



Review

Endothelial cells in dengue hemorrhagic fever

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ABSTRACT

Therapies to prevent or reverse endothelial dysfunction and vascular leak found in dengue hemorrhagic fever (DHF) have not been identified. In this review we summarize dengue viruses and the spectrum of human disease and highlight evidence of endothelial cell dysfunction in DHF based on studies in patients and mouse and tissue culture models. Evidence suggests that both virus antigen and host immune response, can cause endothelial cell dysfunction and weaken endothelial barrier integrity. We suggest possible therapeutic interventions and highlight how therapies targeting altered endothelial function might be evaluated in animal models and in patients with DHF.

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Abbreviations: CLEC5A, C-type lectin domain family 5 member A; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IL, interleukin; MMP, matrix metalloproteinase; NS, nonstructural; S1P, sphingosine-1-phosphatase; SCID, severe combined immunodeficient; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

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1. Introduction

Infection with dengue virus can lead to a wide spectrum of clinical illness from a nonspecific febrile syndrome to dengue hemorrhagic fever (DHF) which is characterized by increased vascular permeability, hemorrhage and shock (Gubler, 1997; Nimmannitya, 1993). The endothelial barrier is of central interest to those

investigating vascular permeability in dengue infections. The barrier consists of a number of elements such as endothelial cells, smooth muscle cells, an extracellular matrix, basement membrane, cytoskeleton and cell–cell junctions (Dejana et al., 2009; Dvorak, 2010), all of which undergo changes both during normal physiology and likely during a dengue infection. Endothelial cells are a critical element of the barrier and much dengue research has focused on endothelial cells in attempts to pinpoint mechanisms of vascular leakage.

In this article we review current concepts of the role of the endothelium in dengue and efforts to develop interventions to prevent or reverse vascular leak. We focus on the role of endothelial cells in vascular leakage in dengue by assessing findings from human studies, animal, and cell culture models. Other factors involved in DHF such as coagulation, host and virus mediators and the autoimmune phenomena found in DHF are considered. We highlight candidate mediators involved in plasma leakage and discuss possible therapeutic interventions targeting altered endothelial function and how they might be evaluated in animal models and in DHF patients.

2. Dengue virus and clinical disease

2.1. Dengue virus

Dengue is caused by dengue viruses (DENV), a group of four serologically distinct positive strand RNA viruses: DENV1, DENV2, DENV3, and DENV4. Dengue viruses belong to the *Flaviviridae* family which includes yellow fever virus and Japanese B encephalitis virus. The DENV viral genome is ten kilobases in length and encodes 10 gene products including structural proteins: C (capsid), prM (membrane), and E (envelope); and nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Gubler et al., 2007; Lindenbach et al., 2007). The E protein plays an important role in viral binding and entry into host cells (Leitmeyer et al., 1999; Modis et al., 2004; Tassaneetrithep et al., 2003). The serologic reactivity to the envelope proteins defines the serotypes of DENV (Gubler et al., 2007).

A number of molecules have been reported to interact with DENV and serve as receptors for viral attachment and entry (Mukhopadhyay et al., 2005). Among these, the best characterized are C-type lectins including DC-SIGN (CD209) and CLEC5A expressed on dendritic cells and macrophages respectively (Chen et al., 2008; Tassaneetrithep et al., 2003). The primary role of DC-SIGN is likely viral attachment since internalization occurs in cells expressing DC-SIGN that lacks internalization sequence (Lozach et al., 2005). Therefore, additional molecules not yet specified are involved in virus internalization. Interaction between viral particles and CLEC5A has been shown to induce production of proinflammatory cytokines which may play a role in dengue pathogenesis (Chen et al., 2008). Although DENV can infect many cell types in vitro including epithelial cells, endothelial cells, hepatocytes, muscle cells, dendritic cells, monocytes and mast cells, the roles of these cells in dengue pathogenesis and the cellular receptors involved in infection are not known (Arevalo et al., 2009; Basu et al., 2011; Brown et al., 2011; Huang et al., 2000; Paes et al., 2009; Salgado et al., 2010).

2.2. Clinical disease

Dengue is transmitted by bites of infected mosquitoes: *Aedes aegypti*, and less commonly *Aedes albopictus* (Gubler et al., 2007). In endemic areas, primary DENV infections occur early in life and are usually mild and often undiagnosed. Primary infections in older children and adults can result in dengue fever (DF). Dengue virus infections were once thought to cause a non-fatal illness before

several severe dengue hemorrhagic fever (DHF) outbreaks that occurred in the 1950–1960s changed this perception (Fresh et al., 1969). Dengue hemorrhagic fever is characterized by fever, thrombocytopenia, hemorrhagic tendency, and plasma leakage (World Health Organization, 1997). Plasma leakage is the clinical feature that distinguishes DHF from DF and is the most important risk factor for severity.

Individuals can be infected more than once with different serotypes of DENV due to the lack of long lasting cross-protective immunity. Epidemiological evidence strongly indicates that a secondary infection poses a higher risk for DHF in comparison to a primary infection. Although the majority of cases with a secondary infection develop DF, which are usually self-limited without requiring significant intervention, a minority of cases develops plasma leakage which occurs around the time of defervescence, resulting in accumulation of fluid in the chest and abdominal cavities (Pramuljo and Harun, 1991; Srikiatkhachorn et al., 2007b). Severe plasma leakage may lead to circulatory insufficiency and death. Increased vascular permeability in other vascular beds such as the kidneys has been suggested on the basis of increased urine protein levels in DHF compared to DF. However, the severity of proteinuria in DHF is mild and not the primary cause of fluid accumulation in the serosal cavities. Hemorrhagic manifestations, ranging from minor skin hemorrhage to mucosal (nose, gum) and gastrointestinal bleeding, are common in both DF and DHF but are more severe in DHF (Nimmannitya, 1993; World Health Organization, 1997).

