Expression of Cytokine, Chemokine, and Adhesion Molecules during Endothelial Cell Activation Induced by Antibodies against Dengue Virus Nonstructural Protein 1

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Expression of Cytokine, Chemokine, and Adhesion Molecules during Endothelial Cell Activation Induced by Antibodies against Dengue Virus Nonstructural Protein 1

Chiou-Feng Lin,* Shu-Chen Chiu,* Yu-Ling Hsiao,* Shu-Wen Wan,* Huan-Yao Lei,* Ai-Li Shiau,* Hsiao-Sheng Liu,* Trai-Ming Yeh,† Shun-Hua Chen,* Ching-Chuan Liu,‡ and Yee-Shin Lin2*

Vascular dysfunction is a hallmark associated with disease onset in dengue hemorrhagic fever and dengue shock syndrome. In addition to direct viral damage, immune responses to dengue virus (DV) infection may also underlie the pathogenesis of disease. We have proposed a mechanism of molecular mimicry in which Abs directed against DV nonstructural protein 1 (NS1) cross-react with endothelial cells and induce damage. In this study, we demonstrated the inflammatory endothelial cell activation induced by anti-DV NS1 via the transcription factor NF-κB-regulated pathway. Protein phosphorylation and NF-κB activation were observed after anti-DV NS1 stimulation in a human microvascular endothelial cell line-1. The cytokine and chemokine production, including IL-6, IL-8, and MCP-1, but not RANTES, in endothelial cells increased after treatment with anti-DV NS1 Abs. The expression of IL-6, IL-8, and MCP-1 was blocked by the preabsorption of anti-DV NS1 with DV NS1 or by the inhibition of NF-κB activation. Furthermore, the increases in both ICAM-1 expression and the ability of human PBMC to adhere to endothelial cells were also observed, and these effects were inhibited by pretreatment with anti-ICAM-1 or anti-MCP-1 Abs. Therefore, in addition to endothelial cell apoptosis, as previously reported, inflammatory activation occurs in endothelial cells after stimulation by anti-DV NS1 Abs. These results suggest the involvement of anti-DV NS1 Abs in the vasculopathy of DV infection. The Journal of Immunology, 2005, 174: 395–403.

Patients with dengue virus (DV) infection present a wide range of diseases from mild dengue fever to life-threatening dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) (1, 2). There is no vaccine against DHF/DSS because its pathogenic mechanisms are still not fully understood (3–6). In dengue pathogenesis, the involvement of viral and immune responses has been hypothesized (3–9). Virus variation and virus load involving Ab-dependent enhancement of infection have been suggested to be responsible for the progression and severity of dengue disease (10–17). Cell activation, impaired proliferation, cytokine and chemokine production, and apoptosis are caused after DV infection by a direct (18–32) or indirect route (33, 34). It is likely that different mechanisms are involved in the pathogenesis of DV infection.

DHF/DSS represents a pathological complication caused by multiple symptoms, including thrombocytopenia, coagulopathy, and vasculopathy (7, 35–37). Plasma leakage, also called the hemorrhagic syndrome, occurs once vessel endothelium is disrupted and is followed by the loss of its barrier function. The pathogenesis of endothelial dysfunction resulting in vascular leakage remains unclear, however. In addition to direct viral damage, such as the induction of apoptosis in endothelial cells as observed in vitro (27), complement activation and cytokine and chemokine production induced by DV infection appear to be involved in the induction of endothelial cell damage (26, 27, 38, 39). DV can also indirectly modulate endothelial cell function through Ab-enhanced infection of DV in peripheral blood monocytes (33). Furthermore, the involvement of anti-endothelial cell autoantibodies in DV pathogenesis has been proposed (40–42). Endothelial cell apoptosis caused by autoantibodies may be related to the transient leakage syndrome in dengue vasculopathy.

Inflammatory immune responses facilitate the progression of disease severity in DHF/DSS (7–9). Lymphocytes, monocytes and macrophages, mast cells, endothelial cells, liver cells, and dendritic cells are the targets of DV, and these cells could produce cytokines or chemokines, or both, after DV infection (20–22, 24–27, 33, 38, 39, 43, 44). Increased levels of cytokines, including IL-2, IL-6, IL-8, IL-10, IL-13, IL-18, IFN-γ, TNF-α, and MCP-1, have been observed in patients with DV infections (8, 27, 45–52). The roles of various cytokines and chemokines in the pathogenesis of DHF/DSS need to be clarified, especially on endothelial cell activation, which is related to vasculopathy.

