Original Article

Synergistic Effect of TNF-α and Dengue Virus Infection on Adhesion Molecule Reorganization in Human Endothelial Cells

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SUMMARY: Dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) is a severe pathological manifestation of dengue virus (DENV) infection. Enhanced production of cytokines in dengue patients is proposed to induce endothelial barrier instability resulting in increased vascular leakage. Tumor necrosis factor (TNF-α) is an inflammatory cytokine that activates endothelial cells and enhances vascular permeability and plasma leakage in DHF/DSS. The present study investigated the in vitro effect of TNF-α and DENV infection on the expression of adherence junction proteins, tight junction proteins, and membrane integrity of human endothelial cell lines. Immunofluorescence staining and western blot analysis demonstrated platelet endothelial cell adhesion molecule-1 (PECAM-1) reorganization and decreased levels of the tight junction protein occludin in human endothelial cells treated with TNF-α and DENV, compared to mock, DENV, or TNF-α-treated cells. Permeability assessed by FITC-dextran as a transport molecule was increased and correlated with the unusual reorganization of PECAM-1. The altered distribution of PECAM-1 and low occludin protein levels in human endothelial cells treated with TNF-α and DENV correlated with increased permeability. In conclusion, the synergistic effect of TNF-α and DENV induced permeability changes in endothelial cells. These results contribute to the understanding of the mechanisms underlying enhanced vascular permeability in DENV infection.

INTRODUCTION

Dengue virus (DENV) infection is one of the most important global health problems currently, and is transmitted through the Aedes aegypti mosquito. It is a major cause of childhood and adult morbidity, particularly in tropical and subtropical areas (1). The severe clinical manifestation of DENV infection is dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (2). The hallmark of DHF/DSS is plasma leakage, which leads to intravascular volume loss and hypovolemic shock, causing death (3). Although vascular leakage is the major pathogenic symptom of DENV infection, the mechanism underlying this is still unclear. Furthermore, plasma leakage in DHF occurs rapidly, and is likely caused by altered vascular function rather than destructive effects on endothelial cells. The elevation of cytokine and immune mediator levels in DHF patients probably has an effect on the functional alterations of endothelial cells (4).

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine with a broad range of biological activity (5). TNF-α affects vascular endothelial cells (EC) and endothelial leukocyte interactions that promote the disruption of cell-cell junctions, disassembly of focal adhesion complexes, and morphological changes leading to increased vascular permeability (6).

Endothelial cells line the lumen of blood vessels and they function as a semi-selective diffusion barrier between the blood and interstitial compartment (7). During vascular damage, cell-cell junctions are dissociated and paracellular permeability is increased. Adherence junctions and tight junctions are suggested to be involved in regulation of the endothelial barrier (8). Platelet endothelial cell adhesion molecule-1 (PECAM-1)/CD31 expressed at the endothelial cell intercellular junction is involved in the regulation of cell-cell junctions of endothelial cells (9), and TNF-α induces changes in the localization and redistribution of PECAM-1 on endothelial cells (5). Ocludin is an integral membrane protein and is implicated in the regulation of paracellular gap junctions (10). The alteration of PECAM-1 and occludin is involved in the activation of endothelial barrier disruption and leads to the increased permeability of endothelial cells.

To gain insights into the pathophysiological processes involved during TNF-α induction and DENV infection, this study analyzed adherence junction and tight junction proteins in human endothelial cells using immunofluorescence staining and western blotting. In addition, the integrity of endothelial cells was investigated via a permeability assay. The results of this study demonstrate that TNF-α and DENV infection induced changes in adherence junctions and tight junctions in human endothelial cells, and are suggested to play a role in increased endothelial permeability during DENV infection.
MATERIALS AND METHODS

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Cultivation of human endothelial cell: Human endothelial cells (EA.hy926) were cultured in DMEM/F-12 (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin G, and 100 mg/ml streptomycin (Sigma, St. Louis, MO, USA) at 37°C in a 5% CO₂ atmosphere.

DENV infection: DENV serotype 2 strain 16681 was used throughout the study. EA.hy926 cells were infected with DENV-2 at a multiplicity of infection (MOI) of 5 for 2 h at 37°C. An MOI of 5 was used, as a previous study indicated an optimized percentage of infection in the absence or presence of DENV-2 were fixed for 1 h and washed twice with PBS, blocked for 1 h with 1% skim milk for 1 h at room temperature. Cells were double stained with FITC-conjugated annexin V and propidium iodide, and analyzed by flow cytometry using FACSscan equipped with CellQuest (BD Biosciences, San Jose, CA, USA).

