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Dengue virus nonstructural protein NS1 binds to prothrombin/thrombin and inhibits prothrombin activation

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Summary *Objectives:* Dengue virus (DENV) infection may result in severe dengue hemorrhage fever (DHF). However the mechanisms to cause hemorrhage during DENV infection are not fully understood. The sera level of secreted DENV nonstructural protein 1 (NS1) is correlated with the development of DHF. However, whether secreted NS1 can interfere with coagulation and contribute to the hemorrhage in DHF is unknown. Since thrombin plays a very important role in the activation of coagulation, we investigated whether NS1 can bind to thrombin and affect its formation or activity.
Methods and results: We first demonstrated that NS1 could bind to thrombin and formed NS1/thrombin complex in dengue patients' sera by enzyme-linked immunosorbent assay (ELISA). The ability of NS1 binding to prothrombin or thrombin was further confirmed using recombinant NS1 (rNS1) by ELISA, co-immunoprecipitation, and rNS1-affinity column purification. Even though the binding of rNS1 to thrombin showed no effect on thrombin activity, rNS1 could inhibit prothrombin activation and prolong activated partial thromboplastin time (APTT) of human platelet poor plasma.
Conclusion: These results suggest secreted DENV NS1 may bind to prothrombin and inhibit it activation, which in turn, may contribute to the APTT prolongation and hemorrhage in DHF patients.

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Introduction

Dengue virus (DENV) infection, which is transmitted by mosquitoes *Aedes aegypti* and *Aedes albopictus*, is prevalent in over 100 countries especially tropical and subtropical areas and threatens the health of more than 2.5 billion people.[1,2] According to World Health Organization, about 100 million DENV infections occur annually. DENV infection may cause mild dengue fever or severe life-threatening dengue hemorrhage fever (DHF) or dengue shock syndrome (DSS).[2,3] In addition to plasma leakage, almost all DHF patients have abnormal hemostasis, which is evidenced by thrombocytopenia, prolonged activated partial thromboplastin time (APTT), decreased fibrinogen level, and increased fibrinogen degradation products.[4,5] The case-fatality rates of DHF/DSS can be up to 20% if untreated. Currently, neither specific treatment nor vaccine is available against DENV infection. Since abnormal hemostasis contributes to the occurrence and severity of hemorrhaging during dengue infections, a better understanding of the mechanisms of the abnormal hemostasis induced by DENV might contribute to the development of a more effective and specific therapy against the development of DHF/DSS.

It is generally believed that soluble mediators such as cytokines produced during the acute phase of infection likely play an important role in the pathogenesis of DHF/DSS.[6–8] However, more and more data support that DENV nonstructural protein (NS1), a 43 kDa glycoprotein, may contribute to the development of DHF/DSS. Unlike other nonstructural proteins, DENV NS1 not only can be expressed on cell surface, but it can also be secreted as a soluble hexamer which forms a lipoprotein particle with an open-barrel protein shell and prominent central channel rich in lipids.[9,10] Secreted NS1 in patients' sera provides a rapid diagnostic marker for DENV infection and the sera level of NS1 is correlated with the viremia level and the development of DHF.[11–13] Recently, it has been identified that NS1 can interact with complement protein C4 and C4b binding protein and promote C4 degradation, which in turn protects DENV from complement-dependent lysis.[14,15] In addition, antibodies against NS1 in dengue patients can cross-react with endothelial cells and platelets.[16–18] Therefore, NS1 may represent an important viral factor to cause DHF. To further explore the pathogenic roles of NS1, we proposed and tested the hypothesis that secreted NS1 may bind to coagulatory factors and affect their functions.

The coagulation cascade consists of two pathways leading to the fibrin formation: the intrinsic and extrinsic pathways.[19] These two pathways converge at the activation of factor X to generate factor Xa ("a" signifies active), and activate prothrombin to thrombin. Thrombin then converts fibrinogen to a fibrin network. Since thrombin plays a key role in the coagulation system, we are interested to know whether DENV NS1 can interfere with thrombin formation or its activity. We first investigated whether or not NS1 in dengue patients' sera can bind to thrombin and formed a complex, then used recombinant NS1 (rNS1) to study its effect on prothrombin activation and thrombin activity.

Materials and methods

Patients' and control sera

Dengue patients' sera were obtained from 32 dengue patients in the acute stage of disease during an outbreak of DENV type 2 infections between August and October 2002 in southern Taiwan (Kaohsiung) and from 48 DENV-infected infants (mostly DENV type 3) during August 1997 to December 2002 with a clinical diagnosis of DHF as described in previous studies.[8,20] In addition, 22 Hepatitis C virus (HCV) positive sera as well as 12 normal human sera were included as negative controls.

Detection of NS1 and NS1-thrombin complex in dengue patients' sera by in-house ELISA

To detect free NS1 (thrombin unbound) in dengue patients' sera binding to thrombin, 50 μL of bovine thrombin (2 NIH units/ml, Sigma–Aldrich, St. Louis, MO) or control protein α-casein (10 μg/ml, Sigma–Aldrich) in carbonate/bicarbonate coating buffer (0.5 M, pH 9.6) was coated on 96-well ELISA plate (GeneDireX, Las Vegas, NV) at 4 °C overnight. The wells were blocked by 0.25% gelatin in phosphate buffer saline (PBS) for 1 h and washed by PBST (0.05% Tween 20 in PBS) 3 times. Dengue patients' sera and control sera were diluted 10 folds in PBST and incubated on wells at 37 °C for 1 h. Bound NS1 was detected by rabbit anti-NS1 polyclonal antibody (1000-fold dilution; GeneTex, San Antonio, TX) for 1 h at 37 °C and captured by horse radish peroxidase (HRP)-conjugated goat anti rabbit IgG antibody (5000-fold dilution; Zymed, San Francisco, CA). After final washes, the color was developed by 3, 3′0.5, 5′-tetramethylbenzedine (TMB) substrate (50 μl) and stopped by equal volume of 2 N sulfuric acid. Optical density (OD) at 450 nm of each well was evaluated by a VersaMax microplate reader (Molecular Devices, Crawley, West Sussex, UK). For NS1/thrombin complex detection, the plate was coated with rabbit anti-thrombin polyclonal antibody (2 μg/ml; GeneTex, San Antonio, TX) followed by the same procedure as described above except mouse anti-NS1 serum (1000-fold dilution) and HRP-conjugated goat anti mouse IgG antibody (5000-fold dilution; Zymed, San Francisco, CA) were used to detect NS1/thrombin complex. In addition, NS1 in dengue patients' sera was also detected by Platelia™ Dengue NS1 Ag-ELISA (Biorad Laboratories, Marnes-La-Coquette, France) following manufacturer's procedure. This test, which uses murine monoclonal antibody to capture DENV NS1 antigen in human serum or plasma, is based on a one-step sandwich format microplate enzyme immunoassay. If NS1 antigen is present in the sample, an immune-complex MAb-NS1-MAb/peroxidase will be formed and detected by chromogenic reaction of substrate.

Preparation and purification of recombinant NS1 protein

Full-length NS1 DNA was amplified from DENV serotype 2 (PL046 strain) using specific primer (forward: