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Endothelial Cell Apoptosis Induced by Antibodies Against Dengue Virus Nonstructural Protein 1 Via Production of Nitric Oxide

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Dengue virus (DV), a member of the genus Flavivirus within the family Flaviviridae, causes a wide range of diseases from mild dengue fever (DF) to severe dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) (1–3). The typical biphasic fever, headache, body pain, and rash characterize clinical manifestations of DF. In some cases, however, patients develop the life-threatening syndrome DHF/DSS, which is characterized by abnormalities of hemostasis and vascular permeability (3). To date, there are no effective strategies to prevent the progression of DHF/DSS, because its pathogenic mechanisms are not fully understood (4, 5). Ab-dependent enhancement of infection has been suggested as an explanation of the high risk for hemorrhage and shock syndrome in the secondary heterologous DV infection (6–10). Virus variation may also be responsible for the severity of dengue disease (11–14). In addition to a direct effect of DV, abnormal immune responses to DV infection, such as the production of cytokines or chemokines and the activation of complement or immune cells, may also underlie the pathogenesis of disease (15–22). Accumulated evidence has shown that host cell apoptosis induced by DV infection may also be involved (22–27). It is likely that several mechanisms are involved in the pathogenesis of DV infection, although their relative importance remains undefined.

Hemorrhagic syndrome, a feature of DHF/DSS, is a hematologic abnormality resulting from multiple factors, including thrombocytopenia, coagulopathy, and vasculopathy (28, 29). The pathogenesis of endothelial dysfunction related to vascular leakage syndrome is not well understood. Ab-enhanced infection of DV in peripheral blood monocytes could modulate endothelial cell function by an indirect route (30). Complement activation and chemokine production induced by DV infection are also suggested to be involved in the induction of endothelial cell damage (22, 31). Several in vitro studies have reported that endothelial cells were the targets of DV (21, 22, 31) and that cell apoptosis was directly caused by DV (22). Thus, endothelial cell apoptosis may be related to the disruption of the endothelial barrier and then lead to the transient leakage syndrome in dengue vasculopathy.

Mouse anti-dengue virus nonstructural protein 1 (NS1) Abs had been shown to cross-react with human endothelial cells (32). In the present study, we demonstrated that endothelial cells underwent apoptosis after being bound by anti-NS1 Abs generated in mice. The signaling pathways were investigated and showed the expression of inducible NO synthase (iNOS) and the production of NO after induction by anti-NS1. Addition of the NO synthase inhibitor Nω-nitro-L-arginine methyl ester decreased in both mRNA and protein levels, whereas p53 and Bax increased after anti-NS1 treatment. Cytochrome c release was also observed. All of these effects could be inhibited by Nω-nitro-L-arginine methyl ester. Taken together, anti-NS1 Abs act as autoantibodies that cross-react with noninfected endothelial cells and trigger the intracellular signaling leading to the production of NO and to apoptosis. Endothelial cell damage may cause vascular leakage that contributes to the pathogenesis of dengue disease. The Journal of Immunology, 2002, 169: 657–664.

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Abbreviations used in this paper: DV, dengue virus; DF, dengue fever; DHF/DSS, dengue hemorrhagic fever and dengue shock syndrome; NS1, nonstructural protein 1; iNOS, inducible NO synthase; eNOS, endothelial NO synthase; L-NAME, Nω-nitro-L-arginine methyl ester; JEV, Japanese encephalitis virus; HMEC-1, human microvascular endothelial cell line-1; MFI, mean fluorescence intensity; z-VAD-fmk, benzoyloxycarbonylvalylalanylaspartic acid fluoromethyl ketone; DEVD-fmk, aspartylglutamylvalylaspartic acid fluoromethyl ketone.
Materials and Methods

Mice
BALB/cByJ breeder mice were obtained from The Jackson Laboratory (Bar Harbor, ME) 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 experiments in the present study.