Although the DF/DHF clinical classification has been in use since the 1960s and has been instrumental in the development of a clinical treatment algorithm that significantly improved case mortality, the 1997 World Health Organization (WHO) guideline defining DF and DHF (World Health Organization, 1997) has been under criticism for its applicability, validity, and ability to identify severe dengue (Bandyopadhyay et al., 2006; Deen et al., 2006). In 2009 the WHO issued a new clinical classification scheme (World Health Organization, 2009) based on information from a multicenter study conducted in Asia and Central and South Americas (Alexander et al., 2011). In this new scheme dengue is classified into dengue and severe dengue. The definitions of severe dengue are: (1) dengue with plasma leakage leading to shock or respiratory distress, (2) severe hemorrhage, and (3) organ failure. This review will be based on the 1997 DF/DHF classification since most studies have until recently utilized this classification scheme.

3. Dengue virus and the endothelial barrier

Since the cardinal manifestations of DHF, namely plasma leakage and hemorrhagic tendency, are suggestive of changes in vascular functions, the roles of the endothelium in the pathogenesis of dengue have long been investigated. Although other cells and structures including perivascular smooth muscle cells, the extracellular matrix and basement membrane, and the glycocalyx participate in the regulation of vascular permeability, the roles of these cells and structures in permeability regulation in dengue have not been intensively investigated. As such, most of the evidence reviewed in this article will be largely related to the roles of endothelial cells. In the following sections we highlight evidence of DENV infection of endothelial cells and subsequent effects of viral antigens and host mediators on endothelial cells in human infections, and in animal and in vitro cell culture models.

3.1. Studies of human infections

Based on human autopsy studies, cells of the immune system including monocytes, tissue macrophages, and lymphocytes have

been shown to express DENV antigens and genomes (Balsitis et al., 2009; Jessie et al., 2004). Using a human skin explant model, some investigators have demonstrated infection of skin dendritic cells following direct inoculation of DENV into the skin explants (Limon-Flores et al., 2005; Wu et al., 2000). Unlike other viral hemorrhagic fevers such as hantavirus where direct infection of endothelial cells has been well documented and implicated in the disease process (Zaki et al., 1995; Zampieri et al., 2007), direct infection of endothelial cells has not been consistently and unequivocally demonstrated in human dengue cases. Endothelial cells in the lungs and spleen have been shown to express viral proteins (Balsitis et al., 2009; Couvelard et al., 1999; Jessie et al., 2004; Salgado et al., 2010). The abundance of endothelial cells stained positive for dengue antigens in these studies was not definitively reported but the frequency appeared to be rather low. It is noteworthy that viral RNA was not detected in endothelial cells in these studies.

A common observation from autopsy or biopsy studies has been diapedesis of circulating mononuclear and red blood cells across the vascular endothelium, perivascular cellular accumulation and oedema (Balsitis et al., 2009; Basilio-de-Oliveira et al., 2005; Bhamarapravati et al., 1967; Boonpucknavig et al., 1979). The extent of endothelial cell death and inflammatory cell infiltration are usually minimal and are not likely the underlying mechanism of increased vascular permeability (Limonta et al., 2007). Although direct measurement of vascular permeability in human dengue has not been performed, indirect measurement using strain gauge plethysmography has demonstrated that the coefficients of capillary filtration were elevated in DHF patients compared to those of healthy controls (Bethell et al., 2001; Vasanwala et al., 2014; Wills et al., 2004). This is consistent with the elevated extracellular water observed during defervescence in patients with DHF in comparison to patients with DF (Libraty et al., 2002b). Increased urinary excretion of small protein molecules such as albumin and transferrin was elevated during shock phase in severe dengue cases in comparison to the excretion rate measured after recovery (Vasanwala et al., 2014; Wills et al., 2004). In addition, increased heparin sulfate urinary excretion was also observed. Although glomerular vascular bed is not the major site where leakage occurs in DHF, these findings may reflect the perturbation of vascular permeability regulation that occurs at other anatomical sites such as the pleural and abdominal cavities.

Despite the minimal changes in endothelial cell morphology several lines of evidence suggest that endothelial cells are activated during a DENV infection. Several biomarkers indicative of endothelial cell activation are elevated in dengue, especially in severe cases. These include soluble vascular cell adhesion molecules (sVCAM-1) and soluble intercellular adhesion molecule 1 (sICAM-1) (Khongphatthanayothin et al., 2006; Koraka et al., 2004). Endothelial cell activation may lead to changes in the coagulation state observed in dengue such as procoagulation state, fibrinolysis and more rarely, disseminated intravascular coagulation (Bhamarapravati, 1989; Carlos et al., 2005; Sosothikul et al., 2007; Weiss and Halstead, 1965; Wills et al., 2002). During normal homeostasis the endothelium produces inhibitors of blood coagulation, such as thrombomodulin, heparin sulfate and plasminogen activators, where upon stimulation by dengue virus and/or cytokines, the endothelium loses its non-thrombogenic protective properties, resulting in the production or release of inducers of coagulation such as von Willebrand factor antigen (vWF:Ag) and tissue factor (TF) (Bauer and Weitz, 1994; Djamiatun et al., 2012; Huang et al., 2003). Although studies have attempted to associate an activated coagulation system with disease severity, the effects of DENV on expression of coagulation factors and related molecules were complex as reflected in the coagulation profiles observed in dengue patients (Butthep et al., 2006; Sosothikul

et al., 2007). The extent to which vasoactive mediators resulting from the activation of the coagulation pathway directly contribute to vascular leakage in DHF is unknown.

