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Elevated Levels of Full-Length and Thrombin-Cleaved Osteopontin During Acute Dengue Virus Infection are Associated with Coagulation Abnormalities

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Abstract

Introduction—Dengue virus (DENV) is transmitted by the mosquito vector, and causes a wide range of symptoms that lead to dengue fever (DF) or life-threatening dengue hemorrhagic fever (DHF). The host and viral correlates that contribute to DF and DHF are complex and poorly understood, but appear to be linked to inflammation and impaired coagulation. Full-length osteopontin (FL-OPN), a glycoprotein, and its activated thrombin-cleaved product, trOPN, integrate multiple immunological signals through the induction of pro-inflammatory cytokines.

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Conflict of interest disclosure

All authors declare that they have no conflicts of interest.

Materials and Method—To understand the role of OPN in DENV-infection, we assessed circulating levels of FL-OPN, trOPN, and several coagulation markers (D-dimer, thrombin-antithrombin complex [TAT], thrombomodulin [TM], and ferritin in blood obtained from 65 DENV infected patients in the critical and recovery phases of DF and DHF during a dengue virus epidemic in the Philippines in 2010.

Results—Levels of FL-OPN, trOPN, D-dimer, TAT, and TM were significantly elevated in the critical phase in both the DF and DHF groups, as compared with healthy controls. During the recovery phase, FL-OPN levels declined while trOPN levels increased dramatically in both the DF and DHF groups. FL-OPN levels were directly correlated with D-dimer and ferritin levels, while the generation of trOPN was associated with TAT levels, platelet counts, and viral RNA load.

Conclusion—Our study demonstrated the marked elevation of plasma levels of FL-OPN and thrombin-cleaved OPN product, trOPN, in DENV-infection for the first time. Further studies on the biological functions of these matricellular proteins in DENV-infection would clarify its pathogenesis.

Keywords

dengue virus; thrombin-cleaved osteopontin; full-length osteopontin; thrombin; inflammation; coagulation

Introduction

Dengue is an acute febrile disease that is caused by the dengue virus (DENV), which is transmitted to the host through the bite of blood-feeding mosquitos [1]. The number of countries reporting dengue cases has been increasing annually. An estimated 50 million dengue infections occur annually worldwide, and approximately 2.5 billion people live in countries where dengue is endemic [2]. In the majority of cases, infection with any of the 4 DENV serotypes is asymptomatic. However, a wide spectrum of clinical symptoms are associated with cases of symptomatic infection. These symptoms range from dengue fever (DF), which is a mild flu-like syndrome, to the more severe dengue hemorrhagic fever (DHF), which is characterized by coagulopathy and increased vascular fragility and permeability. DHF may even progress to hypovolemic shock (dengue shock syndrome) and death [3].

The mechanisms that lead to severe forms of dengue illness are complex, but undoubtedly relate to increased coagulation and fibrinolysis activity during DENV-infection [4, 5]. The activation of coagulation pathways and fibrinolysis have also been reported in DENV-infection, as reflected by increased thrombin-antithrombin complex (TAT), D-dimer (fibrin degradation product), tissue plasminogen activator and prothrombin fragment [6, 7]. In addition, it has been reported that thrombomodulin (TM) is induced in endothelial cells after infection with DENV *in vitro*, and may contribute to anticoagulation pathways in cells during DENV-infection [8]. (TM is an integral membrane protein that is expressed on the surface of endothelial cells, and serves as a cofactor for protein C activation by thrombin.) Nonstructural protein-1 (NS1) is a 43kDa glycoprotein of DENV and is expressed on cell surface or secreted as a soluble hexamer after infection [9, 10]. It is of note that this protein

was reported to bind prothrombin and inhibits its activation into thrombin and it has also been shown that antithrombin antibodies recognize NS1 protein in the sera of patients with dengue [11, 12]. Hemophagocytic syndrome is a final common form of a cytokine storm, which is induced by the uncontrolled proliferation and activation of macrophages, and results in systemic inflammatory responses and multi-organ dysfunction. Elevated ferritin, a marker of hemophagocytic syndrome, has also been reported in patients with dengue [13, 14]. The precise mechanism of DENV activity in the disturbance of capillary permeability is unclear. However, this mechanism is generally thought to be related to the dysregulation of immune and inflammatory factors. There is a need for soluble biomarkers that reflect both inflammation and coagulopathy during DENV-infection. Sosothikul et al demonstrated that von Willebrand factor (vWF) was the best indicator of the hemorrhagic form of dengue fever. Increased levels of soluble TM, vWF antigen, tissue factor and plasminogen activator were reported during the acute phase and were associated with disease severity. In contrast, the levels of ADAMTS-13 were lower in DHF patients compared to DF patients [15, 16]. Here, we studied inflammatory molecule of osteopontin, which have potential cleavage site of thrombin, in DENV-infection.