Abs and reagents
Rabbit Abs specific for the active form of human caspase-3 or both pro (32 kDa) and active caspase-3 (17–22 kDa) were purchased from BD PharMingen (San Diego, CA). Rabbit anti-human Abs to NOS II (iNOS) or NOS III (endothelial NO synthase; eNOS) were from Chemicon (Temecula, CA). Rat anti-mouse CD31 Ab, mouse anti-human Bcl-2 MAb, rabbit anti-human Bax Ab, and mouse anti-human p53 MAb were from Serotec (Oxford, UK); mouse anti-human Bcl-xL MAb and mouse anti-cytokerin ε MAb were from BD PharMingen; and mouse MAb to β-actin was from Sigma-Aldrich (St. Louis, MO). FITC- or HRP-conjugated goat anti-mouse or anti-rabbit IgG were obtained from ICN Pharmaceuticals (Aurora, OH). The caspase inhibitor, benzoylargininmethylvalylalanylaspartic acid fluoromethyl ketone (z-VAD-fmk), and caspase-3 inhibitor, aspartylglutamylvalylalanylaspartic acid fluoromethyl ketone (DEVD-fmk), were purchased from Clontech Laboratories (Palo Alto, CA). The NOS inhibitor Nω-nitro-l-arginine methyl ester (l-NNAME) was obtained from Sigma-Aldrich.

Preparation of recombinant NS1
The full-length DV-2 (New Guinea C strain) NS1 cDNA was cloned to pRSET B expression vector (Invitrogen, Carlsbad, CA) to establish a pRSET-DVNS1 plasmid. After the sequences at the 5′ and 3′ junctions were confirmed, this plasmid was then introduced into Escherichia coli BL21(DE3)pLyS strain (Invitrogen). The recombinant NS1 proteins were induced by a 2 mM final concentration of isopropyl β-D-thiogalactoside and purified with TALON metal affinity resin (Clontech Laboratories). A single band was detected by SDS-PAGE, and the protein sequence was confirmed by Applied Biosystems 477A autosequencer (Foster City, CA).

The plasmid construct expressing full-length Japanese encephalitis virus (JEV) NS1, pET-32a(+)-JNS1, was obtained from Dr. S. L. Hsieh (National Yang-Ming University, Taipei, Taiwan) and Dr. Y. L. Lin (Institute of Biomedical Science, Academia Sinica, Taipei, Taiwan). This plasmid was derived from pcDNA3-JNS1 which was constructed from JEV strain NT109 as previously described (33, 34). The plasmid was transformed into the E. coli BL21(DE3)pLyS strain. The recombinant protein expression and purification followed the procedures described above for DV2 NS1 preparation.

Generation of anti-NS1 Abs
BALB/c mice were immunized i.p. with 25 μg of purified recombinant DV or JEV NS1 proteins emulsified in CFA (Life Technologies, Gaithersburg, MD) and then four times in IFA. Sera were obtained 3 or 4 days after the last immunization. The IgG fractions from hyperimmunized mouse sera were purified with protein G-Sepharose affinity chromatography column (Amersham Pharmacia, Uppsala, Sweden) and recovered with HCl-glycerine. The reactivity of purified IgG against NS1 was confirmed by SDS-PAGE and Western blot. The control IgG was eluted from protein G column loaded with normal mouse sera.

Cell culture
Human microvascular endothelial cell line-1 (HMEC-1) was obtained from the Center for Disease Control and Prevention (Atlanta, GA) (35), and passed in culture plates containing endothelial cell growth medium (Clonetics, Walkersville, MD) composed of 2% FBS, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, and antibiotics. Cells were detached using 1000 U/ml trypsin and 0.5 mM EDTA. Only those cultures from three to five passages with a viability of >95% by eosin Y staining were used for experiments.