Previous studies in our laboratory showed the pathogenic role of Abs against nonstructural protein 1 (NS1) present in dengue patients or generated in mice (40, 41). Anti-DV NS1 Abs cross-reacted with endothelial cells and induced apoptosis via an NO-mediated pathway (40, 42). Pathogenic anti-DV NS1 may contribute to the progression of DHF/DSS (41). In the present...
study, we demonstrated protein tyrosine phosphorylation and NF-κB activation after endothelial cells had been bound by anti-DV NS1 Abs. We also demonstrated that the increased levels of cytokine and chemokine expression were regulated by NF-κB, and that anti-DV NS1 stimulation caused adhesion molecule expression and PBMC adhesion to endothelial cells.

Materials and Methods

Mice

BALB/cByJ breeder mice were obtained from The Jackson Laboratory and maintained on standard laboratory food and water ad libitum in our medical college laboratory animal center. Their 8-wk-old progeny were used for the generation of Abs in the present study.

Abs and reagents

Both purified and FITC-conjugated mAbs against phosphotyrosine (clone PY20) were purchased from Oncogene Research Products. Rabbit Abs specific for NF-κB p65 were purchased from Chemicon International. Anti-human IL-6, IL-8, MCP-1, RANTES, and ICAM-1 mAbs were from BD Pharmingen. Neutralizing anti-human MCP-1 and ICAM-1 mAbs were also from BD Pharmingen, and mouse mAb to β-actin was from Sigma-Aldrich. FITC- or HRP-conjugated goat anti-mouse or anti-rabbit IgG, NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC), protein synthesis inhibitor cycloheximide, caspase inhibitor benzoylargininevalylalanyl-

Polyclonal Abs against DV or Japanese encephalitis virus (JEV) NS1 were obtained from BALB/c mice immunized i.p. with purified recombinant DV-2 (New Guinea C strain) or JEV (NT109 strain) NS1 proteins, as described previously (40). The IgG fractions from hyperimmunized mouse sera were purified with a protein G-Sepharose affinity chromatography column (Amersham Biosciences) and recovered with HCl-glycine. The reactivity of purified IgG against NS1 was confirmed by SDS-PAGE and Western blot. The control IgG was eluted from a protein G column loaded with normal mouse sera. The endotoxin concentration of each of these preparations was <0.03 EU/μg, as determined by a Limulus amebocyte lysate assay (Associates of Cape Cod).

Patient sera

Dengue patient sera were obtained from N. Hung (Department of Dengue Hemorrhagic Fever, Children’s Hospital No. 1). Seventeen serum samples were collected from patients with DHF disease severity grades I–III. Diagnosis of DHF was based on the clinical criteria established by the World Health Organization. Sera from five healthy volunteers were used as the normal controls.

Cell and virus culture

Human microvascular endothelial cell line-1 (HMEC-1) was passed in culture plates containing endothelial cell growth medium (EGM; Cambrex) composed of 2% FBS, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, and antibiotics (40). Only those cultures from three to five passages with a viability of >95% by eosin-Y staining were used for experiments. Baby hamster kidney cell line and C6/36 cells were cultured in DMEM medium containing 10% FBS and antibiotics. PBMC were isolated from normal volunteer blood using Ficoll-Paque isolation (Amersham Biosciences), according to the standard procedures, and were used for adhesion assay.

Dengue-2 virus (PL6M, Taiwan isolated) was maintained in the C6/36 cells. Briefly, monolayers of C6/36 were incubated with DV at a multiplicity of infection of 0.01 and incubated at 26°C in 5% CO2 for 5 days. The cultured 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 the BHK-21 cell line, as described previously (26).

Western blotting and flow cytometry for detection of protein tyrosine phosphorylation

HMEC-1 cells underwent starvation in serum-free EGM for 1 h before the experiment. Next, the cells were treated with mouse anti-DV NS1, anti-JEV NS1, or control IgG for various time intervals. Cells were then harvested and lysed with a buffer containing 1% Triton X-100, 50 mM Tris (pH 7.5), 10 mM EDTA, 0.02% NaN3, and a protease inhibitor mixture (Boehringer Mannheim). After being freeze thawed once, cell lysates were centrifuged at 12,000 rpm at 20 min at 4°C. The supernatants were collected and boiled in sample buffer for 5 min. Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane (Millipore), blocked overnight at 4°C in PBS-T containing 5% skim milk, and probed with Abs against phosphotyrosine at 4°C overnight. After being washed with PBS-T, blots were incubated with a 1:5000 dilution of anti-rabbit IgG (1 h at 4°C). The protein bands were developed with 3-aminono-9-ethylcarbazole (AEC) substrate kit (Zymed Laboratories).