Taken together, evidence from human studies suggests that cells of the reticuloendothelial and immune system appear to be the main *in vivo* targets of DENV. The lack of definitive evidence supporting DENV infection of endothelial cells makes it difficult to conclude whether infection of endothelial cells is responsible for initiating and sustaining vascular permeability in dengue infection. The inherent difficulty of demonstrating DENV infected endothelial cells may be explained by the timing of sample collection, after host immune clearance of virus. It is possible that DENV does indeed infect endothelial cells in human infections but this type of evidence is very difficult to demonstrate. Despite the relative lack of evidence for direct viral infection of endothelial cells, it is likely that the interaction between viral products and the elicited innate and adaptive immune response are critical mechanisms underlying plasma leakage.

3.2. Animal models

Mouse models have been used for immunopathogenesis studies and preclinical testing of dengue vaccine or antiviral candidates with relatively limited success due to their inability to mimic DENV replication kinetics or DF and DHF signs and symptoms without virus or host manipulation. Humanized mouse models with a transplanted human immune system can generate T cells, B cells, macrophages, dendritic cells and NK cells and produce a robust immune response to DENV infection (Akkina, 2013; Kuruvilla et al., 2007; Mota and Rico-Hesse, 2011). Other mouse models including severe combined immunodeficient (SCID), gene knock-outs or mice infected with high dose wild-type or mouse-adapted DENV strains have demonstrated viremia, transmission ability and limited clinical signs and symptoms after infection (An et al., 2004; Atrasheuskaya et al., 2003; Christofferson et al., 2013; Prestwood et al., 2008; Sabin and Schlesinger, 1945; Shresta et al., 2006; Tan et al., 2010).

Studies in mice have demonstrated DENV antigen expressing endothelial cells in various organs including spleen and liver (Chen et al., 2007; Guabiraba et al., 2013). Multifocal endothelial injury and DENV antigen were detected in the capillary endothelium of the central lobular vein area in the liver of BALB/c mice that were intraperitoneally infected with DENV2 (Barth et al., 2006). In a DENV antibody dependent enhancement infection mouse model, sub-neutralizing levels of DENV-specific antibodies promoted massive infection of liver sinusoidal endothelial cells resulting in increased systemic levels of virus (Zellweger et al., 2010).

Minor changes in endothelial cell morphology have been reported in mouse models. In the lungs of BALB/c mice infected with DENV2, blood capillary endothelial cells exhibited phallopoia suggesting activation by the presence of DENV but without direct infection of endothelial cells (Barreto et al., 2007). Hemorrhage manifestations including severe thrombocytopenia and prolonged bleeding time have been demonstrated in mouse models. Endothelial cell damage was accompanied by tissue infiltration of macrophages that secreted TNF- α (Wu-Hsieh et al., 2009; Yen et al., 2008). The roles of dengue virus NS1, TNF- α , and reactive nitrogen and oxygen species in hemorrhage have been reported in some mouse models (Chen et al., 2007; Lin et al., 2002, 2008).

Apart from mouse models, rabbit, gerbil, hamster, chimpanzee and macaque models have been found to be less than ideal to assess DENV infection and subsequent viremia. DENV infected rhesus monkeys demonstrated productive viremia (Freire et al., 2007; Kraiselburd et al., 1985; Scott et al., 1980) and have been used for pre-clinical vaccination (Blaney et al., 2008; Bray et al., 1996;

Guirakhoo et al., 2001; Kraiselburd et al., 1984; Martin et al., 2009; Putnak et al., 2003; Raviprakash et al., 2008; Simmons et al., 2010; Sun et al., 2006) and antiviral studies (Ajariyakhajorn et al., 2005; Malinoski et al., 1990). Studies using these models did not assess if endothelial cells expressed virus antigen after DENV infection in these animal models.

Animal models to study vascular leakage in dengue are limited due to their absence of overt signs of disease. Some degrees of plasma leakage have been observed in an AG129 mouse model, which lacks IFN responsiveness, infected with a mouse-adapted dengue virus type-2 strain (Shresta et al., 2006). As such, comparative studies to assess the potential of wild-type strains to initiate vascular permeability or to infect vascular endothelial cells without system manipulation are not readily possible. In addition, the leakage patterns in mouse models do not reproduce the anatomical pattern observed in human dengue. The clear advantage of mouse models is the ability to allow for a well-defined set of experimental parameters to be systematically tested. Therefore, small animal models may be a useful tool to advance findings from in vitro studies and may help delineate new areas of dengue infection that deserve attention in human studies. Animal models are probably most useful in assessing antiviral compounds as some of these models such as Rhesus monkeys developed significant viremia but these non-human primate models have not been used extensively to study pathogenesis, particularly plasma leakage or endothelial cell dysfunction, as these animals generally do not develop clinical signs of plasma leakage.

3.3. *In vitro* endothelial cell models

Endothelial-like cell lines and primary endothelial cells can be infected by DENV in vitro and produce viral progeny (Arevalo et al., 2009; Azizan et al., 2006; Basu et al., 2011; Dalrymple and Mackow, 2012). The infection process appears to involve heparan sulfate-containing proteoglycan receptors (Dalrymple and Mackow, 2011). The extent of the infection was fairly limited at early time points and neutralization of IFN-beta enhanced the in vitro infection of primary endothelial cells by DENV suggesting that type-I IFN response by endothelial cells limited viral replication and spreading of DENV in endothelial cell monolayers (Dalrymple and Mackow, 2012).

