

Wound Healing and Inflammation/Infection

Crosstalk between coagulation and inflammation during Dengue virus infection

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Summary

Dengue fever is the most prevalent viral disease transmitted by vectors (*Aedes aegypti*, *Aedes albopictus*) in worldwide. More than 100 million cases occur annually with a mortality rate of 5% and no safe vaccine is available. The pathogenesis of Dengue, where host and viral factors participate in the establishment of Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS) remains unresolved. Clinical observations have revealed significant abnormalities in coagulation and inflammation systems, with increased levels of tissue factor (TF) and the chemokine IL-8, correlating with the severity of the disease and implicating damage to endothelial vascular cells (EVC). Here we present

novel insights concerning the crosstalk between the regulatory signaling pathways of the coagulation-inflammation processes, during Dengue virus (DV) infection of EVC. We found that DV up-regulates Protease Activated receptor type-I (inflammation) and TF (coagulation) receptors, via the phosphorylation of p38 and ERK1/2 MAPKs, which favor the activation of NF- κ B transcription factor. This induces pro-inflammatory (IL-8) or pro-adhesive (VCAM-1) gene expression which may lead to EVC activation. The elucidation of the basic principles that signal these processes has important implications for the design of new therapeutic strategies for DHF/DSS.

Keywords

Dengue virus, tissue factor, PAR-1, endothelial cell, crosstalk, coagulation-inflammation

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Introduction

Dengue fever (DF) is the most prevalent viral disease transmitted by arthropod vectors (*Aedes aegypti*, *Aedes albopictus*) in the world (1). More than 100 million cases of DF occur annually with a mortality rate of 1–15% (2), and no safe vaccine is available. Dengue virus (DV) belongs to the genus *Flavivirus* of the *Flaviviridae* family (3). There are four antigenically distinct serotypes (DV 1–4). Infection by any of these may result in either a mild self-limited illness, Dengue shock syndrome (DSS) or Dengue haemorrhagic fever (DHF). DF is characterized by the sudden onset of fever (concurrent with viremia) and a variety of non-specific signs and symptoms including fever, headache, and

weakness, but at the moment of defervescence circulatory failure or haemorrhagic manifestations (DHF/DSS) may occur (4).

Haemostatic changes involve three main factors: vascular damage, thrombocytopenia and multiple defects in the coagulation and inflammation systems (4). Coagulation-inflammation forms an essential part of the host's defensive response during tissue damage (5, 6). Haemostatic serine proteases (thrombin, factors VIIa/Xa) play a central role in the control of both processes and have several converging points for interaction (crosstalk), via the protease-activated receptors (PARs). PARs are molecules coupled to G-proteins which are involved in regulatory signaling pathways, during coagulation-inflammation responses (6). The first link between these processes is mediated by the vas-

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cular endothelium (7), which following injury expresses proteins such as tissue factor (TF) that are able to regulate its activation (8). Transmembrane protein TF is the high-affinity cellular receptor for coagulation factor VII/VIIa that initiates the blood coagulation cascade (8). The extracellular domain of TF contains 219 aminoacids (aa) that share homology with fibronectin-type III modules (9), similar to the class II cytokine receptors (interferon γ and IL-10). The C-terminal of TF contains the transmembrane domain (23 aa) and a short cytoplasmic tail of 21 aa which have three serine residues, representing potential phosphorylation sites that have been shown to act as a substrate for protein kinases, taking part in the receptor signaling associated with inflammatory-coagulation processes (10, 11). Increasingly clinical evidence indicates that proteases of the coagulation system affect inflammatory response, independent of their role in blood clotting (5, 6, 8). Crosstalk between coagulation and infection-inflammation pathways during septic shock is well documented (12). In the case of DSS, the cytokines and activation markers of coagulation-fibrinolysis have been correlated with those involved in bacterial human sepsis (13). TF blockade has been demonstrated to attenuate lethality during septicemia in a baboon sepsis model, where the administration of inhibitors for the ternary complex TF-VIIa-Xa (antibodies to TF, antibodies to the blocked VIIa active site, and TF pathway-inhibitor [TFPI]) caused a reduction in inflammation, rather than attenuation in coagulation (14, 15). Similar findings were reported in an experimental model for Ebola virus infection, where a recombinant inhibitor (rNAPc2) for TF-FVIIa complex reduced the systemic pro-inflammatory response and ameliorate the effects of Ebola haemorrhagic fever among rhesus monkeys (15, 16). Thus, targeting TF appears to aid the antiviral immune response in the case of haemorrhagic fevers. Further studies should be encouraged in order to define how TF-dependent signaling regulates this mechanism during haemorrhagic viral infections. Low-TF mice are also a useful tool for studying the role of TF and the coagulation cascade in other processes, such as inflammation and angiogenesis (17). Low-TF mice manifested reduced survival (compared with wild-type mice), as well as impaired haemostasis and excessive bleeding during vascular injury. The relationship between elevated levels of TF among patients with DHF/DSS and the severity of the disease has also been described recently (18). Sosothikul et al. determined the extent of activation of endothelial cells and the haemostatic system in correlation with the clinical severity, in a prospective cohort study, which included 42 children with DV infection (20 with DF and 22 with DHF), during the three phases of the illness. In the case of DHF patients, TF, plasminogen activator inhibitor (PAI) and von Willebrand factor were significantly elevated during the febrile phase, when compared to DF patients (18). However, the role of these factors during DV infection still needs to be clarified. In this paper, we evaluate the possible participation of the TF and PAR-1 receptors in the signaling pathways involved in the induction of coagulation-inflammation responses, which may favor a pro-coagulant and/or a pro-inflammatory state at endothelial vascular level during DV infection.