Full-length osteopontin (FL-OPN) is a highly phosphorylated and glycosylated matricellular protein. Although FL-OPN is secreted into the extracellular environment or matrix, intracellular form of OPN was also reported [17]. Rather, FL-OPN modulate cell function by interacting with cell-surface receptors, proteases, hormones, other bioeffector molecules, and structural matrix proteins, such as collagens [18]. FL-OPN is an acidic protein that consists of approximately 300 amino acids and is widely expressed in immune cells (for example, macrophages, T cells, and B cells) that are involved in bone resorption, wound repair, immune function, angiogenesis, cell survival, and cancer biology [19–24]. FL-OPN contains the arginine-glycine-aspartic acid (RGD) sequence, a classic cell-binding motif that is recognized by cell surface RGD-recognizing integrins such as $\alpha v\beta 1$, $\alpha v\beta 3$, and $\alpha 5\beta 1$ [25, 26]. In addition to the RGD motif, FL-OPN also contains 2 heparin-binding sites, 1 thrombin cleavage site, and 1 putative calcium-binding site [27]. Proteolytic cleavage of FL-OPN by thrombin (between Arg^{168} and Ser^{169}) generates a functional fragment of N-terminal thrombin-cleaved OPN (trOPN, also known as OPN-R [28]), which contains a cryptic binding site for integrin $\alpha 9\beta 1$ and $\alpha 4\beta 1$ that enhances the attachment of trOPN to integrins [26, 29]. Elevation of trOPN levels has been reported in plasma and tissue of patients with atherosclerotic status, and also in the synovial fluid from knee osteoarthritis [29–31].

A previous study demonstrated that DENV-infection induces *OPN* gene expression in human macrophages [32]. Given the importance of coagulation and inflammation abnormalities in DENV-infection, we designed a prospective clinical study to investigate FL-OPN and trOPN as candidate biomarkers in patient cohorts from Manila, the Philippines, during a dengue epidemic.

Materials and methods

Subjects and study design

During 2010, a study on dengue was conducted at San Lazaro Hospital in Manila, the Philippines. A total of 65 patients with clinical diagnoses of DF (n=53) or DHF (n=12) were enrolled in the study. DF and DHF were defined in accordance with the World Health Organization guidelines. Medical histories, physical examination results, and laboratory examination results were obtained from each of the enrolled patients. For each of the patients infected with DENV and 30 healthy controls (HC), plasma and serum samples were collected during the critical phase (day 4 or 5 of illness) and recovery phase (day 7 or 8 of illness), as described previously [33]. In brief, blood was collected in tubes with or without the anti-coagulant EDTA. EDTA plasma was obtained by centrifugation at 3,000 rpm for 10 min at 4°C. Serum was centrifuged and collected after clot formation at room temperature. Samples were aliquoted and stored at −80°C until use. Multiple thawing was avoided.

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki (Seoul, 2008) and was approved by the Ethics Committees of San Lazaro Hospital, Manila, the Philippines (2009–003), and Tohoku University Hospital, Sendai, Japan (2009–425). Written informed consent was obtained from all study participants.

RNA extraction and DENV quantification

Dengue viral RNA was quantified as previously reported [33]. In brief, genomic viral RNA was extracted from 140 μl of patient serum (critical phase only, n = 65) using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany). The extracted RNA was stored at −80 °C until use. The DENV copy number was measured by a TaqMan® real-time reverse transcription polymerase chain reaction assay (7500 Real-Time PCR System, Applied Biosystems, Foster City, CA, USA) using an *in vitro* transcribed quantitative RNA standard, as described previously [34].

Inflammatory and coagulation marker quantification

OPN levels in plasma were quantified by 2 different commercially available ELISA kits (IBL, Gunma, Japan; R&D Systems, Minneapolis, MN, USA) [35]. In the first kit, polyclonal rabbit antibody (O-17) specific to the N-terminus of OPN (Ile17-Gln31, accession #NP_000573.1) was used as a capture antibody, and a mouse monoclonal antibody (10A16) raised against synthetic peptides corresponding to the internal sequence of human OPN (Lys166-Glu187) was used as a detector antibody. The system does not allow us to detect trOPN. The standard range of this kit is 5–320 ng/ml or 76.9~4920 pmol/L. Here, the result was expressed as pmol/L. In the second ELISA kit, the proprietary capture monoclonal antibody and the detection polyclonal antibodies were both raised against recombinant human OPN (NS0-derived, amino acids Ile17-Asn300). The standard range of this kit is 62.5~4000 pg/ml. Final result was obtained based on dilution factor of 50–200 and expressed as ng/ml.

To detect N-terminal trOPN, a commercially available ELISA kit was used (IBL, Gunma, Japan). The standard range of this kit is 6.25~400 pmol/L. ELISA assay was performed using an anti-trOPN monoclonal antibody (34E3) as the capture antibody, and the O-17 antibody as the detection antibody. This capture antibody specifically reacts to the epitope Ser162–Arg168 exposed by thrombin and dose not reacts to matrix metalloproteinase-3, 7 (MMP-3, 7) cleaved N-terminal trOPN [36]. It is also known that thrombin-activatable fibrinolysis inhibitort (TAFIa) treated trOPN reduce its adherent capacity on Jurkat cells [37], but the treated form was not confirmed to bind the monoclonal antibody [31, 38].

