Alterations in Actin Cytoskeletal Assembly and Junctional Protein Complexes in Human Endothelial Cells Induced by Dengue Virus Infection and Mimicry of Leukocyte Transendothelial Migration

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Received January 22, 2009

Vascular leakage is a hallmark of severe dengue infection. Although extensive studies have been conducted during the past several decades, the molecular mechanisms underlying vascular leakage in dengue shock syndrome (DSS) remain unclear. We thus performed a proteomics study to characterize responses in human endothelial cells (EA.hy926) after DEN-2 virus infection (MOI = 10). Comparative 2-D PAGE analysis revealed significantly altered abundance levels of 15 proteins, which were successfully identified by quadrupole time-of-flight mass spectrometry (MS) and/or tandem MS (MS/MS). These altered proteins were involved in several biological processes, for example, mRNA stability/regulation, transcription and translation regulation, molecular chaperoning, oxidative stress response/regulation, cytoskeletal assembly, protein degradation, and cellular metabolisms. We also performed functional analyses of alterations in actin cytoskeletal assembly and endothelial integrity focusing on adherens junction (VE-cadherin), tight junction (ZO-1) and adhesive molecule (PECAM-1) after 24-h of DEN-2 infection and simulation of transendothelial migration by PECAM-1 cross-linking. Decreased expression and disorganization of the actin-cytoskeleton were observed in the infected cells, whereas the increase in actin stress fibers was found in adjacent noninfected cells. Additionally, a decrease in adhesive protein PECAM-1 was observed. Furthermore, DEN-2 infection caused decreased expression and redistribution of both VE-cadherin and ZO-1, whose changes were enhanced by PECAM-1 engagement. These alterations may potentially be a molecular basis explaining increased endothelial permeability or vascular leakage in DSS.

Keywords: Dengue • Endothelial • Host responses • Pathogenic mechanisms • Proteome • Proteomics • Vascular leakage • Vascular permeability

Introduction

Infection by dengue virus, a member of the Flaviviridae family,1 frequently causes mild febrile illness or dengue fever (DF) but may also lead to life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Outbreaks of dengue virus infection remain an important public health problem worldwide, particularly in tropical/subtropical regions.2–9 Severe dengue virus infection is characterized by thrombocytopenia, vascular leakage and hemorrhage. Because vascular leakage is a crucial mechanism underlying the fatal DSS, one of the major focuses of recent dengue research is an exploratory study of effects of dengue virus infection on endothelial cells.10 As the appropriate animal model that can precisely simulate dengue virus infection in humans is not currently available, evaluation of such effects have been performed mostly in vitro using cultivated endothelial cells.11–13 Several studies have demonstrated that dengue-infected human endothelial cells selectively secrete some proinflammatory chemokines/cytokines (e.g., RANTES, IL-6 and IL-8) in response to dengue virus infection, resulting in endothelial cell...
activation, cell damage,13–15 and altered transendothelial permeability through the cytoskeletal reorganization.16 During the omics era, global analysis of a large number of components in the cells can be performed with a high-throughput manner. The global analysis of components in human endothelial cells in response to dengue virus infection, however, has been conducted only at the transcriptome level.17–19 An initial transcriptomic study of human umbilical vein endothelial cells (HUVECs) employed both differential display RT-PCR and Affymetrix oligonucleotide microarray, and revealed that a majority of differentially expressed transcripts have significant roles in host defense mechanisms, including stress responses, wound healing, inflammatory responses and antiviral pathways.17 Subsequent studies of transcriptome profiles of DEN-2-infected human endothelial-like cells (ECV304) using differential display RT-PCR18 and microarray technology19 revealed that a novel set of altered transcripts included those involved in signal transduction, protein translation and modification, cytoskeletal assembly, cell cycle, and apoptosis.19

Even with the aforementioned data, the information on global changes in endothelial cells at the protein level (altered proteome) remains largely unknown. Also, changes in mRNAs are not always in concordance with alterations in proteins.20 Moreover, some viruses can even modulate protein turnover without any effects on the transcriptional level of such protein.21 The study of altered proteome in endothelial cells is therefore crucial for understanding the molecular mechanisms underlying vascular leakage in severe dengue virus infection. In the present study, we applied a proteomic technology to characterize changes in the proteome of human endothelial cells during an early phase of dengue virus infection. With the use of 2-D PAGE and mass spectrometry, 15 proteins whose abundance levels were significantly altered in response to dengue virus infection were identified. The proteomic data were then confirmed by 2-D Western blot analysis. Moreover, we focused our attention on altered endothelial integrity by investigation on actin cytoskeleton and two intercellular junctional complexes, including adherens and tight junctions. A decrease and disorganization of actin cytoskeleton was observed in the infected cells, whereas an increase in actin bundles known as actin stress fiber formation was observed in adjacent noninfected cells and the dengue-induced decreases in vascular endothelial cadherin (VE-cadherin), Zonula occludens-1 (ZO-1) and platelet-endothelial cell adhesion molecule-1 (PECAM-1) were generalized. In addition, our results also demonstrated that dengue infection and PECAM-1 engagement (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization (to mimic transendothelial leukocyte migration to the inflammatory site) caused decreased expression and reorganization.19

Materials and Methods

Cultivation of EA.hy926 Cell Line. EA.hy926 human endothelial cells originated from the fusion of HUVEC with the lung carcinoma cell line A54922 were grown in D-MEM/F-12 medium (GIBCO; Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO), 100 U/mL penicillin G and 100 mg/mL streptomycin (Sigma; St. Louis, MO). Cells were maintained in a humidified incubator with 5% CO2 at 37 °C. 

