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Secreted complement regulatory protein clusterin interacts with dengue virus nonstructural protein 1

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Abstract

Vascular leakage and shock are the major causes of death in patients with dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). It has been suggested that patients with an elevated level of the free soluble form of dengue virus (DV) nonstructural protein 1 (sNS1) are at risk of developing DHF. To understand the role of sNS1 in blood, we searched for the host molecule with which NS1 interacts in human plasma by affinity purification using a GST-fused NS1. Complement inhibitory factor clusterin (Clu), which naturally inhibits the formation of terminal complement complex (TCC), was identified by mass spectrometry. A recombinant sNS1 produced from 293T cells and sNS1 from DV-infected Vero cells interacted with human Clu. Since an activated complement system reportedly causes vascular leakage, the interaction between sNS1 and Clu may contribute to the progression of DHF.

Keywords: Dengue virus; NS1; Clusterin; Clu; Hemorrhagic fever; Complement; Plasma leakage; Terminal complement complex

Dengue virus (DV) infection is a major cause of morbidity in tropical and subtropical areas. It is estimated that at least 100 million DV infections and >250,000 cases of dengue hemorrhagic fever (DHF) occur annually [1]. DV infection occasionally causes severe illness, namely DHF and dengue shock syndrome (DSS). A pathological feature of these illnesses is vascular plasma leakage [2]. However, the mechanism of plasma leakage caused by the infection remains unknown.

The dengue viruses are enveloped and contain a single, positive-sense RNA genome of about 11 kb that encodes a large polyprotein precursor. Co- and post-translational processing gives rise to three structural and seven nonstructural proteins, encoded by genes in the order (from 5′ to 3′) C, preM, E, nonstructural protein (NS)1, NS2a, NS2b, NS3, NS4, and NS5 [3]. NS1, a 46- to 50-kDa glycoprotein, is unusual for a viral glycoprotein in that it does not form part of the virion structure. However, NS1 is expressed on the surface of, and released from, infected mammalian cells [4–6]. NS1 is initially translocated into the endoplasmic reticulum via a hydrophobic signal sequence encoded in the carboxyl-terminal region of E, where it rapidly dimerizes [7].

NS1 was originally found in a soluble complement-fixing (SCF) antigen in DV-infected cell cultures [8]. The flavivirus NS1 has been recognized as an important immunogen in infections and immunization against NS1 shown to play a role in protection against disease. It has been widely hypothesized that secreted NS1 contributes to the formation of immune complexes which trigger activation of the complement system [9,10]. Indeed, the

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activated complement system has been observed [11,12] and was suggested to induce plasma leakage [9]. However, the physiological functions of membrane-associated (mNS1) and secreted (sNS1) forms of NS have not been fully defined although several studies have shown the clinical relevance of mNS1 and sNS1 [9,13].

In this study, we show that NS1 protein interacts with the human complement regulatory protein clusterin (Clu). Clu is a heterodimer in serum containing two chains of approximately 34 and 36 kDa. Clu is a fluid-phase inhibitor of the terminal pathway of the complement system [14]. Its putative role in serum is to inhibit the formation of terminal complement complex (TCC). Clu is likely to protect cells from attack by complement. On the other hand, an increased level of TCC was observed in the pleural fluid of DHF patients [9]. The findings of this study suggest to us that the NS1/Clu interaction may lead to the onset of plasma leakage.

Materials and methods

Viral infection. Monolayers of Vero cells were seeded in 100-mm plates and cultured in minimum essential medium (MEM) until infected. The cells were infected with the New Guinea C (NGC) strain. They were infected with DV at a multiplicity of infection (MOI) of 1 or 0.2 that was adjusted by dilution in OPTI-MEM (Invitrogen). After adsorption for 2 h at 37 °C, the cells were washed six times to remove residual virus and then incubated with OPTI-MEM at 37°C for 5 days prior to recovery of the culture medium.

Plasmid construction. For construction of the NS1 expression plasmid, total RNA from DV-infected cells was isolated using Trizol reagent (Invitrogen, Carsbad, CA). Reverse transcription was performed with random primers and the resulting cDNA was used as a template for PCR. Gene-specific primer sets were used to amplify specific genes. These primer sets were: (1) NGC NS1, 5′-GGCGGATCCGCCATGGCCGATAGT GGTTGCGTTGTGAGC-3′ and 5′-GCGCTCGAGTCAGGCTGTGAC CAAGGAGTTGACC-3′; and (2) NS1 of Thai isolates (R05-135 and R01-98), Fw 5′-GGCGTCGACCCGATAGTGGTTGCGTTGTGAGC-3′ and 5′-GCGGCGGCCGCTCAGGCTGTGACCAAGGAGTTGACC-3′. The amplified sequences were inserted into the BamHI/XhoI or SalI/NotI sites of pGEX-6P-1 (Invitrogen) and p-FLAG-CMV-3 (Sigma, St. Louis, MO). For immunization, GST-His.NS1 derived from the NGC strain was cloned by insertion of linker 5′-AATTCGC CATGGCCCATCACCAC-CACCACCATGCAGG-3′ and 5′-AATTC TGCATGGTGGTGGTGG TGATGGGCCATGGCG-3′ into the EcoRI site of GST-NS1.

