Both Virus and Tumor Necrosis Factor Alpha Are Critical for Endothelium Damage in a Mouse Model of Dengue Virus-Induced Hemorrhage

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Hemorrhage is a common clinical manifestation in dengue patients. However, the pathogenic mechanism of dengue virus (DV)-induced hemorrhage awaits clarification. We established a mouse model of DV hemorrhage using immunocompetent C57BL/6 mice by injecting DV serotype 2 strain 16681 intradermally. While inoculation of 3 × 10⁵ PFU of DV induced systemic hemorrhage in all of the mice by day 3 of infection, one out of three of those injected with 4 × 10⁷ to 8 × 10⁷ PFU developed hemorrhage in the subcutaneous tissues. The mice that were inoculated with 4 × 10⁷ to 8 × 10⁷ PFU but that did not develop hemorrhage were used as a basis for comparison to explore the pathogenic mechanism of dengue hemorrhage. The results showed that mice with severe thrombocytopenia manifested signs of vascular leakage and hemorrhage. We observed that high viral titer, macrophage infiltration, and tumor necrosis factor alpha (TNF-α) production in the local tissues are three important events that lead to hemorrhage. Immunofluorescence staining revealed that DV targeted both endothelial cells and macrophages. In addition, the production of high levels of TNF-α in tissues correlated with endothelial cell apoptosis and hemorrhage. By comparing TNF-α−/− to IgH−/−, C5−/−, and wild-type mice, we found that TNF-α was important for the development of hemorrhage. In vitro studies showed that mouse primary microvascular endothelial cells were susceptible to DV but that TNF-α enhanced DV-induced apoptosis. Our mouse model illustrated that intradermal inoculation of high titers of DV predisposes endothelial cells to be susceptible to TNF-α-induced cell death, which leads to endothelium damage and hemorrhage development. This finding highlights the contribution of the innate immune response to dengue hemorrhage.

Dengue virus (DV) is a mosquito-borne viral disease that affects humans in both the tropics and subtropics. The mild form of dengue is dengue fever (DF). Approximately one-third of DF patients may have hemorrhage manifestations ranging from mild skin hemorrhage, gingival or nasal bleeding, and gastrointestinal bleeding to severe hemorrhage (9). Severe hemorrhage with low platelet counts, plasma leakage, and pleural or other effusions are the characteristics of dengue hemorrhagic fever (DHF) (9). Therefore, hemorrhage, mild or severe, is clinically significant for DV infection.

Viralentr virus strains and preexisting nonneutralizing heterologous anti-DV antibodies are reported to be risk factors for DHF (10, 22). Immune responses involving the activation of T lymphocytes, the production of cytokines or chemokines, and complement activation are also considered to be important for the pathogenesis of DHF (8, 19, 23). Higher levels of tumor necrosis factor alpha (TNF-α) also correlate with severe disease (2, 8). The production of TNF-α instead of gamma interferon (IFN-γ) by activated T cells was suggested to contribute to the pathogenesis of dengue disease (20, 21, 23). Patients with DHF have subnormal levels of C3, C4, and C5 in the serum and increased complement metabolism (26). From those studies, it appears that both innate as well as adaptive immunity are implicated in severe disease. However, the key factor(s) that induces dengue hemorrhage has not been revealed.

Over the past decade, the study of direct and indirect interactions between endothelial cells (ECs) and DV has become a central focus in the understanding of the pathogenesis of DV hemorrhage. DV antigens (Ags) are detected in vascular endothelium in biopsy tissues from DHF/dengue shock syndrome (DSS) patients, indicating that DV targets ECs in vivo (15). It remains to be clarified, however, whether DV, the host immune response, or the complex interplay between the virus and the host causes EC damage in the infected host. An animal model will be best for the investigation of the complex relationship between DV and the host that results in hemorrhage.

Small-animal models that have been used to study dengue disease include hu-PBL-SCID mice, SCID-K562 mice, SCID-HepG2 mice, STAT-1−/− mice, AG129 mice with IFN-α/β and -γ receptor deficiencies, and immunocompetent BALB/c, A/J, and C57BL/6 mice (4, 14, 16, 18, 28, 32). By intravenous, intraperitoneal, or intracerebral inoculation of DV, the animals had liver pathology, thrombocytopenia, or neurological symptoms. Hemorrhage manifestation was not reported as being a prominent feature in any of these animal models.