Dengue Virus Stock: Production and Titration. C6/36, a cell line from Aedes albopictus (ATCC CRL-1660), and PscloneD, a swine fibroblast cell line, were cultivated at 28 and 37 °C, respectively, in L-15 medium (GIBCO) containing 10% heat-inactivated FBS, 10% tryptose phosphate broth (TPB) (Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma). DEN-2 (strain 16681) was propagated in C6/36 cells. Briefly, the confluent monolayer of C6/36 was incubated with DEN-2 at a multiplicity of infection (MOI) of 0.1 in a maintenance medium (L-15 medium containing 1% FBS, 10% TPB, 100 U/mL penicillin and 100 µg/mL streptomycin) at 28 °C for 3 h with gentle shaking. Subsequently, supernatant was removed and replaced with fresh maintenance medium, and then incubated further at 28 °C until approximately 50% cytopathic effect (CPE) was observed. The culture supernatant was collected by centrifugation at 1500 rpm and 4 °C for 5 min. The virus stock was kept as aliquots at −70 °C until use. Virus titer was then determined by focus forming assay using a swine fibroblast cell line, PscloneD. The stained foci were used for calculation of virus titer (FFU/mL) in the culture supernatant.

Infection of EA.hy926 Cells with Dengue Virus (DEN-2). EA.hy926 cells were infected with DEN-2 at various MOI (1, 5 and 10) and then incubated at 37 °C for 2 h. The supernatant was then removed and replaced with fresh maintenance medium (D-MEM/F-12 containing 5% FBS). The cells were further incubated at 37 °C in 5% CO2 for 12, 24, and 48 h. The parallel uninfected cells served as the mock-control.

Detection of DEN-2 Antigen by Cytoplasmic Staining and Quantitative Analysis of DEN-2 Infectivity. To determine an optimal condition for infection and DEN-2 infectivity in EA.hy926 cells, mock-control and DEN-2-infected cells were detected for dengue viral antigen. The cells were harvested at 12, 24, and 48 h after the infection. The harvested cells were washed with plain D-MEM/F-12 and fixed with 2% formaldehyde (BDH; Poole, U.K.) in PBS for 1 h at room temperature (RT). Cells were permeabilized with 0.1% Triton X-100 (Fluka; Buchs, Switzerland) in PBS. Permeabilized cells were incubated with 3H5 monoclonal antibody (culture supernatant collected from the hybridoma cells clone 3H5) specific to DEN-2 envelope (E) protein for 1 h at RT. Subsequently, cells were washed with 0.1% Triton X-100 in PBS and incubated with FITC-conjugated rabbit anti-mouse immunoglobulin (DAKO; Glostrup, Denmark) at RT in the dark for 30 min. After washing with 0.1% Triton X-100 in PBS, cells were resuspended in PBS and analyzed by flow cytometry using FACScan equipped with CellQuest software (Becton Dickinson; Franklin Lake, NJ). Percentage of DEN-2 infection (%Infection) was calculated according to the formula: %Infection = [(no. of infected cells/ no. of total cells) × 100%].

Flow Cytometric Analysis of Cell Death upon DEN-2 Infection. Mock-control and DEN-2-infected cells were harvested as described above and resuspended in the growth medium (D-MEM/F-12 supplemented with 10% FBS). The cells were pelleted by centrifugation at 2000 rpm for 5 min and washed once in ice-cold annexin V buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2-2H2O; pH 7.4). After washing step, the cells were resuspended with annexin V buffer at a final concentration of 5 × 105 cells/mL and then incubated with FITC-conjugated annexin V (BD Biosciences, San Diego, CA) on ice in the dark for 15 min. Propidium iodide (BD Biosciences) at the final concentration of 0.2 µg/mL was added to the cell suspension prior to analysis by flow cytometry. The
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fixed and permeabilized cells were used as the positive control, whereas the untreated cells were used as the negative control.

**Protein Extraction for 2-D PAGE.** Mock-control and DEN-2-infected cells were harvested from 5 individual culture flasks for each group. The condition chosen for proteome analysis was an infection at the MOI of 10 and a postinfection incubation period of 24 h, at which a majority of the cells were infected by the virus but the cell death did not increase. Cell pellet was collected by centrifugation at 2000 rpm for 5 min and washed three times with PBS. Cellular proteins were extracted using a lysis buffer containing 7 M urea, 2 M thiourea, 40 mg/mL 3-(3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), 120 mM diithiothreitol (DTT), 2% (v/v) ampholytes pH 3–10, and 40 mM Tris-base) at 4 °C for 30 min. Unsolubilized materials were then removed by centrifugation at 12 000 rpm and 4 °C for 5 min and protein concentrations in the supernatants were measured using Bio-Rad Protein Assay (Bio-Rad Laboratories; Hercules, CA) based on the Bradford method.

**Protein Separation by 2-D PAGE, Staining, and Visualization of Protein Spots.** A total of 200 μg of protein was mixed with a rehydration buffer containing 7 M urea, 2 M thiourea, 20 mg/mL CHAPS, 120 mM DTT, 40 mM Tris-base, 2% ampholytes (pH 3–10) and trace amount of bromophenol blue to make a total volume of 150 μL per strip. The mixture was loaded into the Immobiline DryStrip, nonlinear pH 3–10, 7-cm long (GE Healthcare; Uppsala, Sweden) at RT for 16 h. The first-dimensional separation or isoelectric focusing (IEF) was performed at 20 °C using Ettan IPGphor II IEF System (GE Healthcare) with a step-and-hold gradient to reach 9083 Vh. The strips were equilibrated with equilibration buffer I (6 M urea, 130 mM DTT, 112 mM Tris-base, 4% (w/v) SDS, 30% glycerol and 0.002% bromophenol blue) for 15 min, followed by equilibration in buffer II (6 M urea, 135 mM iodoacetamide, 112 mM Tris-base, 4% (w/v) SDS, 30% glycerol and 0.002% bromophenol blue) for 15 min. Thereafter, the second-dimensional separation was performed by placing the strips on top of a 12% polyacrylamide slab gel (8 × 9.5 cm) using the SE260 mini-Vertical Electrophoresis Unit (GE Healthcare) at 150 V for approximately 2 h. 2-D gels were fixed in a solution containing 10% (v/v) methanol and 7% (v/v) acetic acid for 30 min and stained with SYPRO Ruby (Invitrogen-Molecular Probes, Eugene, OR) for 16 h with gently shaking. Image capture of 2-D gels was performed using Typhoon 9200 laser scanner (GE Healthcare). A total of 10 gels (five gels derived from individual culture flasks for each group) were obtained.