GST-fusion proteins and affinity purification. The recombinant glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* BL21 and purified with glutathione sepharose 4B (GE Healthcare, Piscataway, NJ). Human plasma was incubated on ice for 1 h after the addition of Triton X-100 to 0.3%, centrifuged at 20,800*g* for 20 min at 4 °C to remove insoluble debris, and incubated with GST-fusion proteins for at least 2 h at 4°C. The beads bound to proteins were washed with binding buffer, 10 mM Hepes, pH 8.0, 200 mM KCl, and 0.3% Triton X-100, then the bound proteins were eluted with elution buffer containing 10 mM Hepes, pH 8.0, 1.5 M KCl, and 0.3% Triton X-100. The eluted fractions were precipitated by trichloroacetic acid and dissolved in Laemmli's buffer.

Protein staining. Proteins were developed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and visualized with coomassie brilliant blue (CBB) staining (Bio-Safe Coomassie, Bio-Rad, Hercules, CA) and silver staining (Silver Quest Silver Staining kit, Invitrogen).

Mass spectrometry. Protein isolated from the SDS–PAGE gel was digested with trypsin and analyzed by Q-Tof Ultima API (Micromass, Beverly, MA).

Transfection. 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) (HyClone, Logan, UT) containing 10% fetal bovine serum (FBS). The cells were transfected with plasmid using Lipofectamine 2000 (Invitrogen), and incubated with OPTI-MEM to prevent the effect of the FBS.

Co-immunoprecipitation. The culture medium was recovered from transfected-293T cells or infected-Vero cells, and centrifuged at 900*g* for 10 min to remove debris. The supernatant was incubated with or without human plasma for 4 h at 4 °C. The mixtures were incubated with antibody to Clu, FLAG, or NS1 for 2 h at 4 °C, then immunoprecipitated with GammaG Sepharose (GE Healthcare, Piscataway, NJ) for 1 h at 4 °C. The beads were washed with buffer (1% [vol/vol] NP-40, 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.1% protease inhibitors). Precipitated proteins were dissolved in Laemmli's buffer and subjected to SDS–PAGE, then analyzed by Western blotting.

Antibody preparation. GST-His.NS1 was purified with Ni-NTA (Qiagen, Valencia, CA) under denaturing conditions (6 M guanidine hydrochloride) and emulsified with Freund's complete adjuvant (1:1 in vol/vol). A rabbit was then immunized intradermally with the emulsified immunogen mixture. After three booster-immunizations with recombinant GST-His.NS1 without adjuvant, antiserum was obtained. All animal experiments conformed to the guide for the care and use of laboratory animals of the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

Antibodies. Anti-FLAG (M2) and anti-Clu (H-330) antibodies were purchased from SIGMA and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Results

GST-NS1 binds with Clu in human plasma

To identify the host-derived proteins interacting with DV NS1 protein, we performed affinity purification using GST-NS1. GST-fusion proteins were expressed in *E. coli* and purified with glutathione sepharose 4B, then visualized with CBB (Fig. 1A). GST-NS1 was incubated with human plasma and bound proteins were eluted with KCl. As well as GST alone, GST-Tat was used as a negative control since human immunodeficiency virus 1 (HIV-1) Tat was observed to be circulating in HIV-infected patients and associated with the pathogenesis of HIV-1-associated Kaposi's Sarcoma [15]. GST-CycT1 containing human cyclin T1 (CycT1) was also used as a negative control, since the CycT1 binds to Tat [16]. The bound proteins were eluted with KCl from beads bound to GST-fusion protein, and visualized by silver-staining (Fig. 1B). A specific band, exhibiting mobility at 34 to 36 kDa, was observed in the eluate from GST-NS1 (Fig. 1B, lane 2) but not in that from GST alone, GST-Tat, or GST-CycT1 (Fig. 1B, lanes 1, 3 and 4). The NS1-specific band was analyzed by mass spectrometry revealing several candidate peptide fragments, with a human complement inhibitor, Clu, as the most probable candidate (Table 1). To confirm this result, we examined the eluate from GST alone and GST-NS1 by Western blotting with a specific antibody against Clu. As expected, the band with mobility at 34–36 kDa was specifically detected with this antibody (Fig. 1C). A smeared band containing clusterin α and β-chain is characteristic of the glycosylated secreted form of Clu [17]. Based on the data, we concluded that GST-NS1 associates with Clu.