ELISA kits to detect TAT (Abcam, Cambridge, MA, USA), D-dimer (Hyphen BioMed, Neuville-Sur-Oise, France), TM (R&D Systems, Minneapolis, MN, USA) and ferritin (Bio-vendor, Brno, Czech Republic) were used according to the manufacturer's instructions.

Statistical analysis

Data were expressed as medians because the distributions were non-Gaussian. The Kruskal Wallis test was used to assess differences in the plasma FL-OPN and trOPN levels among the HC, DF, and DHF groups. When a significant difference was found among these groups, Dunn's multiple comparison test was used to assess between-group differences for each pair of groups. Differences between the critical and recovery phases were assessed using the Wilcoxon signed-rank test. Relationships between parameters were assessed using Spearman's rank correlation coefficients. Two-tailed tests were used in all appropriate instances, and values of $P < 0.05$ were considered statistically significant. All statistical analyses were performed using GraphPad Prism software, version 6 (GraphPad Software Inc., San Diego, CA, USA).

Results

Elevated levels of plasma FL-OPN in the critical phase of DENV-infection decline during the recovery phase

Plasma levels of FL-OPN were measured in patients with DF and DHF during both the critical and recovery phases. Two different ELISA kits (IBL and R&D Systems) were used to determine FL-OPN levels because it has previously been demonstrated that these ELISA systems can have discordant results [35, 39, 40]. Analysis with the IBL kit showed that the levels of OPN were markedly elevated in both patients with DF (median, 25,951 pmol/L; 9.2-fold increase) and patients with DHF (median, 27,550 pmol/L; 9.7-fold increase), as compared with the HCs (2,814 pmol/L; Figure 1A). The R&D Systems kit also demonstrated elevated levels in patients with DF (median, 540 ng/ml; 7.9-fold increase) and patients with DHF (median, 692 ng/ml; 10.1-fold increase), as compared with the HCs (68 ng/ml; Suppl. Figure 1A). FL-OPN levels significantly differed among the 3 groups ($P < 0.0001$), and multiple-comparison corrected assessments indicated that the FL-OPN levels differed between the patients with DF and the HCs ($P < 0.001$), as well as between the patients with DHF and the HCs ($P < 0.001$), based on measurements from both the IBL and R&D Systems kits. However no significant differences in FL-OPN levels were found between patients with DF and patients with DHF (Figure 1A; Suppl. Figure 1A).

FL-OPN levels were significantly lower during the recovery phase than they were during the critical phase for patients with DF (median:1,199 pmol/L; $P < 0.00001$), as well as for patients with DHF (median: 907 pmol/L; $P < 0.001$) (IBL kit). The FL-OPN levels measured by the R&D Systems kit were also significantly decreased in patients with DF (121 ng/ml; $P < 0.0001$) and patients with DHF (285 ng/ml; $P < 0.01$). Interestingly, the levels were significantly lower than those of HCs, 0.57 fold and 0.68 fold reduction in DF and DHF by using IBL kit, respectively ($P < 0.05$; Figure 1A). However, using the R&D Systems kit, FL-OPN levels remained greater in DENV-infected subjects than they were in HCs during the recovery phase (1.7-fold increase in DF, not significant; 4.2-fold increase in DHF, $P < 0001$; Suppl. Figure 1A). A Spearman rank correlation coefficient revealed a significant correlation between IBL and R&D Systems assessments of the DENV-infected patients' FL-OPN levels during the critical phase. However, no correlation was evident during the recovery phase (Suppl. Figure 1B), indicating that the IBL ELISA kit only measured the FL-OPN, and specifically did not measure the cleaved form, whereas the R&D Systems kit measured both forms, but could not differentiate between them.

Elevated plasma levels of trOPN persist and increase during the recovery phase of DENV-infection

The levels of trOPN were elevated during the critical phase, both in patients with DF (median: 38 pmol/L) and patients with DHF (median: 43 pmol/L), as compared with the HCs (1 pmol/L). As assessed using the Kruskal–Wallis test, the trOPN levels of patients with DF and patients with DHF were significantly different from those of the HCs ($P < 0.0001$ and $P < 0.0001$, respectively; Figure 1B). Interestingly, the levels of trOPN during the recovery phase were significantly higher than those during the critical phase in both the DF and DHF groups (median: 979 and 1348 pmol/L; $P < 0.0001$ and $P < 0.01$, respectively; Figure 1B).

Elevated levels of trOPN are inversely associated with FL-OPN levels during the recovery phase of DENV-infection

We found no correlation between FL-OPN and trOPN during the critical phase (data not shown). During the recovery phase, however, a strong inverse correlation was observed between the trOPN levels and IBL FL-OPN levels in both the DF group ($r = -0.84$, $P < 0.0001$) and the DHF group ($r = -0.73$, $P < 0.05$) (Figure 1C). We did not observe a similar correlation for the R&D FL-OPN levels in recovery phase (Suppl. Figure 1C).