For immunostaining followed by flow cytometric analysis, cells were washed in PBS, fixed with 1% paraformaldehyde in PBS at room temperature for 10 min, and permeabilized with 70% ethanol. After being washed three times, cells were incubated with FITC-conjugated Ab against phosphotyrosine for 1 h at 4°C and analyzed by flow cytometry (FACS Calibur; BD Biosciences) with excitation set at 488 nm.

Western blotting and EMSA for detection of NF-κB activation

Nuclear translocation of NF-κB was detected by Western blotting. Harvested cells were lysed with buffer A (10 mM HEPES (pH 7.8), 5 mM MgCl2, 10 mM KCl, 1 mM ZnCl2, 0.2 mM EDTA, 1 mM NaVO3, 10 mM NaF, 0.5 mM DTT, and 0.5 mM PMSF), incubated on ice for 10 min, and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatants were collected and lysed with a buffer containing 1% Triton X-100, 50 mM NaCl, 1 mM ZnCl2, 0.2 mM EDTA, 25% glycerol, 1 mM NaVO3, 10 mM NaF, 0.5 mM DTT, and 0.5 mM PMSF) and incubated for 15 min on ice with occasional mixing. Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane, blocked overnight at 4°C in PBS-T containing 5% skim milk, and probed with Abs against NF-κB p65 subunit at 4°C overnight. After being washed with PBS-T, blots were incubated with a 1:5000 dilution of HRP-conjugated anti-rabbit IgG for 1 h at 4°C. The protein bands were developed with an AEC substrate kit.

For EMSA, nuclear extracts were collected, as described above, and separated by 3% acrylamide gel. The DIG Gel Shift kit (Roche Diagnostics) was used according to the manufacturer’s instructions. The protocol was specific for NF-κB binding that consisted of a digoxigenin-labeled double-strand oligonucleotide (5′-AGTTGAGGGGACCTTTCCAGGC-3′). Specificity of protein-DNA complexes was detected by immunoreactivity with sheep anti-digoxigenin conjugated with alkaline phosphatase and developed using chemiluminescent substrate disodium 3-(4-methoxyxpyrrole[1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1<sup>3</sup>]-decane]-4-yl) phenyl phosphate (Roche). The generated chemiluminescent signals were recorded on x-ray film by autoradiography.

Immunohistochemistry and flow cytometry for detection of cytokine, chemokine, and adhesion molecule

Monolayers of HMEC-1 cells were cultured on sterile glass slides, followed by treatment with mouse anti-DV NS1, anti-JEV NS1, or control IgG for various time intervals. For flow cytometric analysis, cells were detached using 1000 U/ml trypsin and 0.5% EDTA. Cells were fixed and permeabilized with Cytofix/Cytoperm kit (BD Pharmingen). For immunohistochemical staining, fixed cells were incubated with 0.3% H2O2 in PBS for 5 min to quench endogenous peroxidase activity and then washed again with PBS. Abs specific for IL-6, IL-8, RANTES, MCP-1, and ICAM-1 were added to cells and incubated for 1 h at 4°C. After being washed with PBS, cells were incubated with HRP- or FITC-conjugated anti-primary Ab for 1 h at 4°C. After being washed with PBS, cells were developed using the AEC substrate kit (Zymed Laboratories), counterstained with hematoxylin (Sigma-Aldrich), and viewed with light microscopy or analyzed by flow cytometry with excitation set at 488 nm.

ELISA

The concentrations of cytokines and chemokines, including IL-6, IL-8, and MCP-1, in culture supernatants and DHF patient sera were determined using ELISA kits (R&D Systems). The manufacturer’s instructions were followed, and the concentrations were determined by spectrophotometry at 450 nm (Molecular Devices).