Measurement of cell death: EA.hy926 cells were grown to confluence on glass coverslips. After 0, 8, 16, and 24 h, mock- and TNF-α-treated cells were harvested by centrifugation at 1,610 × g for 5 min and washed twice with cold phosphate buffered saline (PBS). Cells were double stained with FITC-conjugated annexin V and propidium iodide, and analyzed by flow cytometry using FACSscan equipped with CellQuest (BD Biosciences, San Jose, CA, USA).

Confocal laser scanning microscopy: EA.hy926 cells were grown to confluence on glass coverslips. After 0, 8, 16, and 24 h, mock- and TNF-α-treated cells cultured in the absence or presence of DENV-2 were fixed for 10 min using 4% paraformaldehyde in PBS. Cells were washed twice with PBS, blocked for 1 h with 1% bovine serum albumin in PBS, and incubated with anti-PECAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect DENV protein (red color) at a dilution of 1:2,000 for 1 h and then washed with PBS. Coverslips were mounted with 50% glycerol/PBS. Cell nuclei were stained blue with 4',6-diamidino-2-phenylindole. The EA.hy926 cells were double stained with FITC-conjugated annexin V and propidium iodide, and analyzed by flow cytometry using FACSscan equipped with CellQuest (BD Biosciences, San Jose, CA, USA).

Western blot analysis: EA.hy926 cells were infected with DENV-2 at MOI 5 with or without TNF-α-treatment for 24 h, then harvested for western blot analysis. A total of 20 μg protein was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequently transferred to a nitrocellulose membrane. The membrane was blocked with PBS containing 5% skim milk for 1 h at room temperature. The membrane was then incubated with mouse monoclonal anti-occludin (Santa Cruz Biotechnology) and mouse monoclonal anti-GAPDH antibodies at a dilution of 1:1,000 at 4°C overnight. Thereafter, the membrane was washed 3 times in PBS, incubated with HRP-conjugated rabbit anti-mouse immunoglobulin antibody (Dako, Glostrup, Denmark) and visualized by Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Quantitative analysis of protein bands was performed using the Image J program.

In vitro permeability assay: Vascular permeability was evaluated using the ECM 644 In vitro Vascular Permeability Assay kit (Merck Millipore, Billerica, MA, USA). EA.hy926 cells were seeded into collagen-coated, 1.0 μm-pore, polyethylene membrane inserts of transwell plates (1 × 10⁵ cells/insert well) (Merck Millipore). Cells were infected with or without DENV prior to treatment with TNF-α for 24 h. Approximately 150 μl of a 1: 40 dilution of FITC-Dextran (supplied from the assay kit) in complete DMEM/F-12 was added into the insert and incubated for 30 min at room temperature (RT). The inserts were removed, and plate well solution from the lower chambers was collected to determine the fluorescence intensity using a multi-detection microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA). The fluorescence excitation and emission wavelengths were 485 and 530 nm, respectively.

Determination of TNF-α in culture supernatants by ELISA: The level of TNF-α was measured using the Quantikine human TNF-α immunoassay kit (R & D Systems, Minneapolis, MN, USA) according to the supplier’s protocol. Briefly, 50 μl aliquots of assay diluent were added into the wells of a 96-well microplate precoated with monoclonal antibody against human TNF-α. Then, 200 μl of samples or standard was added into each microplate well and incubated for 2 h at RT. After incubation, the plate was washed with buffer for 4 times, and then 200 μl human TNF-α conjugate was added into each well, and the plate was incubated for 2 h at RT. The plate was washed again, and then, 200 μl of substrate solution was added to each well, and the plate was incubated for 20 min. Finally, 50 μl of stop solution was added to each well, and optical density at 450 nm was measured on a microplate reader (Synergy MX; Biotek, Winooski, VT, USA).