**Matching and Quantitative Analysis of Protein Spots.** Image Master 2D Platinum (GE Healthcare) software (version 6.0) was used for matching and analysis of protein spots visualized in individual gels. Parameters used for spot detection were (i) minimal area = 10 pixels; (ii) smooth factor = 2.0; and (iii) saliency = 2.0. A reference gel was created from an artificial gel combining all of the spots presenting in different gels into one image. The reference gel was used for determination of existence and difference of protein expression between gels. Intensity volumes of individual spots were obtained and subjected to statistical analysis. Differentially expressed protein spots were subjected to in-gel tryptic digestion and identification by mass spectrometry.

**In-Gel Tryptic Digestion.** The protein spots whose intensity levels significantly differed between groups were excised from 2-D gels, washed twice with 200 μL of 50% acetonitrile (ACN)/25 mM NH₄HCO₃ buffer (pH 8.0) at room temperature for 15 min, and then washed once with 200 μL of 100% ACN. After washing, the solvent was removed, and the gel pieces were dried by a SpeedVac concentrator (Savant; Holbrook, NY) and rehydrated with 10 µL of 1% (w/v) trypsin (Promega; Madison, WI) in 25 mM NH₄HCO₃. After rehydration, the gel pieces were crushed and incubated at 37 °C for at least 16 h. Peptides were subsequently extracted twice with 50 μL of 50% ACN/5% trifluoroacetic acid (TFA); the extracted solutions were then combined and dried with the SpeedVac concentrator. The peptide pellets were resuspended with 10 µL of 0.1% TFA and purified using ZipTipC₁₈ (Millipore; Bedford, MA). The peptide solution was drawn up and down in the ZipTipC₁₈ 10 times and then washed with 10 μL of 0.1% formic acid by drawing up and expelling the washing solution three times. The peptides were finally eluted with 5 μL of 75% ACN/0.1% formic acid.

**Protein Identification by Q-TOF MS and/or MS/MS Analyses.** The proteolytic samples were preprocessed 1:1 with the matrix solution (5 mg/mL α-cyano-4-hydroxy-cinnamic acid (CHCA) in 50% ACN, 0.1% (v/v) TFA and 2% (w/v) ammonium citrate) and spotted onto the 96-well sample stage. The samples were analyzed by the Q-TOF Ultima mass spectrometer (Micromass; Manchester, U.K.), which was fully automated with predefined probe motion pattern and the peak intensity threshold for switching over from MS survey scanning to MS/MS, and from one MS/MS to another. Within each sample well, parent ions that met the predefined criteria (any peak within the m/z 800–3000 range with intensity above 10 count ± include/exclude list) were selected for CID MS/MS using argon as the collision gas and a mass dependent ±5 V rolling collision energy until the end of the probe pattern was reached. The LM and HM resolution of the quadrupole were both set at 10 to give a precursor selection window of about 4 Da wide.

The instrument was externally calibrated to <5 ppm accuracy over the mass range of m/z 800–3000 using a sodium iodide and PEG 200, 600, 1000, and 2000 mixtures and further adjusted with Glu-Fibrinopeptide B as the near-point lock mass calibrant during data processing. At a laser firing rate of 10 Hz, individual spectra from 5-s integration periods acquired for each of the MS survey and MS/MS performed were combined, smoothed, deisotoped (fast option) and centroided using the ProteinLynx GlobalSERVER 2.0 data processing software (Micromass). This entailed the identification of the monoisotopic, carbon-12 peaks for MS data, and deconvolution of multiply charged spectra to their singly charged equivalents for MS/MS data. MaxEnt 3, a maximum-entropy-based technique, has been designed for this purpose and is an integral part of ProteinLynx GlobalSERVER 2.0.

The combined MS and MS/MS ion meta data were searched in concert against the NCBI mammalian protein database using the ProteinLynx GlobalSERVER 2.0 workflow. Additionally, the MS and MS/MS data were extracted and outputted as the searchable .txt and .pk1 files, respectively, for independent searches using the MASCOT search engine (http://www.matrixscience.com), assuming that peptides were monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. Only 1 missed trypsin cleavage was allowed, and peptide mass tolerances of 100 and 50 ppm were used for peptide mass fingerprinting (PMF) and MS/MS ions search, respectively.

**Western Blot Analyses.** For 1-D Western blotting, equally loaded 20 μg of proteins from each sample was resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. For 2-D Western blotting, totally, 100 μg of proteins from each sample was resolved by 2-D PAGE as described above and then
transferred onto a nitrocellulose membrane. Non-specific bindings were blocked with 5% skim milk in PBS for 1 h. Rabbit polyclonal anti-EF-2 (Santa Cruz Biotechnology; Santa Cruz, CA; 1:500) or mouse monoclonal anti-innRNP K (Santa Cruz Biotechnology; 1:500), anti-β-actin (Santa Cruz Biotechnology; 1:250), anti-VE-cadherin (Santa Cruz Biotechnology; 1:500), or anti-ZO-1 (Zymed Laboratories, San Francisco, CA; 1:500) (all were diluted in in 5% milk in PBS) was used as the primary antibody and incubated with the membrane at RT for 1–3 h. After washing, the membrane was incubated with swine anti-rabbit IgG conjugated with horseradish peroxidase (1:1000 in 5% milk in PBS) (DAKO) or rabbit anti-mouse IgG conjugated with horseradish peroxidase (1:1000 in 5% milk in PBS) (DAKO) at RT for 1 h. Reactive protein bands/spots were then visualized using SuperSignal West Pico chemiluminescence substrate (Pierce Biotechnology, Inc.; Rockford, IL).