Coagulation marker levels in DENV-infected patients

The levels of TAT, D-dimer, and TM were significantly higher in -infected patients than they were in HCs based on the results of a Mann-Whitney test. Ferritin levels appear to be elevated in DENV- infected patents (the reference range was 25–283 ng/ml in HC according to manufacturer). Furthermore, a Wilcoxon signed-rank test indicated that the levels of each of these markers had declined significantly between the critical and recovery phases (Figure 2).

Plasma levels of OPN correlated with hematological and coagulation biomarkers throughout the course of DENV-infection

To study whether plasma FL-OPN levels were correlate with clinical and laboratory markers during DENV-infection, we examined potential correlations using Spearman's rank correlation coefficient from all DENV-infected patients, because the levels of these markers did not differ significantly between patients with DF and patients with DHF. In the critical phase, FL-OPN levels were positively correlated with elevated hematocrit, D-dimer, and ferritin levels ($r = 0.37$, 0.26, and 0.25, respectively) and negatively correlated with platelet count ($r = -0.44$). In the recovery phase, an even stronger correlation was observed between FL-OPN and D-dimer levels ($r = 0.42$), a moderate correlation was observed with TAT ($r = 0.42$), and a negative correlation was observed with lymphocyte levels ($r = -0.29$; Table 1).

TrOPN levels were associated with virological, hematological, and coagulopathy markers in DENV-infection

The Spearman rank correlation coefficient was used to determine the extent to which laboratory findings and coagulation markers were correlated with trOPN in the DENV-infected patients. During the critical phase, monocytes, DENV viral load, and TAT levels were associated with trOPN ($r = -0.26$, 0.46, and -0.37, respectively). During the recovery phase, levels of lymphocyte and ferritin were positively correlated with those of trOPN ($r = 0.28$ and 0.33, respectively) and, additionally, TAT levels and platelet counts were observed to be inversely correlated with trOPN levels ($r = -0.34$ and -0.32, respectively; Table 1).

Discussion

To the best of our knowledge, this is the first study to provide evidence that the plasma levels of matricellular protein FL-OPN and trOPN are elevated in patients with DF and DHF during the critical phase of illness, as compared with healthy subjects. During the recovery phase, FL-OPN levels declined; however, the levels of the thrombin cleaved byproduct trOPN continued to increase significantly.

The magnitudes of the increases in these proteins were much greater in this study than in previous reports on other diseases. Although trOPN has been detected in joint and ocular fluids in local inflammatory diseases, detection of trOPN in plasma (>100 pmol/L) in a disease-specific manner has not been demonstrated [30, 31]. DENV infects a plethora of cell types, including endothelial cells, fibroblasts, and macrophages [41–43]. Because FL-OPN is released from many of these cell types [23], DENV-infection could exacerbate the release of FL-OPN. Our study also demonstrated significant increases in the levels of D-dimer, TAT, TM and trOPN through the course of acute DENV-infection. Activation of coagulation pathways is known to be initiated by endothelial damages caused by DENV-infection [5]. We observed an inverse correlation between TAT and trOPN during the both critical and recovery phases, suggesting that the underlying mechanisms are complex. The involvement of NS1, which is known to inhibit prothrombin activation into thrombin, in delayed increase of trOPN is less likely because the levels of TAT and trOPN were inversely correlated. It is also well known that TAT is a relatively stable thrombin generation marker [44], in contrast trOPN can be substrate for several enzymes such as MMP-3, MMP-7 and

TAFIa [27]. The inverse correlation may indicate the activation of these enzymes with thrombin in inflammatory critical phase. The reason of further increase of trOPN in recovery phase is unclear, but it is possible that trOPN bind to integrins in critical phase in inflammatory tissue. Furthermore, it has been proposed that TAFIa is reduced in DENV-infected patients because of consumption secondary to excessive thrombin generation [16]. More detailed kinetics of the levels of OPN and trOPN with TAFIa would clarify the underlying mechanisms of their generation.

The most unique characteristic of trOPN is the expression of a functional integrin binding site for the integrin α9. The integrin α4 can bind both full-length and trOPN via SVVYGLR^{168} [45, 46]. In contrast, integrin α9 can only bind trOPN at cryptic cleaved site Arg168 [47]. Reportedly, Arg168 is required for α9 binding in addition to Val164, Tyr165, and Leu167 for cell adhesion and migration [48]. Furthermore, overexpression of FL-OPN was shown to regulate tumor metastasis and angiogenesis through the integrin αVβ3 [49]. Indeed, further cleavage of trOPN by TAFIa is believed to lose its inflammatory activities [37]. Therefore, more studies are necessary to understand the roles of OPNs in inflammation.

