grown in MEM supplemented with 10% FBS. Cell monolayers were DENV or mock infected at a MOI of 1 and allowed virus adsorption for 2 hours at 37°C. After three washes with phosphate-buffered saline (PBS), cells were incubated with 2% FBS medium at 37°C in an atmosphere of 5% CO₂ for different times. All experiments were performed with the same number of HUASMC, HUVEC, and LLC-MK2 cells.

Plaque assays for virus quantification. Culture supernatants of HUASMC were collected at 0, 24, 48, and 72 hours postinfection (p.i.) and DENV infectious particles were quantified by plaque assays in BHK-21 cells, as described earlier. Concomitantly, supernatants from HUVEC and LLC-MK2 cell cultures at 72 hours p.i. were titrated.

Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for genome copies quantification.

Culture supernatants of HUASMC cells were collected at 0, 24, 48, and 72 hours p.i. and DENV genomes were quantified by RT-qPCR. Briefly, viral RNA was extracted with the NucleoSpin RNA virus kit (Macherey-Nagel, Düren, Germany) and quantified using the Genesig RT-qPCR advanced kit for dengue virus (Primerdesign, Southampton, United Kingdom) according to the manufacturer's instructions. The reactions were carried out with a StepOne™ real-time PCR system (Applied Biosystems, Carlsbad, CA). Supernatants from HUVEC and LLC-MK2 cell cultures at 72 hours p.i. were also tested.

Indirect immunofluorescence for DENV infected cells quantification. Human umbilical artery smooth muscle cells, HUVEC, and LLC-MK2 cells were cultured on glass coverslips coated with 1% gelatin (Sigma) in 24 well plates seeded with 100,000 cells per well. At 72 hours p.i., cells were fixed with cold acetone for 10 minutes, washed with PBS, and stored at -20°C. Afterward, the slides were treated with 50 mM NH₄Cl for 10 minutes and incubated with a 1:300 dilution of mouse anti-DENV 1, 2, 3 and 4 envelope protein monoclonal antibody (GTx29202; GeneTex, Irvine, CA) or a 1:800 dilution of rabbit anti-DENV NS3 protein polyclonal antibody (GTx124252; GeneTex) for 1 hour at 37°C. After washing, the coverslips were incubated for 30 minutes at 37°C with 1:75 diluted fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) (DAKO, Glostrup, Denmark) in 0.02% Evans blue or 1:400 dilution of Alexa Fluor 647 goat anti-rabbit IgG (Invitrogen, CA) in PBS. Stained slides were mounted with Prolong Gold with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA) and images were acquired with a Cytation 3 Cell Imaging Multi-Mode Reader (BioTeK, Winooski, VT). Image analysis of the whole coverslip was performed with the software CellProfiler 2.0 (<http://www.cellprofiler.org>; Broad Institute, Cambridge, MA).

Statistics. Data are expressed as mean ± standard deviation of three independent experiments. Statistical significance of the differences between mean values was determined by using an unpaired Student's *t*-test. The level of significance is denoted in each figure.

RESULTS

All four dengue serotypes are able to replicate in HUASMC cells. To test the permissiveness of HUASMC to DENV infection, confluent cell monolayers were infected at a MOI of 1 with each of the four DENV serotypes. Virions were quantified in culture supernatants every 24 hours for 72 hours. The supernatants of HUASMC monolayers infected with the four DENV serotypes exhibited an increasing number of plaque-forming units (PFU) after 48 hours p.i. (Figure 1A). However, there were significantly higher replication efficiencies of the different DENV serotypes in HUVEC and LLC-MK2 cell line when compared with HUASMC at 72 hours after infection (Figure 1B). These results demonstrate that the HUASMC line is permissive to DENV infection by all four serotypes; however, virion production is lower in these cells than in the frequently used HUVEC and LLC-MK2 cells.

