DENGUE VIRUS INFECTS HUMAN ENDOTHELIAL CELLS AND INDUCES IL-6 AND IL-8 PRODUCTION

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Abstract. In this study dengue virus (DV) was found to infect primary endothelial cells derived from human umbilical cord veins (HUVEC) and alter their cytokine production. Dengue virus infection of HUVEC was confirmed by an increase in plaque-forming units in the culture supernatant and by immunofluorescence assay. HUVEC produced large amounts of interleukin (IL)-6 and IL-8 but not IL-1β after DV infection. Both the replication of DV and the production of IL-6 and IL-8 by HUVEC after DV infection were inhibited by ribavirin, an antiviral synthetic guanosine analogue. Additionally, increased serum levels of IL-6 and IL-8 were observed in patients with dengue hemorrhagic fever but not dengue fever. Therefore, our results suggest that endothelial cells can be a target for DV infection, and that DV-induced IL-6 and IL-8 production by endothelial cells may contribute to the pathogenesis of dengue hemorrhagic fever.

INTRODUCTION

Dengue viruses are mosquito-borne flaviviruses which are subgrouped into four antigenically-related serotypes.1 Primary infection generally results in mild dengue fever (DF). However, secondary infection with different serotypes might cause fatal dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS).2 There are at least two different hypotheses which have been proposed to explain the pathogenesis of DHF/DSS. The first is the antibody-dependent enhancement (ADE) theory, which is focused on the immune status of the host.3 According to the ADE, preexisting anti-DV antibodies can potentiate subsequent virus infection of monocytes through Fc receptors, resulting in increased viremic titers and the development of DHF/DSS. The other hypothesis is focused on the differences in viral virulence between serotypes and/or between strains within serotypes.4 There is epidemiologic and laboratory evidence to support both of these hypotheses.

The clinical symptoms of DHF/DSS, which include hemorrhage, thrombocytopenia, increased vascular permeability, decreased blood pressure, and hypovolemic shock suggest that defects in hemostasis are probably involved in its pathogenesis. Endothelial cells are known to play an important role in regulating vessel permeability and maintaining hemostasis.5 Certain pathogens can infect endothelial cells and alter functions such as cytokine production, adhesion molecule expression, permeability, and procoagulation activity,6-8 resulting in a deregulation of hemostasis. Therefore, in this study, we examined whether DV could infect HUVEC and the cytokine production by HUVEC in response to the infection.

MATERIALS AND METHODS

Preparation of virus stock and virus titration. Dengue type 2 (strain PL0046 and 16681) and type 3 viruses were propagated in C6/36 cells. Briefly, monolayers of C6/36 were inoculated with the dengue virus at multiplicity of infection (MOI) of 0.01 and incubated at 26°C in 5% CO₂ in air for 5 days. The culture medium was harvested and cell debris was removed by centrifugation at 900×g for 10 min. After further centrifugation at 16,000×g for 10 min, the virus supernatant was collected and stored at −70°C until use. Virus titer was determined by plaque assay using BHK-21 cell line. Briefly, a 10-fold serial dilution of virus was added to BHK-21 monolayer, and incubated at 37°C in 5% CO₂, in air for 5 days. Plaque numbers were counted after staining with crystal violet.

Isolation of endothelial cells. HUVEC were isolated and cultured as described previously by Jaffe and others9 with a slight modification. Briefly, umbilical veins from individual cords were cannulated, washed with phosphate buffered saline (PBS), and treated with 0.1% collagenase type V (Sigma, St. Louis, MO) in M-199 medium (Gibco, Gaithersburg, MD) at 37°C for 15 min. Detached cells were removed by flushing with M-199 supplemented with 10% fetal bovine serum (FBS). After washing, the cells were resuspended in growth medium containing M-199 supplemented with 20% FBS, 1% endothelial cell growth supplement (ECGS), 1% heparin, and 1% penicillin. The cells were grown to confluence and were detached using trypsin (1,000 units/ml) and EDTA (0.5 mM). Subcultures of HUVEC were performed by seeding the cells in flasks precoated with 1% gelatin. Only cells with 2 to 4 passages were used for experiments. The cell monolayers exhibited the typical cobblestone appearance of endothelial cells and expressed LDL receptors.10