In this study, we successfully developed a mouse model...
of dengue hemorrhage. Immuno competent mice receiving intradermal inoculations of DV developed hemorrhage. High viral titers, macrophage infiltration, and the production of TNF-α correlated with EC apoptosis and hemorrhage. Moreover, results of gene knockout experiments clearly demonstrated that TNF-α was key to the development of hemorrhage. In vitro studies employing primary mouse microvascular ECs demonstrated that TNF-α enhanced DV-induced apoptosis. Together, these results demonstrate that DV predisposes EC to TNF-α-induced apoptosis, which leads to hemorrhage.

MATERIALS AND METHODS

Mice. C57BL/6, IgH (Igh-fms-gen), TNF-α−/− (TNFα−/−), A/HeJ (He/J), and AJ mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME) and bred at the Laboratory Animal Center of the National Taiwan University College of Medicine. All mice were housed in sterile cages fitted with filtered cage tops and fed with sterilized food and water. Four- to five-week-old mice were used for experiments.

Virus. DV serotype 2 (DV-2) strain 16681 was used throughout this study. DV-2 16681 was originally isolated from a Thai patient who suffered from DHF (24). The virus was propagated in the C6/36 insect cell line cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Grand Island, NY) containing 2% heat-inactivated fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) at 28°C. Culture supernatants were collected 5 days after virus infection. The virus titer was then determined by plaque assay on a BHK cell line. To prepare high titers of DV, virus supernatant was concentrated on a Centriplus device (10-kDa cutoff) (Amicon; Millipore) by centrifugation before the plaque assay. The virus titer could reach 10^{10} PFU/ml after concentration. The inoculum was prepared by diluting virus stocks in phosphate-buffered saline (PBS) immediately before inoculation. To inactivate the virus, virus stocks were treated with UV light at 50 mJ/cm^2 for 3 min in the SPECTROLINKER XL-1000 CROSSLINKER (Spectronics Corporation, Westbury, NY). Loss of infectivity was confirmed by plaque assay. Japanese encephalitis virus (JEV) (strain B1PS3) was also propagated in the C6/36 cell line by following the same protocol as that used for DV.

Intradermal infection. Mice at 4 to 5 weeks of age were inoculated with DV (in 0.4 ml) intradermally at four sites on the upper back. Mice given PBS, mock C6/36 culture supernatant, an equivalent titer of UV-inactivated DV (UV-DV), or viable JEV through the same route were used as controls. Western blot analysis for E and PreM proteins was used to confirm that Mac-1 (F4/80)-conjugated rat anti-mouse Mac-1 antibody (Ab) (clone M1/70), PE-conjugated rat anti-mouse TNF-α Ab (clone 1D4B), and FITC-conjugated anti-fluorescein Ab was added, and 3,3'-diaminobenzidine was used as a substrate for color development. Hoechst 33258 dye (Sigma Aldrich) was used to stain nuclei. All antibodies for immunofluorescence staining were obtained from Sigma-Aldrich.

In situ detection of DNA fragmentation. Tissue sections or cell monolayers were fixed in 4% paraformaldehyde at room temperature for 10 min and then treated with 3% H_2O_2 in methanol. Cryosections or monolayers were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate on ice for 2 min. To detect apoptotic cells in tissues, an FITC-conjugated terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling (TUNEL) mixture (In Situ Cell Death Detection kit; Roche Applied Science, Indianapolis, IN) was added to tissue sections. PE-conjugated anti-Mac-1 or PE-conjugated anti-C3-Dab 1 was used then to further determine the types of cells undergoing apoptosis. To detect cell death in primary EC monolayers, Conve rtase-POD (peroxidase-conjugated anti-fluorescein Ab) was added, and 3,3'-diaminobenzidine was used as a substrate for color development. Hoechst 33258 dye was used to stain nuclei.