Dengue virus infection of endothelial cells was associated with changes in gene transcription and protein expression of several functional pathways including type I IFN, chemokines, cytokines, angiogenesis, complement, and coagulation (Avirutnan et al., 1998; Azizan et al., 2009; Warke et al., 2003). Endothelial cells infected with DENV secreted IL-6, IL-8, CXCL10, CXCL11, and RANTES (Dalrymple and Mackow, 2012; Huang et al., 2000). NS5 and NS4B have been shown to induce expression of IL-8 in dendritic cells and a macrophage cell line and likely also in endothelial cells (Kelley et al., 2012; Medin et al., 2005). Mediators induced by DENV possess permeability enhancing effects and chemoattractant properties that may contribute to loss of vascular integrity and recruitment of inflammatory cells. In regards to the coagulation pathway, up-regulation of tissue factor expression by DENV infected cells have been consistently demonstrated while the effects of DENV on endothelial expression of thrombomodulin and PAI-1 have been conflicting (Huerta-Zepeda et al., 2008; Jiang et al., 2007; Yeh et al., 2013). These data are consistent with findings from human studies investigating the coagulation system (Bethell et al., 2001; Sosothikul et al., 2007).

Studies on the effects of DENV on endothelial cell survival and integrity have shown conflicting results (Bonner and O'Sullivan, 1998; Bunyaratvej et al., 1997; Liu et al., 2009). No changes in cell morphology have been reported in some studies while others have demonstrated apoptosis of DENV infected endothelial cells through

mechanisms including fas-fasL and TNF-alpha induced nitric oxide production (Liao et al., 2010; Yen et al., 2008). DENV infection at low multiplicity of infection also acted synergistically with TNF-alpha in increasing permeability of an endothelial cell monolayer (Dewi et al., 2004). In addition, endothelial cells from different vascular beds exhibited different patterns of adhesion molecule expression when infected with DENV (Peyrefitte et al., 2006) which may have implications on the anatomically specific pattern of plasma leakage found in dengue. Loss of endothelial cell barrier integrity and disrupted cell-cell junctions resulted from disassembly of VE-cadherin junction protein, decreased expression of tight junction protein ZO-1 and rearrangement of the actin cytoskeleton after DENV infection (Dewi et al., 2008; Kanlaya et al., 2009).

Apart from the sparse evidence in human studies to rationalize in depth in vitro studies of DENV infection of endothelial cells, major caveats of these in vitro studies are the ability of in vitro cultured endothelial cell to reproduce cellular response in vivo and the lack of other elements of the blood vessels which regulate vascular permeability in a highly coordinated manner in these in vitro models. For example, the endothelium is supported by a basement membrane composed of collagen IV and laminin, among other structurally related proteins. Changes in the basement membrane composition induced changes in endothelial cells and modified their interactions with leukocytes (Ruiz-Torres et al., 2006), which would be relevant to host immune response and possibly vascular leakage in dengue. In this regard, results from in vitro studies should be interpreted within the context of this limitation.

The evidence from human, animal and in vitro models suggests the possibility that endothelial cells can be infected by DENV however the impact of the infection on vascular leakage is unknown. Moreover, evidence also indicates that host derived mediators likely contribute towards plasma leakage and coagulopathy in dengue. Due to conflicting findings from human studies demonstrating altered levels of mediators and coagulation factors, and the lack of methodology that can reliably measure vascular permeability in humans, findings from human studies must be interpreted with caution. Nevertheless since both the virus and host mediators probably contribute to altered endothelial cells function, it is logical that therapeutics targeting virus and/or host mediators could mitigate severe vascular leakage.

3.4. *Effects of viral antigens on endothelial cells*

High circulating levels of virus and viral antigen have been associated with DHF in many studies (Libraty et al., 2002a,c; Vaughn et al., 2000). The higher viral burden may be due to a relatively impaired type I IFN response that allows for more viral replication or may be mediated by cross-reactive, non-neutralizing antibodies that enhance viral uptake into susceptible cells. In the presence of antibodies such as during a secondary infection, viral antigens may form immune complexes with dengue-specific antibodies and activate the complement via the classical pathway (Fig. 1). Low levels of complement components and increased levels of activated complement components reported in dengue are indicative of complement activation during a dengue virus infection (Bokisch et al., 1973; Malasit, 1987). Dengue-specific antibodies have been shown to bind DENV infected endothelial cells and activate complement leading to deposition of non-lytic complement complexes (Avirutnan et al., 1998). In addition, terminal complement complexes have been found in pleural fluids of DHF patients (Avirutnan et al., 2006). These findings indicate that the complement pathway is activated during dengue virus infection and interacts directly with endothelial cells (Boom et al., 1989; Malasit, 1987).