To confirm the permissiveness of HUASMC to DENV infection and estimate the replicative fitness of the virus in this cell line, monolayers were infected at a MOI of 1 with the four DENV serotypes. Dengue virus RNA was quantified from culture supernatants every 24 hours for 72 hours. The supernatants of HUASMC monolayers infected with the four DENV

FIGURE 1. Human umbilical artery smooth muscle cells (HUASMC) are permissive to dengue virus (DENV) infection. Virion production and release was quantified by plaque assays of culture supernatants at different time points post-infection (p.i.) of HUASMC, human umbilical vein endothelial cells (HUVEC), and LLC-MK2 (macaque kidney cells) infected with each of the four DENV serotypes (multiplicity of infection:1). (A) Infection kinetics of HUASMC cells (24–72 hours p.i.) with DENV 1–4 measured by plaque assays (plaque-forming units [PFU]/mL). (B) DENV 1–4 titers in culture supernatants of HUASMC, HUVEC, and LLC-MK2 cells at 72 hours p.i. Data are expressed as the mean ± standard deviation of three independent experiments. *** $P < 0.001$ compared with its HUVEC and LLC-MK2 cells counterparts. (D) l. = assay detection limit.

serotypes showed an increase in DENV genomic RNA copies at 48 hours p.i. (Figure 2A). Nevertheless, the production of infectious virions and genomic RNA from the different DENV serotypes was significantly higher in HUVEC and LLC-MK2 cells than in HUASMC cells at 72 hours after infection (Figure 2B). Thus, the replicative fitness of DENV in HUASMC cells was significantly lower than that observed in HUVEC and LLC-MK2 cell lines, based on the genome-to-PFU ratios calculated at 72 hours p.i. (Figure 2C).

Dengue virus antigens are detected by immunofluorescence in HUASMC. Human umbilical artery smooth muscle cells' monolayers infected with the four DENV serotypes were stained by indirect immunofluorescence to

quantify cellular infection. After 72 hours of infection, HUASMC, HUVEC, and LLC-MK2 cells were stained with an anti-DENV 1, 2, 3 and 4 envelope protein monoclonal antibody and fluorescence images were analyzed using the software CellProfiler 2.0. In contrast to the mock control (Figure 3A), infected HUASMC showed cytoplasmic green fluorescence staining (Figure 3B and C, white arrows), which was automatically identified by image analysis (Figure 3D, green outlines) to calculate the percentage of infected cells against the total number of identified cellular nuclei (Figure 3D, white outlines). As expected, the LLC-MK2 cell line showed higher percentages of positive cells with all four DENV serotypes compared with HUASMC cells (Figure 3E). By contrast,

FIGURE 2. Human umbilical artery smooth muscle cells (HUASMC) release dengue virus (DENV) genomes on infection. Dengue virus genomic RNA was quantified by real-time qRT-PCR from cell culture supernatants of HUASMC, human umbilical vein endothelial cells (HUVEC), and LLC-MK2 (macaque kidney cells) infected with DENV (multiplicity of infection:1). (A) Dengue virus 1–4 infection kinetics (24–72 hours post-infection [p.i.]) of HUASMC cells measured by real-time genomic qRT-PCR (copies/mL). (B) Genome copies present in culture supernatants of HUASMC, HUVEC, and LLC-MK2 cells at 72 hours p.i. with the four DENV serotypes. (C) Calculated genome-to-plaque-forming unit (PFU) ratios of HUASMC, HUVEC, and LLC-MK2 cells supernatants at 72 hours p.i. with each DENV serotype. Data are expressed as the mean $\pm$ standard deviation of three independent experiments. * P < 0.05, ** P < 0.005, and *** P < 0.001 calculated to its HUASMC counterpart.

