Platelet Apoptosis and Apoptotic Platelet Clearance by Macrophages in Secondary Dengue Virus Infections

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Background. The mechanisms of thrombocytopenia and platelet phagocytosis in dengue illness are not fully understood.

Methods. A prospective hospital-based study was conducted to examine the relationships between platelet counts, serum thrombopoietin (TPO) levels, and platelet apoptosis and phagocytosis in 81 patients with secondary dengue virus (DV) infections and 38 healthy volunteers. The apoptosis and phagocytosis of cultured platelets after exposure to DV were also examined.

Results. Platelet apoptosis, platelet phagocytosis, and serum TPO levels were increased significantly in patients during the acute and early convalescence phases compared with levels observed in patients during the convalescence phase and in healthy volunteers. A significant correlation between platelet apoptosis and platelet phagocytosis was also observed in these patients. Platelet phagocytosis was inhibited significantly by the D89E mutant, which carries a point mutation in the RGD motif of milk fat globule–epidermal growth factor 8, a phosphatidylserine-recognizing bridge molecule. DV-induced platelet apoptosis and increased phagocytosis of DV-induced apoptotic platelets was confirmed using in vitro assays.

Conclusions. Our data suggest an increased phagocytosis of DV-induced apoptotic platelets by macrophages via a phosphatidylserine-recognizing pathway in secondary DV infection. Accelerated platelet clearance, however, was overcome by TPO-induced enhanced thrombopoiesis in these patients.

Clinical Trials Registration. UMIN000004835.

Dengue virus (DV) is a mosquito-borne human viral pathogen with 4 distinct serotypes: DV1, DV2, DV3, and DV4 [1, 2]. The rapid increase in the number of human cases of DV infection has become a major public health concern during the past 3 decades in tropical and subtropical countries. Transient thrombocytopenia associated with acute febrile illness and hemorrhagic manifestations is a hallmark of DV infections. The common severe form, dengue hemorrhagic fever (DHF), is characterized by a sudden increase in vascular permeability. Secondary DV infections, which are observed commonly in dengue-endemic areas, are more likely to constitute a risk factor for DHF than are primary DV infections [3].

Studies undertaken >40 years ago suggested that the suppression of megakaryocytopenia in bone marrow was responsible for the thrombocytopenia and hemorrhagic manifestations in severe dengue illness [4, 5]. However, the precise mechanisms underlying...
DV-induced bone marrow suppression during the acute phase of this disease remain unclear. Thrombopoietin (TPO) is a cytokine that specifically regulates megakaryocytopoiesis and platelet production by activating c-MPL, the receptor of TPO [6, 7]. Because TPO is elevated when platelet production is reduced, serum TPO levels may be a useful indicator of megakaryocytopoiesis in this disease.

In contrast, our previous observations using flow cytometric analysis demonstrated an in vitro increase in the phagocytosis of platelets by macrophages in patients with secondary DV infections [8]. However, no significant inhibition of platelet phagocytosis was observed using antibodies directed against Fcγ receptors or complement receptor 3. Furthermore, we reported previously that high-dose intravenous immunoglobulin treatment had no effect on the severe thrombocytopenia of patients with secondary DV infections [9]. Therefore, the mechanisms of accelerated platelet phagocytosis in this disease remain uncertain.

Previous in vitro and in vivo studies suggest that apoptotic cell death is involved in the cytopathological mechanism of DV infections [10, 11]. Platelets undergo an apoptotic program that regulates their normal circulatory lifespan [12] and is induced after platelet activation by agonists such as thrombin [13]. Apoptotic platelets express phosphatidylserine (PS) on their surfaces and also include activation of caspase 3, a key effector of apoptosis. A previous study also reported morphological changes in aged platelets, as assessed using electron microscopy, similar to those observed during granulocyte apoptosis [14]. The apoptotic cells are recognized by phagocytes through “tethering” ligands such as the milk fat globule–epidermal growth factor 8 (MFG-E8), PS receptors such as TIMD4, and scavenger receptors such as CD36 [15, 16]. The apoptotic platelets are then degraded in the phagocytes.

In this study, we hypothesized that DV induces platelet apoptosis during the acute phase of secondary DV infection, with subsequent acceleration of phagocytosis of the apoptotic platelets mediated by a PS-recognizing pathway.