Materials and methods

Cell cultures and virus

Two different isolates of the Dengue virus serotype 2 were employed; one isolated from a fatal case of Dengue haemorrhagic fever (DHF-DV/1013) and the other from a patient with classical Dengue fever (DF-DV/D2NGC). Both samples were evaluated in terms of their virulence ability in mice (19). DV isolates were kindly donated by Dr. Duane Gubler from The Center for Disease Control (CDC) in Fort Collins, CO, USA. These were named: DHF-DV (elevated virulence ability) and DF-DV (low-medium virulence), respectively. Cultures of LLC-MK2 cells from African green monkey kidney (ATCC) were used for the propagation, titration and purification of the DV isolates. The viral serotype was confirmed using a specific monoclonal antibody (Den TypeTM Green Kit, Globio).

Endothelial cell isolation from human umbilical cord veins

Isolation of human umbilical vein endothelial cells (HUVEC) was performed by applying a modification of a previously published method (20). Briefly, umbilical cord veins were cannulated and washed, using saline solution with HEPES [0.75 M NaCl, 5 mM KCl, 5 mM HEPES, 6 mM glucose, pH 7.5 (Sigma, St. Louis, MO, USA)]. Subsequently, 5 ml of 0.075 % trypsin-versene solution (Gibco) were added. Cells were incubated 10 minutes (min) in a water-bath at 37°C. Cell suspension was centrifuged at 1,200 rpm for 8 min and resuspended in M 199 culture medium (Gibco), supplemented with 10% fetal bovine serum (FBS, Gibco), 5 U/ml of heparin (Gibco), 50 U/ml of penicillin/streptomycin (Gibco) and 25 mg/ml of vascular endothelial growth factor (Sigma). Culture medium was renewed three times a week. Endothelial cells were identified according to their cobblestone morphology and by the immunofluorescent pattern resulting from the reaction of antibodies against von Willebrand Factor (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). In all the experiments only HUVEC with three passages were used.

DV propagation and titration

Confluent LLC-MK2 monolayers were inoculated, using varying doses of DF-DV or DHF-DV at multiplicity of infection (MOI)=0.1 and 1.0 and incubated at 37°C with 5% CO₂ until cytopathic effect (CPE) was above 90% (6–8 days). The culture supernatants (SN) were harvested, and debris was removed by centrifugation at 900 g for 10 min. Subsequently, the samples were centrifuged at 16,000g for 10 min, and SN was collected and stored at –70°C until use. DV titer was determined by lytic plaque assay (21). LLC-MK2 cells were seeded into a 24-well culture plate and incubated at 37°C with 5% CO₂ until confluence. Cultures were inoculated with 100 μ l of serial log dilutions (10-fold variations) of DF-DV or DHF-DV (in serum free medium of 10⁰ up to 10⁻⁹) by duplicate and incubated for 2 h at 37°C with 5% CO₂. Viral inoculum was eliminated and cultures were washed three times. Monolayers were overlaid with 1 ml of incomplete medium, containing 2% methylcellulose and incubated until CPE was observed (6–8 days) followed by staining

with 1% crystal violet for 15 min. The virus titer was calculated and expressed as plaque forming units (pfu) per milliliter.

HUVEC DV infection

The primary endothelial cells were infected with both viral isolates at MOI = 0.1 and 1 when confluence was around 90%. After 2 hours (h) of adsorption at 37°C in 5% CO₂, the viral inoculum was removed, and cells were washed once with a salt solution supplemented with 20 mM HEPES, pH 7.4. Cells were suspended in serum-free growth medium, supplemented with endothelial growth factor and incubated at 37°C with 5% CO₂.

Interleukin (IL)-8 determination by ELISA assay

The ELISA for IL-8 determination was performed as previously described by Kittigul et al. (22). Briefly, a microtitration plate was covered with 100 µl of anti-IL-8 monoclonal antibody (R&D Systems) at 10 µg/ml in bicarbonate buffer pH 9.6 and incubated at 4°C overnight. The plate was washed with 0.05% phosphate-buffered saline (PBS)-Tween (PBST) pH 7.4. Non-specific binding sites were blocked by adding 200 µl of 1% BSA-PBS solution for 2 h at room temperature (RT). Samples were washed four times with PBST and 100 µl of rh-IL-8 protein were added (R&D Systems Inc.), as well as the SN of the HUVEC cultures infected with both DF-DV and DHF-DV isolates, or the SN of cultures stimulated with 10 ng/ml of tumour necrosis factor (TNF)-α and SN of the cells which had been pre-incubated with the anti-TF full-length human recombinant antibody (American Diagnostica, Stamford, CT, USA). The plate was then incubated for 1.5 h at 37°C and washed four times. Subsequently, 100 µl/well of the biotinylated anti-IL-8 antibody (R&D Systems Inc.) at 5 µg/ml were added, and samples were incubated for 1.5 h at 37°C, washing the plate as previously described. Subsequently, 100 µl/well of streptavidin-peroxidase (Sigma USA) in a 1:5,000 dilution were added to each well and incubated for 1 h at 37°C. Finally, the samples were washed four times, and color was developed by the addition of 100 µl of tetramethylbenzidine substrate (Sigma). After 15 min, the reaction was halted by adding 50 µl/well of 0.05 M sulfuric acid. Optical density was determined using an ELISA reader (Thermolab System, Beverly, MA, USA) at 450 nm.