RT-PCR

The expression of MCP-1 and RANTES mRNA was determined using RT-PCR analysis. Total cellular RNA from endothelial cells was extracted using TRizol reagent (Invitrogen Life Technologies), according to the manufacturer’s instructions. The concentration of RNA was quantified by spectrophotometry at 260 nm (U-2000; Hitachi). The cDNA was prepared
by reverse transcription, as previously described (40), and PCR was performed using a PCR controller (GeneAmp PCR System 2400; PerkinElmer). PCR was conducted in 50 μl of the reaction mixture (1.5 mM MgCl₂ and 0.2 mM each of dATP, dGTP, dCTP, and dTTP) containing primers at 1.5 μM each, 0.2 μg/ml RNase A (Sigma-Aldrich), and 1 U of TaqDNA polymerase (Promega), as follows: 94°C for 1 min; 30 thermal cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and a final cycle at 72°C for 5 min. The oligonucleotide primers for human MCP-1 (sense, 5'-GAGGTGAACTCTGCCGAGGACTG-3' and antisense, 5'-ATGCTCTCCTCGG-3'), RANTES (sense, 5'-TGCC TCCCCATATTCTCGG-3' and antisense, 5'-TCATGGTGTCGCACTCCG-3'), and antisense, 5'-ATGCTCTCCTCGG-3') were used according to previously published sequences (27, 40). DV primer set (sense, 5'-GATATGG TCTGTTGATCCTGTA-3' and antisense, 5'-CTGATTTCCATCCGGTA-3') was used as an infected control (26). The PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and viewed with UV light.

**Transient transfection**

In experiments using dominant-negative mutant, HMEC-1 cells were cotransfected with the dominant-negative IKK-β (the kind gift of C. Chen, Department of Pharmacology, National Taiwan University) or the empty vector pcDNA3.1 (the kind gift of M. Lai, Department of Biochemistry, National Cheng Kung University). Briefly, HMEC-1 cells were grown to 50% confluent in six-well plate, then transfected with the dominant-negative IKK-β (0.5 μg) or the empty vector (1.0 μg) using Lipofectamine 2000 reagent (Invitrogen Life Technologies). After 24 h of transfection, the cells were used for experiments.

**Adhesion assay**

HMEC-1 cells (5 x 10⁴ cells/well) were plated into eight-well glass chamber slides (Nalge Nunc International). When monolayers were confluent, the cells were stimulated with anti-DV NS1, anti-JEV NS1, or control IgG in serum-free culture medium. After 24 h of incubation, the cells were washed once with medium and incubated for 2 h at 37°C with isolated PBMC (1 x 10⁵ cells/well) in a total volume of 250 μl/well. At the end of the incubation period, the nonadherent cells were removed by washing twice with 0.1% BSA in PBS. Adherent cells were stained with Liu’s stain (TONYAR Biotech) and viewed with light microscopy. The adherent cells were counted on three consecutive microscopic fields (53, 54).

**Statistical analysis**

Comparisons between various treatments were performed by Student’s t test with SigmaPlot version 4.0 for Windows (Cytel Software). Values were considered statistically significant at p < 0.05.

**Results**

Protein tyrosine phosphorylation and NF-κB activation induced by anti-DV NS1 Abs in human endothelial cells

In a previous study (40), we showed that anti-DV NS1 cross-reacted with endothelial cells. To assess the signal transduction induced by anti-DV NS1 in endothelial cells, we investigated protein phosphorylation and NF-κB activation in the present study. We used Western blotting to assay the levels of protein tyrosine phosphorylation from Ab-treated HMEC-1 cells (Fig. 1A, upper panel). We also determined the relative ODs of bands (Fig. 1A, lower panel). Results showed tyrosine phosphorylation of proteins in HMEC-1 after binding with anti-DV NS1, but not with anti-JEV NS1 or control IgG. EGM-treated cells were used as the positive control for protein phosphorylation induced by growth factors. Protein tyrosine phosphorylation was further analyzed by flow cytometry as presented by the percentages of fluorescence-positive cells (Fig. 1B, upper panel). The time-kinetic changes of phosphorylated cells after Ab stimulation are shown in Fig. 1B (lower panel). Results indicated that protein tyrosine phosphorylation reached its highest level 30 min after anti-DV NS1 treatment. The specificity of Ab stimulation was demonstrated by the blockage of protein tyrosine phosphorylation when anti-DV NS1 Abs were pretreated with rDV NS1 proteins (data not shown). Tyrosine phosphorylation of cellular proteins was also observed in HMEC-1 cells after they had been treated with DHF patient sera (data not shown).