MATERIALS AND METHODS

Patients and Study Design
A prospective hospital-based study of DV infection was conducted at San Lazaro Hospital, Manila, Philippines, between September 2009 and December 2010. Ninety-seven patients aged ≥10 years and with a clinical suspicion of DV infection were enrolled during the study period. The age restriction was imposed because 10 mL of blood was required for the analyses of platelets of each patient in this study. Among the patients enrolled, 86 were confirmed as having acute-phase DV infection (3–7 days after the onset of illness) on the basis of results of reverse-transcription polymerase chain reaction (RT-PCR) or dengue immunoglobulin M (IgM)–capture enzyme-linked immunosorbent assay (ELISA) for DV. Subsequently, 81 patients were found to have secondary DV infection based on a dengue hemagglutination-inhibition assay [2, 17]. DF or DHF was diagnosed according to the World Health Organization (WHO) guidelines [18]. The patients with grade III or grade IV DHF and patients requiring platelet transfusion were excluded. Because most patients had secondary DV infection, we decided to examine patients with secondary infection, and not primary infection, in this study.

Platelet-rich plasma and serum from the patients enrolled were prepared. Peripheral platelet counts, platelet apoptosis, serum TPO levels, and platelet phagocytosis were determined in these patients at the time of enrollment (acute phase), 4 days after the first blood collection (early convalescence phase), and ≥4 days after the second blood collection (convalescence phase). Thirty-eight age-matched healthy control subjects were enrolled during the same period at St Luke’s Medical Center, and the same assays were performed as those performed for the patients. Serum TPO levels were measured using a sandwich ELISA (R&D Systems) in 79 patients and 27 controls only because of insufficient volume of serum samples.

The research proposal for this study was approved by the Bioethics Committee of the San Lazaro Hospital and the Institutional Ethics Review Board of St Luke’s Medical Center. All patients and controls provided written informed consent.

Platelet Phagocytosis and Inhibition Assay
Platelet phagocytosis by differentiated THP-1 cells was examined in platelet samples from 81 patients and 38 controls, as described previously [8, 19]. In brief, washed platelets were stained with CellTracker Orange CMTMR (CTO; Molecular Probes), and the frequency of platelet phagocytosis was determined by counting the CTO-positive, CD61 (a platelet-specific marker)–negative cells using flow cytometry. As a positive control for each assay, the apoptotic pathway was activated in platelets obtained from healthy donors by incubation with human thrombin (5 NIH units/mL; Sigma-Aldrich) [13]. The mean frequency (SD) of phagocytosis of thrombin-treated apoptotic platelets was 50.47 (6.15). The frequency of platelet phagocytosis was expressed as a percentage, as follows: [(the frequency of phagocytosis of the test platelets/the frequency of thrombin-treated apoptotic platelets) × 100]. Assays of inhibition of platelet phagocytosis were performed in 7 patients with DHF, 17 patients with DF, and 20 controls. FLAG-tagged recombinant D89E of the MFG-E8 protein, carrying a point mutation in the RGD motif, was prepared for an inhibition assay of platelet phagocytosis, as described previously [20]. The D89E mutant protein inhibits the phagocytosis of apoptotic cells in vitro and in vivo.

CTO-stained platelets were pretreated with phosphate-buffered saline (PBS) or D89E (1.0 µg/mL) in PBS for 30 minutes at 37°C.
Platelet Apoptosis

Platelet apoptosis was examined in 58 patients and 28 controls. Platelets were prepared from the patients or controls, as described previously [8]. Purified platelets were stained with a platelet marker (phycocerythrin [PE]/Cy5-conjugated anti-CD41; BioLegend) and then stained with PE-conjugated annexin V (BD Pharmingen) for PS exposure. Platelets were also stained with FAM-DEVD-FMK (Immunochemistry Technologies) for active caspase 3 [13].

In Vitro Platelet Apoptosis and Phagocytosis of Apoptotic Platelets

DV1 (Mochizuki strain), DV2 (16681 strain), DV3 (H87 strain), or Japanese encephalitis virus (JEV; Nakayama strain) was propagated in C6/36 mosquito cells in Leibovitz L-15 medium (Gibco) containing 2% fetal bovine serum and 0.29% tryptose phosphate broth. Human platelets isolated from healthy donors at a concentration of 10^6/mL were incubated in L-15 medium containing DV of each serotype or JEV at a multiplicity of infection of 1 or in medium only for 96 hours at 37°C.

Platelets were prepared from the patients or controls, as described previously [8]. Purified platelets were stained with a platelet marker (phycocerythrin [PE]/Cy5-conjugated anti-CD41; BioLegend) and then stained with PE-conjugated annexin V (BD Pharmingen) for PS exposure. Platelets were also stained with FAM-DEVD-FMK (Immunochemistry Technologies) for active caspase 3 [13].