Staining for DV Ag. Cryosections were fixed with 4% paraformaldehyde at room temperature for 10 min. To detect viral Ag on cryosections, polygonal rabbit anti-DV antiseraum was used, and the sections were left at 4°C overnight. PE-conjugated anti-rabbit Ab (Invitrogen) was then added to the sections and left at 4°C for 1 h. To detect DV target cells, PE-conjugated anti-Mac-1 or PE-conjugated anti-C3-Dab 1 and rabbit anti-DV Ab were added to the same section simultaneously. After incubation at 4°C overnight, FITC-conjugated anti-rabbit Ab was added to determine whether DV Ag-expressing cells were apoptotic. Tissue sections were double stained with C3-Dab 1-conjugated TUNEL mixture and rabbit anti-DV Ab by following the same protocol.

Mouse microvascular EC isolation. The method of isolation of mouse microvascular ECs was adopted from a previously described method (33). Briefly, brains from mice at 8 to 12 weeks of age were collected. Cerebral cortices were
harvested, and the solid tissues were homogenized in RPMI medium containing 2% inactivated fetal calf serum by use of a glass tissue grinder. The homogenates were separated by 15% dextran (average molecular mass of 68,800 Da; Sigma-Aldrich) and treated with a collagenase-dispase mixture (Boehringer Mannheim, Indianapolis, IN) at 0.05% (wt/vol) at 37°C for 6 h with occasional agitation. Cells were washed with buffer and resuspended in RPMI medium containing 30% 2% inactivated fetal calf serum by use of a glass tissue grinder. The homogenates were separated by 15% dextran (average molecular mass of 68,800 Da; Sigma-Aldrich). Cells were used to assess the effect of DV-infected macrophage culture supernatants on ECs. The percentage of apoptotic cells was determined by using the In Situ Cell Detection system as described above. To neutralize the effect of TNF-α on ECs. The percentage of apoptotic cells was determined by using the In Situ Cell Detection system as described above. To neutralize the effect of TNF-α using antibody (clone MP6-XT3; eBioscience) before addition to the culture media. The cells were cultured in RPMI medium containing 1% inactivated fetal calf serum at 37°C for 2 h. Viable DV or UV-DV was then added to the monolayers at a multiplicity of infection (MOI) of 5 or 10 and incubated at 37°C for 2 h with gentle shaking every 15 min. The monolayer was washed with Hank’s balanced salt solution and cultured in the respective fresh growth medium. Culture supernatants from infected macrophage monolayers were harvested after infection and stored at −70°C before the assay. Undiluted supernatants were used to assess the effect of DV-infected macrophage culture supernatants on ECs. The percentage of apoptotic cells was determined by using the In Situ Cell Detection system as described above. To neutralize the effect of TNF-α, supernatants from DV-infected macrophages were preincubated with neutralizing anti-mouse TNF-α Ab (clone MP6-XT3; eBioscience) before addition to the EC culture. To assay the effect of TNF-α, ECs with or without DV infection were cultured in medium containing human recombinant TNF-α (Biosource, Camarillo, CA) at 30, 300, or 3,000 pg/ml.

**Statistical analysis.** The Statistical Package for the Social Sciences (SPSS Inc.) program was employed for statistical analysis. The difference between the means of experimental groups was analyzed using analysis of variance and the Tukey post hoc test. Statistical significance was determined at P values of <0.01 and <0.05.