We next investigated the involvement of NF-κB in the anti-DV NS1-triggered signaling pathways. After Ab treatment for 3 or 24 h, endothelial cell extracts were separated for cytoplasmic and nuclear fractions, and then followed by immunoblotting for NF-κB. The expression of NF-κB decreased in the cytoplasmic fraction and increased in the nuclear fraction in cells with anti-DV NS1 treatment compared with those with anti-JEV NS1 or control IgG treatment (Fig. 2A). Activation of NF-κB as indicated by its translocation from cytoplasm to nucleus was also observed by immunohistochemical staining (data not shown). The DNA-binding ability of NF-κB was determined by EMSA, which showed positive signals in the anti-DV NS1-treated cells compared with the untreated, anti-JEV NS1, or control IgG group (Fig. 2B). The DNA-binding ability of NF-κB was inhibited when anti-DV NS1 was preabsorbed with rDV NS1 proteins. The specificity of the cross-reactivity of anti-NS1 Abs was thus confirmed. To further demonstrate whether NF-κB activation is requisite for its DNA-binding ability mediated by anti-DV NS1, the effect of NF-κB inhibitor PDTC was examined. Results showed that the addition of PDTC to cell cultures caused an inhibition of the DNA-binding activity of NF-κB in anti-DV NS1-treated cells (data not shown).

**Production of cytokines and chemokines in endothelial cells after anti-DV NS1 stimulation**

Immune activation of endothelial cells along with the induction of proinflammatory responses underlie dengue vascular disorders (7, 9). We therefore examined whether anti-DV NS1 Abs could induce inflammatory factors in endothelial cells. Results from immunohistochemical staining revealed the expression of IL-6, IL-8,
and MCP-1 by HMEC-1 cells after they had been treated with anti-DV NS1 for 24 h (Fig. 3A). The expression of RANTES, however, could not be detected in anti-DV NS1-treated cells. Expression of IL-6, IL-8, and MCP-1 was further quantified using both flow cytometric analysis (Fig. 3B) and ELISA (Table I). Treatment with anti-JEV NS1 Abs showed only basal levels of IL-6, IL-8, MCP-1, and RANTES, similar to those with control IgG. The ELISA (Table I) and immunohistochemical staining (data not shown) showed IL-6, IL-8, and MCP-1 production after anti-DV NS1 treatment for as short as 3–6 h. The specificity of the cross-reactivity of anti-NS1 Abs was further confirmed by the inhibitory effect of preabsorption of anti-DV NS1 with recombinant DV NS1, but not JEV NS1 proteins (Fig. 3B). Further studies showed that the mRNA expression of MCP-1, but not RANTES, was elevated after anti-DV NS1 stimulation in HMEC-1 cells, as detected via RT-PCR (Fig. 4). By contrast, cells infected with DV showed increased mRNA expression in RANTES, but not in MCP-1. The ELISA quantification demonstrated high levels of MCP-1 in DHF patient sera at different disease grades compared with healthy controls (Table II), similar to results in a study of DSS patients (27).

Requirement for NF-κB in anti-DV NS1-induced cytokine and chemokine production

Anti-DV NS1 induced NF-κB activation (Fig. 2). We further examined the involvement of NF-κB modulation on the expression of IL-6, IL-8, and MCP-1. After treatment with anti-DV NS1 in HMEC-1 cultures for 24 h, the protein levels of IL-6, IL-8, and MCP-1 increased. NF-κB inhibitor PDTC, but not NO synthase inhibitor l-NAME or caspase inhibitor zVAD-fmk, was able to inhibit the anti-DV NS1-mediated elevations of IL-6, IL-8, and MCP-1 expression (Fig. 5, left). Further confirming the requirement for NF-κB activation, HMEC-1 cells transfected with dominant-negative IKK-β showed a blockage in IL-6, IL-8, and MCP-1 expression compared with the vector control (Fig. 5, right). These results revealed that NF-κB in anti-DV NS1-induced endothelial cell activation up-regulates cytokine and chemokine expression.

MCP-1 facilitates the expression of ICAM-1 induced by anti-DV NS1 in endothelial cells

The activation of endothelial cells involves not only the induction of cytokines and chemokines; our data showed that the expression of adhesion molecules was also augmented. After anti-DV NS1
stimulation, an increase in the expression of ICAM-1 was detected with immunohistochemical staining (Fig. 6A). The increase in ICAM-1 expression was confirmed using flow cytometric analysis (see Fig. 6B for time kinetics and dose-response data). To further investigate the regulation of ICAM-1 expression by NF-kB in anti-DV NS1-treated cells, PDTC was used. Results showed a reduction of ICAM-1 expression once cells were pretreated with PDTC (data not shown). MCP-1 elevates ICAM-1 expression (55–57).

We therefore investigated the relationship between MCP-1 and ICAM-1 expression after anti-DV NS1 stimulation. Pretreatment with MCP-1-neutralizing Abs reduced the expression of ICAM-1, suggesting that the ICAM-1 expressed was regulated, at least in part, by MCP-1 in anti-DV NS1-treated endothelial cells. Similar results were observed using both immunohistochemical staining (Fig. 6A) and flow cytometric analysis (Fig. 6B). Further studies using anti-DV NS1 F(ab)_2 confirmed that the activation of endothelial cells, as detected by ICAM-1 expression, was not via an Fc-mediated signaling pathway (data not shown).