Statistical Analysis

All data are shown as the mean (SD) for continuous variables with normal distribution or as the median (interquartile range) for those with nonnormal distribution. Platelet counts, platelet apoptosis, serum TPO levels, and platelet phagocytosis in patients during the acute, early convalescence, and convalescence phases and in controls were analyzed using repeated measures analysis of variance (ANOVA) and Scheffe’s method for the post hoc multiple comparisons test. Data from the assays of inhibition of platelet phagocytosis were analyzed using the independent samples t test. In vitro experiments aimed at examining the induction of apoptosis and platelet phagocytosis using healthy donor cells in different culture conditions were performed using ANOVA and Tukey multiple comparisons test. The significance of the correlations was estimated using the Pearson coefficient. P < .05 was considered significant. The SPSS statistical software version 16.0 (SPSS, an IBM Company) was used for all data analyses.

RESULTS

Patient Characteristics

Among the 81 patients, 57 were diagnosed with DF and 24 with DHF (Table 1). Patients with DHF were classified further into DHF grade I (n = 5) or grade II (n = 19). No fatal cases were included. The mean increase in hematocrit was significantly greater in DHF patients than in DF patients (P < .0001). The mean period from the onset of fever to the admission of the patients was 3.7 (1.0) days.

Platelet Counts and TPO

The peripheral platelet counts were significantly lower in patients during the acute phase compared with controls (P < .0001; Table 1) and were significantly lower in DHF patients compared with DF patients (P < .05; Table 1). The low baseline platelet counts during the acute phase increased significantly to normal levels during the early convalescence and convalescence phases (P < .0001; Figure 1A). In contrast, serum TPO levels were significantly higher in patients during the acute phase compared with controls (P < .0001; Table 1). Serum TPO levels decreased significantly during the early convalescence.
and convalescence phases ($P < .0001$; Figure 1B). No correlation was found between platelet counts and serum TPO levels during the acute and convalescence phases, but a weak inverse correlation was found between these 2 parameters during the early convalescence phase (Figure 1C).

**Platelet Apoptosis**

Representative density plots of the frequency of platelet apoptosis, as measured using annexin V binding and levels of active caspase 3, are shown for controls and patients with DF or DHF during the acute phase (Figure 2). Platelet apoptosis determined via annexin V binding and the levels of active caspase 3 was significantly increased in patients during the acute phase compared with controls ($P < .0001$; Table 1). Platelet apoptosis was also significantly greater in DHF patients than in DF patients ($P < .05$; Table 1). The increased apoptosis of the platelets isolated from patients, as evidenced by both annexin V binding and the levels of active caspase 3, decreased significantly during the early convalescence and convalescence phases ($P < .01$ for early convalescence phase, $P < .0001$ for convalescence phase; Figure 3A and 3B). The number of apoptotic platelets exposing PS was significantly elevated in patients during the early convalescence ($P < .0001$) and convalescence phases ($P < .0001$) but not during the acute phase ($P = .173$) compared with that observed in controls (Figure 3C).

**Platelet Phagocytosis and Correlation With Platelet Apoptosis**

Platelet phagocytosis was significantly increased in patients compared with controls ($P < .0001$; Table 1) and differed significantly between patients with DF and those with DHF ($P < .05$; Table 1). The significantly elevated platelet phagocytosis values during the acute phase returned to normal levels during the early convalescence ($P < .01$) and convalescence phases ($P < .0001$; Figure 4A). During the acute phase, significant correlations were observed between platelet phagocytosis and platelet apoptosis ($P < .0001$ for both annexin V binding and active caspase 3; Figure 4B and 4C). Weak but significant correlations between these 2 parameters were also found during the early convalescence phase; however, during the convalescence phase, they were only significant for active caspase 3.

**Inhibition of Platelet Phagocytosis**

Next, we determined whether the apoptotic platelets were phagocytosed by macrophages via the PS-recognition pathway.
by pretreating platelets with the D89E mutant protein to block the phagocytosis of apoptotic platelets. This treatment led to a significant inhibition of platelet phagocytosis in 7 patients with DHF (Figure 5A) and 17 patients with DF (Figure 5B) during the acute and early convalescence phases. In contrast, no inhibition of phagocytosis was observed in patients during the convalescence phase or in controls (Figure 5C).