Recent studies have focused on the role of dengue NS1 protein in the pathogenesis of dengue. NS1 is produced both as membrane

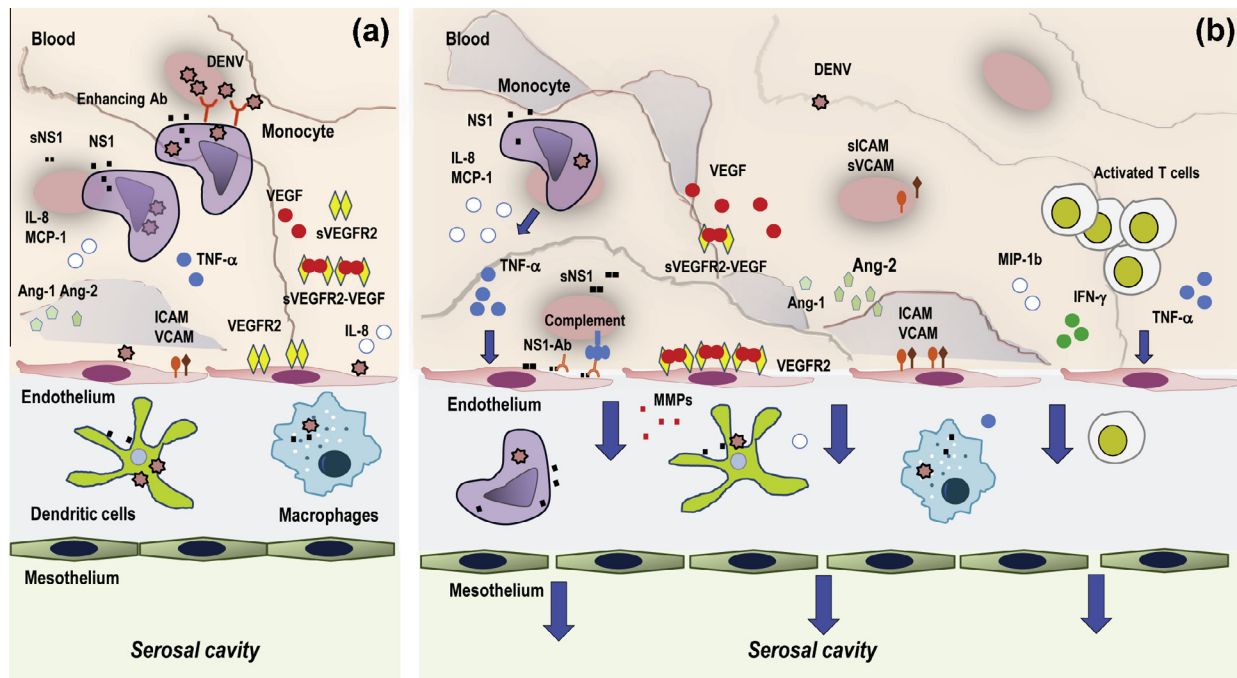


Fig. 1. Proposed scenario for the progression of plasma leakage and involvement of endothelial cells during DENV infection. (a) Acute phase (pre-leakage). DENV infects monocytes, dendritic cells (DCs), macrophages (and possibly endothelial cells) resulting in increased viremia. Infected cells produce chemokines such as IL-8 and MCP-1 and cytokines including TNF- α , triggering an innate immune response. Dengue virus NS1 is expressed on the surface of infected cells and sNS1 can be detected in the blood. Basal levels of angiopoietin (ang)-1 and ang-2, VEGF and sVEGFR2 are found in the blood and expression of ICAM and VCAM on the surface of endothelial cells. During this early phase the endothelium is not compromised and there is no plasma leakage. (b) Plasma leakage phase. Soluble NS1 and anti-NS1-antibody/NS1 complexes can interact with endothelial cells and activate the complement system. Cytokines such as TNF- α , MIP-1 β , IFN- γ and other permeability-enhancing mediators are produced by DENV-infected cells and memory T cells, activated by conserved or altered peptide ligands. Elevated VEGF and suppressed levels of sVEGFR2 correlate with the extent of plasma leakage. Ang-1, which can antagonize the permeability-enhancing effects of VEGF, is decreased, while levels of its functional antagonist, ang-2, are increased. DENV-induced secretion of matrix metalloproteinases (MMPs) by DCs can damage endothelial cells while differential expression of ICAM and VCAM between quiescent and activated endothelial cells may influence adhesion and transmigration of circulating leukocytes which can alter plasma leakage. Circulating levels of sICAM and sVCAM provide evidence of endothelial cell activation and/or damage. The net results of this scenario are compromised endothelial cells and a weakened barrier, leading to leakage of albumin-rich fluid into serosal cavities.

and soluble proteins. The circulating levels of soluble NS1 have been shown to correlate with disease severity (Avirutnan et al., 2006; Libraty et al., 2002c). NS1 binds mesothelial and endothelial cells, preferentially microvascular endothelial cells, via interaction with heparan sulfate or chondroitin sulfate E (Avirutnan et al., 2007). Further, NS1 protein has been shown to modify complement activation by binding to C4 and C1s and enhances C4b degradation (Avirutnan et al., 2010). As such, NS1 both activates the classical complement pathway by forming immune complexes with anti-NS1 antibodies, and inhibits the complement mediated lysis of virions leading to heightened viral/antigen burden. Moreover, given that terminal complement components possess permeability enhancing activities, most likely either via deposition of immune complexes and/or attraction of leukocytes and their downstream effects (Boom et al., 1989), it is apparent that an altered innate immune response including activated complement pathway has potential effects leading to vascular leakage in DHF.

4. Autoimmunity in dengue

A series of studies have demonstrated DENV-induced autoantibodies against endothelial cells, platelets, and molecules in the coagulation and fibrinolytic pathways in humans and experimental animals (Lin et al., 2003, 2002; Liu et al., 2011). The majority of these antibodies reacted to NS1 antigen although some were E and prM specific. The host molecular targets of these antibodies include H⁺-transporter/ATP synthase, protein disulfide isomerase, vimentin, heat shock protein 60, fibrinogen and plasminogen (Chuang et al., 2011; Lin et al., 2011; Liu et al., 2011). These anti-

bodies have been shown to induce abnormal activation and functions of platelets and endothelial cells leading to endothelial cell apoptosis in vitro and hemorrhage in experimental animals (Falconar, 1997; Sun et al., 2007). It is unclear how long these antibodies persist after an acute dengue virus infection and how clinically important these antibodies are in dengue pathogenesis since patients usually have a complete recovery without autoimmune manifestations as long term complications particularly in children. However, one study has suggested an increase in constitutional complaints and possibly elevated autoimmune markers (C-reactive protein, antinuclear factors, immune complex levels) in adults with symptomatic dengue two years after an infection (Garcia et al., 2011). More definitive studies will be needed to clarify the role of autoantibodies on endothelial cell disruption and dengue pathogenesis.