Prothrombin activation (F1+2) immunoassay

Quantitative determination of prothrombin fragments (F1+2) were evaluated in the SN of infected endothelial vascular cells (EVC) with DF-DV and DHF-DV isolates (MOI=1) using the ELISA Enzygnost F1+2 kit, according to the manufacturer's instructions (Dade Behring, Deerfield, IL, USA). For the assay, we used plasma from healthy volunteers. In the case of the negative control, the SN of HUVEC cultures plus plasma were used. A parallel inhibitory assay in presence of hirudin (specific thrombin inhibitor) was performed.

Western blot assays

Cell lysates were separated by SDS-PAGE at 12% and transferred to nitrocellulose membranes, which were blocked with 5% non-fat dry milk-PBS buffer for 1 h at RT and incubated with the following primary antibodies: phospho-ERK1/2, constitutive ERK, and phospho-p38, diluted 1:1,000 in Tris Buffer Saline

(TBS) overnight at 4°C. Membranes were washed three times over with PBS. The secondary antibody (peroxidase conjugated) was added and incubated for 1 h at RT. The samples were washed as previously described. The bands were observed using lumi-light plus ECL substrate and exposure to film.

Flow cytometry assay

Confluent monolayers of HUVEC were grown in 25 cm² culture flasks and infected with DF-DV or DHF-DV isolates at MOI = 1. Likewise, inactivated DV (boiled) isolates were used as controls. HUVEC at 8 h post-infection (p.i.), MOI = 1 with DF-DV or DHF-DV were detached using PBS and incubated with a specific monoclonal antibody to human TF Fluorescein Isothiocyanate conjugated, (American Diagnostica), PAR-1 (ATAP, FITC conjugated) or VCAM-1 phycoerythrin conjugated antibody (PE, Santa Cruz, Biotechnology Inc.), diluted at 1:100, 2 h at 4°C. Unbound antibody was removed by washing with 0.025% PBS-BSA. Finally, cells were fixed with 2% paraformaldehyde. Control cells were incubated with FITC- (Fluorescein Isothiocyanate) or PE- (Phycoerythrin) conjugated, non-specific antibodies. Mean fluorescence intensity (MFI) was detected by flow cytometry assay (FACS). In parallel experiments, cells were pre-treated for 2 h with p38-specific inhibitor (SB203580, Sigma) 1 µM and ERK1/2 specific inhibitor (PD98059, Sigma USA) 10 nM. As a positive control, we used TNF-α (Sigma) 10 ng/ml (strong TF inducer) or thrombin (Dade Behring) 5 U/ml (principal activator of PAR-1).

Immunofluorescence NF-κB assay

NF-κB activation assay was developed as described by Huang et al. (23). Briefly, HUVEC infected with the DHF-DV or stimulated with TNF-α were fixed on slides with methanol. Subsequently, the samples were hydrated, and incubated with the anti- NF-κB p65 polyclonal antibody conjugated with FITC (Santa Cruz Biotechnology Inc.) for 1 h at RT in a humidified chamber. Staining was performed as described in the manufacturer's protocol (Santa Cruz Biotechnology Inc.). Cells were analyzed by microscopy.

Statistical analysis

The data were analyzed using Statistical (version 6) software. Continuous data were expressed as the mean ± SD and evaluated using Student's t test and Kolmogorov-Smirnov test (Spreadsheet2). Statistical significance was recognized when $p < 0.05$. Nominal data were presented as a percentage (%).

Results and discussion

TF expression in DV-infected EVC

To date, no animal model for DHF/DSS has been established, and all concepts concerning the pathogenesis of severe forms of the disease have been derived from experimental in-vitro observations. Endothelial cells have been proposed as an appropriate model for studying DSS (24). Accordingly, we employed the HUVEC as our model. Exposition of TF on damaged endothelium (this protein is only expressed during endothelial injury) is a key event in the induction of coagulation-inflammation responses. We evaluated the presence of TF on the surface of

Table 1: TF, PAR-1, VCAM-1 expression and prothrombin activation during DV infection of endothelial cells. A) Detection of TF, PAR-1 and VCAM-1 in DV-infected EVC. TF, PAR-1 and VCAM-1 were analyzed by FACS assay in HUVEC infected with DF-DV and DHF-DV (MOI=1) or stimulated with TNF- α (positive control for TF and VCAM-1). As positive control for PAR-1, we used 5 U/ml of thrombin. The presence of TF, PAR-1 and VCAM-1 was correlated according to the mean fluorescence intensity (MFI). Values represent MFI means \pm SD of nine independent experiments. Non-infected EVC were used as negative

control. * Indicates p-values with respect to the negative control. B) Quantitative determination of prothrombin fragments 1+2 (F1+2). F 1+2 were evaluated in the SN of EVC infected with DF-DV and DHF-DV MOI = 1 (a, b) by means of ELISA assay (Enzygnost F1+2 kit). We performed a parallel inhibitory assay in the presence of hirudin (Hir: c, d), the specific thrombin inhibitor. Also was performed a free EVC assay using only human plasma (pl) plus DV isolates (e, f). Values represent means \pm SD of at least 4 independent experiments assayed by triplicate.

