Increased dengue virus-infected endothelial cell apoptosis caused by antibodies against nonstructural protein 1

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Abstract

Vascular dysfunction is a hallmark of severe dengue haemorrhagic fever (DHF) in dengue virus (DENV) infection. Both viral infection and immunopathogenesis are involved in the vasculopathy of DHF. We previously showed that antibodies against DENV nonstructural protein 1 (NS1) may cross-react with endothelial cells. A further study demonstrated the pathogenic role of anti-NS1 antibodies on endothelial dysfunction by inducing cell death and inflammation. In this study, we investigated the effect of anti-NS1 antibodies on DENV-infected human endothelial cells. We found increased binding activity of anti-NS1 antibodies to endothelial cells after DENV infection. Either DENV infection or anti-NS1 treatment caused endothelial cell apoptosis. Importantly, co-treatment with anti-NS1 antibodies increased DENV-induced endothelial cell apoptosis. The generation of nitric oxide (NO) could be detected in anti-NS1-stimulated, but not DENV-infected, endothelial cells. Furthermore, anti-NS1-induced endothelial cell apoptosis was NO-dependent, whereas DENV infection-induced apoptosis was NO-independent. These results suggest an additive effect but distinct mechanisms between anti-NS1 antibodies and DENV in endothelial cell damage. These findings indicate that both viral infection and cross-reactive antibodies may be involved in dengue pathogenesis by causing endothelial dysfunction.

Keywords: Dengue virus (DENV); Autoantibody; NS1; Endothelial cells; Apoptosis.

Introduction

Dengue virus (DENV) causes human diseases like mild dengue fever (DF) and severe dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS) in most tropical and subtropical areas of the world. Sequential infection with different DENV serotypes may influence the severity of the disease. Antibody-dependent enhancement (ADE) of DENV infection is responsible for DHF in the secondary infection due to the presence of cross-reactive and non-neutralizing antibodies from prior infection. In addition to ADE, viral variation and immunopathogenesis are involved in the progression of DHF. We previously found that anti-DENV nonstructural protein 1 (NS1) antibodies cross-reacted with endothelial cells and induced these cells to undergo apoptosis. Autoimmunity-mediated endothelial cell damage may, therefore, also

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contribute to the pathogenesis of dengue disease.

Vasculopathy, coagulopathy and thrombocytopenia are the hallmarks of severe dengue haemorrhagic syndrome.\textsuperscript{[3,5,6]} Previous studies showed the manifestation of vasculopathy characterized by endothelial dysfunction caused by direct viral cytotoxicity as well as immune-mediated inflammatory responses and cellular damage.\textsuperscript{[15,16]} In addition, we demonstrated that anti-NS1 antibodies present in dengue patient sera caused endothelial cell apoptosis.\textsuperscript{[11,12]} Furthermore, NS1 protein can be expressed in a glycosylphosphatidylinositol (GPI)-linked form on the surface of DENV-infected cells.\textsuperscript{[17]} In the present study, we investigated the apoptotic effect of DENV infection in human endothelial cells in the presence of anti-NS1 antibodies.

Materials and methods

Cell cultures

Human umbilical cord vein endothelial cells (HUVECs) were cultured in modified M-199 medium as previously described.\textsuperscript{[11]} Human microvascular endothelial cell line-1 (HMEC-1) was passed in culture plates containing endothelial cell growth medium M200 (Cascade Biologics) composed of LSGS (2% fetal bovine serum, 1 $\mu$g/ml hydrocortisone, 10 ng/ml epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 $\mu$g/ml heparin, and antibiotics. C6/36 cells were cultured in DMEM medium containing 10% fetal bovine serum and antibiotics. Fetal bovine serum was pre-incubated at 56 $^\circ$C for 30 minutes to inactivate complement before cell culture. For experiments, 1000 U/ml trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA) were used to detach cells.

Viruses

DENV-2 (PL046, Taiwan-isolated) was maintained in C6/36 cells. Briefly, a monolayer of C6/36 cells was incubated with DENV-2 at a multiplicity of infection (MOI) of 0.01 and incubated at 26 $^\circ$C in 5% CO$_2$ for 5 days. The cultured medium was harvested and cell debris was removed by centrifugation at 900 x g for 10 minutes. After further centrifugation at 16 000 x g for 10 minutes, the virus supernatant was collected and stored at -70 $^\circ$C until use. The virus titre was determined using a plaque assay.