Confocal Laser Scanning Microscopy. EA.hy 926 monolayers were grown on coverslip 1 day prior to infection with DEN-2 at MOI of 10 for 2 h. Cells left untreated were performed in parallel and served as the control. After 24 h postinfection, cells were rinsed with PBS and fixed/permeabilized with ice-cold ethanol for 3 min at −20 °C with an exception for actin and PECAM-1 stainings. For actin staining, cells were fixed with 3.7% formaldehyde/PBS for 10 min at RT and permeabilized with 1% Triton X-100/PBS at the same condition. For surface staining of PECAM-1, cells were fixed only. After washing with PBS, cells were blocked with 1% BSA/PBS for 1 h at RT. Cells were incubated with mouse monoclonal antibody to ZO-1 (Zymed Laboratories Inc., San Francisco, CA), VE-cadherin (Santa Cruz Biotechnology), or PECAM-1 (Santa Cruz Biotechnology) at dilution of 1:100 in blocking buffer at 4 °C overnight. Goat anti-mouse IgG conjugated with Cy3 was added at a dilution of 1:2500 (DAKO) at RT for 1 h. Unbound secondary antibody was removed by rinsing with PBS and coverslips were mounted with 50% glycerol/PBS. Images were taken under a laser scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany). Actin was stained by rhodamine-conjugated phalloidin (Invitrogen, Carlsbad, CA) at a dilution of 1:40 for 1 h at RT. Culture supernatant derived from hybridoma cells clone 3H5 producing antibody against DEN-2 envelope (E) protein was used to detect dengue viral antigen using 3H5 monoclonal antibody, which is specific for DEN-2 envelope protein. The data were obtained from three independent experiments. Increasing the virus inoculums (MOI) at all time-points was associated with the increased %Infection (Figure 2A). At 12 h postinfection, only modest degree of infectivity was observed, whereas the longer postinfection incubation periods (at 24 and 48 h postinfection) had greater %Infection. However, the %Infection at 24 h versus at 48 h postinfection was comparable at all MOI used. For example, at the MOI of 10, %Infection was 93.59 ± 1.28% and 89.29 ± 5.18%, for 24 and 48 h postinfection, respectively, p was not significant by ANOVA with Tukey’s post-hoc multiple comparisons (Figure 2A).

Quantitative analysis of DEN-2-induced cell death was performed using double staining with FITC-conjugated annexin V and propidium iodide, which in principle can be used to determine early and late phases of apoptotic cells, respectively. Propidium iodide can be also used to detect necrotic cells. Percentage of cell death was calculated using the formula: % Cell death = [(no. of all death cells/no. of total cells) × 100%]. At 12 and 24 h postinfection, % cell death was comparable between mock-control cells and DEN-2-infected cells, regardless of the MOI used (Figure 2B). However, massive cell death was observed at 48 h postinfection, particularly with the MOI of 5 and 10 (38.63 ± 4.19% and 55.96 ± 7.84%, respectively).

In this study, we aimed to evaluate early host responses in human endothelial cells upon dengue virus infection, as determined by the altered cellular proteome at an early phase of DEN-2 infection in EA.hy926 cells. The optimal condition for our present study should be the one that provided the greatest %Infection, while had no significant increase in % cell death. We therefore selected the MOI of 10 at 24 h postinfection incubation period as the optimal condition for subsequent proteome analysis (Figure 2).

Altered EA.hy926 Cellular Proteome Induced by DEN-2 Infection. 2-D PAGE was utilized to resolve proteins extracted from 5 individual culture flasks per group of mock-control and DEN-2-infected EA.hy926 cells (total number of gels was 10). Approximately 800 protein spots were detected in each gel using SYPRO Ruby, a fluorescence stain (Figure 3). Differential proteome analysis (Figure 2).

Results

Morphology of EA.hy926 Cells upon DEN-2 Infection. To screen for an optimal condition for proteome analysis of changes in DEN-2-infected EA.hy926 cells, we infected the cells with various MOI, including 1, 5, and 10 and with 12, 24, and 48 h postinfection periods. Mock-control cells were used as the negative control. Cell morphology was evaluated at each postinfection time-point using an inverted microscope. The DEN-2-infected cells at 12 and 24 h postinfection showed similar morphology to the mock-control cells and the cells did not show obvious changes by increasing MOI or doses of the virus used (Figure 1). However, at 48 h postinfection, the cells had dramatic changes in morphology: floating (detached) cells with rounding shape representing death cells were clearly observed, particular at the MOI of 5 and 10 (Figure 1). The optimal condition of DEN-2 infection in EA.hy926 cells for subsequent proteome analysis was then finally determined by flow cytometric analyses of DEN-2 infectivity and cell death.

Flow Cytometric Analyses of DEN-2 Infectivity and Cell Death. DEN-2 infection was confirmed by cytoplasmic staining to detect dengue viral antigen using 3H5 monoclonal antibody, which is specific for DEN-2 envelope protein. The data were from 5 individual culture flasks per group of mock-control and DEN-2-infected cells, respectively. Propidium iodide can be also used to detect necrotic cells. Percentage of cell death was calculated using the formula: % Cell death = [(no. of all death cells/no. of total cells) × 100%]. At 12 and 24 h postinfection, % cell death was comparable between mock-control cells and DEN-2-infected cells, regardless of the MOI used (Figure 2B). However, massive cell death was observed at 48 h postinfection, particularly with the MOI of 5 and 10 (38.63 ± 4.19% and 55.96 ± 7.84%, respectively).

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proteomics analysis using a 2-D image analysis software revealed 15 differentially expressed protein spots between the two groups (mock-control vs DEN-2-infected). All of these differentially expressed protein spots were consistently present or absent in all 5 gels within each group, and had statistically significant p values (< 0.05). And all of them were successfully identified by Q-TOF MS and/or MS/MS analyses. Identities, quantitative data, p values, degrees of changes, and other detailed information are summarized in Table 1.

