Original Article
Association DENV1 and DENV2 infection with high serum levels of soluble thrombomodulin and VEGF in patients with dengue fever and dengue hemorrhagic fever

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Abstract: Infection by dengue virus (DENV) can be asymptomatic or manifest in two clinically differentiated forms: dengue fever (DF) and denguehemorrhagic fever (DHF). The principal pathophysiological characteristic of DHF is the increase in vascular permeability and the loss of plasma caused by the malfunction of the vascular endothelium that induces the release of chemical mediators. However, so far there is nothing that allows for the identification the patients that are at risk of developing the more severe form of the illness. The objective of this study was to investigate the relationship between the serum levels of soluble thrombomodulin (sTM) and VEGF with the severity of dengue and the viral serotype. 231 serum samples were analyzed, 70 DF, 80 DHF and 81 control group, all were residents of Guerrero state in Mexico. The infection by dengue virus as well and the levels of sTM and VEGF were determined using the ELISA sandwich, while the serotype was determined by real time RT-PCR. Our results show that the concentrations of sTM correlate with the degree of severity of the disease given that they are significantly higher ($p<0.001$) in the DHF group (median = 10.2 ng/mL) than in the DF group (median = 7.2 ng/mL), and these in turn higher than those of the control group (median = 3.3 ng/mL). The concentration of sTM was significantly higher ($p=0.0002$) in the patients infected with DENV2. For the VEGF, the highest levels were found in DF (median = 291.3 pg/mL) and did not correlate with the severity of the disease. In conclusion, our results indicate that sTM is a good marker for the severity of the infection by DENV, better than VEGF, and with higher sensibility and specificity.

Keywords: Thrombomodulin, VEGF, dengue fever, dengue hemorrhagic fever, DENV1, DENV2

Introduction

Dengue is a viral infection caused by four different serotypes (DENV 1-4) transmitted by the *Aedes aegypti* and *Aedes albopictus* mosquitoes that are present in all tropical and subtropical regions in the planet. The World Health Organization (WHO) in its most recent revision of the classification scheme of dengue, established that the patients must be classified with dengue (D) or sever dengue (DS). According to the new classification, patients that recover without complications are diagnosed with dengue, whereas those that develop conditions (plasma extravasation, accumulation of serous fluid, severe bleeding and severe impairment of organs) are diagnosed with severe dengue [1]. However, the previous classification (where they were classified as dengue fever and hemorrhagic dengue fever I, II, III, and IV) is still widely used despite its limitations [2]. Dengue is a global health problem as it is endemic in approximately 100 countries, where it causes 50 to 100 million cases each year [3]. In 2010 in the Americas 1.5 million cases were reported; while in Mexico 50,368 confirmed cases were reported in 2012 of which 17,706 were dengue hemorrhagic fever (DHF) which caused 64 deaths [4].

The clinical management is complicated due to the lack of specific antiviral drugs, and although the majority of the patients recover after 5-7 days of the acute phase a small proportion develops plasma extravasation, thrombocytopenia and acute mucosal bleeding indicating DHF which can quickly lead to hypotension, cardiovascular collapse and finally death [5]. The pathophysiological mechanisms responsible of the development of DHF are not entirely understood, which is why predictive biomarkers have been sought that would allow to identify in early stages the patients that potentially could develop the disease´s severe form. Besides the routine clinical laboratory biomarkers have been reported others, such as cytokines [6, 7], nitric oxide [8], elastase [9], hyaluronic acid [10], endotelial function mediators [11], components of coagulation [12, 13], adhesión molecules [14], and circulating endotelial cells [15], as predictive markers of DHF. However, not all can be used as predictive markers of DHF given that some are highly variable, others very costly or methodologically difficult to determine and for some the sensibility and specificity are unknown. Because the dengue virus can infect endothelial cells both *in vitro* and *in vivo* and induce apoptosis and thereby cause damage to the endothelium mainly in the DHF [16], vascular injury markers are good candidates for analysis.