DV infection of endothelial cells. Monolayers of HUVEC were trypanized and resuspended in growth medium. Approximately 1×10⁴ HUVEC were seeded into each well of 12-well tissue-culture plates (Falcon, Helena, MT). After overnight incubation, DV was added to the cells at the MOI of 1 and allowed to adsorb for 2 hours. Unbound viruses were removed by washing with PBS. Infected cells and culture supernatants were collected at different time intervals after infection. HUVEC without infection (medium alone [med]) or inoculated with heat-inactivated virus (56°C, 30 min) (i.DV) were used as controls. In addition, Japanese encephalitis virus (JEV) strain NIH was also used as a negative control. In some experiments, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (Sigma)) was added to HUVEC with DV for 2 hours at 37°C, for 1 hour at 4°C after virus adsorption, or for 2 hours before DV infection. In other experiments, ribavirin was included in the culture medium throughout the infection.

Human sera. Sera were collected from 24 patients at the
cells were cultured on glass slides coated with 1% gelatin. After infection with cold acetone for 10 min, washed with PBS, and stored at°C. Mouse anti-dengue monoclonal antibody (Chemicon, Temecula, CA) at a 1:400 dilution was applied overnight. After washing, the slides were incubated with 1:200 diluted FITC conjugated anti-mouse IgG (Sigma) for 1 hour. Slides were viewed and photographed using fluorescent microscopy (Olympus, Tokyo, Japan).

Immunofluorescence assay. HUVEC (10^4) were cultured on glass slides coated with 1% gelatin. After infection with DV (PL0046) at the MOI of 1 for 24, 48, or 72 hours, cells were fixed with cold acetone for 10 min, washed with PBS, and stored at°C. Mouse anti-dengue monoclonal antibody (Chemicon, Temecula, CA) at a 1:400 dilution was added to the slides and incubated at 4°C in a moisture chamber overnight. After washing, the slides were incubated with 1:200 diluted FITC conjugated anti-mouse IgG (Sigma) for 1 hour. Slides were viewed and photographed using fluorescent microscopy. HUVEC grown on slides were infected with dengue virus (PL0046) for 72 hours. IFA was performed as described in Materials and Methods. The result is representative of three similar experiments.

RESULTS

Infection of HUVEC by dengue virus. HUVEC supernatants were collected every 24 hours after DV infection and the plaque-forming units (PFUs) were determined. An increase in PFU in the supernatants of DV-infected-HUVEC was observed 48–72 hr after infection (Figure 1). A similar increase in PFU was also found in the cell lysates of DV-infected HUVECs (data not shown). In addition, there were different replication efficiencies among different strains and types of DV. Strain 16681 infected-HUVEC produced more virus progeny as compared with strain PL0046 and type 3 dengue virus 72 hours after infection.

DV infection confirmed by immunofluorescence assay. Immunoreactive DV proteins were detected in the cytoplasmic region of DV-infected HUVEC 24 hours after infection by immunofluorescence staining with mAb to DV. The most intense fluorescence staining was observed at 72 hours (Figure 2). The number of fluorescent-positive cells also increased with increased duration of infection. Uninfected cells were consistently negative.

Increase in IL-6 and IL-8, but not IL-1β after DV infection. To analyze the cytokine production of HUVEC in response to DV infection, culture supernatants of HUVEC were collected 24, 48, and 72 hours after infection. IL-6, IL-8, and IL-1β in the culture supernatants were measured by ELISA (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer’s instructions. Samples with levels above the maximum optical density were diluted and retested. The detection limits of the ELISAs were as follows: IL-1β < 1 pg/ml, IL-6 < 1 pg/ml, IL-8 < 2 pg/ml. Sera from patients and normal individuals were also assayed using the same procedures.