Of these altered proteins, 9 proteins had increased abundance levels in response to DEN-2 infection, including a set of heterogeneous nuclear ribonucleoproteins (hnRNP K, hnRNP H1 and hnRNP C1/C2), proteasome β subunit, peroxiredoxin 1, tubulin-specific chaperone A (or cofactor A), and β-actin. In contrast, 5 other proteins had decreased abundance levels upon DEN-2 infection, including eukaryotic translation elongation factor-2 (EF-2), chaperonin-containing TCP1 (CCT) subunit 7, thioredoxin reductase 1 (TXNRD1), glutamate dehydrogenase 1, and purine nucleoside phosphorylase. The information available in the protein databases and literature search on these altered proteins revealed that they play important roles in mRNA stability/processing, transcription and translation regulation, molecular chaperoning, oxidative stress response/regulation, cytoskeletal assembly, protein degradation, and cellular metabolisms (see Table 2). A protein spot that was detected only in the DEN-2-infected group was identified as dengue nonstructural protein 1 (NS1).

Confirmation of the Proteomic Data by 2-D Western Blot Analysis. We performed 2-D Western blotting to confirm alterations in some of the identified proteins in response to DEN-2 infection. EF-2 served as a representative for the proteins with decreased levels, whereas hnRNP K isoforms were increased in response to DEN-2 infection in EA.hy926 cells (Figure 4).

Altered Expression and Redistribution of Actin Cytoskeleton, Cadherin, ZO-1 and Surface PECAM-1 after Dengue Virus Infection. Even though a set of altered proteins in response to DEN-2 infection were identified by proteomic analysis, we did not find any proteins that could be directly linked to the impaired membrane integrity and pathology of blood vessels in DHF/DSS. However, we found the increase in actin cytoskeleton in response to DEN-2 infection. Therefore, we performed indirect immunofluorescence to evaluate the organization of actin cytoskeleton. In comparison to mock-control cells, the infected cells as indicated by positive E proteins in the cytoplasm (stained in green) showed the decreased expression and reorganization of the actin cytoskeleton. However, actin stress fiber formation was increased in the adjacent noninfected cells (Figure 5A). Because endothelial integrity is maintained mainly by adherens junction, we next observed the adhesive protein cadherin, a major protein in adherens junction, upon dengue virus infection. Comparing to the mock-control cells, the infected cells revealed weaker staining of vascular endothelial cadherin (VE-cadherin), indicating a marked reduction of cadherin expression after dengue infection (Figure 5A). As the connector protein ZO-1 is a marker of tight junction and serves as a linker between adherens junction and actin cytoskeleton, we also investigated on ZO-1 expression and distribution in dengue-infected cells in order to determine the barrier function of endothelial monolayer. The results showed that ZO-1 was markedly decreased or almost absent in the infected cells (Figure 5A). We finally examined the expression of platelet-endothelial-cell adhesion molecule-1 (PECAM-1 or CD31) (generally, PECAM-1 is concentrated at cell-cell connection site and plays a role in intercellular adhesion, transendothelial migration of leukocytes, and cellular

Figure 1. Morphology of EA.hy926 cells upon DEN-2 infection. EA.hy926 cells were infected with varying MOI (1, 5 and 10) and the cell morphology was observed at various postinfection (p.i.) time-points (h). At 12 and 24 h postinfection, the infected cells had no obvious changes in their morphology as compared to the mock-control cells. At 48 h postinfection, the infected cells (particularly with the MOI of 5 and 10) showed obviously changes such as dense granules in the cytoplasm, rounding shape, and detachment. The images were captured with an original magnification of ×400 in all panels, each of which is a representative of three independent experiments.
modulator via signal transduction). Marked decrease in PECAM-1 expression was found in the infected cells when compared to the mock-control cells (Figure 5A).

The decreased expression levels of β-actin, VE-cadherin and ZO-1 were confirmed by 1-D Western blot analyses (Figure 5B). Please note that, although we identified an increased level of one spot of β-actin by 2-D PAGE analysis, the data from 1-D Western blot analysis, which was consistent with the immunofluorescence data, showed the decreased level of β-actin. These disparate results were not surprising as β-actin was expressed as multiple spots in the 2-D gel and change in one spot did not represent overall change in multiple forms of the same protein, which could be observed in 1-D Western blot analysis.

Effects of PECAM-1 Cross-Linking on Expression and Distribution of Actin Cytoskeleton, VE-cadherin and ZO-1. Transendothelial migration is a multistep process that requires a serial involvement of adhesion molecules. Homotypic interaction of PECAM-1 is employed later in a cascade when leukocytes migrate through the junction between endothelial cells (paracellular migration). It is plausible that the recruitment of leukocytes to inflammatory sites and cytokine storm that occurs in response to dengue infection may have a synergistic effect on the endothelial integrity and permeability. We thus simulated transendothelial migration event, particularly at a step of PECAM-1 engagement after cells were challenged with dengue virus. We hypothesized that remodeling of the actin cytoskeleton, cadherin and ZO-1 may be a molecular basis that underlies the increased vascular permeability during DHF/DSS.