Thrombomodulin (TM) is a membrane glycoprotein expressed by endothelial cells from arteries, veins, capillaries and lymphatic vessels that can be released to circulation forming soluble thrombomodulin (sTM). This protein works as an anticoagulant competing with fibrinogen to bind thrombin and inhibit the fibrin formation [17]. It has been reported that serum levels of sTM are altered in various pathologies which involve endothelium damage [18]. On the other hand, the vascular endothelium growth factor (VEGF) is a cytokine with an important role in the disruption of endothelium during inflammation, promoting vasodilation and permeability [19]. To date, very few studies exist with samples of dengue patients that relate the thrombomodulin with the severity of the disease in a significant number of biological samples and analyze its potential as a predictive biomarker of DHF [15, 20, 21], while the reports for VEGF and its relationshipto dengue virus infection are contradictory [22-25]. The objective of this work was to evaluate the levels of sTM and VEGF as biomarkers of vascular damage in patients infected with dengue virus, and to correlate them with the severity of the disease and the viral serotype to propose them as potential biomarkers for the identification of patients at risk of developing DHF before serious complications arise. Our results indicate that sTM serum levels correlate with the severity of the disease with a high degree of sensitivity and specificity mainly in patients infected with DENV2.

Materials and methods

Selection of the population and sample collection

The patients were recruited captured between different dengue outbreaks in the month of July 2011 and October 2012 in the state of Guerrero, Mexico as part of dengue epidemiologic vigilance according to national guidelines that instructs to sample 30% of individuals with fever and 100% of those with bleeding. Patients attended different hospitals distributed in the health districts of the state of Guerrero, Mexico, where clinical diagnosis as carried out and were classified as having dengue according to WHO criteria, 1997 [26]. A sample of peripheral blood was taken from each patient from which serum was obtained which were sent to the State Laboratory of Public Health "Galo Soberon y Parra" (LESP) where dengue virus infection was confirmed and the virus was typified. Informed consent from patients was not required for the realization of the study because the research committees of bioethics, biosafety and biosecurity of LESP gave their approval for the use of the samples. However, the anonymity of patients and the confidentiality of the results were completely preserved following all administrative guidelines according to the laboratory quality management program as required by the Ministry of Health in Mexico. Clinical and sociodemographic data were obtained from epidemiological studies conducted by the LESP.

Participants of the control group (without fever at the time of, or within 10 days prior to sampling) were volunteers who were recruited by the laboratory of molecular biomedicine of Universidad Autónoma de Guerrero during the same period as the patients with dengue.All participants answered a questionnaire on socio-demographic and health data in general; they also signed an informed consent form

according to the principles of the Helsinki declaration. Exclude people were those who had fever or any symptoms suggestive of dengue fever, as well as those who mentioned that they had a chronic degenerative disease such as diabetes and hypertension.

Confirmation of infection by DENV

Due to the samples being taken during the acute phase of the disease between 1 and 10 days after the beginning of the fever, they were divided in two groups: those 0-5 days after fever and those ≥ 6 days according the algorithm established by the health authorities [27]. Serum samples were analyzed using the ELISA technique for detection of specific antibodies IgM or IgG against the dengue virus with the ELISA kit (Panbio, Brisbane, Quesland, Australia), according to the manufacturer's instructions. The result (expressed in units PanBio) was determined for each sample by dividing the absorbance value of the test sample between the cut-off value and multiplied by the dilution of base 10. A sample was considered as positive for IgM or IgG when the PanBio units were ≥ 11 o ≥ 22, respectively. The presence of NS1 protein was determined qualitatively by ELISA with the Platelia™ NS1 Ag (Bio-Rad Laboratories, Marnes-la-Coquette, France) kit according to the manufacturer's instructions. Samples ≥ 1 units were considered positive.