A				
	Negative control	Positive control	DF-DV	DHF-DV
TF	512 \pm 2.55	34.19 \pm 7.12 *(p<0.02)	22.75 \pm 3.97 *(p<0.02)	41.26 \pm 6.51 *(p<0.02)
PAR-1	6.94 \pm 3.32	36.10 \pm 7.63 *(p<0.0003)	28.57 \pm 8.59 *(p<0.0003)	49.41 \pm 9.71 *(p<0.0003)
VCAM-1	1.81 \pm 0.23	71.14 \pm 4.81 *(p<0.001)	61.33 \pm 5.71 *(p<0.000)	85.80 \pm 5.98 *(p<0.000)

B						
	a	b	c	d	e	f
Negative Control	HUVEC+ PI+DF-DV	HUVEC+ PI+DHF-DV	HUVEC+ Hir+PI+DF-DV	HUVEC+ Hir+PI+DHF-DV	Plasma + DF-DV	Plasma+ DHF-DV
98.62 \pm 5.4	*804.93 \pm 11.1	*1141 \pm 42.3	96.25 \pm 1.7	105 \pm 6.06	*262.87 \pm 22.4	*494.25 \pm 44.8

*p value vs. negative control p<0.0000 (a vs. c p<0.0001), b vs. d p<0.0001).

HUVEC cultures infected with the DF-DV or DHF-DV by means of FACS analysis. The positive control (stimulated EVC with TNF- α) showed a MFI value of 34.19 \pm 7.12 (Table 1A).

In HUVEC infected with the DHF-DV, TF expression was 41.26 \pm 6.51 (Table 1A), similar to that obtained in presence of the strong TF inducer (TNF- α). However cultures inoculated with the DF-DV presented a lower fluorescence (22.75 \pm 3.97). The MFI for the negative control was 5.1 \pm 2.55. These results suggest that the DV was able to up-regulate TF at the surface of the endothelial cells and that the DHF-DV isolate induced a strong TF response. Geisbert et al. stated that the over-expression of TF in primate monocytes/macrophages plays a crucial role in triggering the pro-coagulant and/or haemorrhagic complications which characterize Ebola virus infection (15). Nonetheless during DV infection, the participation of TF in these processes remains undefined.

Procoagulant state of DV-infected EVC

In order to examine whether DV infection of EVC is able to promote the shift from a non-thrombogenic surface to a procoagulant state, we measured the conversion of prothrombin into thrombin (the final enzyme produced in the blood coagulation pathway) by the quantitative determination of human prothrombin fragments (F1+2) in the SN of DV-infected HUVEC cultures, using an ELISA assay (Table 1B). The concentration of F1+2 of EVC inoculated with the DHF-DV (MOI=1) was 1141.11 \pm 42.3 pM, while for the DF-DV (MOI=1) it was 804.93 \pm 11.1 pM. As a control, we performed a parallel assay in the presence of hirudin (specific thrombin inhibitor). We observed an inhibitory ef-

fect in the presence of both viral samples (Table 1B), thus verifying the specificity of the phenomenon. The statistical significance of each evaluated group is shown in Table 1B. Likewise, we assessed the possible role played by DV, in the activation of prothrombin using a free endothelial cell system (only human plasma plus both DV isolates). We observed that the concentration of prothrombin activation fragments was 262.87 \pm 22.4 pM in the presence of DF-DV and 494.25 \pm 44.8 pM for DHF-DV (Table 1B, lines e, f). These interesting results may hint at an additive effect favored by DV in the generation of thrombin. It is acknowledged that thrombin production is triggered by vascular damage and is strictly limited to neighboring cells expressing TF. This result correlates with the clinical observations (13, 18). It has been reported that concentrations of the prothrombin activation fragments (F1+2) were higher among patients with DHF/DSS (who later died) than among those who survived (median activation fragments). This data suggests that fatal cases course with excessive thrombin formation (13).

Participation of PAR-1 in DV-infected EVC

The haemostatic proteases, generated at the endothelial level, can mediate cell signaling and influence the inflammatory balance within the vasculature. This proteolytic activity is detected by its specific receptors (PARs). Therefore, the TF-initiation reaction produces highly efficient and specific signaling through PARs. Either, thrombin or the complex TF-VIIa-Xa activates PAR-1. The activation mechanism contemplates PAR cleavage by a serine protease at its amino-terminal (NH₂) extracellular domain, generating a new NH₂ that functions as a tethered ligand which