**RESULTS**

**DV induces hemorrhage in C57BL/6 mice.** To mimic natural infection in humans, immunocompetent C57BL/6 mice were injected intradermally with DV. Hemorrhage developed at different sites including the subcutaneous tissue, abdomen, and intestine (Fig. 1A), the skin, and lymph nodes (data not shown) at day 3 after virus inoculation. The incidence, severity, and site of hemorrhage development correlated with the size of virus inoculum. While 100% and 73% of the mice receiving 3 × 10⁹ and 1 × 10⁸ PFU, respectively, developed systemic hemorrhage, 33% of those given 8 × 10⁷ PFU exhibited hemorrhage at the subcutaneous tissue (Table 1 and Fig. 1A and B). Notably, subcutaneous hemorrhage developed at approximately the diagonal crossing of the lines connecting the four injection sites, and the degree of hemorrhage ranged from mild to severe (Fig. 1A and B). Interestingly, although mice exhibited severe hemorrhage after receiving a high viral inoculum (Fig. 1A), they appeared to be healthy, and no sign of paralysis was observed. To confirm that hemorrhage is DV specific, mice inoculated with PBS, UV-DV (3 × 10⁹ and 4 × 10⁹ PFU), mock C6/36 supernatant, and viable JEV (4 × 10⁷ PFU) were examined for hemorrhage development. Data showed that only the mice that were inoculated with viable DV but not any other inoculum developed hemorrhage, indicating that hemorrhage development is SV-DV specific. Moreover, microscopic examination of the subcutaneous hemorrhage tissues revealed red blood cell extravasation (Fig. 1C). These results demonstrate that intradermal inoculation of DV induces hemorrhage in mice and that hemorrhage is accompanied by vascular leakage.

**Mice with severe thrombocytopenia manifest hemorrhage.** Next, we collected peripheral blood from mice receiving 4 × 10⁹ PFU of DV and tested whether thrombocytopenia is associated with hemorrhage. Since about two-thirds of mice receiving 4 × 10⁹ PFU did not develop hemorrhage, they were used as nonhemorrhage (nH) controls. At day 3 after inoculation, the platelet counts in mice injected with DV were significantly lower (P < 0.01) in both hemorrhage mice (H mice) (732.2 ± 109.5 × 10⁻³ platelets/μl) and nH mice (772.7 ± 49.9 × 10⁻³ platelets/μl) than in control mice (923.3 ± 73.5 × 10⁻³ platelets/μl). At day 7, the counts in nH mice (727.5 ± 36.5 × 10⁻³ platelets/μl) remained at the same level as that on day 3, but that in H mice (578.0 ± 71.9 × 10⁻³ platelets/μl) was further reduced (P < 0.05). These data demonstrate that DV infection induces thrombocytopenia, and mice with severe thrombocytopenia manifest hemorrhage.

**TABLE 1. Correlation between virus inoculum and hemorrhage**

<table>
<thead>
<tr>
<th>Virus titer (PFU)</th>
<th>No. of mice with hemorrhage/</th>
<th>(% of mice with hemorrhage)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of mice inoculated</td>
<td></td>
</tr>
<tr>
<td>3 × 10⁹</td>
<td>16/16 (100)</td>
<td></td>
</tr>
<tr>
<td>1 × 10⁸</td>
<td>11/15 (73)</td>
<td></td>
</tr>
<tr>
<td>8 × 10⁷</td>
<td>11/33 (33)</td>
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</tbody>
</table>

<sup>a</sup> C57BL/6 mice were inoculated intradermally with the indicated titers of viable DV at four sites on the upper back. Hemorrhage was observed at day 3 after inoculation. The data from three to four experiments are pooled.

<sup>b</sup> Mice developed systemic hemorrhage as shown in Fig. 1A.

<sup>c</sup> Mice developed hemorrhage in the subcutaneous tissue as shown in Fig. 1B.
DV distribution in mice after intradermal inoculation. To study the relationship between virus titer and hemorrhage, we compared virus capsid gene expression in tissues from H and nH mice. At day 3 after virus inoculation, the viral capsid gene was detectable in spleen, liver, brain, skin, and serum in both H and nH mice but the level of expression was significantly higher in H than in nH mice (Fig. 2A and B). Although viral capsid RNA was no longer detectable in the subcutaneous tissues at day 3 after inoculation, it was expressed at day 1 (Fig. 2A), showing that DV was present in subcutaneous tissues soon after intradermal inoculation but was cleared thereafter. Furthermore, while viral capsid gene copy number increased from day 1 to day 3 in the sera of H and nH mice, it was significantly higher in H than in nH mice (Fig. 2B). Immunofluorescence staining also showed the presence of viral antigen in the skin (increased from day 1 to day 3) (Fig. 2C) and subcutaneous tissues (at day 1) (Fig. 2D) after inoculation. Together, these data indicate a strong correlation between high viral titers and hemorrhage development.