Detection and typification of DENV

Viral RNA was extracted from 140 uL of serum using QIAamp® Viral RNA Mini Kit columns (QUIAGEN) following the manufacturer's instructions. The RNA was eluted in 50 uL of nuclease free water and stored at -80°C for subsequent analysis. Viral typing was done by real time Multiplex RT-PCR with TaqMan probes based on the method developed by Jeff Chang et al, 2006 [28]. Real time multiplex RT-PCR determines the presence of RNA of the four dengue virus serotypes in the serum and plasma. A positive result is indicative of recent infection caused by the identified serotype. The following reference strains were used as positive controls: a) DEN-1 Hawaii, b) DEN-2 New Guinea, c) DEN-3 H87 or PR-6 and d) DEN-4 H-241. All PCR reactions were done in a ABI-7500 real time PCR system (Applied Biosystems). This procedure has been validated by the CDC

Dengue Branch in San Juan, Puerto Rico, who also kindly donated the reference strain. Amplification values are expressed as Ct, where one Ct is the cycle at which the relative fluorescence values exceed the cut off or threshold. The Ct is inversely proportional to the amount of RNA present in the sample. Samples with Ct values less than or equal to 35 are positive for related serotypes.

Quantification of serum levels of sTM and VEGF

Serum levels of sTM and VEGF was quantified by ELISA sandwich whit Thrombomodulin Human ELISA kit (Abcam, USA) and VEGF Human ELISA kit, (Invitrogen Corporation, USA) according to the manufacturer's instructions respectively. In both cases the secondary antibody coupled to HRP was added and was read in automated ELISA equipment (TECAN minilyser model) at 450 nm. Calculations were made based on a standard curve generated with controls provided by the manufacturer. The following sTM reference values were taken for normal human serum: 4.46±1.36 ng/mL (range of 2.4 to 7.9 ng/mL). The VEGF reference values suggested by the manufacturer are from 40 to 600 pg/mL (median 270 pg/mL) in human serum of clinically healthy people. All samples were analyzed in duplicate and following the quality control standards and relevant biosafety guide lines, insufficient and lipemic serum samples were removed.

Statistical analyses

The clinical information of each patient and the control group was stored captured in a database using the STATA Version 11.1 software. Means and medians were calculated for the quantitative variables and qualitative variables are reported as frequencies. Because the variables did not have a normal distribution we proceeded to stratify the sTM and VEGF values in tertiles for better analysis. Comparisons of the medians among groups were made by Krus Calwalis or Mann-Whitney U tests. The correlation between the markers studied was determined using Spearman's correlation coefficient and the association between markers and DF or DHF was evaluated using multinomial logistic regression models. A statistical significance of $p < 0.05$ was used. Indices of validity (sensitivity and specificity) were calculated for the

Table 1. Characteristics of the studied people according to diagnosis

Characteristics	Control group n=81	DF n=70	DHF n=80	P^*
Age				
Median (IR)	21 (19-23)	23 (16-37)	25 (14-34)	0.003*
Gender				
Male	37 (45.6)	40 (57.1)	43 (53.7)	0.313*
Female	44 (54.4)	30 (42.9)	37 (46.3)	
Viral Serotype (%)				
DENV1	--	27 (38.6)	29 (36.2)	0.023
DENV2	--	43 (61.4.)	51 (63.75)	
Days of illness				
Median (IR)	--	2.5 (1-3)	4 (3-5)	0.0006*
sTM (ng/mL)				
Median (IR)	3.3 (2.8-3.9)	7.9 (6.5-10-5)	10.2 (7.4-13.1)	0.0001
VEGF (pg/mL)				
Median (IR)	134.9 (73-5-191.4)	291.3 (158.4-449.9)	201.3 (132.7-332.7)	0.0001

*Kruskal-Wallist test. IR = Interquartile range.

markers studied taking as reference the diagnosis of dengue.

Results

Characteristics of the population studied

A total of 231 serum samples were analyzed, (70 patients with DF, 80 with DHF and 81 individuals of the control group). Of the total study population, 51.9% were male and 48.1% female, age ranges were from 0-95 years and the medians of 21, 23 and 25 years for control group, DF and DHF respectively. Statistical analysis showed significant differences in terms of age and days of disease progression between groups (**Table 1**).