Regulation of ICAM-1 and MCP-1 on the adherence of PBMC to anti-DV NS1-treated endothelial cells

The ability of immune cells to adhere to DV-infected endothelial cells was investigated next. Based on the findings shown in Fig. 6, anti-DV NS1 Ab-induced expression of ICAM-1 was down-regulated by anti-MCP-1. To explore the effects of ICAM-1 up-regulation on endothelial cell and immune cell interaction, the adherence of PBMC to anti-DV NS1-treated HMEC-1 cells was assessed. Higher levels of PBMC adhering to endothelial cells could be observed in cells treated with anti-DV NS1 for 24 h, compared with those with anti-JEV NS1 or control IgG (Fig. 7A). The increase in PBMC adherence was quantified by counting the numbers of adherent cells under a microscope (see Fig. 7B for time kinetics and dose-response data). We further investigated whether the increase in ICAM-1 expression was related to the ability of PBMC to adhere to endothelial cells. Pretreatment with ICAM-1-neutralizing Abs inhibited the adherence of PBMC, suggesting that ICAM-1 expressed on endothelial cells was involved in this binding effect.

We also tested the potential regulatory role of MCP-1 on ICAM-1-mediated PBMC adherence to endothelial cells. A reduction in PBMC adherence was demonstrated when endothelial cells were pretreated with anti-MCP-1 Abs (Fig. 7). These results thus suggested that the ICAM-1-mediated ability of PBMC to adhere to endothelial cells was at least partially regulated by MCP-1. To further investigate whether the up-regulation of cell adherence was dependent on new protein synthesis, HMEC-1 cells were pretreated with protein synthesis inhibitor cycloheximide. Results showed that cycloheximide treatment inhibited PBMC adhesion, suggesting a requirement for protein synthesis of ICAM-1 or MCP-1 to increase PBMC adherence to HMEC-1 cells (Fig. 7B).

Discussion

Dengue disease is an emerging public health problem, and an increasing number of cases are being reported (5, 6, 58). Vascular leakage, liver abnormality, and hemorrhagic diathesis are the life-threatening complications that occur in dengue patients with DHF/DSS. Their pathogenic mechanisms, however, are not well understood. We have proposed a mechanism of molecular mimicry in which Abs directed against DV NS1 would cross-react with endothelial cells and cause damage. Our studies showed anti-endothelial cell Abs (AECAs) in dengue patient sera (41). Moreover, endothelial cell damage induced by anti-DV NS1 by the production of NO may play a role in the disruption of vessel endothelium and contribute to the AECAs-induced pathogenesis of vasculopathy (40, 42). When taken together with previous findings that anti-DV NS1 Abs also cross-reacted with platelets (59) and expressed on endothelial cells was involved in this binding effect.

We also tested the potential regulatory role of MCP-1 on ICAM-1-mediated PBMC adherence to endothelial cells. A reduction in PBMC adherence was demonstrated when endothelial cells were pretreated with anti-MCP-1 Abs (Fig. 7). These results thus suggested that the ICAM-1-mediated ability of PBMC to adhere to endothelial cells was at least partially regulated by MCP-1. To further investigate whether the up-regulation of cell adherence was dependent on new protein synthesis, HMEC-1 cells were pretreated with protein synthesis inhibitor cycloheximide. Results showed that cycloheximide treatment inhibited PBMC adhesion, suggesting a requirement for protein synthesis of ICAM-1 or MCP-1 to increase PBMC adherence to HMEC-1 cells (Fig. 7B).

Table I. Production of IL-6, IL-8, and MCP-1 by endothelial cells after treatment with anti-DV NS1 Abs for various time periodsa

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ab Dose (µg)</th>
<th>Control IgG</th>
<th>Anti-JEV NS1</th>
<th>Anti-DV NS1</th>
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<tr>
<td></td>
<td></td>
<td>IL-6 (pg/cell)</td>
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<tr>
<td>0</td>
<td></td>
<td>0.12 ± 0.02</td>
<td>0.14 ± 0.10</td>
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<td>3</td>
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<td>6</td>
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<td>0.35 ± 0.14</td>
<td>5.33 ± 1.34**</td>
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<td>0.39 ± 0.11</td>
<td>7.65 ± 2.52**</td>
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<td>24</td>
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<td>MCP-1 (ig/cell)</td>
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<td>0.71 ± 0.01</td>
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* ELISA kits were used for the assay. Three individual cultures were performed in each group, and the averages of values were expressed as mean ± SD; *, p < 0.05 vs control IgG; **, p < 0.01 vs control IgG.