Generating anti-NS1 antibodies

Recombinant DENV-2 NS1 was expressed and purified as previously described.\textsuperscript{[13]} To generate antibodies against DENV NS1, we immunized BALB/c mice intraperitoneally, once with 25 $\mu$g of recombinant NS1 protein emulsified in complete Freund’s adjuvant and then four times with 25 $\mu$g of recombinant NS1 protein emulsified in incomplete Freund’s adjuvant. Sera were obtained 3 or 4 days after the last immunization. The immunoglobulin G (IgG) fractions from hyperimmunized mouse sera and normal mouse sera used for the control were purified using a protein G-Sepharose affinity chromatography column (Amersham Pharmacia, Uppsala, Sweden).

Detecting binding activity

Endothelial cells ($5 \times 10^5$) were washed with phosphate-buffered saline (PBS) and fixed with 1% formaldehyde in PBS at room temperature for 10 minutes, then washed again with PBS. Mouse anti-NS1 IgG or control IgG were then incubated with cells at 4 $^\circ$C for 1 hour. After the cells had been washed three times with
PBS, they were incubated with 1 μl of 1 mg/ml fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 4 °C for 1 hour and then washed again with PBS. The binding activity of mouse anti-NS1 or control IgG to endothelial cells was analysed using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA) with excitation set at 488 nm.

**Detecting cell death**

Cells were fixed with 70% ethanol in PBS for a terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) reaction using the ApoAlert DNA Fragmentation Assay Kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions, and then analysed using flow cytometry.

**Detecting caspase-3 activity**

Caspase-3 activation was determined using the ApoAlert caspase-3 colorimetric assay kit (Clontech) according to the manufacturer’s instructions. Relative optical density (OD) measurements were performed using a microplate reader (Molecular Devices).

**Detecting nitric oxide production**

The production of nitric oxide (NO) was assessed as the accumulation of nitrite (NO$_2^-$) in the medium using a colorimetric reaction with the Griess reagent. Briefly, the culture supernatants were collected and mixed with an equal (1:1) volume of Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H$_3$PO$_4$). The absorbance was measured at 540 nm using a microplate reader. NaNO$_2$ was dissolved in double-distilled H$_2$O and used for the standard control (from 1 to 50 μM).

**Statistical analysis**

Using the Student’s unpaired t-tests in SigmaPlot version 8.0 for Windows (Cytel Software Corporation, Cambridge, MA), P-values were determined for all data from three independent experiments. Statistical significance was set at $P < 0.05$.

**Results**

**Binding activity of anti-NS1 to DENV-infected human endothelial cells**

Previous studies in our laboratory showed the presence of anti-endothelial cell autoantibodies in dengue patient sera and that the levels of anti-endothelial cell autoantibodies in DHF/DSS patient sera were higher than those in DF patient sera.[11,12] Further studies demonstrated that anti-NS1 antibodies accounted for the cross-reactivity and the induction of endothelial cell death. After DENV infection, the expression of NS1 protein was detected in patient plasma and on cell surface.[13-19] In this experiment, HUVECs were infected with DENV-2 at MOIs of 1 and 10 for 12, 24, and 48 hours, and the binding ability of anti-NS1 antibodies was analysed using flow cytometry. We found greater anti-NS1 time-dependent endothelial cell binding activity in DENV-infected cells than in mock-infected cells (Figure 1).

**Effect of anti-NS1 in DENV-induced endothelial cell apoptosis**

Our previous studies[11-14] showed that anti-NS1 antibodies induced endothelial cell apoptosis. Another study[15] showed that DENV infection caused endothelial cell cytotoxicity. In this experiment, we further investigated the effect
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First, HUVECs were infected with DENV-2 at an MOI of 1 for 12, 24, and 48 hours, or treated with 5 or 25 μg of anti-NS1 for 24 hours. Cell apoptosis was measured using a TUNEL reaction and then flow cytometric analysis. Consistent with previous studies, both DENV infection and anti-NS1 antibodies induced apoptosis (data not shown). We then assessed the effect of DENV-induced endothelial cell apoptosis in cells co-treated with anti-NS1 antibodies. We found that anti-NS1 treatment time-dependency increased DENV-induced endothelial cell apoptosis (Figure 2).