Macrophages infiltrate and produce TNF-α in the hemorrhage tissue. To investigate the contribution of immune cells to hemorrhage development, subcutaneous tissues from H and nH mice were examined for the presence of NK cells, neutrophils, macrophages, dendritic cells, and T cells. The results revealed that no appreciable numbers of NK cells (NK1.1+), T cells (CD3+), dendritic cells (CD11c+), Langerhans cells (CD207+), or neutrophils (Gr.1+) were present (data not shown). Only macrophages (Mac-1+/F4/80+/CD14+) were de-
results showed a significant increase in TNF-α mRNA in H mice over the PBS control at day 3 after DV infection (Table 2). It was further increased in H but not in nH mice at day 3 (Table 2). Quantitation of in situ TNF-α protein in tissue homogenates showed that while TNF-α levels increased from day 1 on, the level in H mice (155.2 ± 65.7 pg/ml) was about eight times higher than that in nH mice (19.2 ± 1.1 pg/ml) at day 3 after infection (Table 2). These results indicate a strong association between macrophage infiltration, TNF-α production, and hemorrhage.

Immunofluorescence staining of subcutaneous hemorrhage tissues showed that TNF-α colocalized with macrophages (Fig. 4A), indicating that macrophages are a likely source of TNF-α. In vitro experiments were performed to determine whether DV-infected macrophages produce TNF-α. Figure 4B shows that DV induced macrophage production of TNF-α at as early as 6 h (122.1 ± 32.8 pg/ml) after infection. The level of TNF-α reached the peak at 12 h (329.0 ± 86.2 pg/ml) and declined thereafter. These results together revealed that macrophage infiltration and production of TNF-α in the tissues after DV infection are two important events that lead to hemorrhage.

**TNF-α is key to the development of dengue hemorrhage in mice.** TNF-α−/− mice were employed to ascertain the role of TNF-α in hemorrhage development. Since Ab and complement have been implicated in the protection as well as the pathogenesis of hemorrhage in dengue (23) and DV infection-induced DV-specific immunoglobulin M (IgM) and IgG production and C3 deposition were observed in hemorrhage tissues (data not shown), mice with IgH and complement defects were used as controls. Table 3 shows that the percentages of TNF-α−/− mice (5%) that developed hemorrhage were significantly lower (P < 0.05) than those of wild-type, IgH−/−, and A/HeJ (C5−/−) mice. The percentages of hemorrhage development in IgH−/− (29%) and C5−/− (36%) mice were not much different from those of their respective wild-type mice (35% and 33%, respectively). These data clearly indicate that TNF-α, but not Ab production or complement deposition, is key to the development of hemorrhage in DV-infected mice.

**ECs are targets of DV and become apoptotic in the hemorrhage tissue.** To determine whether hemorrhage is accompanied by cell death, subcutaneous tissues were stained with TUNEL reagents. The results show an overwhelming presence

![FIG. 3. Macrophage infiltration in hemorrhage tissue. The cryosections of subcutaneous tissues collected from mice injected with PBS, 4 × 107 PFU of viable DV (Live-DV), or an equivalent titer of UV-DV at days 1 and 3 after inoculation were stained with PE-conjugated rat anti-mouse Mac-1 Ab. Cryosections of tissues from H mice were stained with PE-conjugated anti-mouse isotype IgG2b Ab as a staining control. H mice or nH mice were determined at day 3 after inoculation. The number + standard deviation on the top of each picture indicates the mean percentage of Mac-1+ cells in each group. The percentage of Mac-1+ cells was determined by dividing the count of Mac-1+ cells by the total number of Hoechst-positive cells in the same field. The data were pooled from counting cells in four sections obtained from four different mice. Three to five fields in each section and a total of 750 to 800 cells were counted. **, P < 0.05, by comparing the number of infiltrating macrophages in tissue from H mice that to in tissue from nH mice at day 3 after infection.](http://jvi.asm.org/)