Statistical analysis. Statistical analysis of data was performed using Student’s t-test, and differences were considered significant if P < 0.01.
Dengue virus induces cytokine production of HUVEC

**Figure 3.** Dengue virus induces IL-6 and IL-8 production by human umbilical cord veins endothelial cells (HUVEC). HUVEC were infected with different types and strains of dengue virus (PL0046, DV3, or 16681) and culture supernatants were collected every 24 hours, and assayed by cytokine ELISA. Controls included cells without infection (med), cells treated with heat-inactivated DV (iDV), and cells incubated with Japanese encephalitic virus (JEV). Results represent the accumulative amounts of IL-6 (A) and IL-8 (B). Data are displayed as the mean ± SD of three experiments.

**Figure 4.** Cytokines in the sera of dengue patients. Serum IL-6 and IL-8 concentrations were assayed in dengue fever (DF) patients (n = 16), dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) patients (n = 8), normal controls (n = 19), and patients with other febrile illness (OFI, n = 16) by cytokine ELISA kits. Bar represents the mean ± SD. (*P < 0.01 as compared to normal controls).

Ribavirin inhibits DV replication in HUVEC. Ribavirin co-incubated with HUVEC during infection exerted a dose-dependent inhibition on virus replication with a maximal effect at a dose of 100 µg/mL (inhibition from 2.4 × 10^5 PFU/mL to 5.5 × 10^2 PFU/mL) (Figure 5). However, DV could not be completely eradicated at higher doses of ribavirin. Ribavirin added with DV for 2 hours and then washed away exerted a lower inhibitory effect in virus replication (from 2.4 × 10^5 PFU/mL to 1 × 10^4 PFU/mL at 100 µg/mL). The inhibition of viral replication was not due to a toxic effect of ribavirin on HUVEC because ribavirin up to 1,000 µg/ml showed no toxicity to HUVEC as demonstrated by trypan blue exclusion (> 95% viable).

Ribavirin inhibits DV-induced IL-6 and IL-8 production by HUVEC. Ribavirin not only inhibited DV replication in HUVEC but also inhibited DV-induced IL-6 and IL-8 in a dose-dependent manner (Figure 6). When ribavirin was added and maintained in the medium at a dose of 100 µg/mL, IL-6 and IL-8 production were inhibited to 18.16% and 19.74%, respectively as compared to controls. When ribavirin was added at the same time as infection but washed away 2 hours later, the decrease in cytokine production was less (IL-6: 56.7%; IL-8: 59.4% as compared to untreated controls). In addition, pretreatment of HUVEC with ribavirin for 2 hours before DV infection with subsequent removal of the drug had no effect on DV-induced cytokine production (IL-6: 102.8%; IL-8: 100.6% compared with untreated controls).

**Discussion**

DHF/DSS is a major health problem in Southeast Asia and Central America because of the lack of early diagnostic markers and effective therapeutic regimens. No suitable animal model has yet been established to study its pathogenesis. Hemostatic defects and vascular leakage are the major abnormalities in DHF/DSS patients. In this study, we examined the effect of DV infection on cytokine production by human endothelial cells because these cells play a crucial role in the regulation of vascular permeability and in the control of hemostasis. Monocyte/macrophages were previously believed to be the major targets of dengue virus infection. Nevertheless, an increasing number of reports have indicate that DV can infect human endothelial cells as well. In addition, chimeric DV envelope protein has been shown to bind to endothelial cells, indicating the endothelium can be a target for DV infection. However, the results of these studies are questionable because many used a human endothelial cell line (ECV 304) that is a derivative of...
FIGURE 5. Ribavirin inhibits dengue virus (DV) replication in human umbilical cord veins endothelial cell (HUVEC). Human endothelial cells (1 × 10⁵) were infected with DV (strain PL0046) at a MOI of 1 in the presence or absence of ribavirin at infection and persisted in the medium. Viral titers were determined by plaque assay 48 hours after infection. Viral titer of DV-infected HUVEC incubated with no ribavirin was designated as 100%. Data represent mean ± SD of three experiments.