After extensive washing step, we observed that the viral particles (stained in green with clone 3H5 monoclonal antibody specific to DEN-2 envelope protein) bound to the cell surface and some of them were distributed throughout the cytoplasm after a challenge with DEN-2 for 2 h without further incubation, indicating that these cells were infected by the virus at this
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<td>Heterogeneous nuclear ribonucleoprotein K</td>
<td>gi</td>
<td>55958547 MS, MS/MS</td>
<td>106, 48</td>
<td>45, 2</td>
<td>13, 1</td>
<td>5.43</td>
<td>42.01</td>
<td>0.0542 ± 0.0062</td>
<td>0.0887 ± 0.0065</td>
<td>1.64</td>
<td>0.0049</td>
</tr>
<tr>
<td>4</td>
<td>Chaperonin containing TCP1, subunit 7 isoform a</td>
<td>gi</td>
<td>545360 MS</td>
<td>82, NA</td>
<td>39, NA</td>
<td>11, NA</td>
<td>7.55</td>
<td>59.84</td>
<td>0.1053 ± 0.0094</td>
<td>0.0737 ± 0.0073</td>
<td>0.70</td>
<td>0.0290</td>
</tr>
<tr>
<td>5</td>
<td>TXNRD1 (Thioredoxin reductase 1)</td>
<td>gi</td>
<td>49168498 MS, MS/MS</td>
<td>107, 33</td>
<td>34, 3</td>
<td>13, 1</td>
<td>6.07</td>
<td>55.28</td>
<td>0.0450 ± 0.0027</td>
<td>0.0355 ± 0.0016</td>
<td>0.79</td>
<td>0.0165</td>
</tr>
<tr>
<td>6</td>
<td>HNRPH1 (Heterogeneous nuclear ribonucleoprotein H1)</td>
<td>gi</td>
<td>48145673 MS, MS/MS</td>
<td>91, 73</td>
<td>42, 3</td>
<td>11, 1</td>
<td>5.79</td>
<td>49.38</td>
<td>0.0162 ± 0.0099</td>
<td>0.0451 ± 0.0044</td>
<td>2.78</td>
<td>0.0288</td>
</tr>
<tr>
<td>7</td>
<td>Glutamate dehydrogenase 1</td>
<td>gi</td>
<td>4885281 MS, MS/MS</td>
<td>106, 16</td>
<td>37, 3</td>
<td>14, 1</td>
<td>7.66</td>
<td>61.70</td>
<td>0.1053 ± 0.0094</td>
<td>0.0737 ± 0.0073</td>
<td>0.70</td>
<td>0.0290</td>
</tr>
<tr>
<td>8</td>
<td>Heterogeneous nuclear ribonucleoprotein H1</td>
<td>gi</td>
<td>5031753 MS, MS/MS</td>
<td>96, 59</td>
<td>38, 3</td>
<td>13, 1</td>
<td>5.89</td>
<td>49.48</td>
<td>0.0247 ± 0.0163</td>
<td>0.0959 ± 0.0242</td>
<td>3.88</td>
<td>0.0402</td>
</tr>
<tr>
<td>9</td>
<td>Nonstructural protein [Dengue virus type 2]</td>
<td>gi</td>
<td>3349832 MS</td>
<td>44, NA</td>
<td>23, NA</td>
<td>7, NA</td>
<td>6.41</td>
<td>40.53</td>
<td>0.0000 ± 0.0000</td>
<td>0.1041 ± 0.0170</td>
<td>DIV/0</td>
<td>0.0003</td>
</tr>
<tr>
<td>10</td>
<td>Proteasome (prosome, macropain) subunit, beta type</td>
<td>gi</td>
<td>2653343 MS, MS/MS</td>
<td>112, 51</td>
<td>65, 11</td>
<td>14, 2</td>
<td>8.27</td>
<td>26.67</td>
<td>0.0591 ± 0.0182</td>
<td>0.1115 ± 0.0080</td>
<td>1.89</td>
<td>0.0297</td>
</tr>
<tr>
<td>11</td>
<td>Peroxiredoxin 1</td>
<td>gi</td>
<td>4505591 MS, MS/MS</td>
<td>79, 101</td>
<td>52, 15</td>
<td>6, 2</td>
<td>8.27</td>
<td>22.32</td>
<td>0.3510 ± 0.0245</td>
<td>0.4717 ± 0.0435</td>
<td>1.34</td>
<td>0.0421</td>
</tr>
<tr>
<td>12</td>
<td>Tubulin-specific chaperone A (Cofactor A)</td>
<td>gi</td>
<td>4759212 MS/MS</td>
<td>NA, 53</td>
<td>NA, 10</td>
<td>NA, 1</td>
<td>5.25</td>
<td>12.90</td>
<td>0.0708 ± 0.0233</td>
<td>0.1402 ± 0.0160</td>
<td>1.98</td>
<td>0.0398</td>
</tr>
<tr>
<td>13</td>
<td>Heterogeneous nuclear ribonucleoprotein C (C1/C2)</td>
<td>gi</td>
<td>4249959 MS/MS</td>
<td>NA, 58</td>
<td>NA, 15</td>
<td>NA, 3</td>
<td>5.00</td>
<td>32.43</td>
<td>0.0329 ± 0.0164</td>
<td>0.0777 ± 0.0084</td>
<td>2.36</td>
<td>0.0411</td>
</tr>
<tr>
<td>14</td>
<td>Beta actin</td>
<td>gi</td>
<td>4501885 MS, MS/MS</td>
<td>71, 193</td>
<td>27, 17</td>
<td>10, 4</td>
<td>5.29</td>
<td>42.05</td>
<td>0.0291 ± 0.0189</td>
<td>0.0871 ± 0.0143</td>
<td>2.99</td>
<td>0.0400</td>
</tr>
<tr>
<td>15</td>
<td>Purine nucleoside phosphorylase</td>
<td>gi</td>
<td>387033 MS, MS/MS</td>
<td>84, 23</td>
<td>53, 4</td>
<td>12, 2</td>
<td>7.29</td>
<td>32.38</td>
<td>0.4238 ± 0.0204</td>
<td>0.1818 ± 0.0568</td>
<td>0.43</td>
<td>0.0039</td>
</tr>
</tbody>
</table>

*PMF = peptide mass fingerprinting; %cov = %sequence coverage [(number of the matched residues/total number of residues in the entire sequence) × 100%]; DIV/0 = divided by zero NA = not applicable; pI and MW were theoretical values.*
time-point (Figure 6). Cross-linking of PECAM-1 molecules resulted in markedly decreased expression and redistribution of VE-cadherin and ZO-1 (from continuous lines into discontinuous dot lines) in the infected cells but slightly affected the mock-control cells, indicating the synergistic effects between DEN-2 infection and PECAM-1 cross-linking. However, we did not observe the obvious alteration in actin cytoskeleton arrangement after PECAM-1 cross-linking (Figure 6).