Immunohistochemistry and flow cytometry for detection of endothelial cell binding activity
HMEC-1 cells were seeded either on monolayers or in small glass slides coated with 1% gelatin for confocal microscopy or in suspensions at 5 × 10⁴ cells for flow cytometric analysis. For preparation of a single-cell suspension, cells were detached from plates with 1000 U/ml trypsin and 0.5 mM EDTA. Cells were washed briefly in PBS, fixed with 1% paraformaldehyde in PBS at room temperature for 10 min, and washed again with PBS. Mouse anti-NS1 or control IgG were then incubated with cells at 4°C for 1 h. The glass slides for confocal microscopy detection were incubated in a humidified chamber. After three washings with PBS, cells were incubated with 1 μl of 1 mg/ml FITC-conjugated goat anti-mouse IgG at 4°C for 1 h and washed again with PBS. The binding activity of mouse anti-NS1 or control IgG to endothelial cells was observed with a confocal laser microscope (Leica TCS SP2, Nussloch, Germany) or analyzed by flow cytometry (FACScan; BD Biosciences, San Jose, CA) with excitation set at 488 nm. For confocal microscopy, 20-μm-thick serial sections of HMEC-1 cells were analyzed. Optical sections were obtained in 0.6-μm steps along the z-axis.

Binding activity of anti-NS1 with mouse vessel endothelium ex vivo
Mouse vessels, obtained from the small veins of BALB/c mice, were perfused with anti-CD31, anti-DV2 NS1, anti-JEV NS1, or control IgG and incubated at 4°C for 1 h. After they were washed three times with PBS, the vessels were perfused with OCT compound, and tissue blocks were prepared. The vessels were sliced into 3-μm-thick sections and fixed with 1% paraformaldehyde in PBS at room temperature for 10 min. After being washed with PBS, vessel sections were incubated with 0.3% H₂O₂ in PBS for 5 min to quench endogenous peroxidase activity and then washed again with PBS. Next, 1 μl of 1 mg/ml HRP-conjugated goat anti-mouse IgG was added and incubated at 4°C for 1 h. The binding ability was observable by following the manufacturer’s instructions for the AEC substrate kit (Zymed Laboratories, San Francisco, CA) and then using light microscopy.

TUNEL assay
HMEC-1 cells were cultured in plates and incubated with mouse anti-NS1 or control IgG in the culture medium for various time intervals. Cells were harvested from plates containing endothelial cells by adding lysis buffer (5 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 0.5% Triton X-100) at 4°C for 15 min. After centrifugation at 10,000 rpm for 10 min, the supernatant containing fragmented DNA was incubated with 50 μg/ml RNAse (Sigma-Aldrich) at 37°C for 2 h, followed by the addition of a final concentration of 100 μg/ml proteinase K (Sigma-Aldrich), after which the mixture was incubated at 50°C overnight. After phenol-chloroform extraction, DNA was precipitated in 70% isopropanol and washed with 70% ethanol and then octanedithiol and ethanol. The resulting pellet was resolved in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.1% SDS) and viewed with UV light.

DNA ladder analysis
HMEC-1 cells were cultured in plates and incubated with mouse anti-NS1 or control IgG. After 24 h, 5 × 10⁵ cells were harvested from the culture plates by adding lysis buffer (5 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 0.5% Triton X-100) at 4°C for 15 min. After centrifugation at 10,000 rpm for 10 min, the supernatant containing fragmented DNA was incubated with 50 μg/ml RNase (Sigma-Aldrich) at 37°C for 2 h, followed by the addition of a final concentration of 100 μg/ml proteinase K (Sigma-Aldrich), after which the mixture was incubated at 50°C overnight. After phenol-chloroform extraction, DNA was precipitated in 70% isopropanol and 20 μg/ml glycerol for 3 h at −70°C. The resulting pellet was resolved in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA), and the DNA sample was analyzed by 2% agarose gel electrophoresis in buffer containing 90 mM Tris-acetate (pH 8.0) and 4 mM EDTA. Gel was stained with ethidium bromide at 1 μg/ml and viewed with UV light.

Immunohistochemical staining for detection of caspase-3 and iNOS
A monolayer of HMEC-1 cells was cultured on sterile glass slides followed by treatment with mouse anti-NS1 or control IgG for various time intervals. Cells were washed with PBS, then incubated with 0.3% H₂O₂ in PBS for 5 min to quench endogenous peroxidase activity and then washed again with PBS. One microliter of 0.1 mg/ml rabbit Abs, specific for active-form caspase-3 or for iNOS, was added to cells and incubated for 1 h at 4°C. After being washed three times with PBS, cells were incubated with 1 μl of 1 mg/ml HRP-conjugated goat anti-rabbit IgG for 1 h at 4°C. After being washed with PBS, cells were developed using the AEC substrate kit and viewed with light microscopy.