DENV infection and circulating serotypes

All patient samples with dengue tested positive for NS1, IgG or IgM as determined by ELISA, which confirmed dengue virus infection. Circulating serotypes found were DENV1 and DENV2 and the analysis showed that DENV2 serotype was the most frequent in DF as DHF (**Table 1**). No multiple infections or DENV3 and DENV4 serotypes were detect.

### Serum levels of sTM and VEGF are elevated in patients with DENV

sTM levels were determinate in 81 individuals of the control group, 70 patients with DF and 80 patients with DHF. Our results show that concentrations of sTM correlate with the degree of severity of the disease and that are significantly higher (P<0.001) in the group of DHF (mean = 10.22 ng/mL) than in the group of DF (median = 7.90 ng/mL) and these in turn higher than in controls (median = 3.31 ng/mL) (**Table 1** and **Figure 1A**). Moreover, by determining the serum levels of VEGF it was found that they were higher in DF (median = 291.29 pg/mL) than in DHF (median = 201.29 pg/mL). Although the three groups studied showed significant differences (P=0.0001) VEGF serum levels did not correlate with the severity of the disease (**Table 1** and **Figure 1B**). When analyzing sTM levels in relation to viral serotypes, they were significantly higher (P=0.00024) in patients infected with DENV2 (10.1 ng/mL) than in patients with DENV1 (7.99 ng/mL) (**Figure 2A**). While for VEGF no significant difference was found in relation to the serotype (**Figure 2B**).

sTM serum levels strongly correlated to VEGF (r=0.25; P=0.0005). We found significant associations of tertiles of sTM with having DF or DHF and this association was more significant in the third tertile for DHF (OR=7, CI 95% 4.2-8.8; p<0.001). We also found significant association of VEGF tertiles with having DF and, although less robust than sTM, this association was more significant in the third tertile for DF (OR=2.7, CI 95% 1.7-3.6, p<0.001) (data no shown).

Finally, we determined the sensitivity and specificity of the two tests analyzed using the confir-
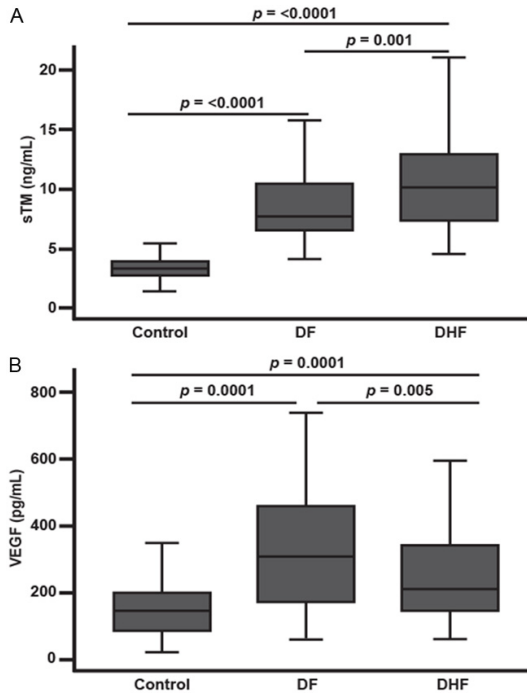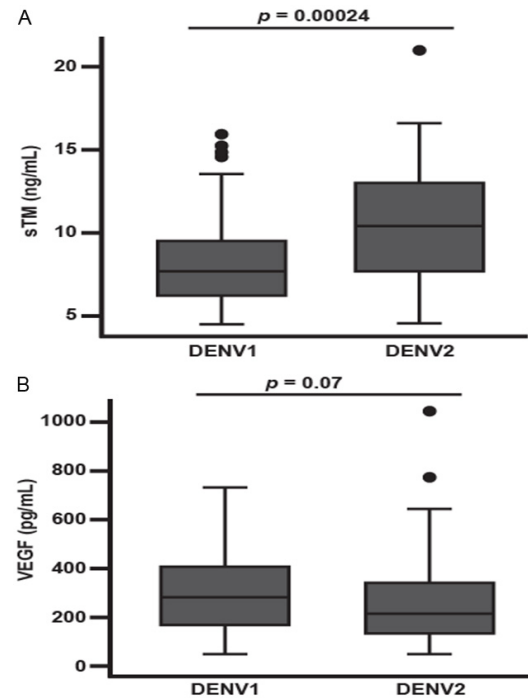
Figure 1. Soluble thrombomodulin and VEGF levels in patients with dengue and control group. Dengue patients are grouped according to the clinical diagnosis, all were positive for NS1 and DENV1 or DENV2. Serum levels of soluble thrombomodulin correlate with disease severity (A) while the highest serum levels of VEGF were found in DF (B). Box-and-whisker plots shown median values (Horizontal line in the box), 25-75% interquartile range (upper-lower limits of the box).