We have observed that FL-OPN levels are associated with both hematocrit levels and platelet counts, which suggests that FL-OPN levels may reflect the relative level of plasma leakage and thrombocytopenia during the critical phase of DENV-infection. Because FL-OPN was also positively correlated with D-dimer levels during both the critical and recovery phases (and with ferritin in the critical phase), these results suggest that plasma levels of FL-OPN may track the progression of inflammation and coagulopathy during DENV-infection. In the recovery phase, a positive correlation between trOPN and ferritin was also noted, and an inverse correlation was observed with platelet count. TrOPN is known to bind αVβ3 integrin on platelets and contributes to their migration to inflammatory sites [50]. Further, trOPN acts as a chemo-attractant for hematopoietic stem cells and possibly progenitor cells [36].

Taken together, our study demonstrated the marked elevation of plasma levels of FL-OPN and thrombin-cleaved OPN product, trOPN, in DENV-infection for the first time. Further studies on the biological functions of these matricellular proteins in DENV-infection would clarify its pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DENV	dengue virus
DF	dengue fever
DHF	dengue hemorrhagic fever
TAT	thrombin-antithrombin complex
TM	thrombomodulin
NS1	nonstructural protein-1
FL	full-length
tr	thrombin-cleaved
OPN	osteopontin
RGD	arginine-glycine-aspartic acid
TAFIa	thrombin-activatable fibrinolysis inhibitor
HC	healthy control
MMP	matrix metalloproteinase

References

1. Henchal EA, Putnak JR. The dengue viruses. Clinical microbiology reviews. 1990; 3:376–96. [PubMed: 2224837]
2. Nathan, MBD-DR.; Guzman, M. Epidemiology, burden of disease and transmission. Geneva: World Health Organization; 2009. New edition
3. Abel S, Liautaud B, Cabie A. Dengue. The New England journal of medicine. 2012; 367:180–1. [PubMed: 22784126]
4. Avila-Aguero ML, Avila-Aguero CR, Um SL, Soriano-Fallas A, Canas-Coto A, Yan SB. Systemic host inflammatory and coagulation response in the Dengue virus primo-infection. Cytokine. 2004; 27:173–9. [PubMed: 15304247]
5. Huerta-Zepeda A, Cabello-Gutierrez C, Cime-Castillo J, Monroy-Martinez V, Manjarrez-Zavala ME, Gutierrez-Rodriguez M, et al. Crosstalk between coagulation and inflammation during Dengue virus infection. Thromb Haemost. 2008; 99:936–43. [PubMed: 18449425]
6. Wills BA, Oragui EE, Stephens AC, Daramola OA, Dung NM, Loan HT, et al. Coagulation abnormalities in dengue hemorrhagic Fever: serial investigations in 167 Vietnamese children with Dengue shock syndrome. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2002; 35:277–85. [PubMed: 12115093]
7. Suharti C, van Gorp EC, Setiati TE, Dolmans WM, Djokomoeljanto RJ, Hack CE, et al. The role of cytokines in activation of coagulation and fibrinolysis in dengue shock syndrome. Thromb Haemost. 2002; 87:42–6. [PubMed: 11858187]
8. Chen LC, Shyu HW, Lin HM, Lei HY, Lin YS, Liu HS, et al. Dengue virus induces thrombomodulin expression in human endothelial cells and monocytes in vitro. The Journal of infection. 2009; 58:368–74. [PubMed: 19307023]
9. Gutsche I, Coulibaly F, Voss JE, Salmon J, d'Alayer J, Ermonval M, et al. Secreted dengue virus nonstructural protein NS1 is an atypical barrel-shaped high-density lipoprotein. Proc Natl Acad Sci U S A. 2011; 108:8003–8. [PubMed: 21518917]
10. Flamand M, Megret F, Mathieu M, Lepault J, Rey FA, Deubel V. Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. J Virol. 1999; 73:6104–10. [PubMed: 10364366]