Discussion

Vascular leakage is one of the important hallmarks for severe dengue virus infection, particularly DSS. To address molecular mechanisms underlying vascular leakage in dengue virus infection, we performed a proteomics study to identify changes in cellular proteome of human endothelial cells upon DEN-2 infection. EA.hy926 was derived from the fusion between HUVECs and the lung carcinoma cell line A549 and the study by Unger et al.23 has shown that overall characteristics of this cell line are closely related to those of the primary HUVECs. The optimal condition of dengue virus infection for proteome analysis in our present study was carefully defined as we would like to address changes that were affected solely by the host response to dengue virus infection, not by apoptosis or necrosis of cells after a certain phase of infection. We therefore selected the infection condition by varying doses of virus inoculums (MOI) and postinfection incubation periods, and examining degrees of infectivity and cell death. The condition that provided the greatest %Infection and unchanged % cell death (MOI of 10 and 24 h postinfection period) was then chosen for proteome analysis (Figure 2). On the basis of our findings in EA.hy926 cells, DEN-2 viral progeny began to release into the culture supernatant at 8 h postinfection and its release was significantly greater during 16–24 h postinfection periods (data not shown). We presumed that only a few replication cycles occurred during this period. In this study, we therefore defined our infection condition as the early phase of infection.

Differential proteomics and mass spectrometric analyses identified significant alterations (either increase or decrease in levels) of 15 proteins (Figure 3 and Table 1), all of which are novel findings as compared to the data reported in the previous transcriptomic studies.17–19 These inconsistencies were not unexpected as there were so many factors varied in these different studies that could definitely affect the results; for example, differences in cell type, virus serotype/strain, and the infection condition (MOI and postinfection incubation period). Moreover, to the best of our knowledge, the present study is the first that reports changes in the proteome of human endothelial cells in response to dengue virus infection, whereas the aforementioned studies have reported the alterations only

Table 2. Functional classification of the altered proteins in DEN-2-infected EA.hy926 cells

<table>
<thead>
<tr>
<th>Functional classification</th>
<th>Down-regulated proteins</th>
<th>Up-regulated proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein involved in mRNA stability/processing and transcription regulation</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Translation regulatory protein</td>
<td>–</td>
<td>• Heterogeneous nuclear ribonucleoprotein H</td>
</tr>
<tr>
<td>Molecular chaperone</td>
<td>• Eukaryotic translation elongation factor2 (EF-2)</td>
<td>• Heterogeneous nuclear ribonucleoprotein K</td>
</tr>
<tr>
<td>Oxidative stress regulatory protein</td>
<td>• Chaperonin containing TCP1</td>
<td>• Heterogeneous nuclear ribonucleoprotein C1/C2</td>
</tr>
<tr>
<td>Cytoskeletal protein</td>
<td>• Thioredoxin reductase 1 (TXNRD1)</td>
<td>–</td>
</tr>
<tr>
<td>Protein involved in degradation of other proteins</td>
<td>–</td>
<td>• Tubulin-specific chaperone A (Cofactor A)</td>
</tr>
<tr>
<td>Metabolic enzymes</td>
<td>• Glutamate dehydrogenase 1</td>
<td>• Peroxiredoxin 1</td>
</tr>
<tr>
<td>Viral protein</td>
<td>• Purine nucleoside phosphorylase</td>
<td>• Beta actin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Proteasome subunit, beta type</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Nonstructural protein</td>
</tr>
</tbody>
</table>

Figure 4. 2-D Western blot analyses of elongation factor-2 (EF-2) (A and B) and hnRNP K (C and D). Extracted proteins (100 µg of total protein for each gel) were resolved by 2-D PAGE and transferred onto nitrocellulose membranes. The membranes were incubated with rabbit polyclonal anti-EF-2 or mouse monoclonal anti-hnRNP K primary antibody, and then with respective secondary antibody conjugated with horseradish peroxidase. The immunoreactive spots were then visualized by chemiluminescence and autoradiography.
Therefore, it is difficult to directly compare our data to those of other previous studies. On the other hand, the information obtained from diverse conditions of the infection at both transcript (transcription) and protein (translation) levels would be complementary to yield the comprehensive picture to explain how endothelial cells respond to dengue virus infection.

Recently, secretome profiling of dengue-infected HepG2 cells reported by Higa et al. showed that some interesting proteins, for example, alpha-enolase, superoxide dismutase, peptidyl-
propyl isomerases A and B, tissue inhibitor of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2) and macrophage migration inhibitory factor (MIF), might play important roles in hepatic involvement in the progression of dengue infection. In addition, several studies have demonstrated that the infected endothelial cells produced proinflammatory cytokines and chemokines such as TNF-α, IFN-γ, RANTES, IL-6 and IL-8, which in turn regulated the cell permeability.14,16,25 Moreover, cytokine profiling in dengue-infected patient sera revealed the significant elevation of IL-6, IL-8 and TGF-β1 in DHF/DSS patients.26 In our present study, we did not characterize the altered secretion of proteins and chemokines/ cytokines. However, we expected that some secreted proteins and chemokines/cytokines were also altered in the DEN-2-infected endothelial cells. Therefore, further secretome and/or metabolome analyses will be beneficial and complementary to our present study.

Unfortunately, we did not observe the alterations in proteins that are directly responsible for the occurrence of vascular leakage using proteomic approach. However, we found the altered actin expression upon dengue virus infection. Actin plays a predominant role in maintaining cell shape and membrane integrity through interaction with protein components of the actin cytoskeleton.33 Cadherin is recognized as the core component of adherens junction and mediates local control of the actin cytoskeleton through its cytoplasmic tail by linking to catenin complexes.28 It was evidenced that inhibition of vascular endothelial cadherin (VE-cadherin) resulted in an increased permeability and augmented transmigration of neutrophils through the endothelium.34 In our present study, we observed a similar pattern of the VE-cadherin distribution in both mock-control and infected cells; however, VE-cadherin expression was suppressed in the infected cells (Figure 5). Previous study demonstrated that VE-cadherin was down-regulated after co-culture of endothelial cells with peripheral blood mononuclear cells (PBMC).32 We demonstrated herein that direct dengue infection dramatically caused cadherin suppression within 24 h post-infection. This alteration would support the increased permeability of EA.hy926 monolayer.