HUASMC displayed a small percentage of cells (5–15%) with positive staining, indicating that this cell line has a low permissiveness to DENV infection with all four serotypes. However, only DENV-2 and DENV-3 led to a higher antigen production in HUVEC cell line compared with HUASMC cells (Figure 3E). Finally, no difference was observed in the identification of HUASMC-infected cells by immunostaining with an anti-DENV 1–4 envelope protein-specific monoclonal antibody and an anti-NS3 polyclonal antibody (Figure 3F), which indicates that the detected antigens are produced *de novo* during the infection. These results demonstrate that DENV antigens can be detected in HUASMC despite the low permissiveness as shown by the low percentage of infected cells compared with HUVEC and LLC-MK2 cells.

DISCUSSION

Research on DENV pathophysiology has been hampered by the lack of competent animal models for reproducing the *in vivo* human infection.⁷ Therefore, most conclusions regarding the pathophysiological mechanisms of this disease in humans rely on postmortem studies or are extrapolations from the *in vitro* permissiveness of primary cells to DENV.^{8,11} A key remaining question regarding DENV pathophysiology is the role of alterations in the different cellular components of the blood vessel. This has been addressed for endothelial cells because they are the major component of capillary blood vessels, and the microvascular dysfunction is closely associated with the severity of dengue.¹⁵ Our findings confirm that DENV do infect the endothelial cell line HUVEC, as shown previously.¹⁶ Indeed, future work is necessary to assess whether direct dengue viral infection of endothelium is the major cause of the extensive vascular leakage, which has been previously observed in patients with dengue hemorrhagic fever and dengue shock syndrome.¹⁹

A neglected component of the tissue response to this extensive vascular leakage has to do with the physiological compensatory mechanisms associated with the response of arterioles and particularly with the regulation of vascular diameter by smooth muscle cells present in the arteriolar wall. Significant vascular leakage and the resulting hypovolemia trigger vasoconstriction of arterioles to compensate for hypotension.²⁴ During hemorrhagic shock, the vascular hyporeactivity is related to a desensitization to calcium and mitochondrial dysfunction in smooth muscle cells in blood vessels.^{25,26} In addition, damage to lymphatic smooth muscle cells in collecting lymphatic vessels leads to an impairment in lymph formation and interstitial fluid balance, generating edema and thereby perturbing blood volume recovery.²¹ Therefore, the observed effect of DENV infection in smooth muscle cells could play an important role in precipitating the outcome of severe shock due to a deficient compensatory response to hypotension. Our observations in cell culture conditions may thus reveal a hitherto unexplored mechanism of vascular pathology in DENV infection.

In the present work, we compared the permissiveness of primary HUASMC cells, primary HUVEC cells, and the model cell line LLC-MK2 with DENV strains of the four serotypes. The results demonstrate that HUASMC cells are permissive to DENV infection by all four serotypes. However, virus production and replicative fitness are significantly lower in this cell line than in HUVEC and LLC-MK2 cells. Indeed, on infection, HUASMC cells displayed a small percentage of DENV

FIGURE 3. Dengue virus (DENV) antigens are detected in human umbilical artery smooth muscle cells (HUASMC). Epifluorescence images of immunostained cells with an anti-DENV 1-4 envelope protein-specific monoclonal antibody (green), and a cytoplasmic (red) and nuclei (blue) counterstains. The images were captured at 72 hours post-infection (p.i.) with each DENV serotype at a multiplicity of infection (MOI) of 1. (A) Representative image of mock-infected HUASMC cells at $\times 100$ magnification (scale bar = 50 μm). (B and C) Representative images of DENV-infected HUASMC (arrows) at $\times 400$ (scale bar = 20 μm) and $\times 100$ (scale bar = 50 μm) magnification, respectively. (D) Image analysis for quantifying infected cells (green outline) vs total cell nuclei (white outlines) with the software CellProfiler 2.0. (E) Percentages of infected cells in HUASMC, human umbilical vein endothelial cells (HUVEC), and LLC-MK2 (macaque kidney cells) cell lines. (F) Comparison of DENV labeling by immunostaining with an anti-DENV 1-4 envelope protein-specific monoclonal antibody (green) and an anti-NS3 polyclonal antibody (red) in HUASMC cells at 72 hours p.i. with DENV-2 and DENV-3 at a MOI of 1. Magnification of $\times 400$ (scale bar = 20 μm). Data are expressed as mean $\pm$ standard deviation of three independent experiments. ** $P < 0.005$, *** $P < 0.001$ calculated to its HUASMC cells counterpart. This figure appears in color at www.ajtmh.org.