5. Candidate mediators involved in plasma leakage

Studies comparing levels of biological mediators in patients with either DF or DHF have been conducted in order to gain insights into the pathogenesis of severe dengue. The heterogeneity in study designs, times of sample collection, differences in patient populations, and the clinical classification have complicated the interpretation of conflicting findings from these studies. The difficulties in evaluating the biological relevance of cytokines in dengue pathogenesis lie in the lack of animal models that mimic the full spectrum of clinical dengue. However, although not providing a full clinical picture of DHF, recent immunocompromised mice and/or mouse adapted DENV models have demonstrated some

Table 1

Examples of mediators associated with endothelial cell activation and plasma leakage in dengue hemorrhagic fever.

	Comment	References
<i>Proinflammatory mediators</i>		
IL-8	Chemo-attractant that enhances leukocyte extravasation across endothelium; possesses permeability-enhancing effect; correlation between increased serum levels of IL-8 and DHF; documented in pleural effusions of DHF cases	Huang et al. (2000), Avirutnan et al. (1998)
Matrix metalloproteases (MMPs)	Can damage endothelial cells through loss of expression of cell–cell adhesion proteins	Luplertlop et al. (2006)
TNF-alpha	Permeability enhancing and pro-coagulation effects, possibly through production of reactive oxygen species; conflicting reports on levels in DHF cases	Braga et al. (2001), Dewi et al. (2004), Shresta et al. (2006)
<i>Coagulation factors</i>		
Thrombomodulin	Present in large quantities on the surface of endothelial cells; acts as an anticoagulant; increased levels of soluble thrombomodulin have been shown in dengue shock syndrome (DSS)	Butthep et al. (2006), Sosothikul et al. (2007)
Tissue plasminogen activator (tPA) von Willebrand factor antigen (vWF:Ag)	Inducer of fibrinolysis; correlation between sera levels of tPA and DHF Acts to stabilize adhesion of platelets and promote coagulation; increased levels found in DHF and DSS	Sosothikul et al. (2007), Huang et al. (2003) Sosothikul et al. (2007), Djamiatun et al. (2012)
<i>Angiogenic factors/receptors</i>		
VEGF, VEGFR1 and VEGFR2	Elevated levels of plasma VEGF associated with suppressed levels of soluble VEGFR2 at the time of plasma leakage in DHF cases. VEGF levels correlated with the extent of plasma leakage sVEGFR-1 found higher in DHF than DF patients	Srikiatkachorn et al. (2007a, 2007b), Tseng et al. (2005), Seet et al. (2009)
Angiopoietin-1 and angiopoietin-2	Ang-1 can antagonize permeability enhancing effects of VEGF; levels decreased in DHF along with increased levels of its antagonist, ang-2	Michels et al. (2012)
<i>Other</i>		
Soluble nonstructural protein 1 (NS1) and anti-NS1 antibodies	NS1 binds to endothelial cells; correlation between sNS1 levels and severe disease; modifies complement activation	Avirutnan et al. (2006), Libraty et al. (2002a, 2002b, 2002c)
Soluble adhesion molecules, ICAM-1 and VCAM-1	VCAM-1 found elevated in DSS patients compared to acute dengue fever patients; less conclusive for ICAM-1	Khongphattananayothin et al. (2006), Koraka et al. (2004)
Complement components: C5a, terminal SC5b-9 complex	Direct permeability-enhancing activity, or membrane-lytic activity, and/or attraction of leukocytes and downstream effects	Avirutnan et al. (2006)

clinical features of dengue including fever, thrombocytopenia, rash, hemorrhage and vascular leakage (Balsitis et al., 2009; Mota and Rico-Hesse, 2009; Paes et al., 2005; Shresta et al., 2006; Tan et al., 2011). Studies in these models have identified candidate cytokines in plasma leakage and hemorrhage.

Levels of several mediators have been shown to be elevated in DHF in comparison to DF. These include: (1) mediators released from DENV infected cells including IL-6, IL-8, IL-10 and chemokines (CXCL9, 10, and 11), (2) mediators produced by cells of the innate immune system such as NK cells, (3) mediators produced by cells of the adaptive immune system such as T lymphocytes. Elevated levels of both Th1 (IFN-gamma) and Th2 (IL-4, IL-13, IL-10) type cytokines have been reported in DHF. Elevated levels of some these mediators such as IL-8 have been documented in pleural effusions of DHF cases (Avirutnan et al., 1998) suggesting their involvement in plasma leakage. Among cytokine studied in dengue, TNF-alpha with its permeability enhancing and pro-coagulation effect, has been extensively studied and there have been conflicting reports on TNF-alpha levels in severe dengue (Azeredo et al., 2006; Braga et al., 2001). Studies in mouse models have implicated TNF-alpha in mediating plasma leakage and hemorrhage, possibly through the production of reactive oxygen species (Shresta et al., 2006; Wu-Hsieh et al., 2009).