<table>
<thead>
<tr>
<th>Mice</th>
<th>mRNA (TNF-α/β-actin)</th>
<th>Protein (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live DVE</td>
<td>0.43 ± 0.09</td>
<td>28.4 ± 4.5†</td>
</tr>
<tr>
<td>UV-DVE</td>
<td>ND</td>
<td>65.5 ± 15.2*</td>
</tr>
<tr>
<td>PBS</td>
<td>ND</td>
<td>15.2 ± 1.0†</td>
</tr>
<tr>
<td>Live DVE</td>
<td>0.63 ± 0.05</td>
<td>65.5 ± 15.2*</td>
</tr>
<tr>
<td>UV-DVE</td>
<td>ND</td>
<td>15.2 ± 1.0†</td>
</tr>
<tr>
<td>PBS</td>
<td>ND</td>
<td>14.1 ± 0.2†</td>
</tr>
</tbody>
</table>

a The relative levels of TNF-α mRNA expression were determined by RT real-time PCR.

b The concentration of TNF-α protein in the supernatants of tissue homogenates was quantified by ELISA.

c Mice were inoculated intradermally with 4 × 107 PFU of viable DV (Live DV), UV-inactivated DV (UV-DV), or PBS on the upper back.

d Subcutaneous tissues were collected from mice at days 1, 2, and 3 after live DV inoculation and at day 3 after PBS or UV-DV inoculation. Data at each time point were pooled from six mice. The averages of values are expressed as means ± standard deviations. * and † indicate P values of <0.01 and <0.05, respectively, compared to PBS-inoculated mice. †, P < 0.01 compared to nH mice at day 3, ND, not done.

e No hemorrhage was observed in UV-DV- or PBS-inoculated mice.
of apoptotic cells in tissues from H mice (37.2% ± 18.0%) but not nH mice (1.0% ± 0.4%) at day 3 after infection (P < 0.05). Double staining revealed that apoptotic cells were DV Ag positive (Fig. 5). Further analysis of the types of cells undergoing apoptosis revealed that as many as 94.0% of CD31+ ECs (1) and only 23% of macrophages (F4/80+/Mac-1+) in the hemorrhage tissues were apoptotic, indicating a strong link between EC apoptosis and hemorrhage. In addition, double staining unveiled that both ECs and macrophages are targets for DV at the early phase of infection (Fig. 6).

** Table 3. TNF deficiency diminishes DV-induced hemorrhage **

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>No. of mice with hemorrhage/ no. of mice inoculated (% of mice with hemorrhage)</th>
</tr>
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<tbody>
<tr>
<td>C57BL/6</td>
<td>........................................................................7/20 (35)</td>
</tr>
<tr>
<td>IgH−/−</td>
<td>........................................................................4/14 (29)</td>
</tr>
<tr>
<td>TNF−/−</td>
<td>........................................................................1/20 (5)**</td>
</tr>
<tr>
<td>A/J</td>
<td>........................................................................6/18 (33)</td>
</tr>
<tr>
<td>A/HeJ</td>
<td>........................................................................8/22 (36)</td>
</tr>
</tbody>
</table>

a IgH−/− mice, TNF−/− mice, and their wild-type C57BL/6 counterparts as well as A/HeJ mice and their wild-type A/J counterparts were injected intradermally with 4 × 10^5 PFU of viable DV. The data from three experiments are pooled.

b Mice developed hemorrhage in the subcutaneous tissue as shown in Fig. 1B.