In Vitro Platelet Apoptosis and Phagocytosis of Platelets
The apoptosis of platelets pretreated with CTR increased significantly in medium containing DV4 after incubation for 72 hours and 96 hours \( (P < .0001) \) compared with that of platelets pretreated with CTR observed in medium only or in medium containing JEV (Figure 6A). Pretreatment with a caspase 3 inhibitor reduced significantly the apoptosis of platelets cultured in medium containing DV4 or JEV or in medium only after incubation for 72 hours and 96 hours \( (P < .05) \) compared with platelets pretreated with CTR. Our Western blot results confirmed the enhanced activation of caspase 3 in platelets cultured in medium containing DV4 compared with platelets cultured in medium only or in freshly isolated platelets (Figure 6B). The phagocytosis of platelets pretreated with CTR cultured in medium containing DV4 for 72 hours was significantly higher than that observed in platelets pretreated with CTR cultured in medium only \( (P < .0001); \) Figure 6C). Pretreatment with a caspase 3 inhibitor reduced significantly the phagocytosis of platelets pretreated with CTR cultured in medium only \( (P = .01) \) or in medium containing DV4 \( (P < .0001) \). Culture in the presence of DV1, DV2, or DV3 increased the apoptosis of platelets pretreated with CTR similarly after incubation for 72 hours and 96 hours compared with that observed in medium only \( (P < .0001); \) Figure 6D). Pretreatment with a caspase 3 inhibitor reduced significantly the apoptosis of platelets cultured in medium containing DV1,
DV2, or DV3 or in medium only after incubation for 72 hours and 96 hours compared with platelets pretreated with CTR (P < .0001).

**DISCUSSION**

In this study, we demonstrated a significant increase in platelet apoptosis in patients during the acute and early convalescence phases of secondary DV infections compared with the increase observed in patients during the convalescence phase or in controls. Although the frequency of apoptosis has been reported as <0.1% in peripheral blood mononuclear cells of pediatric patients with DF or DHF during the acute phase [21], the frequency of apoptotic platelets was >10% in patients during the acute and early convalescence phases. Platelet apoptosis was significantly higher in DHF patients than in DF patients. Because plasma viremia is higher in DHF patients than in DF patients during the acute phase [22], the induction of platelet apoptosis is presumably attributable to dengue viremia. Although a recent study reported that erythrocyte-derived microvesicles released PS to the surface of nucleated cells during erythrocyte lysis [23], we did not use a procedure of erythrocyte lysis to separate platelets in this study. Therefore, the results in our study may not involve this false marking with PS as apoptotic.

Our in vitro assays demonstrated that all serotypes of DV enhanced the spontaneous apoptosis of human platelets. The phagocytosis of cultured platelets after exposure to DV4 was also increased. In contrast, no enhancement of spontaneous apoptosis was found in JEV-exposed platelets. This finding suggests the DV-specific induction of platelet apoptosis and a direct interaction between DV and platelets, although the precise nature of these molecular interactions remains unclear. Detection of dengue virus RNA and the electron microscopy-based finding of DV-like particles in platelets isolated from dengue patients may, in part, support our in vitro results [17, 24]. Previous studies have reported increased levels of tumor necrosis factor α in the sera of DHF patients [25, 26] and suggested that this enhanced apoptosis of platelets is triggered by the extrinsic death pathway in the blood circulation of these patients [16]. Because the in vitro exposure of human platelets to DV2 led them to bind positively in the presence of a virus-specific antibody [27], DV may bind to platelets in patients with secondary DV infections who have increased serum IgG levels that recognize the infecting DV serotype.

Some similar biochemical pathways are used during platelet activation and platelet apoptosis [28]. In activated platelets, P-selectin is translocated from the α-granule and exposed on the platelet surface [29]. A previous in vitro study reported that exposure of DV2 increased expression of P-selectin, which is a marker of platelet activation and fibrinogen-binding properties in platelets [30]. In our preliminary study performed in 2008, we assessed the extent of the expression of P-selectin in 19 patients with secondary DV infections and 15 controls. The frequency of P-selectin expression on platelets was increased significantly in patients during the acute phase compared with controls, increased further during the early convalescence phase, and decreased slightly during the convalescence phase (data not shown). Therefore, the kinetics of the expression of P-selectin were distinct from those of platelet apoptosis observed in the present study, which is in agreement with a previous report of an in vitro study [31].

Platelet apoptosis correlated significantly with the platelet phagocytosis in our patients, as assessed using an ex vivo assay. This increased platelet phagocytosis was inhibited significantly in patients during the acute and early convalescence phases by pretreatment with the D89E mutant protein. These findings suggest that apoptotic platelets are phagocytosed via a PS-recognizing pathway in the systemic circulation of these patients.
patients. A recent report of the engulfment of platelets by monocytes in a nonhuman primate model of DV infection supports the present findings in our patients [32].