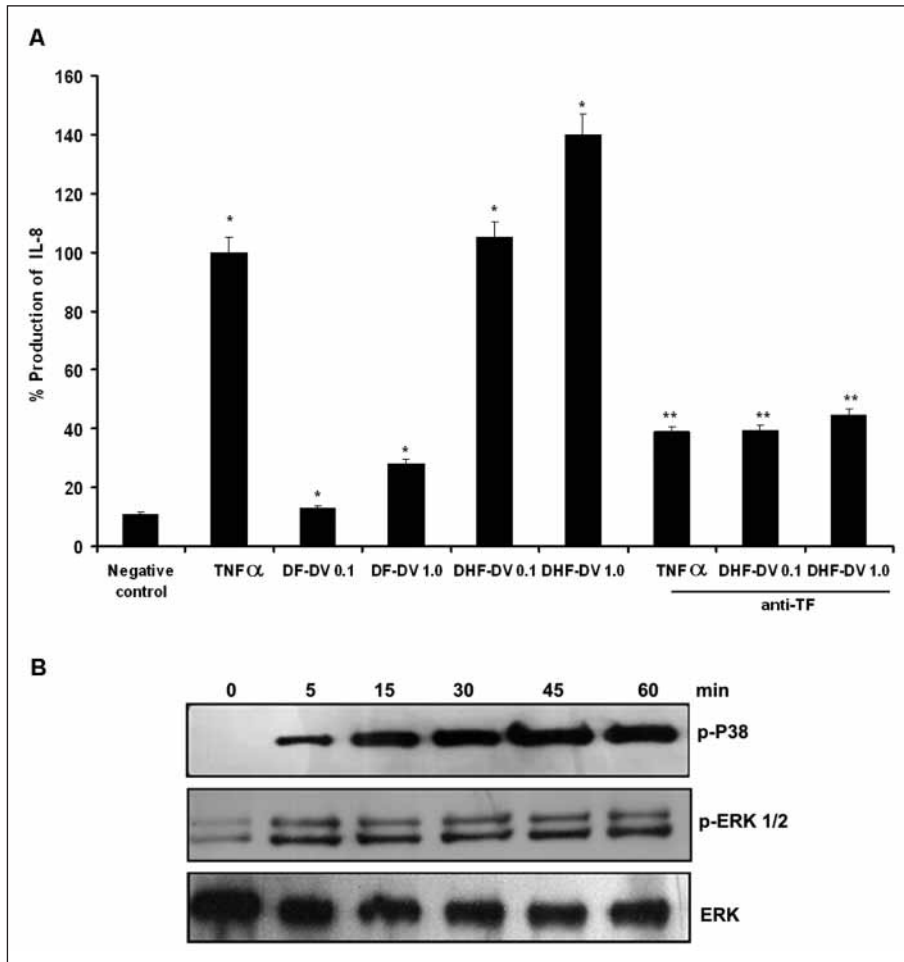


Figure 1: TF participation in pro-inflammatory response in DV-infected HUVEC.

A) IL-8 production (and IL-8 inhibitory) ELISA assay in DV-infected EVC. ELISA assay conditions were optimized by the titration of the recombinant human IL-8 (rh IL-8) and a specific anti-IL-8 antibody. Parallel assay was performed in HUVEC pre-treated for 2 h with (1 μ g/ml) full-length recombinant human anti-TF antibody before being infected with the DHF-DV. Results are shown as the mean \pm SD for at least three independent experiments assayed by triplicate. *Indicates p-values with respect to the negative control ($p < 0.001$), ** $p < 0.001$ when compared samples in the presence of anti-TF antibodies with respect to the samples in absence of anti-TF antibodies. B) Kinetic assay of MAPKs p38 and ERK1/2 phosphorylation in DV-infected EVC. Phosphorylation of p38 and ERK1/2 MAPKs was measured by Western blot assay at different time intervals (0, 5, 15, 30, 45, 60 min). Constitutive ERK was determined as control. The data shown is representative of a further three that rendered similar results.

binds to (and activates) the receptor, in order to accomplish trans-membrane signaling. We detected PAR-1 by FACS, with the help of a specific monoclonal antibody which recognizes the cleavage site of PAR-1 at the NH₂-terminal region (activated form of the receptor). For the positive control, we performed a parallel assay in the presence of thrombin (5 U/ml), which is the main activator of PAR-1. Table 1A shows that thrombin-cleavage of PAR-1 displayed a MFI value of 36.10 ± 7.63 . In endothelial cells infected with the DF-DV isolate, the level of activation was 28.57 ± 8.59 . Cultures infected with the DHF-DV show the most elevated levels of fluorescence (49.41 ± 9). This outcome suggests that PAR-1 can be involved in EVC activation mediated by haemostatic serine proteases, during DV infection.