Table II. MCP-1 levels in DHF patient seraa

<table>
<thead>
<tr>
<th>Group</th>
<th>MCP-1 (pg/ml)</th>
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<tbody>
<tr>
<td>Controls (n = 5)</td>
<td>72.9 ± 18.9</td>
</tr>
<tr>
<td>DHF patients (n = 17)</td>
<td>2695.6 ± 444.0***</td>
</tr>
<tr>
<td>Grade I (n = 1)</td>
<td>3009.5 ± 351.4***</td>
</tr>
<tr>
<td>Grade II (n = 13)</td>
<td>2610.8 ± 383.2***</td>
</tr>
<tr>
<td>Grade III (n = 3)</td>
<td>2958.5 ± 618.6***</td>
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</tbody>
</table>

* ELISA kits were used for the assay. Three individual cultures were performed for each person, and the averages of values are expressed as mean ± SD; *** p < 0.001 vs controls.
that transient thrombocytopenia in DV-infected mice was associated with the generation of anti-platelet Abs (60), our results suggest that the onset of autoimmune responses in DV infection may have implications in DHF immunopathogenesis (9). Several other reports have also shown the autoimmune responses in DV infection (61, 62).

In addition to cell apoptosis (40), we showed in this study that immune activation in endothelial cells also occurred after anti-DV NS1 stimulation. Protein tyrosine phosphorylation and NF-κB activation in HMEC-1 cells were observed. There was also an increase in the expression of cytokines and chemokines, including IL-6, IL-8, and MCP-1, but not RANTES. The involvement of NF-κB in the expression of IL-6, IL-8, and MCP-1 was also demonstrated. Up-regulation of ICAM-1 expression and ICAM-1-mediated adhesion of PBMC to endothelial cells were observed after anti-DV NS1 stimulation. The expression of ICAM-1 was modulated, at least in part, by MCP-1 and by NF-κB. Taken together, these facts indicate that endothelial cell activation that involves NF-κB-regulated cytokine and chemokine production and adhesion molecule expression, as well as adherence of immune cells to endothelial cells, may represent the inflammatory mechanisms mediated by anti-DV NS1 Abs. The signaling pathways induced by anti-DV NS1 in endothelial cells leading to immune activation and inflammation are shown in Fig. 8. Although our previous study showed an NO-mediated mechanism of apoptosis induced by anti-DV NS1, immune activation is NF-κB dependent, but NO and apoptosis independent.

Several in vitro studies have shown that endothelial cells are the targets of DV and that induction of proinflammatory responses and apoptotic cell death are directly caused by DV (26, 27, 63). We have shown somewhat similar effects mediated by anti-DV NS1 Abs, specifically that endothelial cells underwent apoptosis (40–42) and, in the present study, induced proinflammatory effects. The combined effects of DV and anti-NS1 Abs on endothelial cells related to dengue pathogenesis remain to be investigated. Recently, DV NS1 has been reported to express in a GPI-linked form that results in signal transduction, as evidenced by tyrosine phosphorylation of cellular proteins (64). In this study, we showed tyrosine phosphorylation and NF-κB activation after anti-DV NS1 stimulation in endothelial cells without DV infection. The role of protein tyrosine phosphorylation in the NF-κB-mediated immune activation requires further clarification. To explore the signaling pathways mediated by anti-DV NS1, the surface molecules of endothelial cells, GPI linked or not, recognized by these autoantibodies need to be identified. Studies on potential autoantigens using anti-DV NS1 Abs and DHF/DSS patient sera are in progress.

Various autoimmune diseases associated with vascular dysfunction may be related to the generation of AECAs (42). Vasculopathy in autoimmune diseases involves both cytotoxicity and inflammation facilitated by AECAs via a direct or an indirect route. The elevated expression of inflammatory mediators, including cytokines (IL-1 and IL-6), chemokines (IL-8 and MCP-1), and adhesion molecules (ICAM-1, VCAM-1, and E-selectin), has been shown in AECAs-stimulated endothelial cells (65–68). However, the molecular mechanisms of AECA-mediated endothelial cell activation remain poorly defined. Studies on the mechanisms of endothelial activation have shown that degradation of I-κB followed by NF-κB activation might contribute to the induction of proinflammatory responses (67). Activation of NF-κB was also detected when anti-DV NS1 cross-reacted with uninfected endothelial cells.