11. Chuang YC, Lin YS, Liu HS, Wang JR, Yeh TM. Antibodies against thrombin in dengue patients contain both anti-thrombotic and pro-fibrinolytic activities. Thromb Haemost. 2013; 110:358–65. [PubMed: 23740201]
12. Lin SW, Chuang YC, Lin YS, Lei HY, Liu HS, Yeh TM. Dengue virus nonstructural protein NS1 binds to prothrombin/thrombin and inhibits prothrombin activation. The Journal of infection. 2012; 64:325–34. [PubMed: 22138554]
13. Szyper-Kravitz M. The hemophagocytic syndrome/macrophage activation syndrome: a final common pathway of a cytokine storm. The Israel Medical Association journal: IMAJ. 2009; 11:633–4. [PubMed: 20077953]
14. Tan LH, Lum LC, Omar SF, Kan FK. Hemophagocytosis in dengue: comprehensive report of six cases. Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology. 2012; 55:79–82. [PubMed: 22789140]
15. Butthep P, Chunhakan S, Tangnararatchakit K, Yoksan S, Pattanapanyasat K, Chuansumrit A. Elevated soluble thrombomodulin in the febrile stage related to patients at risk for dengue shock syndrome. The Pediatric infectious disease journal. 2006; 25:894–7. [PubMed: 17006283]
16. Sosothikul D, Seksarn P, Pongsewalak S, Thisyakorn U, Lusher J. Activation of endothelial cells, coagulation and fibrinolysis in children with Dengue virus infection. Thromb Haemost. 2007; 97:627–34. [PubMed: 17393026]
17. Shinohara ML, Kim JH, Garcia VA, Cantor H. Engagement of the type I interferon receptor on dendritic cells inhibits T helper 17 cell development: role of intracellular osteopontin. Immunity. 2008; 29:68–78. [PubMed: 18619869]
18. Bornstein P. Matricellular proteins: an overview. Journal of cell communication and signaling. 2009; 3:163–5. [PubMed: 19779848]
19. Steitz SA, Speer MY, McKee MD, Liaw L, Almeida M, Yang H, et al. Osteopontin inhibits mineral deposition and promotes regression of ectopic calcification. The American journal of pathology. 2002; 161:2035–46. [PubMed: 12466120]
20. Mori R, Shaw TJ, Martin P. Molecular mechanisms linking wound inflammation and fibrosis: knockdown of osteopontin leads to rapid repair and reduced scarring. J Exp Med. 2008; 205:43–51. [PubMed: 18180311]
21. Koguchi Y, Kawakami K, Uezu K, Fukushima K, Kon S, Maeda M, et al. High plasma osteopontin level and its relationship with interleukin-12-mediated type 1 T helper cell response in tuberculosis. American journal of respiratory and critical care medicine. 2003; 167:1355–9. [PubMed: 12574077]
22. Matusan-Ilijas K, Behrem S, Jonjic N, Zarkovic K, Lucin K. Osteopontin expression correlates with angiogenesis and survival in malignant astrocytoma. Pathology oncology research: POR. 2008; 14:293–8. [PubMed: 18493866]
23. Chagan-Yasutan H, Tsukasaki K, Takahashi Y, Oguma S, Harigae H, Ishii N, et al. Involvement of osteopontin and its signaling molecule CD44 in clinicopathological features of adult T cell leukemia. Leukemia research. 2011; 35:1484–90. [PubMed: 21645921]
24. Dai J, Peng L, Fan K, Wang H, Wei R, Ji G, et al. Osteopontin induces angiogenesis through activation of PI3K/AKT and ERK1/2 in endothelial cells. Oncogene. 2009; 28:3412–22. [PubMed: 19597469]
25. Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. Journal of cell science. 2006; 119:3901–3. [PubMed: 16988024]
26. Oldberg A, Franzen A, Heinegard D. Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. Proceedings of the National Academy of Sciences of the United States of America. 1986; 83:8819–23. [PubMed: 3024151]
27. Uede T. Osteopontin, intrinsic tissue regulator of intractable inflammatory diseases. Pathology international. 2011; 61:265–80. [PubMed: 21501293]
28. Sharif SA, Du X, Myles T, Song JJ, Price E, Lee DM, et al. Thrombin-activatable carboxypeptidase B cleavage of osteopontin regulates neutrophil survival and synoviocyte binding in rheumatoid arthritis. Arthritis and rheumatism. 2009; 60:2902–12. [PubMed: 19790060]