In addition to proinflammatory cytokines, recent studies have demonstrated changes in the levels of angiogenic factors and their receptors that may contribute to enhanced permeability (Table 1). These molecules include vascular endothelial growth factor (VEGF) and its soluble receptors: VEGF receptor-1 (VEGFR1) and VEGFR-2, and angiopoietin-1 and angiopoietin-2. Elevated levels of plasma VEGF (Seet et al., 2009; Tseng et al., 2005) were associated with suppressed levels of soluble VEGFR2 at the time of plasma leakage in DHF cases and correlated with the extent of plasma leakage (Seet et al., 2009; Srikiatkachorn et al., 2007a). Angiopoietin-1 antagonizes the permeability enhancing effects of VEGF and its lev-

els have been reported decreased in DHF along with increased levels of its functional antagonist, angiopoietin-2 (Michels et al., 2012). These findings suggest that alterations in angiogenic cytokines may be an important mechanism in the dengue pathogenesis and vascular leakage (Fig. 1).

Endothelium interaction with immune cells may be a mechanism leading to altered vascular integrity. Increased permeability of an endothelial monolayer has been demonstrated in co-culture experiments with DENV infected monocytes (Carr et al., 2003). TNF-alpha released from monocytes has been implicated as the key mediator in this process (Bonner and O'Sullivan, 1998; Carr et al., 2003; Dewi et al., 2004; Kelley et al., 2012). Dengue virus induced matrix metalloproteases (MMPs) production and secretion by dendritic cells damaged an endothelial cell layer through loss of expression of the cadherin cell–cell adhesion proteins leading to increased permeability (Luplertlop et al., 2006). Differential expression of the ICAM-1 and VCAM-1 between quiescent and activated endothelial cells influences the degree of adhesion and transmigration of circulating leukocytes which can alter vascular permeability through extravasation through the endothelial barrier (Aird, 2007).

Taken together, this evidence suggests several mediators may participate in the changes in endothelial cell functions and vascular integrity in dengue. It is quite likely that the multi-system manifestations of dengue are mediated by several cytokines and other mediators. A summary of candidate mediators highlighted throughout this review can be found in Table 1. The early and late events that may lead to plasma leakage are depicted in Fig. 1.

6. Rationale for therapeutic intervention for dengue

Currently there is no specific treatment for dengue. The primary treatment is careful fluid replacement to maintain effective intra-

vascular volume and tissue perfusion (World Health Organization, 1997, 2009). Since there are no validated clinical or laboratory predictors that can identify potentially severe cases early in the course of the illness, close monitoring of dengue cases for signs of plasma leakage and compromised circulation is essential in dengue care.

Several potential therapeutic strategies and agents to modify clinical course in children have been advocated; these include blood component transfusion, carbazochrome sodium sulfonate, corticosteroids, or intravenous immunoglobulin, and recombinant-activated factor VII (Chuansumrit et al., 2005; Dimaano et al., 2007; Panpanich et al., 2006; Tassniyom et al., 1997). Very little evidence was available to support complete adoption of these strategies. Since high viral burden is often associated with severe dengue, an important strategy is to reduce viral burden by antiviral agents. The timely administration of antiviral agents is critical and may be problematic since studies have shown that patients usually develop peak viremia at the time of presentation. This is an active area of investigation and animal studies have identified candidate molecules that will likely enter clinical trials in the future (Perry et al., 2013; Schul et al., 2007; Stein et al., 2011). Herein our discussion will focus on therapeutic interventions that may mitigate vascular leakage either by neutralizing host mediators or enhancing the integrity of the endothelial barrier.

6.1. Interventions targeting host mediators and endothelial cell permeability

Identifying therapies that target host mediators may prove effective in preventing dengue related vascular leakage and mortality. The anti-inflammatory and vasoactive effects of corticosteroids provide a rationale for their use in dengue. In an early randomized control study (Min et al., 1975), hydrocortisone was administered in varying doses for 3 days to children having DSS and results demonstrated a statistically significant decrease in mortality, setting the stage for additional clinical studies. However, subsequent studies failed to demonstrate steroid efficacy in improving clinical outcomes (Kularatne et al., 2009; Panpanich et al., 2006; Sumarmo et al., 1982; Tassniyom et al., 1993). Notably, most clinical studies on corticosteroids have been conducted in patients who already had plasma leakage (DHF or DSS) and therefore little is known if steroids would be effective in preventing vascular leakage if administered during the early stages of illness. Well designed trials in children and adults are needed to tease out the possible therapeutic benefits of corticosteroids. Along the same rationale, there has been interest in lovastatin due to its potential anti-inflammatory effects on the endothelium although no published reports regarding its efficacy are currently available.

On the basis of findings from animal studies, TNF- α appears to be a prime target for intervention given that there are currently approved anti-TNF- α antibodies and inhibitors already in clinical use (Brown et al., 2011). Other potential targets for the angiogenic cytokines include VEGF and angiopoietins. The pattern of elevated VEGF and angiopoietin-2 levels along with suppressed levels of angiopoietin-1 is consistent with the changes in vascular integrity and coagulation abnormalities in severe dengue. Effective neutralizing antibodies for VEGF are currently in clinical use for the treatment of malignancy (Ferrara et al., 2005) and retinopathy (Zehetner et al., 2013) and are readily available candidates for trials. In addition, inhibitors of VEGF signaling pathways are in development and may be useful for dengue treatment. Mediators that enhance vascular integrity through various mechanisms such as angiopoietin-1 and sphingosine-1-phosphatase (S1P) are also potential therapeutic agents. Vasculotide, an experimental compound and an angiopoietin-1 receptor agonist, reduced the loss of endothelial cell VE-cadherin and cytoskeleton rearrangement *in vitro*. Another compound that is a natural plasmin digest prod-