Western blot analysis
HMEC-1 cells were harvested from cultures after treatment with mouse anti-NS1 or control IgG for various time intervals. Cells were 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,
Mannheim, Germany). After being freeze-thawed once, cell lysates were centrifuged at 12,000 rpm for 20 min at 4°C. The supernatants were collected and boiled in sample buffer for 5 min. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), blocked overnight at 4°C in PBS-T (PBS plus 0.1% Tween 20) containing 5% skim milk, and probed with Abs against caspase-3, Bcl-2, Bcl-xL, Bax, p53, cytochrome c, and β-actin at 4°C overnight. After being washed with PBS-T, blots were incubated with a 1/5000 dilution of HRP-conjugated goat anti-mouse or anti-rabbit IgG for 1 h at 4°C. The protein bands were developed with an AEC substrate kit.

Flow cytometric analysis for NOS expression

A monolayer of HMEC-1 cells was cultured on sterile glass slides followed by treatment with mouse anti-NS1 or control IgG. Cells were detached using 1000 U/ml trypsin and 0.5 mM EDTA. Cells were then washed in PBS, fixed with 1% paraformaldehyde in PBS at room temperature for 10 min, and permeabilized with 70% ethanol. Rabbit anti-iNOS or anti-eNOS Abs were added to cells (5 × 10⁵) and incubated for 1 h at 4°C. After being washed three times with PBS, cells were incubated with FITC-conjugated goat anti-rabbit IgG for 1 h at 4°C and analyzed by flow cytometry.

**Determination of NO concentration**

HMEC-1 culture medium was replaced by DMEM without phenol red (Sigma-Aldrich) before the cells were incubated with mouse anti-NS1 or control IgG. After various time intervals, the culture supernatant was collected, and NO production was measured by the Griess diazotization reaction to spectrophotometrically detect nitrite formed by the spontaneous oxidation of NO under physiological conditions. The Griess reagent kit for nitrite determination (Molecular Probes, Eugene, OR) was used according to the manufacturer’s instructions. Briefly, culture supernatant was mixed with an equal volume of Griess reagent, incubated at room temperature for 10 min, and then spectrophotometrically quantified by an Emax microplate reader at 548 nm (Molecular Devices, Sunnyvale, CA). NaNO₂ was used as a standard and the standard curve of nitrite concentration against its OD was plotted.

**Annexin V staining**

A modified staining assay of apoptotic cells with annexin V was performed according to the method of Vermes et al. (36). HMEC-1 cells (10⁶ per test) were collected from the glass slide after incubation with mouse anti-NS1 or control IgG for various time intervals, washed twice with PBS, and resuspended in annexin V binding buffer (10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂). After centrifugation, cells were incubated in 100 µl of the same buffer containing 5 µl FITC-conjugated annexin V (BD Pharmingen) at room temperature for 15 min in the dark and then analyzed by flow cytometry.

**RT-PCR**

Total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The concentration of RNA was quantified by spectrophotometry at 260 nm (Biotecx, Houston, TX) following the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions. The total cellular RNA was extracted by an Ultraspec-II RNA isolation system (Biotecx, Houston, TX) according to the manufacturer’s instructions.

**Results**

Anti-NS1 Abs generated in mice cross-react with human endothelial cells and induce apoptosis

In the present study, we showed that anti-dengue NS1 could cross-react with endothelial cells. The binding activity of anti-NS1 IgG from hyperimmunized mouse sera with HMEC-1 cells was viewed with confocal microscopy (Fig. 1A); a subsequent flow cytometric analysis showed the percentages of fluorescence-positive cells (Fig. 1B). Unlike anti-dengue NS1 IgG, the anti-JEV NS1 IgG did not show the cross-reaction with HMEC-1 cells exerting a level...
similar to that of the control IgG. Anti-dengue NS1 binding activity could also be observed in HUVEC (data not shown) and in the vascular endothelium of a mouse vessel ex vivo (Fig. 2). Staining with CD31 marker was used as the positive control for vessel endothelium, whereas anti-JEV NS1 showed a negative result similar to that of the control IgG.