Pro-adhesive response in DV-infected EVC

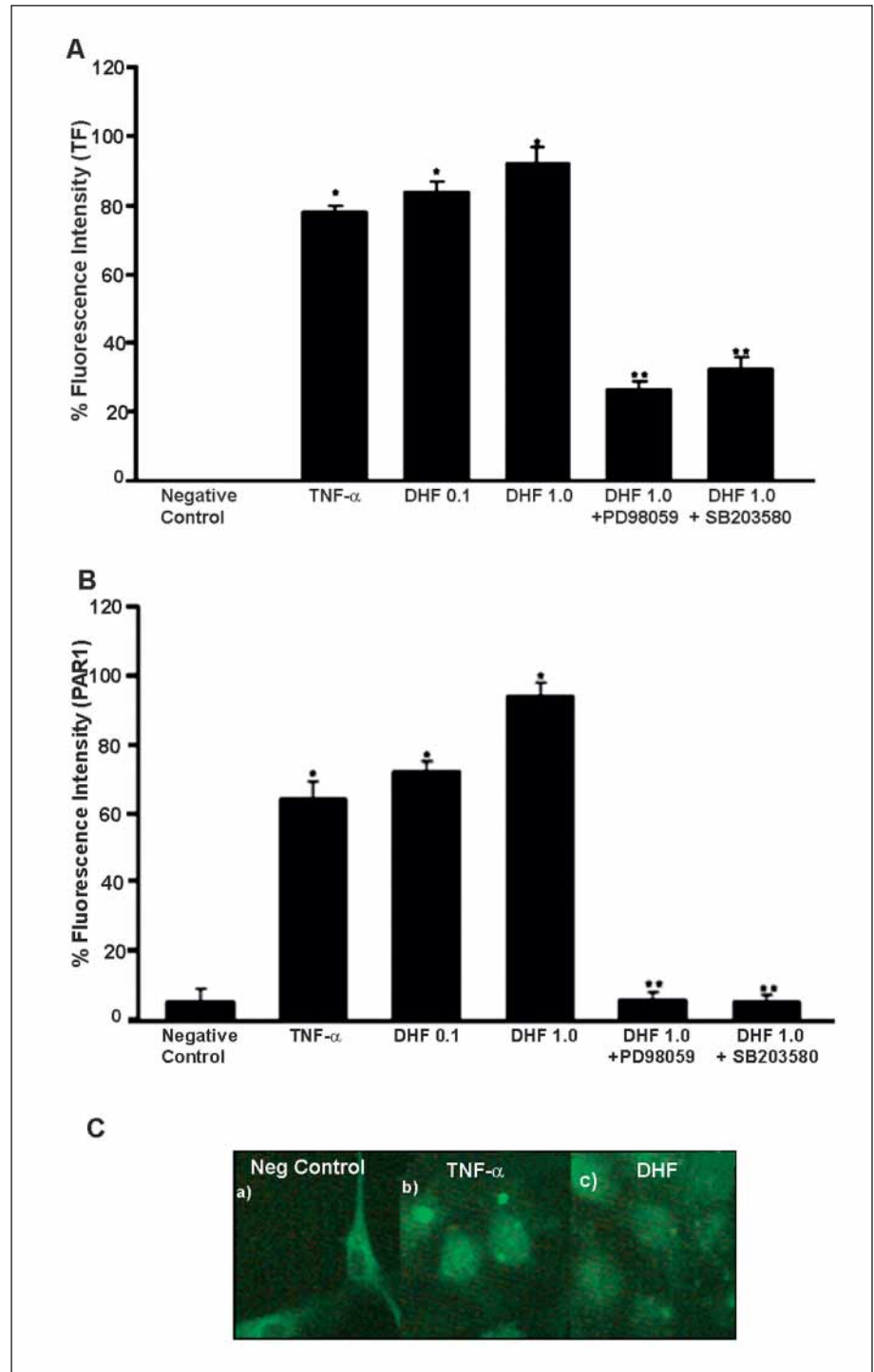
Activated endothelial cells express several pro-adherent proteins (ICAM, PECAM, E-selectin and VCAM-1). Among these, the vascular cell adhesion molecule-1 (VCAM-1) plays an essential role as a leukocyte-binding element. The influx of leukocytes results in a local inflammatory response, endothelial damage, plasma leakage, and shock. Among patients with DHF/DSS, elevated levels of adhesion molecules are further increased (25). We evaluated the expression of VCAM-1 in HUVEC cultures infected with DF-DV or DHF-DV (MOI=1). HUVEC cultures

stimulated with 10 ng/ml of TNF- α (a well known VCAM-1 agonist) were used as a positive control. We observed that (Table 1 A) both of the DV isolates were able to induce the expression of VCAM-1 (DF-DV 61.33 ± 5.71 and DHF-DV 85.80 ± 4.81). The DHF-DV exhibited the strongest response when compared with the DF-DV, with the negative control (1.81 ± 0.23 , $p < 0.000$) and also to TNF- α (71.14 ± 4.49 , $p < 0.001$). These results match clinical observations reported in patients with DHF/DSS (25).

TF participation in the pro-inflammatory (IL-8) response in DV-infected EVC

It has been reported that the chemokine IL-8 plays a key role in the endothelial damage produced by inflammatory response. There is a striking correlation between increased levels of IL-8 and the occurrence of grade III and IV DHF, and the absence of this chemokine in DF patients (26). To date, the mechanisms which explain the induction of IL-8 in endothelial cells during DHF remain unknown. We evaluated the relationship between IL-8 production and the possible pathway involved in this process during DV infection. To elucidate the possible participation of TF in IL-8 expression, we performed an IL-8 production (and IL-8 inhibitory) ELISA assay. Initially, we detected the levels of this chemokine in the SN of HUVEC cultures, stimulated with

Figure 2: p38, ERK 1/2 and NF- κ B in TF and PAR-1 expression during DV infection of EVC. A) Participation of p38 and ERK 1/2 kinases in TF expression. To evaluate the possible role of MAPKs p38 and ERK1/2 during TF expression, HUVEC were pre-incubated with the specific MAPKs inhibitors SB203580 (p38) or PD98059 (ERK1/2) and subsequently infected with the DHF-DV at different doses (MOI = 0.1, 1). TF was measured by FACS assay. Results stand the MFI mean \pm SD of three independent experiments assayed by triplicate. *Indicates $p < 0.001$ when compared to the negative control, ** $p < 0.002$ when compared DHF-DV infected HUVEC in presence of inhibitors versus DHF-DV infected HUVEC without inhibitors. B) Participation of p38 and ERK1/2 MAPKs in PAR-1 expression. To examine the possible role of MAPKs p38 and ERK1/2 during PAR-1 expression, HUVEC cultures were pre-incubated with the specific inhibitors SB203580 (p38) or PD98059 (ERK1/2) and subsequently infected with the DHF-DV at different doses (MOI = 0.1, 1). PAR-1 was detected by FACS assay. Results represent the mean \pm SD of at least three experiments. *Indicates $p < 0.005$ when compared to the negative control, ** $p < 0.002$ when compared DHF-DV (MOI = 1) without inhibitor. C) NF- κ B activation in DV-infected endothelial cells. HUVEC were infected with DHF-DV (MOI = 1) or stimulated with TNF- α (5 ng/ml). Cells were fixed on slides with methanol and incubated with the specific anti-NF- κ B p65 antibody. The assay was representative of the three independent experiments assayed by triplicate: a) Negative control, b) TNF- α and c) DHF-DV.