A recent study provided a possible molecular mechanism of AECA’s regulation of ICAM-1 expression through ERK1/2 activation in Behcet’s disease (69).

In dengue pathogenesis, elevated levels of inflammatory IL-6 and IL-8 were correlated with clinical presentation of DHF (46–48, 52). Also, endothelial cells infected with DV induced IL-6 and

**FIGURE 5.** The involvement of NF-κB in the expression of IL-6, IL-8, and MCP-1 in endothelial cells after anti-DV NS1 stimulation. In the presence or absence of PDTC (100 μM), L-NAME (10 μM), or zVAD-fmk (10 μM) for 1-h pretreatment or transfected with dominant-negative IKK-β (ΔIKK-β) or empty vector, HMEC-1 cells were treated with 5 μg of anti-DV NS1, anti-JEV NS1, or control IgG for 24 h. The expression of IL-6, IL-8, and MCP-1 was detected using ELISA, and the averages are shown (mean ± SD).

**FIGURE 6.** MCP-1 regulates the expression of ICAM-1 in endothelial cells after anti-DV NS1 stimulation. A HMEC-1 cells were treated with 5 μg of anti-DV NS1, anti-JEV NS1, or control IgG for 24 h. Cells were labeled with mouse anti-human ICAM-1 Abs, followed by HRP- or FITC-conjugated goat anti-mouse IgG. Cells treated with anti-DV NS1 in the presence of 5 μg of neutralizing anti-MCP-1 were also tested. The ICAM-1 expression was detected using immunohistochemical staining. B, HMEC-1 cells were treated with 1, 5, or 25 μg of anti-DV NS1, anti-JEV NS1, or control IgG for 6, 12, or 24 h. The ICAM-1 expression was detected using flow cytometry analysis. Three individual cultures were performed, and the percentages of positive cells are shown (mean ± SD).
The numbers of adherent cells were quantified in each tested culture. In anti-DV NS1-treated groups, cells cultured with 5 μg of neutralizing anti-ICAM-1 or anti-MCP-1 were also tested. Cells were washed with PBS, and then cocultured with fresh human PBMC for 2 h. Cell adhesion was observed using Liu’s stain.

In the present study, we showed that anti-DV NS1 promoted proinflammatory endothelial cell activation causing increases in IL-6 and IL-8 protein expression and secretion. Furthermore, anti-DV NS1 induces the generation of MCP-1 in endothelial cells. A previous study showed MCP-1 in DSS patient plasma and pleural fluid, but not in DV-infected endothelial cells (27). We found, in the present study, MCP-1 production by endothelial cells after anti-DV NS1 stimulation; MCP-1 in DHF patient sera was also confirmed. In addition to the two chemokines IL-8 and MCP-1, RANTES was also checked in this study and was found not induced by anti-DV NS1 stimulation, although it was produced in endothelial cells after DV infection (27). MCP-1-mediated elevation of ICAM-1 expression and facilitation of leukocyte transmigration have been suggested (53–55). Results in this study showed that MCP-1 up-regulated ICAM-1 expression in anti-DV NS1-treated HMEC-1 cells. Endothelial cell activation followed by elevated expression of ICAM-1 may contribute to the adherence of immune cells to endothelial cells in the inflammatory responses associated with dengue disease pathogenesis. The involvement of other cytokines, chemokines, and adhesion molecules is currently under investigation.

In the present study, we showed the inflammatory initiator role of anti-DV NS1 resulting in endothelial cell activation. In addition to the increased expression of cytokines and chemokines, a possible chemotactic effect on PBMC adherence may contribute to the indirect damage in endothelial cells. Activation of endothelial cells via DV-infected peripheral blood monocytes has been reported (33). Endothelial cell activation and apoptosis might play a role in the fulminant, but short-lived vascular leakage of DHF pathogenesis (27). The clinical features indicate that vascular endothelia in serosal tissues are preferentially affected by dengue pathogenetic mechanisms, resulting in ascites and pleural effusion for most of the plasma leakage in dengue shock syndrome (70). Tissue culture fluid from DV-infected monocytes has activated endothelial cells (33). Serum inflammatory cytokines may relate to the development of plasma leakage and disease severity (8, 52). Given all of these data, endothelial vascular dysfunction in dengue pathogenesis involves multiple factors, including the dengue virus itself and anti-DV NS1, direct and indirect effects on endothelial cells, and immune activation and apoptosis.

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References