**Figure 1: Binding activity of anti-NS1 to DENV-infected endothelial cells**

[HUVECs were infected with DENV-2 (MOI 1 and 10) for 12, 24, and 48 hours, then incubated with 5 μg of mouse anti-NS1 IgG or control IgG and then FITC-conjugated anti-mouse IgG. The binding ability was analysed using flow cytometry. A set of representative histograms (A) and the percentages of HUVECs reactive with anti-NS1 IgG are shown as mean ± SD of triplicate cultures (B). The data related to their histograms are labeled (a-h). #: statistically significant compared to the group treated with anti-NS1 alone. * and #: P < 0.05; **: P < 0.01; *** and ###: P < 0.001]

**Figure 2: Additive effect of DENV and anti-NS1 antibodies on endothelial cell apoptosis**

[HUVECs were treated with 5 μg of mouse anti-NS1 IgG or control IgG for 12 and 24 hours with or without DENV-2 (MOI 1) pre-infection for 24 hours. They were then analysed using a TUNEL reaction and flow cytometric analysis. A set of representative histograms (A) and the percentages of apoptotic cells are shown as mean ± SD of triplicate cultures (B). The data related to their histograms are labelled (a-h). #: P < 0.05; **: P < 0.01; ***: P < 0.001]
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Effect of nitric oxide in anti-NS1- and DENV-induced endothelial cell apoptosis

Our previous studies showed that anti-NS1 caused endothelial cell apoptosis via a nitric oxide (NO)-mediated pathway. In the present study, the effect of DENV infection on NO production was investigated. First, anti-NS1 but not DENV infection caused NO production in endothelial cell HMEC-1 (Figure 3). Furthermore, there was no effect of DENV infection on anti-NS1-induced NO production. Using NO synthase inhibitor L-NAME, we next examined the effects of NO on anti-NS1- and DENV-induced endothelial cell apoptosis by determining the activation of caspase-3. The results demonstrated only anti-NS1- but not DENV-induced endothelial cell apoptosis via a NO-regulated signalling (Figure 4).

Discussion

In the present study, we demonstrated that anti-NS1 antibodies increased DENV-infected endothelial cell damage. A previous study had showed that DENV caused complement activation and apoptosis in cultured endothelial cells. A recent study further demonstrated that anti-NS1 antibodies increased complement activation. Here we showed an additive effect between anti-NS1 antibodies and DENV in endothelial cells. DENV infection increased the binding activity of anti-NS1 antibodies to endothelial cells. There are at least two possible explanations for this. One is the NS1 expression on the surface of DENV-infected cells as previously described, and the other, which still needs to be confirmed, is the increased expression of self-antigens caused by DENV infection. These two possibilities are not mutually exclusive. Our preliminary study (unpublished data) using proteomic analysis...
identified several candidate autoantigens present on the surface of endothelial cells. We are currently investigating whether DENV infection may upregulate the expression of these candidate autoantigens.

Although anti-NS1 binding activity was increased after DENV infection, further mechanistic studies indicated that the causes of endothelial cell apoptosis by anti-NS1 antibodies and DENV infection were distinct. Anti-NS1 treatment without DENV infection caused cell apoptosis through a NO-regulated pathway. DENV infection-induced endothelial cell apoptosis was, however, not mediated by NO. There are at least two possibilities for the distinct mechanisms of apoptotic induction between anti-NS1 and DENV infection. One is that DENV infection may induce apoptosis via a NO-independent manner as evidenced in Figure 4. The other, which remains to be investigated, is that GPI-linked NS1 protein on the surface of DENV-infected cells does not involve a NO-mediated pathway by anti-NS1 stimulation. Because anti-NS1 but not DENV causes a NO-mediated apoptotic pathway, it is likely that DENV-increased autoantigen expression is not involved in the additive effect of endothelial cell apoptosis after anti-NS1 binding. This yet needs to be confirmed.

In this study, the induction of endothelial cell cytotoxicity was demonstrated. We previously showed that anti-NS1 antibodies caused inflammatory activation that was mediated by the production of cytokines, chemokines and adhesion molecules. A previous study also showed that DENV infection led to chemokine production in endothelial cells. Whether the combination of DENV and anti-NS1 antibodies may increase the inflammatory response requires further investigation.

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