29. Breyne J, Juthier F, Corseaux D, Marechaux S, Zawadzki C, Jeanpierre E, et al. Atherosclerotic-like process in aortic stenosis: activation of the tissue factor-thrombin pathway and potential role through osteopontin alteration. Atherosclerosis. 2010; 213:369–76. [PubMed: 20732681]
30. Kurata M, Okura T, Kumon Y, Tagawa M, Watanabe H, Nakahara T, et al. Plasma thrombin-cleaved osteopontin elevation after carotid artery stenting in symptomatic ischemic stroke patients. Hypertension research: official journal of the Japanese Society of Hypertension. 2012; 35:207–12. [PubMed: 22113358]
31. Hasegawa M, Segawa T, Maeda M, Yoshida T, Sudo A. Thrombin-cleaved osteopontin levels in synovial fluid correlate with disease severity of knee osteoarthritis. The Journal of rheumatology. 2011; 38:129–34. [PubMed: 21041276]
32. Moreno-Altamirano MM, Romano M, Legorreta-Herrera M, Sanchez-Garcia FJ, Colston MJ. Gene expression in human macrophages infected with dengue virus serotype-2. Scandinavian journal of immunology. 2004; 60:631–8. [PubMed: 15584975]
33. Chagan-Yasutan H, Ndhlovu LC, Lacuesta TL, Kubo T, Leano PS, Niki T, et al. Galectin-9 plasma levels reflect adverse hematological and immunological features in acute dengue virus infection. Journal of clinical virology: the official publication of the Pan American Society for Clinical Virology. 2013; 58:635–40. [PubMed: 24239423]
34. Kubo T, Agoh M, Mai le Q, Fukushima K, Nishimura H, Yamaguchi A, et al. Development of a reverse transcription-loop-mediated isothermal amplification assay for detection of pandemic (H1N1) 2009 virus as a novel molecular method for diagnosis of pandemic influenza in resource-limited settings. Journal of clinical microbiology. 2010; 48:728–35. [PubMed: 20071551]
35. Vordermark D, Said HM, Katzer A, Kuhnt T, Hansgen G, Dunst J, et al. Plasma osteopontin levels in patients with head and neck cancer and cervix cancer are critically dependent on the choice of ELISA system. BMC cancer. 2006; 6:207. [PubMed: 16911785]
36. Grassinger J, Haylock DN, Storan MJ, Haines GO, Williams B, Whitty GA, et al. Thrombin-cleaved osteopontin regulates hemopoietic stem and progenitor cell functions through interactions with alpha9beta1 and alpha4beta1 integrins. Blood. 2009; 114:49–59. [PubMed: 19417209]
37. Myles T, Nishimura T, Yun TH, Nagashima M, Morser J, Patterson AJ, et al. Thrombin activatable fibrinolysis inhibitor, a potential regulator of vascular inflammation. The Journal of biological chemistry. 2003; 278:51059–67. [PubMed: 14525995]
38. Kon S, Ikesue M, Kimura C, Aoki M, Nakayama Y, Saito Y, et al. Syndecan-4 protects against osteopontin-mediated acute hepatic injury by masking functional domains of osteopontin. J Exp Med. 2008; 205:25–33. [PubMed: 18158320]
39. Constantinescu D, Vornicu M, Grigoriu C, Cozmei C, Grigoriu BD. Assaying for circulating osteopontin in practice: a technical note. The European respiratory journal: official journal of the European Society for Clinical Respiratory Physiology. 2010; 35:1187–8.
40. Plumer A, Duan H, Subramaniam S, Lucas FL, Miesfeldt S, Ng AK, et al. Development of fragment-specific osteopontin antibodies and ELISA for quantification in human metastatic breast cancer. BMC cancer. 2008; 8:38. [PubMed: 18237408]
41. Jessie K, Fong MY, Devi S, Lam SK, Wong KT. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. The Journal of infectious diseases. 2004; 189:1411–8. [PubMed: 15073678]
42. Kurane I, Janus J, Ennis FA. Dengue virus infection of human skin fibroblasts in vitro production of IFN-beta, IL-6 and GM-CSF. Archives of virology. 1992; 124:21–30. [PubMed: 1571018]
43. Halstead SB. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. Reviews of infectious diseases. 1989; 11 (Suppl 4):S830–9. [PubMed: 2665015]
44. Romisch J, Paques EP. Thrombin-hirudin complex stability: a comparison with the thrombin-antithrombin III complex. Blood coagulation & fibrinolysis: an international journal in haemostasis and thrombosis. 1991; 2:643–6. [PubMed: 1782334]
45. Green PM, Ludbrook SB, Miller DD, Horgan CM, Barry ST. Structural elements of the osteopontin SVVYGLR motif important for the interaction with alpha(4) integrins. FEBS letters. 2001; 503:75–9. [PubMed: 11513858]

46. Barry ST, Ludbrook SB, Murrison E, Horgan CM. Analysis of the alpha4beta1 integrin-osteopontin interaction. Experimental cell research. 2000; 258:342–51. [PubMed: 10896785]

47. Smith LL, Cheung HK, Ling LE, Chen J, Sheppard D, Pytela R, et al. Osteopontin N-terminal domain contains a cryptic adhesive sequence recognized by alpha9beta1 integrin. J Biol Chem. 1996; 271:28485–91. [PubMed: 8910476]

48. Ito K, Kon S, Nakayama Y, Kurotaki D, Saito Y, Kanayama M, et al. The differential amino acid requirement within osteopontin in alpha4 and alpha9 integrin-mediated cell binding and migration. Matrix biology: journal of the International Society for Matrix Biology. 2009; 28:11–9. [PubMed: 19000758]

49. Standal T, Borset M, Sundan A. Role of osteopontin in adhesion, migration, cell survival and bone remodeling. Experimental oncology. 2004; 26:179–84. [PubMed: 15494684]

50. Helluin O, Chan C, Vilaire G, Mousa S, DeGrado WF, Bennett JS. The activation state of alphavbeta 3 regulates platelet and lymphocyte adhesion to intact and thrombin-cleaved osteopontin. J Biol Chem. 2000; 275:18337–43. [PubMed: 10751402]