It is important to determine the role of the accelerated phagocytosis of apoptotic platelets in thrombocytopenia in this disease. With an adult blood volume of 5.0 L, approximately $5.0 \times 10^{10}$ apoptotic platelets may be cleared from the blood circulation each day because the concentration of apoptotic platelets exposing PS was approximately $1.0 \times 10^{10}$/L of blood in controls (Figure 3C). In contrast, the concentration of apoptotic platelets in our patients was $0.7 (0.4–1.10) \times 10^{10}$/L of blood in the acute phase, $1.3 (0.8–2.3) \times 10^{10}$/L of blood in the early convalescence phase, and $2.4 (0.8) \times 10^{10}$/L of blood in the convalescence phase. Collectively, our data indicate that approximately $6.5–12.0 \times 10^{10}$ apoptotic platelets may be cleared from the blood circulation in patients during the early convalescence and convalescence phases. However, the accelerated clearance of apoptotic platelets by macrophages appeared to be overcome rapidly by enhanced thrombopoiesis.

Figure 6. Effect of dengue virus 4 (DV4) or Japanese encephalitis virus (JEV) on platelet apoptosis determined using annexin V binding (A) and activation of caspase 3 (B) and on platelet phagocytosis (C) in vitro, and effect of DV1, DV2, or DV3 on platelet apoptosis in vitro (D). Percentage of apoptosis of platelets pretreated with a caspase inhibitor negative control (open bar) or platelets pretreated with a caspase 3 inhibitor (filled bar) from healthy donors cultured in L-15 medium containing DV of each serotype or JEV or in medium only. Percentage of phagocytosis of platelets pretreated with a caspase inhibitor negative control (open bar) or platelets pretreated with a caspase 3 inhibitor (filled bar) cultured in L-15 medium containing DV4 or in medium only for 72 h. $^{*}P<.0001$ (vs pretreatment with a caspase inhibitor negative control in medium only or in medium containing JEV or pretreatment with a caspase 3 inhibitor in medium containing DV4). $^{**}P<.05$ (vs pretreatment with a caspase 3 inhibitor in medium only or in medium containing JEV).

$^{*}P<.01$ (vs pretreatment with a caspase 3 inhibitor in medium only). $^{**}P<.0001$ (vs pretreatment with a caspase inhibitor negative control in medium only or in medium containing JEV).

$^{*}P<.0001$ (vs pretreatment with a caspase inhibitor negative control in medium only or pretreatment with a caspase 3 inhibitor in medium containing DV after 72 h and 96 h incubation). Data are expressed as the mean (SD) (9 wells per group from 3 independent experiments).
because platelet production can increase &gt;10-fold under the conditions of increased demand [33]. Accelerated platelet clearance, therefore, may not have affected the severity of thrombocytopenia in our patients.

Although the patients enrolled in this study were diagnosed based on the WHO case definition of 1997 [18], Deen et al emphasized the necessity of a reassessment of this WHO case definition because of several difficulties regarding its practical use [34]. Based on this background, the new WHO guidelines, published in 2009, include cases with severe bleeding and severe organ involvement [35], and a recent revised and expanded edition of dengue guidelines, published in 2011, includes dengue fever without unusual hemorrhage and expanded dengue syndrome [36]. However, our cohort did not include such cases with unusual manifestations; thus, the application of these new guidelines does not alter the findings of the present study. Further studies are required to determine the role of apoptotic platelet clearance in patients with primary infection or dengue shock syndrome and unusual manifestations classified in the new guidelines [35, 36].

TPO is produced at a constant rate by the liver and enters the circulation where TPO receptors on platelets bind to and degrade this protein. Therefore, serum TPO levels correlate inversely with platelet production rate [37]. The levels of TPO were increased in the sera of our patients during the acute phase, which is consistent with the results of a previous study [38]. This finding may be explained by the suppression of megakaryocytes and reduction in platelet production, which have been reported in DHF patients in earlier studies [3, 4]. However, we found no correlation between platelet counts and serum TPO levels during the acute and convalescence phases in this study. The regulation of serum TPO levels in these patients appears to be complex and its underlying mechanisms remain uncertain.

In conclusion, the present data suggest the increased clearance of DV-induced apoptotic platelets by macrophages primarily via a PS-recognizing pathway in patients with secondary DV infection. To the best of our knowledge, this is the first evidence of platelet apoptosis and accelerated clearance of apoptotic platelets in this disease.

**Notes**

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