Apoptotic HMEC-1 cell death induced by anti-NS1 IgG was demonstrated by TUNEL assay followed by flow cytometric analysis (Fig. 3A). Apoptosis could be detected at 12 h; it increased at 24 h. Treatment with caspase inhibitor zVAD-fmk inhibited cell death. Similar results were obtained when fragmented DNA was detected on gel (Fig. 3B).

**Activation of caspase-3 during anti-NS1-induced apoptosis in HMEC-1 cells**

Fig. 3 shows that the induction of endothelial cell apoptosis by anti-NS1 was caspase dependent, so the involvement of caspase-3 in this process was further examined. Immunohistochemical staining revealed the presence of active form caspase-3-positive cells in HMEC-1 cells treated with anti-NS1 but not with control IgG (Fig. 4A). The increase in 17-kDa active caspase-3 fragments and the decrease in 32-kDa procaspase-3 could be detected in anti-NS1-treated HMEC-1 cells by Western blot analysis (Fig. 4B). The caspase-3 inhibitor DEVD-fmk inhibited the formation of 17-kDa caspase-3 cleavage products (Fig. 4B) and active-form positive cells (Fig. 4A). The percentages of active form caspase-3-positive cells were further quantified by flow cytometry and showed a positive correlation with the percentages of apoptotic cells as determined by TUNEL reaction (Fig. 4C). To further demonstrate whether caspase-3 activation is requisite for the endothelial cell apoptosis mediated by anti-NS1, the effect of DEVD-fmk was examined. Results showed that the inhibition of caspase-3 caused a blockage of endothelial cell apoptosis (Fig. 4C).

**NOS expression and NO production after anti-NS1 treatment of HMEC-1 cells**

In an attempt to investigate the signaling pathways in anti-NS1-induced apoptosis, iNOS and eNOS expression and NO production were monitored in HMEC-1 cells. Results showed that after treatment with anti-NS1 IgG, there was a prominent increase in iNOS expression as demonstrated by both the percentages of positive cells and the mean fluorescence intensity (MFI) (Fig. 5A, left). Although an increase in eNOS expression was also detectable, the increase in MFI was very small when compared with the group without anti-NS1 treatment and to the IgG control group (Fig. 5A, right). The expression of iNOS could be observed after anti-NS1 treatment for as short as 3 h and with as low as 1 μg (Fig. 5, B and C). Expression of iNOS was also confirmed by immunohistochemical staining; it showed a time-dependent expression of iNOS after treatment with anti-NS1 but not with control IgG (Fig. 5D). The production of NO showed positive time- and dose-dependent correlations with iNOS expression (Fig. 5, B and C). Furthermore, the addition of l-NAME to cell cultures caused an inhibition of NO production (Fig. 5B). Interestingly, the iNOS expression was also inhibited at 24 h (Fig. 5, B and D), but not at early stages (data not shown). Studies are under way to explore the possible mechanisms.
Requirement of NO production in HMEC-1 cell apoptosis

Whether the increase in NO production was related to the induction of endothelial cell apoptosis was queried. Both a change in cell morphology and an increase in the percentage of TUNEL-positive cells could be observed in endothelial cells treated with anti-NS1 for 24 h (Fig. 6A). Treatment with the NOS inhibitor L-NAME inhibited the change of morphology and reduced the percentage of TUNEL-positive cells, suggesting that NO was involved in HMEC-1 cell apoptosis. In the time-kinetic study, the exposure of phosphatidylserine (determined by annexin V staining) occurred as early as 3 h after anti-NS1 treatment, whereas DNA fragmentation (determined by TUNEL staining) was detectable at 12 h (Fig. 6B). L-NAME blocked both the exposure of phosphatidylserine and the fragmentation of DNA. Treatment with control IgG for 24 h exhibited basal levels similar to those of the nontreated group.