antigen-positive cells, indicating that this cell line has a low permissiveness to DENV infection by all four serotypes. Although it is evident that new infectious viral particles were produced by infected HUASMC (Figure 1), the genome copies

did not increase (for DENV1 and DENV2) or only increased one log after 48 hours p.i. (for DENV3 and DENV4), as shown in Figure 2. This observation suggests that most of the genomes detected in the supernatant are from defective particles

produced by these cells or genomes released from dead cells, which occlude the expected elevation associated with the increased infectious particles.

In the context of a viral infection with low permissiveness, a possibility to explain this phenomenon arises if the infected cells are able to replicate the viral genomes, but there is a problem with virion assembly or maturation in a high proportion of infected cells. These genomes would be eventually released from the cells, explaining the high genome copies at all-time points assessed. Only the viral particles produced from a subpopulation of infected cells with relatively higher permissiveness would represent the PFUs, which are increasing progressively over time. This is probably due to a viral morphogenesis problem in infected HUASMC cells and, therefore, the viral antigens are detected only in a small proportion of cells. Nevertheless, the immunofluorescence data demonstrate that new viral proteins are produced at least in the subpopulation of cells that produce viable viral particles (Figure 3). Indeed, permissiveness was very similar for HUASMC and HUVEC cells, both of which derive from the umbilical cord, where they form functional blood vessels.²⁰ Altogether, these results support a model where DENV induces the dysfunction of smooth muscle cells, thereby contributing to the vascular hyporeactivity in vivo.

The compensatory mechanisms to hypovolemia include an early sympathetic response characterized by increased heart rate and systemic increments in vascular resistance, which are mostly mediated by the action of catecholamines, especially noradrenaline, in cardiac muscle and in arteriolar smooth muscle cells.^{27,28} In addition, this compensatory vasoconstriction is mediated by thromboxane A₂-triggered signaling in smooth muscle cells.²⁹ It has been demonstrated that plasma levels of thromboxane A₂ are significantly lower in dengue shock syndrome patients than in healthy populations and patients with dengue hemorrhagic fever but without shock.³⁰ This suggests that smooth muscle cells are already hyporeactive during DENV-induced shock. Thus, the infection of those cells by DENV would further contribute to vascular dysfunction in vivo. In support of this contention, work by Balsitis and collaborators displays a splenic artery highly positive for NS3 staining located within the muscular layer in the arterial wall,¹¹ suggesting that the infection of smooth muscle cells might occur in vivo during the DENV infection in humans.

This is the first report of DENV-permissive infection of smooth muscle cells. Despite the limitations of an in vitro model of infection, our results suggest that the infection of arteriolar, arterial, or lymphatic smooth muscle cells could have important implications for DENV-induced shock. Further work is required to demonstrate the infection and dysfunction of these cells in vivo and to design strategies to protect them for cardiovascular homeostatic mechanisms. This protection may represent a new approach in the treatment of DENV-induced hypotension.

Received February 27, 2018. Accepted for publication August 14, 2018.

Published online November 5, 2018.

Acknowledgments: We thank Carlos Vargas Eduarte for his invaluable technical support and assistance, as well as José María Gutiérrez Gutiérrez from Instituto Clodomiro Picado (Universidad de Costa Rica) for his scientific advice and critical reading of the manuscript. We are

also grateful to Christine Carrington (The University of West Indies) for English proofreading of the manuscript.

Financial support: This work was supported by Universidad de Costa Rica (project VI-803-A5-025), Consejo Nacional para Investigaciones Científicas y Tecnológicas (project FI-182-10), and the Florida Ice and Farm Co.

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