Analysis of non-structural (NS) 1 level in culture supernatants: The NS1 level was determined in culture supernatants using ELISA. Briefly, 0.1 ml of 10 μg/ml of monoclonal antibody against Flavivirus-cross reactive NS1 was coated on 96-well ELISA plates and incubated at 4°C overnight. After washing and blocking, the samples were diluted to 1:5, 1:25, and 1:125 in diluent (4% BSA in PBS). One hundred microliters of samples were added to the plates and incubated at 37°C for 2 h. Then, 100 μl of 25 μg/ml monoclonal antibody against DENV-2 NS1 was added and incubated further at 37°C for 1 h. Finally, 100 μl of substrate solution (1-Step™ Ultra TMB-ELISA, Thermo Fisher Scientific, Rockford, IL, USA) was added and incubated for 5 min. The reaction was stopped by adding 50 μl of 2 N sulfuric acid. The plate was measured for the absorbance at 450/620 nm on a microplate reader (Anthos 2010, Salzburg, Austria) (12,13).

Statistical analysis: An unpaired t test was performed
to compare the data between treatment and control groups using StatView software version 5.0. The criteria for defining the data of significant differences included $p$ values $<0.05$ or $p$ values $<0.0001$.

**RESULTS**

**Optimization of TNF-α treatment:** Various concentrations of TNF-α were administered to EA.hy926 cells at $37^\circ$C for 24 h to determine the concentration of TNF-α that induced the minimum percentage of cell death. Approximately 10% cell death was observed using 0.01–1 ng/ml TNF-α, which was the level observed in untreated control cells, whereas 10–100 ng/ml TNF-α caused 30–40% cell death (Fig. 1). These concentrations of TNF-α were also used for in vitro vascular permeability assays to observe its effects on human endothelial cells. TNF-α concentration between 0–1 ng/ml induced a slight change in membrane permeability, whereas 10–100 ng/ml TNF-α had a greater effect on membrane permeability (Fig. 2). Therefore, TNF-α at a concentration of 1 ng/ml was determined to be optimal for use in all further studies.

**Combined effect of TNF-α and DENV infection redistributes PECAM-1 in human endothelial cells:** The combined effect of TNF-α and DENV infection, which is observed in the serum of dengue patients during viremia, on PECAM-1 and occludin in human endothelial cells was investigated. PECAM-1 expression gradually increased in cells treated with TNF-α alone, DENV-2 infection alone, and TNF-α plus DENV-2 infection. PECAM-1 expression showed time-dependent progressive changes when infected with DENV-2 as assessed by immunofluorescence staining (Fig. 3A). At 0 h, PECAM-1 expression was localized to the periphery of cells in all 3 treatment groups. Treatment with 1 ng/ml TNF-α or DENV-2 at MOI 5 infection at 8, 16, and 24 h destabilized PECAM-1 in EA.hy926 cells. In contrast, PECAM-1 was redistributed, and the formation of intercellular gaps indicated cell-cell junction disruption in EA.hy926 cells infected with DENV-2 at MOI 5 combined with 1 ng/ml TNF-α (Fig. 3A). The potential effect of TNF-α induction and DENV-2 infection on tight junction proteins was analyzed. Occludin is an integral membrane protein involved in cell-cell interactions and regulates barrier functions. Immunoblotting showed that occludin levels were markedly decreased after treatment with 1 ng/ml TNF-α or infection with DENV-2 at MOI 5 for 24 h, and significantly decreased in the combined treatment group (1 ng/ml TNF-α and DENV-2 at MOI 5 for 24 h) as compared with control cells (Fig. 3B and Fig. 3C). The redistribution of PECAM-1 and down-regulation of occludin may be involved in increasing endothelial cell permeability.

**Effect of TNF-α induction and DENV infection on permeability of human endothelial cells:** To confirm the effects of combined TNF-α and DENV-2 stimulation on endothelial cell permeability, FITC-dextran was used with endothelial monolayers as described above. The results showed that treatment of cells with 1 ng/ml TNF-α alone, infection with DENV-2 alone, or 1 ng/ml TNF-α combined with DENV-2 infection significantly increased the permeability of human endothelial cells (Fig. 4), indicating a synergistic effect between TNF-α and DENV infection.