10 ng/ml of TNF- α (positive control) or infected with different doses of DF-DV or DHF-DV isolates (MOI=0.1 and 1.0). Figure 1A shows IL-8 production at 48 h p.i. The concentration of IL-8 in DHF-DV infected cultures was greater (159.5 pg/ml = 140%) than in TNF- α stimulation (110 pg/ml = 100%), whereas IL-8 production in the presence of the DF-DV resembled background production. These results correlate with previously published clinical observations (26). Parallel assays were performed using a specific anti-TF recombinant antibody (which neutralizes its

activity) as follows: HUVEC monolayers were serum-starved overnight and subsequently incubated with a specific anti-TF antibody for 2 h before being infected with the DHF -DV (MOI = 0.1 and 1.0) or stimulated with 10 ng/ml of TNF- α . We found that IL-8 production was reduced in the presence of the anti-TF antibody (Fig. 1A), decreasing as much as 60% among the DHF-DV infected cells (39 pg/ml and 44.61 pg/ml at MOI = 0.1 or 1.0, respectively). Our observations suggest that TF is partially involved in the inflammatory response during DV infection.

p38, ERK 1/2 and NF- κ B in TF and PAR-1 expression during DV infection

Recently, an alteration of the procoagulant/anticoagulant balance during the haemorrhagic fever caused by bluetongue virus was reported, which was mediated by the inflammatory response related to the activation of p38 and ERK1/2 MAP kinases (27). The same authors demonstrated that the pharmacologic inhibition of MAPK p38 reduces the viral induction of cytokines and vascular permeability in human micro-vascular endothelial cells. Similarly, it was documented that TF/FVIIa induces the activation of certain members of the MAP-kinase family (6, 28), which are well-known mediators of inflammatory gene expression, by means of the phosphorylation of transcription factors (28). We therefore assessed the possible participation of MAPKs p38 and ERK1/2 in the regulatory signaling pathway of the coagulation-inflammation processes present during DV infection. We carried out a kinetic study of p38 and ERK1/2 MAPKs phosphorylation in HUVEC infected with the DHF-DV (MOI=1) or stimulated with 10 ng/ml of TNF- α by Western blot assay, using the specific antibodies anti-phospho-p38 and anti-phospho-ERK1/2. We observed that in the presence of the DHF-DV, both protein kinases (p38 and ERK1/2) were quickly phosphorylated, and remained in this state for 45 to 60 min (Fig. 1B), which suggests their participation during DV infection. We also explored the possible activation of MAPKs in the expression of TF on infected HUVEC as follows: EVC were incubated for 2 h in the presence or absence of the specific inhibitors for p38 (SB203580; 1 μ M) and ERK1/2 (PD98059; 10 nM) kinases, followed by the infection of HUVEC cultures with the DHF-DV (MOI=0.1 and 1.0). TF was detected by FACS assay. We observed (Fig. 2A) that in cultures treated with both MAPKs inhibitors, TF expression was decreased at endothelial surface level, showing an MFI value of 13.37 (MOI=1.0) in the presence of SB203580 and 16.14 (MOI=1.0) with PD98059 ($p < 0.002$, when compared to the sample without inhibitor). These results suggest that the up-regu-

lation of TF during DV infection can be mediated by p38 and ERK1/2 kinases. We also tested the possible participation of MAPKs (p38 and ERK1/2) in the up-regulation of PAR-1 expression, as observed in the present study. Parallel assays were performed as described for TF in EVC cultures, infected with different doses (MOI=0.1 and 1.0) of the DHF-DV. We found that in the presence of the SB203580 and PD98059 MAPKs inhibitors, the level of PAR-1 (Fig. 2B) was drastically decreased by both compounds, obtaining MFI values of only 1.43 (p38) and 2.63 (ERK1/2) ($p < 0.002$ when compared to the sample without inhibitor). These important findings indicate the leading role of MAPKs p38 and ERK1/2 in PAR-1 expression and the possible crosstalk between both, coagulation (TF) and inflammation (PAR) receptors.

As mentioned previously, MAPKs are able to signal inflammatory response (IL-8, VCAM-1) gene transcription, via the phosphorylation of the transcriptional factor NF- κ B. This nuclear protein (heterodimer of 50-KDa and 65KDa subunits) is retained in the cytoplasm in association with sequestered proteins (I κ B α) which mask the NF- κ B nuclear localization signal. Its stimulation with pro-inflammatory mediators (TNF- α , thrombin, VIIa/Xa among other haemostatic proteases) results in the serine phosphorylation of the I κ B α kinase (29). The released NF- κ B translocates to the nucleus where it binds to cis-regulatory elements in gene promoters. Recently, it has been reported that PAR-1 mediates NF- κ B activation and ICAM-1 gene transcription (29). We examined the NF- κ B activation in our endothelial cell model, in the presence of the DHF-DV (MOI=1) and TNF- α -stimulated cultures, using immunohistochemistry assay. We observed that in DHF-DV infected HUVEC and also with TNF- α , fluorescence was located mainly in the nucleus, suggesting NF- κ B translocation (Fig. 2C).