uct of fibrin, peptide Bbeta15-42, reduced vascular leak and mortality in a DHF animal model and in rats injected with lipopolysaccharide (Groger et al., 2009). Bbeta15-42 prevented thrombin-induced stress fiber formation, myosin light chain phosphorylation and RhoA activation in endothelial cells, all of which stabilized barrier function. Apart from dengue and sepsis models, data from influenza research demonstrated that a small-molecule agonist of the S1P receptor is sufficient to protect against a lethal influenza infection by reducing pro-inflammatory cytokine production and inhibiting recruitment of neutrophils, macrophages and monocytes to the lung (Teijaro et al., 2011). More studies are needed to determine the generalizability of these compounds in targeting endothelial cell dysfunction found in DHF. The pattern of plasma leakage in DHF, which localizes only in serous cavities, is unique and the underlying mechanism for this localization is poorly understood. As such, agents with demonstrated efficacy in other models of plasma leakage may not be effective in dengue related plasma leakage. It is conceivable that multiple pathways may have to be targeted in order to effectively attenuate vascular leakage.

Since the current mortality rate of DHF is less than 1% with appropriate supportive care, the investigational agents must possess a high safety profile. Some of these potential intervention strategies are associated with significant side effects. For example, anti-VEGF treatment can cause hemorrhage (Gordon et al., 2001; Yang et al., 2003) which will be problematic in dengue. Anti-TNF- α treatment has been associated with reactivation of tuberculosis (Miller and Ernst, 2009). Although these side effects have been associated with long termed administration and may not occur with a brief period of treatment, their side effects must be carefully considered along with potential clinical benefits. Studies that can identify cases with genetic or biological profiles at risk for severe disease may be critical in selecting patients who will benefit from such interventions.

7. Directions for future research

The expansion of dengue to new geographical areas and populations with distinct genetic and host factors underscores the importance of further studies that will delineate the natural history, clinical manifestations, and underlying pathological mechanisms leading to disease severity. Although dengue has been considered a childhood disease in endemic areas, recent studies have indicated a growing trend of DENV infections in older individuals and even in the elderly. Clinical manifestations in these populations will likely be different from the known classical dengue due to additional risk factors such as underlying chronic conditions and medications. It is imperative that prospective studies with well planned data and specimen collection schemes be conducted to gain information necessary for the understanding of the disease mechanisms and the development of effective therapeutic measures. An important challenge in clinical care of dengue is to identify patients at risk for severe disease. Presently there are no clinical and laboratory parameters that can identify these patients early in the course of the infection. The lack of such predictors results in the need for close monitoring of dengue cases, a task that can be overwhelming, particularly in the setting of major outbreaks. Such parameters will also be critical in identifying cases for clinical trials for specific interventions which may be associated with significant side effects.

Since vascular leakage and hemorrhage are the two important severe manifestations future research will focus on the roles of endothelial cells in these two related events. Controversy remains as to whether viruses directly affect vascular permeability or immune mediators elicited by DENV cause enhanced permeability.

Evidence suggests that DENV has the potential to cause direct and indirect effects on the endothelial barrier, activating it and inducing vascular leak. However, the timing of vascular leakage which typically occurs after plasma viral clearance and coincides with elevated levels of several immune mediators in conjunction with the lack of in vivo evidence of virus infected endothelial cells, suggests that a virus induced immune response is probably the main mechanism of vascular leak in DHF. Pertinent research will aim to understand mechanisms of DENV-associated vascular leak, diagnose barrier dysfunction and vascular leak during severe dengue, and identify and test both antivirals and compounds that improve endothelial barrier integrity. A number of potential dengue antiviral compounds have been identified but none have been developed for clinical use. Even if an effective antiviral compound was available it may not be sufficient to prevent and/or control vascular leak particularly if peak viral levels in plasma/tissue have been reached prior to diagnosis. As such, administration of a therapy that enhances endothelial barrier integrity or controls endothelial cell activation combined with the current standard of care, fluid replacement, may have the potential to significantly improve severe dengue outcomes.

Drugs found to improve endothelial barrier integrity and which are safe and effective in mouse models or ideally in other human trials would need to be tested in a clinical intervention study. Ideally, the extent of vascular leak should be directly demonstrated and measured by visualizing techniques such as ultrasound (Colbert et al., 2007; Srikiatkachorn et al., 2007b; Venkata Sai et al., 2005), possibly together with alternative techniques that measure vascular permeability such as strain gauge plethysmography and urine protein/creatinine ratio (Bethell et al., 2001; Vasanwala et al., 2014). Current mouse models to study DHF-associated vascular leak are less than ideal. However, mouse models of sepsis and influenza has been employed to study vascular leak and therapies that improve endothelial barrier identified (Groger et al., 2009; Kumpers et al., 2011; Teijaro et al., 2011). Even though mechanisms of pathogenesis differ in these diseases, it may be feasible to utilize the drugs tested in these models to investigate endothelial barrier function during DHF in the best available animal models and eventually in a clinical setting. Concurrently, therapies that target virus antigens and host factors, both of which can activate endothelial cells and damage the endothelial barrier, should be pursued. Careful consideration must be given, however, to therapeutic interventions targeting host mediators as it is difficult to determine the contribution of mediators in vascular leakage, host defense and repair. Such evaluation could only be performed in animal models in which the timing of interventions can be accurately controlled. It is conceivable that an effective regimen to modify clinical course and severity of dengue will be a combination of antiviral treatments and treatments that focus on restoring endothelial barrier integrity.

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