FIGURE 6. Ribavirin inhibits cytokine production by human umbilical cord veins endothelial cell (HUVEC) induced by dengue virus (DV) infection. Human endothelial cells (1 × 10⁵) were infected with DV (strain PL0046) at a MOI of 1 in the presence or absence of ribavirin at infection and persisted in the medium. IL-6 (A) and IL-8 (B) levels in the supernatants were measured by ELISA 24 hours after infection. Cytokine levels of DV-infected HUVEC without ribavirin were designed as 100%. Data represent mean ± SD of three experiments.

human bladder cell line T24 from the American Type Culture Collection. Therefore, in this study, we used primary HUVEC instead of cell lines to determine whether DV could infect human endothelial cells and their cytokine responses. We demonstrated an increase in plaque-forming units in the supernatants of DV-infected HUVEC 48 to 72 hours after infection. In addition, DV antigen was detected in the cytoplasm of DV-infected HUVEC by IFA. This result is consistent with previous reports showing the presence of DV antigen in the cytoplasm of infected endothelial cells. 18,19 Taken together, these results confirmed that DV can infect and replicate in HUVEC, and they provide a model in which to study the vasculopathy during DV infection.

Dengue virus-infected HUVEC showed a significant increase in the production of IL-6 and IL-8 but not IL-1β. It has been reported that the levels of tumor necrosis factor α (TNF-α), IL-6, and IL-8 but not IL-1β in patients with DHF/DSS were relatively higher than those of healthy persons. 16,20,21 TNF-α production by endothelial cells has not been reported. However, TNF-α released from DV-infected monocytes could modulate endothelial cell function by an indirect route. 22 In the present study, increased IL-6 and IL-8 were observed both in DHF/DSS patients’ sera and supernatants of DV-infected HUVEC, suggesting the involvement of these cytokines in the progress of hemorrhage and shock syndrome. IL-6 could increase vessel permeability and fluid-phase endocytosis and transcytosis of CNS-derived endothelial cells. 23 In addition, IL-6 could amplify leukocyte recruitment and thus plays a positive role in local inflammatory reactions. 24 IL-8 is a chemoattractant for neutrophils, basophils, and lymphocytes. 25 Therefore, local secretion of IL-6 and IL-8 by DV-infected endothelial cells may augment the recruitment of leukocytes and activate or damage endothelial cells themselves. Collectively, the cross-talk between inflammatory cells and the endothelium through cytokines may play an important role in the hemostasis defects in DHF/DSS.

The findings that different strains of dengue virus exhib-
ited different replication efficiency in HUVEC, and that higher MOI of virus induced higher cytokine levels suggest a difference in viral competence to replicate in HUVEC and that virus load contributes to the clinical outcome of DV infection. Even though host factors such as genetic susceptibility and immune status cannot be ruled out, infection with a virus strain having a high replication efficiency or at high virus load may lead to massive IL-6 and IL-8 production and contribute to the development of the severe immunopathology in DHF/DSS.

Ribavirin, a synthetic guanosine analogue that possesses a broad spectrum of activity against DNA and RNA viruses, is currently used in the combination with IFN for the treatment of hepatitis C virus. Ribavirin had a dose-dependent inhibitory effect on DV replication, as well as IL-6 and IL-8 productions of DV-infected HUVECs. These results are consistent with the notion that IL-6 and IL-8 production by HUVEC was induced by DV infection and that inhibition of DV replication in HUVECs resulted in decreased production of cytokines. Since cytokine production plays a causal role in DHF, treatment with ribavirin would have to be given very early in infection in order to prevent virus replication, cytokine release, and subsequent development of DHF. Ribavirin, on the other hand, showed no prophylactic effect on DV infection in a rhesus monkey model. The discrepancy may reflect different anti-viral activities of ribavirin in different assay systems. It also suggests that ribavirin is less active against dengue virus in vivo than in vitro. In summary, our results indicate that DV can infect human endothelial cells and induce IL-6 and IL-8 production which may contribute to the pathogenesis of DHF/DSS.

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