Effect of NO production on Bcl-2, Bcl-xL, Bax, and p53 expression and cytochrome c release in HMEC-1 cells

To understand the possible mechanisms of NO modulation in the apoptotic signaling pathway, expression of the Bcl-2 family proteins Bcl-2, Bcl-xL, and Bax was analyzed by Western blot (Fig. 7A). After treatment with anti-NS1 IgG in HMEC-1 cultures, the protein levels of Bcl-2 and Bcl-xL decreased, whereas that of Bax increased time dependently. The expression of p53 also increased. The release of cytochrome c from mitochondria was observed. Furthermore, L-NAME was able to inhibit the anti-NS1-mediated changes of Bcl-2, Bcl-xL, Bax, and p53 expression and cytochrome c release. These results revealed that the role of NO production in anti-NS1-induced endothelial cell apoptosis is the up-regulation of Bcl-2 and Bcl-xL and the down-regulation of p53 and Bax expression. The mRNA expression was further determined by RT-PCR, which showed the decrease in Bcl-2 and Bcl-xL and the down-regulation of p53 and Bax expression. The mRNA expression was also inhibited by the presence of L-NAME. Therefore, the effects of NO on the expression of these molecules were on both mRNA and protein levels.

Discussion

The pathogenesis of hemorrhage in dengue disease is not well understood. Vascular leakage is one of the hallmarks of dengue
hemorrhagic syndrome (3, 28). We propose a mechanism of molecular mimicry in which Abs directed against DV NS1 could cross-react with endothelial cells and induce these cells to undergo apoptosis. Evidence for this hypothesis has been obtained from mouse Abs against DV NS1 but not JEV NS1, as shown in the present study, and also from dengue patient sera (39). The signaling pathways of anti-NS1-induced apoptosis were further investigated and suggested the involvement of NO during this process. Production of NO further caused up-regulation of p53 and Bax and down-regulation of Bcl-2 and Bcl-xL, the pro- and anti-apoptotic factors that lead to cytochrome c release and caspase-3 activation. The modulations of p53, Bax, Bcl-2, and Bcl-xL by anti-NS1 IgG were on both mRNA and protein expression levels.

The endothelial cell-binding ability of anti-NS1 was demonstrated in HMEC-1 cells (Fig. 1), HUVEC (data not shown), and mouse vessel endothelium (Fig. 2). We have previously shown the cross-reaction of anti-NS1 Abs with platelets, which may explain the characteristic of thrombocytopenia (40). Liver damage is also one of the clinical features of DHF (29, 41). However, anti-NS1 did not cross-react with liver cell lines when both patient sera and mouse Abs were tested (unpublished data).

Activation of caspases is involved in the apoptotic pathway (42, 43). Our results indicated that anti-NS1 induced endothelial cell death via a caspase-dependent pathway. Caspase-3 is synthesized as a 32-kDa proform that is composed of two subunits of 17 kDa and 12 kDa (44). Western blot analysis demonstrated that anti-NS1 caused an increase in the expression of 17-kDa caspase-3 cleavage products and a decrease in the 32-kDa proform. The presence of active form caspase-3 was also confirmed by immunohistochemistry and flow cytometric analysis. Further study revealed that using specific inhibitor DEVD-fmk resulted in the blockage of endothelial cell apoptosis and inhibition of the formation of 17-kDa proteins. Activation of caspase-3 has also been shown to be inhibited by acetylglutamate and o-vanilloylglycine (45, 46), which is consistent with the inhibition of 17-kDa protein formation by DEVD-fmk.