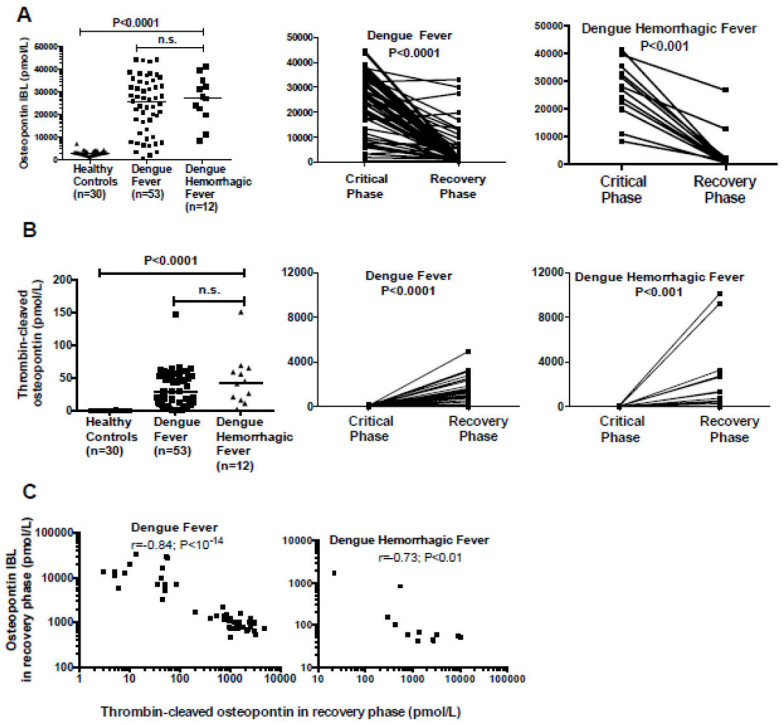

Figure 1. Elevated plasma full-length osteopontin (FL-OPN) and thrombin-cleaved OPN (trOPN) levels in patients infected with dengue virus

(A & B) The levels of FL-OPN (measured by the IBL ELISA kit) and trOPN differed significantly between patients with dengue fever, patients with dengue hemorrhagic fever, and healthy controls during the critical phase of dengue virus infection. The levels of FL-OPN declined in the recovery phase, while those of trOPN increased. (C) A significant inverse correlation was observed between the recovery phase OPN (measured by the IBL ELISA kit) and trOPN levels.

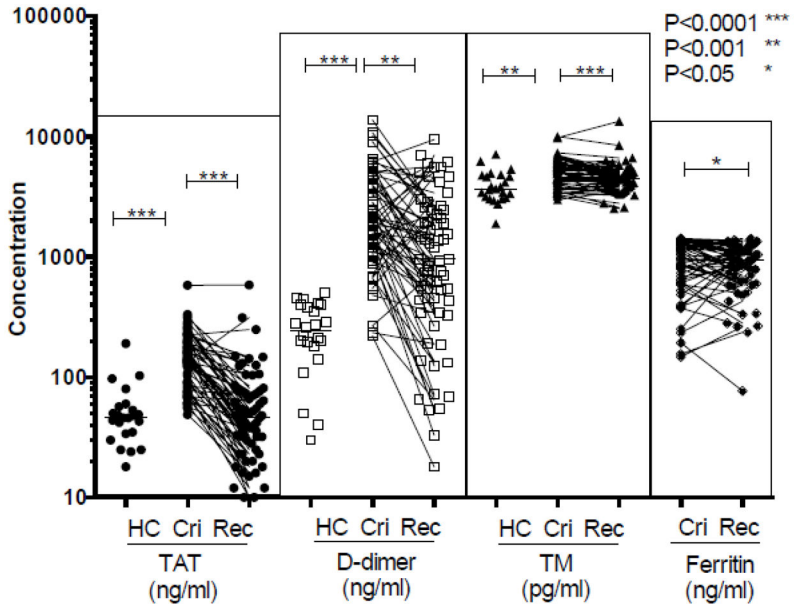

Figure 2. Coagulation marker levels

Plasma levels of thrombin-antithrombin complexes (TAT), and D-dimer, as well as serum levels of thrombomodulin (TM) are shown for healthy controls (HCs) and dengue virus (DENV)-infected patients during the critical (cri) and recovery (rec) phases. Ferritin levels from HCs were not measured because of a sample shortage; the reference range was 25–283 ng/ml in healthy individuals.

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Table 1

Correlation of full-length and thrombin-cleaved OPN with laboratory and coagulation markers.

Laboratory findings/Coagulation markers	Critical Phase				Recovery Phase			
	FL-OPN (IBL)		trOPN		FL-OPN (IBL)		trOPN	
	r	P	r	P	r	P	r	P
Increase of Hct (%)	0.37	<0.01	-	n.s.	-	n.s.	-	n.s.
Plt (10^3/ul)	−0.44	<0.001	-	n.s.	-	n.s.	−0.32	<0.05
Ly (%)	-	n.s.	-	n.s.	−0.29	<0.05	0.28	<0.05
Mono (%)	-	n.s.	−0.26	<0.05	-	n.s.	-	n.s.
Viral RNA (copy/ml)	-	n.s.	0.46	<0.001				
TAT (ng/ml)	-	n.s.	−0.37	<0.01	0.42	<0.001	−0.34	<0.01
D-dimer (ng/ml)	0.26	<0.05	-	n.s.	0.42	<0.001	-	n.s.
TM (pg/ml)	-	n.s.	-	n.s.	-	n.s.	-	n.s.
Ferritin (ng/ml)	0.25	<0.05	-	n.s.	-	n.s.	0.33	<0.01

Abbreviation: TAT, thrombin anti-thrombin complex; TM, thrombomodulin; OPN, osteopontin; FL, full-length; tr, thrombin-cleaved.