We then investigated the endothelial barrier function that mediates via a tight junction, which acts as a gate that provides a paracellular passage of ions and solutes. Transmembrane proteins, occludins and claudins, are the two types of proteins which contribute to this junction. Moreover, tight junction also comprises an associated ZO protein that provides a scaffold and links this junction to adherens junctions and actin cytoskeleton.33 We found that expression of the connector protein ZO-1 was significantly reduced from the cell border and ZO-1 expression was disrupted into a discontinuous line after 24 h of dengue infection (Figure 5). Accumulating studies have supported that disruption of tight junction is a possible mechanism for increasing endothelial permeability. Coxsackievirus B3 could alter endothelial permeability by induced reorganization of F-actin and ZO-1 discontinuity.32 Down-regulation of ZO-1 and occludin contributed to a transient increase of blood-tumor barrier mediated by bradykinin.36 Moreover, several pathogens take advantages by perturbation on tight junctions, including human immunodeficiency virus (HIV),37 simian immunodeficiency virus (SIV),37,38 hepatitis C virus (HCV),39 and rhinovirus.40 We postulated that marked decrease in ZO-1 expression and its redistribution induced by dengue infection may be related to the altered vascular permeability found in dengue-infected patients.

We also studied the alteration in expression level and redistribution of PECAM-1 (also known as CD31). PECAM-1 is a transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily. It is found mainly at the intercellular junctions of endothelial cells and mediates interaction between endothelial cells and adherent leukocytes.41 In our present study, remarkable down-regulation of PECAM-1 was observed in dengue-infected cells. It is plausible that the down-regulated PECAM-1 in dengue-infected cells might be mediated by soluble mediators produced in response to infection. It is therefore interesting to further study on PECAM-1 in response to dengue infection because recent studies have demonstrated roles of PECAM-1 in signal transduction and adhesive function.52,43

Transendothelial migration of leukocytes to inflammatory site is a multistep process that requires interaction between adhesion molecules presenting on both endothelial cells and leukocytes. These adhesion proteins include ICAM-1 and PECAM-1. PECAM-1 engagement is mediated later in a cascade of leukocyte transmigration. In addition, paracellular migration of leukocytes involves remodeling of protein components of cellular junctions and may mediate endothelial permeability under uncontrolled inflammatory condition. To avoid the effect of soluble mediators, we simulated transendothelial migration of leukocytes by PECAM-1 cross-linking after cells were challenged with dengue virus and addressed whether VE-cadherin, ZO-1 and the actin cytoskeleton were rearranged. It has been previously reported that stimulation of dengue-infected endothelial cells with PBMC resulted in the decrease of transendothelial electrical resistance, representing an increase in membrane permeability of the cell monolayer.32 This was concomitant with the down-regulation of VE-cadherin.32 In this notion, our results revealed not only a suppressed expression, but also a punctuation of cadherin to express as a discontinuous line. In our present study, the decreased VE-cadherin expression after PECAM-1 engagement supported the physiological findings that reduction of VE-cadherin is required during the transmigration of neutrophils.44 Unlike the study by Dewi et al.,32 PBMC was not included in our experiment; therefore, lower expression and redistribution of VE-cadherin.
Dengue-Induced Changes in Vascular Permeability

was caused solely by PECAM-1 engagement. In addition, cells were infected for only 2 h before PECAM-1-cross-linking; thus, it is too early for the alteration to be induced by the production of soluble mediators in response to the infection. Moreover, the decreased expression and redistribution of ZO-1 at the cell border was found after PECAM-1 cross-linking, suggesting the disruption of tight junctions.

In summary, we identified a set of altered proteins in EA.hy926 human endothelial cells in response to dengue virus infection. These alterations, as the host responses, are involved in several biological phenomena, which may protect cells from deteriorating effects of the viral infection or, on the other hand, may benefit virus and its replication. Factors determining whether the changes will predominantly benefit the host cells or the virus most likely rely on degree and timing of the infection. Our data reported herein may lead to further highly focused study to address specific molecular pathways in human endothelial cells to better understand the pathophysiology of vascular leakage during severe dengue virus infection. As an example, our functional data provide evidence that dengue virus infection affects the organization of actin cytoskeleton and other molecules involved in the maintenance of endothelial integrity, that is, VE-cadherin and ZO-1. Additionally, constitutively expression of PECAM-1 was also suppressed in response to dengue infection. Moreover, PECAM-1 cross-linking to simulate the event during leukocyte transendothelial migration demonstrated that protein components at the intercellular junctions, particularly adherens junction and tight junction, were down-regulated and reorganized. Taken together, alteration in expression and redistribution of these molecules may serve as a molecular basis of the impaired endothelial integrity and increased vascular permeability observed in severe dengue infection.

Abbreviations: 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis; ACN, acetonitrile; CHAPS, [3-(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate; CID, collision-induced dissociation; CPE, cytopathic effect; DEN-2, dengue virus serotype-2; DF, dengue fever; DSS, dengue shock syndrome; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FFU, focus forming unit; HCV, hepatitis C virus; hnmRN, heterogeneous nuclear ribonucleoprotein, HUVEC, human umbilical vein endothelial cell; IEF, isoelectric focusing; IL, interleukin; MOI, multiplicity of infection; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NS1, nonstructural protein 1; PECAM-1, platelet-endothelial cell adhesion molecule-1; Q-TOF, quadrupole time-of-flight; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; RNA, ribonucleic acid; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; VE-cadherin, vascular endothelial cadherin; ZO-1, Zonula occludens-1.

Acknowledgment. We are grateful to Drs. Prida Malasit, Sansanee Noisakran, and Panisadee Avirutman for their valuable suggestions. We also thank Ms. Patcharee Songprakon for her technical assistance. This work was supported by the National Center for Genetic Engineering and Biotechnology (BT-B-02-MG-B4-5003) (to S.P. and V.T.) and by Siriraj Graduate Thesis Scholarship (to R.K.).

References