**TNF-α levels during DENV infection and/or TNF-α induction in EA.hy926 cells:** To determine if the TNF-α production was dependent on DENV-2 infection, we assessed the levels of TNF-α in the culture supernatants of mock, DENV-2, TNF-α, and DENV-2 plus TNF-α treated EA.hy926 cells. The TNF-α level was $\sim 44 \pm 9$ pg/ml and $48 \pm 9$ pg/ml in TNF-α and DENV-2 plus TNF-α after 24 h post-treatment, respectively (Fig. 5). No detectable level of TNF-α was observed for mock and DENV-2 infection alone. However, at 36 h and 48 h post-infection the TNF-α level showed slightly decreased. Statistical analysis showed no significant difference in the test groups.

**NS1 level in culture supernatants of DENV-2 infection:** To confirm whether the permeability changes in EA.hy926 cells were affected by NS1 production induced by DENV infection. The NS1 level was determined in culture supernatants of mock, DENV-2, and
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Fig. 3. (Color online) Reorganization of platelet endothelial cell adhesion molecule (PECAM)-1 and expression of occludin in EA.hy926 cells infected with DENV-2 and TNF-α induction. A. The effect of tumor necrosis factor (TNF)-α and dengue virus (DENV)-2 on reorganization of platelet endothelial cell adhesion molecule (PECAM)-1. EA.hy926 cells were infected with DENV-2 (multiplicity of infection = 5) and treated with 1 ng/ml TNF-α at the indicated times. PECAM-1 was stained with Alexa 488-conjugated secondary antibody against PECAM-1 (green color). Dengue E proteins (E) were stained with 4G2 monoclonal antibody (red color) and the blue color is nuclear staining (n = 3). B. Representative western blot analysis of occludin expression which showed decreased intensity of the occludin band in the DENV-2 infection and TNF-α treatment group compared to mock control cells. GAPDH was an internal control and unchanged by all treatments. C. Quantitative analysis of occludin expression calculated from 3 experiments using Image J software showed a significant difference between TNF-α versus control and DENV-2 infection plus TNF-α versus control. *p < 0.05 between treated cells versus control cells using Student’s t-test analysis (n = 5, mean ± SEM).

Fig. 4. Effect of dengue virus (DENV)-2 and tumor necrosis factor (TNF)-α on EA.hy926 cell monolayer permeability. The amount of FITC-conjugated dextran increased after DENV-2 infection and in the presence of TNF-α treatment. Endothelial permeability was significantly increased when stimulated with TNF-α together with DENV infection. *p < 0.05, **p < 0.0001 between treated cells versus control cells using Student’s t-test analysis (n = 5, means ± SEM).

Fig. 5. TNF-α production in culture supernatants of DENV-2, TNF-α, and DENV-2 plus TNF-α treated EA.hy926 cells. The TNF-α level was detected only in culture supernatants of TNF-α and DENV-2 plus TNF-α treated EA.hy926 cells at 24, 36, and 48 h. No significant difference of TNF-α was observed among these groups of all time tested in 3 different experiments. NS in the figure was non-significant difference of statistical analysis.

Fig. 6. The NS1 level production from culture supernatants of mock, DENV-2, and DENV-2 plus TNF-α treated EA.hy926 cells. Production of NS1 was detected only in culture supernatants of DENV-2 and DENV-2 plus TNF-α treated cells. The NS1 was slightly increased as time-dependent fashion during 24 h to 48 h. No significant difference of NS1 production during time course was observed among each group in 3 different experiments. NS in the figure was non-significant difference of statistical analysis.
DENV-2 plus TNF-α treatment. The NS1 was ~465 ± 98 ng/ml and 519 ± 68 ng/ml in DENV-2 and DENV-2 plus TNF-α treatment, respectively (Fig. 6). The NS1 was slightly increased at 36 h and 48 h post-treatment as compared to that at 24 h, but the NS1 level in DENV-2 plus TNF-α was slightly decreased compared to that in DENV-2 infection alone. No detectable level of NS1 was observed in the mock control and statistical analysis showed no significant difference in these test groups.

**DISCUSSION**

Hemorrhagic diathesis is one of the common clinical manifestations associated with the severe form of DHF/DSS. Several hypotheses have been proposed to explain the progression of vascular endothelial dysfunction, which is an important characteristic of severe infection in dengue patients (14). The overproduction of inflammatory cytokines is a key factor that may have a role in disease severity. High levels of cytokines during the viremia of dengue patients suggests that both cytokines and virus are correlated with the increased vascular permeability observed in dengue patients (15).