The mechanism involved in the pathogenesis of DHF/DSS remains unexplained. Host and viral factors have been found to enhance the development of severe manifestations. Here we

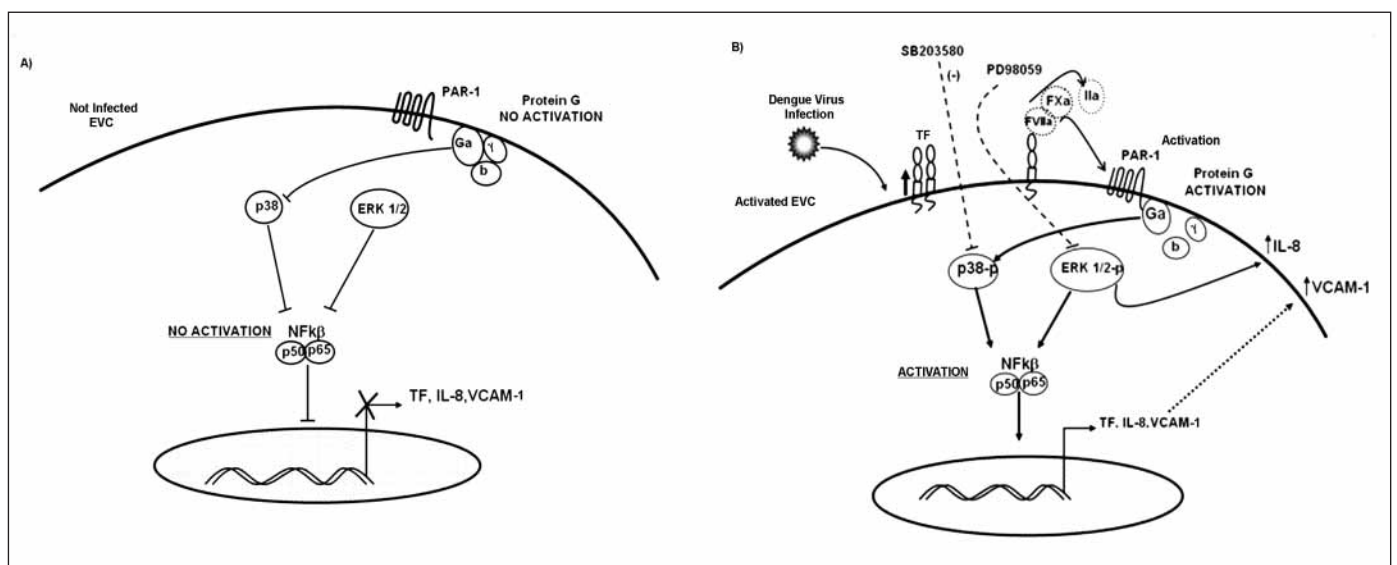


Figure 3: Crosstalk between coagulation-inflammation during DV infection of endothelial vascular cells. In this model we summarize the possible signaling regulatory pathways involved in endothelial cell activation which might explain different aspects of DHF/DSS pathogenesis. We observed that DHF-DV isolate can favor pro-inflammatory and pro-coagulant responses which involve the phosphorylation of MAPK-p38 and ERK1/2 in the up-regulation of TF and PAR-1 receptors during DV infection of endothelial vascular cells (for details see main text).

present very novel insights, concerning the participation of DV in the induction of signaling pathways which may crosstalk in the inflammation-coagulation processes at the vascular endothelial level. Figure 3 shows a diagram of our experimental findings, which may explain different aspects of DHF/DSS pathogenesis.

In the present study, we observed that the DHF-DV up-regulates TF in EVC, which triggers the generation of haemostatic proteases (thrombin) that can promote the activation of protease-activated receptors. PARs in turn, induce signaling inflammatory pathways via MAPKs p38 and ERK1/2, probably by the transcription of the NF- κ B factor, whose activation leads to the up-regulation of adhesion (VCAM-1) or pro-inflammatory (IL-8) molecules. In addition, we observed the participation of TF in IL-8 expression (by blocking assay using a monoclonal specific anti-TF antibody). We also documented a down-regulation of PAR-1 and TF in the presence of specific inhibitors for MAPKs (p38 and ERK1/2), suggesting their participation in the expression of PAR-1 and TF receptors, and a possible link between the coagulation and inflammation processes during DV infection of endothelial cells. Our data support the involvement of MAPKs p38 and ERK1/2 in the up-regulation of TF and PAR-1 receptors in DV-infected EVC.

The present study provides new perspectives concerning the crosstalk of coagulation-inflammation processes during DV infection. These findings have important implications for the de-

sign of different appropriate therapy which will target the thrombo-inflammatory responses present in DHF/DSS. Pharmacological agents that block TF and/or PAR activities represent an important therapeutic approach, since 50–60% of all present therapeutic agents, (directly or indirectly) target G-proteins (30). PARs are G-coupled proteins. A greater understanding of the interplay of coagulation-inflammation in sepsis has stimulated the use of novel therapies (31).

Finally, we propose that the inflammation-coagulation cycle bears great importance in the installation of DHF/DSS episodes. The elucidation of the basic principles that governing the coupling and signaling of pro and anti-coagulant-inflammatory pathways have diverse implications for the development of adequate strategies for DHF/DSS therapy, since the interruption of the vicious inflammation-coagulation cycle may be of prime importance in the control of DSS.

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