NO, generated from L-arginine by NOS, is an important multifunctional mediator of physiologic and pathologic processes, including vasodilatation, inflammation, thrombosis, immune response, and neurotransmission. Generation of NO may result in either initiation or suppression of apoptosis (47–49). The involvement of NO in apoptosis induction has been reported in different cell systems, although the mechanisms are not fully understood. The involvement of several mechanisms in NO-mediated apoptosis has been proposed: peroxynitrite generation (50, 51); an increase in Bax levels (51, 52); increased expression of p53 (52, 53); activation of c-Jun N-terminal kinase/stress-activated protein kinase and p38 kinase (54, 55); ceramide formation (49); and up-regulation of Fas (56). The answers to many questions remain elusive, for example, why NO-mediated apoptosis can be both p53-dependent and -independent (57) and whether the decreased Bcl-2 expression plays a protective role or only represents a secondary phenomenon; both have been proposed (58). Until now, the effects of NO on apoptosis in endothelial cells have not been fully reported. Physiologically relevant concentrations of peroxynitrite have induced apoptosis in HL-60 and U-937 cells, but they appeared ineffective in inducing HUVEC apoptosis (50). A combination of NO and calcium mobilization has been shown to potentiate apoptosis in adrenal vascular endothelial cells (59). In the present study, NOS expression and NO production could be detected in HMEC-1 cells after binding with anti-NS1. Production of NO showed a positive correlation with the enhanced expression of iNOS. Expression of eNOS showed only a slight shift in fluorescence intensity. Interestingly, endothelial cell apoptosis induced by anti-NS1 was blocked by the addition of L-NAME. In other words, the ability of anti-NS1 to trigger endothelial cell apoptosis was a result of the production of NO. Furthermore, up-regulation of p53 and Bax expression as well as down-regulation of Bcl-2 and Bcl-xL were also blocked by the addition of L-NAME. The ratio of death antagonists (Bcl-2 and Bcl-xL) to agonists (Bax) determines whether a cell will undergo apoptosis (60). In the present study, the
modulations of these molecules at both mRNA and protein levels promoted apoptotic pathways.

The intracellular redox state causes an inhibitory or enhancing effect of NO-mediated apoptosis (45, 47). NO and O$_2^-$ that resulted in the reaction product peroxynitrite has been associated with apoptosis (48–51). NO can cause apoptosis by triggering a mitochondrial permeability transition that involves the hyperpolarization of reactive oxygen species (61). N-Acetylcysteine interfered with NO-mediated apoptosis (58). However, the radical NO and O$_2^-$ interaction may also confer protection against apoptotic stimuli (48). Whether the reactive oxygen species are produced and involved in anti-NS1-induced NO-mediated apoptosis requires further investigation.

It has recently been reported that DV NS1 is expressed in a GPI-linked form that results in signal transduction, as evidenced by tyrosine phosphorylation of cellular proteins (62). Our preliminary results (unpublished) showed tyrosine phosphorylation after anti-NS1 stimulation in endothelial cells. The signal transduction pathways from tyrosine phosphorylation to iNOS expression leading to the changes in p53 and Bcl-2 family proteins require further delineation. Endothelial cells have been shown to become apoptotic in response to antagonists of integrin $\alpha_{v}$B3 that cause p53 activation and p21WAF1/CIP1 expression (63). The surface molecules of endothelial cells, GPI-linked or not, recognized by anti-NS1 still need to be identified.

High levels of TNF-α, IL-6, and IL-8 have been observed in the sera of patients with DHF/DSS (22, 64, 65). DV-infected endothelial cells showed an increase in IL-6, IL-8, and RANTES secretion (21, 22). Whether anti-NS1 can elicit cytokine and chemokine production associated with the pathogenesis of DHF/DSS remains to be investigated.

Immune responses to infectious agents are the critical defense mechanisms in a host, yet abnormal immunity may also be involved in the pathogenesis of disease (20, 66, 67). We demonstrated in the present study that the generation of anti-DV NS1 Abs can cross-react with endothelial cells and induce cell damage. When taken together with previous findings that anti-NS1 present in dengue patient sera also cross-reacted with platelets (Ref. 40 and our unpublished data) and that transient thrombocytopenia in DV-infected mice was associated with the generation of anti-platelet Abs (68), this finding suggests that the onset of autoimmune responses in DV infection may have implications in DHF pathogenesis. NO-modulated endothelial cell apoptosis may play a role in the disruption of vessel endothelium and contribute to the pathogenesis of vascular leakage in DHF. Some questions remain: 1) whether these in vitro findings are involved in the in vivo mechanism; 2) whether a mechanism similar to the one found in DV-2 studies would occur in all DV serotypes. Studies are in progress to address this issue. Furthermore, studies are ongoing to identify the endothelial cell surface molecules that can be recognized by anti-NS1 Abs. Whether the epitopes shared between endothelial cell surface molecules and NS1 are conserved in all four dengue virus remains to be assessed.

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References