Figure 2. Serum levels of soluble thrombomodulin and VEGF and its relationship with viral serotypes. Dengue patients are grouped according to viral serotype; all were positive for NS1 and were typed by Real Time RT-PCR. DENV2 patients had soluble thrombomodulin serum levels higher than those patients with DENV1 (A), while VEGF serum levels did not vary according to serotype (B). Box-and-whisker plots shown median values (Horizontal line in the box), 25-75% interquartile range (upper-lower limits of the box).

matory diagnosis of dengueas reference. As shown in **Table 2** the most sensitive and specific test was the determination of serum sTM.

Discussion

In this study we analyzed the levels of sTM and VEGF in serum samples from patients diagnosed with DF and DHF infected with DENV2 and DENV1, as well as people without fever, and related them to the severity of the disease and serotype in order to assess their potential usefulness as early markers of vascular damage that can help identify patients at an early stage before developing severe disease. Our results indicate that the determination of sTM serum is a good marker due to the correlation between sTM levels and disease severity, as well as high sensitivity and specificity which our study showed. Furthermore, VEGF is a less sensitive and specific marker with greater variability in the population.

Dengue is endemic en Mexico and despite the efforts of health authorities to control the disease incidence has increased alarmingly in the last ten years [29]. The state of Guerrero is located in the southern part of the country in a tropical region of the highest incidence of DENV infection. According to the reports of the special system of epidemiological surveillance of dengue in 2012 in Mexico 50,368 confirmed cases were reported, of which 4,505 were in the state of Guerrero, representing 8.9%. However, of the 64 deaths due to dengue hemorrhagic fever in the country during the same year, 8 were in the state of Guerrero, representing 12.5% [4]. Considering that the state of Guerrero is one of 28 states that have reported dengue cases, these figures are alarming. It is well known that the differential diagnosis and clinical management of dengue patients is complicated mainly because they do not always have all the symptoms characteristic of the disease or become apparent until the critical stag-

Table 2. Sensitivity and specificity for serum levels of soluble thrombomodulin and VEGF in patients with dengue

	Control group (n)	DF (n)	Sensitivity (%)	Specificity (%)	DHF (n)	Sensitivity (%)	Specificity (%)
sTM (ng/mL)							
1° tercil (1.4-4.8)	70	3	--	--	3	--	--
2° tercil (4.9-8.7)	10	38	92.7	87.5	29	90.6	98.6
3° tercil (8.8-21.1)	1	29	90.6	98.6	48	94.1	98.6
VEGF (pg/mL)							
1° tercil (7.8-136.4)	48	11	--	--	19		
2° tercil (136.4-281.3)	31	18	62.1	60.8	27	58.7	60.8
3° tercil (281.4-1047)	12	41	78.8	80	24	55.8	80

sTM = Soluble Thrombomodulin. VEGF = Vascular Endothelial Growth Factor.

es. Therefore, it is of utmost importance to identify patients who started with symptoms of DHF to give them the proper management and avoid major complications or even death [26, 30].