TNF-α is a potent cytokine that activates endothelial cells and enhances capillary permeability. Previous in vitro studies have reported significantly increased levels of TNF-α and mRNA levels in DHF/DSS patient serum (16–20), and it was proposed to have a central role in the development of plasma leakage (1,21). Several studies have reported that TNF-α causes an increase in endothelial permeability and is involved in the disruption and reorganization of the apical junction complex and cytoskeleton proteins (22–25). Vascular endothelial cells respond to TNF-α by promoting alterations in the molecular organization of E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 (VCAM-1), all of which are involved in paracellular permeation (8). In this study, human endothelial cell lines (EA.hy926) were used to investigate whether exposure to TNF-α and dengue virus is responsible for the vascular leakage phenomenon observed in severe dengue patients. EA.hy926 cells express membrane proteins including Von Willebrand factor, coagulation factor VIII (anti-hemophilic factor), tissue plasminogen activator, and thrombomodulin. Their phenotype closely resembles primary vascular endothelial cells including fibrinolysis, angiogenesis, and inflammation (26). Talavera et al. described that IL-8 secretion during dengue virus infection in human dermal microvascular endothelial cells caused actin cytoskeleton and tight junction occludin reorganization (27). Kanlaya et al. also proposed that decreasing levels of vascular endothelial cadherin and Zonula occludens-1 in addition with alteration of actin assembly might be responsible for increased permeability in dengue virus-infected human endothelial cells (11). Recently, it was reported that treatment for recombinant dengue NS1 produced from human embryonic kidney 293 cells in human endothelial cell monolayers (HPMECs and HUVECs) caused disruption of endothelial cell permeability in a dose- and time-dependent manner (28). The authors also suggested that this effect might have been derived from the hexameric NS1 produced from eukaryotic cells. These results contradicted our results that indicate no difference in NS1 levels produced from DENV infection alone or DENV plus TNF-α. Other studies have reported that large amounts of cytokines, i.e. TNF-α, IL-6, and MCP-1 are triggered by dengue virus infection. Treatment with anti-TNF-α antibody also suppressed IL-6, MCP-1, and IFN-α levels, suggesting that severe vascular leakage is triggered by TNF-α (29).

Combined treatment with TNF-α and infection with DENV induced the redistribution of PECAM-1 in EA.hy926 cells that was time-dependent, suggesting that both factors had an enhancing effect on modulating the altered vascular permeability by remodeling endothelial membrane proteins. The discontinuous PECAM-1 staining was a phenomenon of cell-cell junction distortions and caused the paracellular gap, resulting in vascular leakage. Moreover, the decreased levels of occludin in tight junctions observed in the 3 treatment groups (DENV infection, TNF-α treatment, and TNF-α + DENV infection) suggest that TNF-α and the dengue virus play a role in inducing vascular permeability by causing the loss of tight junction proteins. These results also correlated well with the significant increase of FITC-dextran in TNF-α treated cells combined with DENV infection. These results support a synergistic effect between TNF-α and DENV to increase the severity of plasma leakage. DENV alone did not affect permeability of endothelial cells since TNF-α could not be detected during DENV infection at MOI 5 in this study. A previous study showed that DENV infection and cytokines alter the transendothelial electrical resistance of endothelial cells resulting in increased permeability (30). Recently, Raekiansyah et al. also suggested that DENV infection alone does not directly affect endothelial or vascular permeability (31).

In summary, the redistribution of PECAM-1 and down-regulation of occludin after treatment with 1 ng/ml of TNF-α, infection with DENV-2 at MOI 5, or infection with DENV-2 at MOI 5 combined with 1 ng/ml of TNF-α increased endothelial permeability in EA.hy926 cells. TNF-α and DENV-2 infection induced vascular permeability, adherence junction and tight junction reorganization, and highly enhanced effects were observed when a combination of TNF-α and DENV-2 was used. Several studies have reported that the overproduction of cytokines and chemokines have a role in increasing plasma leakage in DHF/DSS. The interplay between DENV and multiple cytokines might explain the induction of endothelial permeability in DHF/DSS pathogenesis. TNF-α is a cytokine that activates and enhances endothelial cell permeability. Further investigation is required to determine the involvement of cytokines in the pathogenesis of vascular leakage in DENV infection and DHF/DSS.

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**Conflict of Interest** None to declare.
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