** indicates that the P value when TNF−/− mice were compared to wild-type, IgH−/−, A/J, or A/HeJ mice each was < 0.05.

In vitro experiments demonstrate that TNF− in the DV-infected macrophage culture supernatant induces EC apoptosis. Primary microvascular ECs were isolated to study their susceptibility to DV- and TNF−-induced cell death. The results demonstrate that DV alone at MOIs of 5 and 10 induced 11.0% ± 1.1% and 29.9% ± 5.6% EC apoptosis, respectively, at 24 h of infection (Fig. 7A). Recombinant TNF− at 3,000 pg/ml but not at 30 or 300 pg/ml induced EC death (Fig. 7A). Interestingly, the addition of 300 pg/ml of TNF− to DV-infected cells increased cell death from 11.0% ± 1.1% to 21.0% ± 3.8% (P < 0.01) at an MOI of 5 and from 29.9% ± 5.6% to almost threefold to as high as 85.4% ± 8.3% (P < 0.05) at an MOI of 10, and the addition of 3,000 pg/ml of TNF− increased cell death to 92.0% ± 0.3% at an MOI of 10. It is worth noting that the addition of 300 pg/ml of TNF− to UV-DV-treated cells did not increase the percentage of cell death compared to that of TNF−-treated uninfected cells, indicating that EC death is not a result of overstimulation by high concentrations of Ag. Moreover, treatment with 12-h supernatants from DV-infected macrophage cultures completely destroyed EC monolayers preinfected with DV (Fig. 7B). Over 95% of DV-infected ECs at an MOI of 10 were apoptotic after intradermal inoculation. The cryosections of subcutaneous tissues collected from mice receiving 4 × 10^5 PFU of viable DV at day 1 after inoculation were stained with (A) rabbit anti-DV antiserum plus FITC-conjugated goat anti-rabbit IgG, FITC-conjugated TUNEL mixture, and Hoechst 33258 stain.
treatment with 12-h supernatants (Fig. 7C). Importantly, the effect of macrophage culture supernatants on ECs was completely neutralized by anti-TNF-α Ab pretreatment, regardless of the virus titer with which the ECs were infected (Fig. 7B and C). These results together demonstrate that DV induces macrophage production of TNF-α and that TNF-α enhances DV-induced EC death.

**DISCUSSION**

It has been documented that high levels of TNF-α are associated with severe dengue disease (2, 13, 34). There is also a positive relationship between soluble TNF receptor (sTNFR)/sTNFR-II levels and the severity of DHF (2, 12). Single-nucleotide polymorphism analysis identified TNF-α polymorphism at TNF allele 308A to be a possible risk factor for hemorrhagic manifestations in DF patients (6). The TNF-α promoter with a polymorphism at position −308 is a stronger transcriptional activator, which is responsible for the production of higher levels of TNF-α (31). The TNF 308A-positive patients are more likely to develop DHF than TNF 308A-negative patients (6). These observations together strongly indicate that high levels of TNF-α are critical for the pathogenesis of severe dengue illness. However, the direct causal relationship between TNF-α and DV hemorrhage has not been clearly established. We showed in this study the direct relationship between high in situ levels of TNF-α and hemorrhage in a DV-infected host (Table 2) and that without TNF-α, the chances to develop hemorrhage are greatly diminished (Table 3). In addition, we observed that tissue viral titers in TNF−/−mice were higher than those in wild-type mice, yet mice did not develop hemorrhage (data not shown). These data together provide direct evidence for the critical role of TNF-α in dengue hemorrhage.

Our results demonstrated that DV infection induces mouse macrophages to produce TNF-α (Fig. 4A and B). It has been reported that human monocytes infected by DV produce TNF-α (3, 5). Sensitized T cells from dengue patients also produce TNF-α in culture upon restimulation (7, 20). A recent publication reported the cytokine responses of DV-specific memory CD4+ T cells in peripheral blood mononuclear cells of volunteers who received experimental live attenuated monovalent DV vaccines (21). While Ags from homotypic DV elicit the highest IFN-γ response, peptide sequences from heterotypic DV elicits CD4+ T-cell responses with higher TNF-α+/−to-IFN-γ+ T-cell ratios. In our mouse model, hemorrhage develops at as early as day 3 after virus inoculation, a time too early for T cells to mount a significant response. Thus, our model illustrates the importance of innate immune responses to DV infection in the pathogenesis of hemorrhage. However, treatments. Other infected ECs were treated with 12-h supernatants collected from DV-infected macrophage cultures with or without anti-TNF-α Ab pretreatment. Infected ECs treated with anti-TNF-α Ab were used as controls. Isotype rat IgG1 was used to demonstrate the specificity of anti-TNF-α Ab-mediated block. Percent EC apoptosis was calculated as described above (A). Data were pooled from three separate experiments. h.p.i., hours postinfection. * and ** indicate P values of <0.05 and <0.01, respectively.
it is entirely possible that inappropriate T-cell activation resulting in the production of TNF-α in later stages of primary infection or in secondary infection may worsen the disease.