During the development of DENV infection damage to the vascular endothelium is produced, although no solid evidence exist showing that the virus infects endothelial cells *in vivo* in humans, histopathological changes were detected in studies of microvasculature [31, 32]. It has also been demonstrated *in vitro* that the infection by dengue virus increases the expression of thrombomodulin on endothelial cells and that this increase is through the induction of inhibitory macrophage migration factor of the MAPK and PI3K signaling pathway [21, 33]. Various *in vivo* studies have reported various activation biomarkers and endothelial dysfunction as Angiopoietin 1 and 2 [11], von Willebrand factor and ADAMTS13 [12], thrombomodulin [15, 34], sICAM-1, sVCAM-1, VEGF and sFlt-1 [14], are altered in the blood of dengue patients [35]. However, more studies are needed in different populations around the world to show the consistency of these results.

Serum sTM has been evaluated in previous studies; however, the determinations were made in a small number of samples and the viral serotype is not reported, there is only one report from the American continent and the rest have been done in an Asian child population [15, 20]. On the other hand, reports of VEGF are controversial. Whereas in some of the studies high levels of this cytokine have been found in patients with DHF compared to patients with DF [23, 36], other authors have reported that the levels of VEGF decrease with the severity of the disease [7, 22, 23]. Unlike previous studies, we determined viral serotypes in diseased patients and correlate them to the levels of sTM and VEGF, finding that patients infected with DENV2 showed significantly higher sTM levels than those infected with DENV1 (P=0.00024). Our results clearly indicate that serum levels of sTM increased according to the severity of the disease, since according to the statistical analysis a strong association was found between having DHF and the third tertile (OR=7, IC 95% 4.2-8.8, p<0.001). Also significant differences were found between the three groups (P<0.001) and the sensitivity and specificity of the method was greater than 90%; these results agree with previous reports for Asian child population and support the utility of serum sTM as a marker of the severity of dengue. Our study was conducted in a very wide range of ages (from 0-95 years) without finding any association between age and serum levels of sTM, ruling out the possibility that age alters the results. We also found no association with sex for either of the two markers, indicating that it could be used for both men and women. sTM levels in the control group were on average lower than those reported for other populations [34, 35], which could be due to genetic variability of ethnicity itself, which justifies these studies in different populations. Increased serum sTM in patients with dengue fever suggests direct damage to the endothelium from early stages of the disease since the sTM is a specific marker of vascular damage and not only of endothelial activation as VEGF and other cytokines [18, 37]. It has been reported that the soluble form of the protein is released into the plasma only by direct

damage to endothelial cells during the development of vascular complications [36]. The finding of high levels of sTM in patients who did not have the severe form of the disease is very interesting because this suggests early damage to the endothelium which could increase and lead them to develop DHF. It has been estimated that a high percentage of patients with dengue fever bleeding episodes may occur; however, the pathogenesis of this complication in this group of patients is not fully understood [30, 34, 38]. Our results indicate that VEGF levels are significantly higher in dengue patients compared to the control group (P<0.0001); however, patients with DHF had lower levels than patients with DF, showing no correlation with disease severity. In previous reports similar behavior has been foundand is attributed to the interaction of VEGF with its receptor 1 with which it forms a complex allowing its endocytosis and thus decreases in plasma levels [22, 23]. While other studies [24, 25] found higher levels of VEGF in DHF than in DF attributing it to the activation of the fibrinolytic system that happens in patients with hemorrhagic dengue and the participation of mastocytes in the production of VEGF as a result of the interaction with the virus [39]. We do not exclude the possibility that other factors both viral and hosts that were not analyzed could affect VEGF levels in the study population. Taken together, our results indicate that thrombomodulin is a better marker of severity in DENV1 and DENV2 infection that VEGF because sTM levels are significantly higher in DHF than in DF and its sensitivity and specificity is very high. Therefore, we might suggest that determination of serum sTM in patients with suspected dengue would be useful for the identification of those who are at higher risk of developing the severe form of the disease and thus contribute to improved clinical management of patients mainly in endemic areas for this disease as the southern region of Mexico.

Disclosure of conflict of interest

None

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