Vascular ECs line the inner surface of blood vessels and play an important role in vascular functions (17). It is thus a logical assumption that hemorrhage or altered vascular permeability is a result of EC damage. Whether DV targets ECs in dengue patients has been a point of debate for decades until recently, when Jessie et al. reported their immunohistochemical staining and in situ hybridization of biopsy and autopsy specimens from DF/DHF/DSS patients (15). Their report clearly showed the presence of DV Ags in sinusoidal ECs of the liver as well as in the vascular endothelium of the lung (15). Our study of the hemorrhagic mouse tissue also showed that DV targets ECs (Fig. 6). Double staining of hemorrhagic tissues with anti-NS1 and anti-CD31 or anti-CD14 antibodies revealed that viral NS1 Ag was detectable in CD31 + cells at 6 h and the intensity increased at 12 h and 24 h after inoculation, but it was not in CD14 + cells at any of these early time points, confirming that DV replicates in ECs soon after infection. In addition, unlike what was observed for human skin, neither CD11c + nor CD207 + cells stained for DV Ag at these early time points (data not shown). Thus, it appears that DC does not make a major contribution to dengue hemorrhage in our mouse model and that ECs, as an early target of DV, are predisposed by DV to the deleterious events that occur subsequently.

The loss of endothelium integrity in DHF/DSS has long been attributed to the inflammatory mediators released by mononuclear phagocytes, of which TNF-α is the primary candidate (10, 11). However, macrophage production of TNF-α is common to a variety of infections (29) in which a profound alteration of endothelial permeability may not be a prominent feature. It remains a question why TNF-α is specifically of importance to dengue hemorrhage. In their report showing a positive relationship between high sTNFR levels and disease severity, Bethell et al. proposed that the effect of systemic cytokines on the endothelium is determined by the local events that occur, such as viral infection (2). It is interesting that no viral RNA was detectable at day 3 when hemorrhage develops at the subcutaneous tissues, although the presence of virus was documented at day 1 (Fig. 2A). Thus, it appears that the presence of DV predisposes the endothelium to vascular damage triggered by TNF-α, yet its concurrent presence is not required for hemorrhage development.

Nonhuman primates have been used to study dengue (9). The animals showed no signs of disease but developed viremia. The mouse model of DV hemorrhage that we reported in this study does not show all the characteristics of a natural DV infection in humans, but the mice develop hemorrhage and thrombocytopenia. After a person is bitten by an infective mosquito, the virus undergoes replication and circulates in the circulation (Fig. 2A and B), and hemorrhage developed within only 3 days. It appears that introducing a high DV inoculum intradermally quickly meets the threshold of virus titer that is required to stage a pathogenic event in the murine host. Therefore, our mouse model models only the immediate events that lead to hemorrhage but not the entirety of the pathogenesis of human dengue hemorrhage.

It is noteworthy that hemorrhage development is DV specific, and virus viability is essential. Introducing neither UV-DV nor viable JEV, another mosquito-borne flavivirus, through the same route at an otherwise equivalent titer did not result in hemorrhage. In addition, injecting recombinant TNF-α (8 μg) along with mock C6/36 supernatants through the same route did not induce hemorrhage (data not shown), excluding the possibility that injury (as might be induced through needle sticks) is involved in the process. Based on these observations, we speculate that a specific interaction between DV and cells, especially at high DV titers, perhaps through the activity of a specific viral component(s), is critical for the development of hemorrhage.

In the present study, we established a mouse model of DV hemorrhage through the intradermal inoculation of high titers of virus. By employing this mouse model, we identified TNF-α as being one of the key host factors causing dengue hemorrhage, which is consistent with the recent observation reported by Shresta et al., who found that TNF-α is the key mediator of severe DV-induced disease in mice (27). In our model, hemorrhage develops within 3 days after virus inoculation, a time that is too early for the adaptive immune response to take effect. Although our finding emphasizes the importance of the innate immune response in hemorrhage development, it does not rule out the contribution of nonneutralizing Ab-dependent enhancement in increasing infection or the involvement of TNF-α-producing activated T cells in the pathogenesis of DV hemorrhage in secondary infection. Experiments to address these questions are currently being undertaken.

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