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Microparticles as prognostic biomarkers in dengue virus infection

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ABSTRACT

Promising biomarkers which may help predict the risk of developing severe dengue virus infection (DVI) are lacking and will be helpful. Thus the main aim of this study was to analyze the role of cell-derived microparticles (MP) in DVI. Sixty patients with DVI i.e. 18: dengue with warning signs (DWS); 1: DSS and 41: dengue without warning signs (DWOS); along with 15 controls (other febrile illness) were included in the study. The following MPs were assessed: annexinV, platelet (CD41a), red blood cell (RBC) (CD235a) and activated endothelial (CD62e) MPs. Patients with profound thrombocytopenia without bleeding had statistically elevated platelet MP (PMP) levels when compared to patients with profound thrombocytopenia with bleeding ($p < .001$). RBC MPs were found to be significantly elevated in the 2nd phase in patient with DWS which was seen earliest on day 4 of infection with a cut off of ≥ 2200 MPs/μl when compared to patients with DWOS ($p < .0001$). PMPs may prove to be a promising novel biomarker which helps discriminate patients in need of prophylactic platelet transfusion from those who do not. RBC MPs, on the other hand could be potential biomarkers capable of identifying potentially severe patients who require immediate care. Thus, MPs seem to be a promising important biomarker in many aspects of DVI.

1. Introduction

Dengue is an infectious, flu- like systemic disease transmitted by *Aedes* mosquitoes to humans. Across the world, approximately 3.6 billion people are at risk of getting infected with dengue virus infection (DVI); 50 million being infected per year globally (Bhatt et al., 2013) with 34% being contributed by India (Chakravarti et al., 2012). From 2006 to 2012, an average of six million individuals per year in India had symptomatic illness with DVI; many cases go unreported (Shepard et al., 2014).

DVI manifests as a spectrum of illnesses ranging from mild febrile illness to the severe forms, i.e. dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). According to the recent WHO guidelines, cases are now classified as dengue without warning signs (DWOS), dengue with warning signs (DWS) and severe dengue (World Health Organization, 2009). There are no vaccines or specific therapeutics currently available. Rapid diagnosis along with appropriate immediate fluid replacement is what is essential to achieve success in these cases. Promising biomarkers which may help predict the risk of developing severe DVI are lacking.

Microparticles (MPs) are small (0.1–1 μm) cell-derived phospholipid membrane vesicles produced either spontaneously or in response to various stimuli such as cell activation, apoptosis or stress (Piccin et al., 2007). There are very limited studies on the role of MPs in DVI (Hottz et al., 2013; Punyadee et al., 2015), suggesting that MPs may act as promising biomarkers for predicting the severity. In India, till date there are no studies on the role of MPs in DVI.

2. Material and methods

2.1. Ethics approval

The study was approved by the Institutional Ethics Committee Review Board i.e. "Institutional Committee for Research on Human Subjects, National Institute of Immunohaematology (ICMR)". A written informed consent was obtained from all participants and all investigations were conducted according to the principles expressed in the Declaration of Helsinki.

2.2. Patients and controls

During the monsoon season from July 2016 to October 2016, patients with fever visiting the Department of Medicine, KEM Hospital are generally tested for a panel of infections by the clinicians. Being a

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preliminary study, 60 DVI patients who tested positive for both- specific IgG and IgM capture ELISA and dengue NS1 antigen test were included in the study. Controls were 15 patients showing symptoms but tested negative for DVI, i.e. other febrile illness. The day of fever was calculated according to the onset of fever and not from the day of enrollment in the hospital. The main aim was to analyze the role played by circulating microparticles in DVI. The normal ranges of different MP levels in healthy subjects were assessed in our earlier studies (Patil et al., 2013).

2.3. Study design

Citrate blood samples were collected at least twice as the infection progressed, either in febrile phase which is from Day 1–3, critical phase: Day 4–7 or recovery phase: ≥8 days. MP assessment by flow cytometry (standardized by participating in the International Society of Thrombosis and Haemostasis workshop) was carried out as described earlier (Patil et al., 2013). In short, Megamix beads (Biocytex, Marseille, France) which contains 0.5, 0.9 and 3 μm beads are used so as to make the microparticle gate on forward scatter (FSC) versus side scatter (SSC) graph which includes all events 0.9 μm and below and excludes all events 1 μm and above as seen in our earlier study (Patil et al., 2013). AnnexinV, platelet derived, red blood cell (RBC) and activated endothelial MPs were assessed using fluorescein isothiocyanate (FITC) labeled annexin V, phycoerythrin (PE) labeled CD 41 (CD41-PE, IgG_1, K, clone HIP8), PE labeled CD235a (CD 235a-PE, IgG_{2b}, K, clone GA-R2 (HIR2)) and PE labeled CD62e (CD 62e- PE, IgG1, K, clone 68-5H11) respectively. All the antibodies and buffers were provided by BD Biosciences, San Jose, CA. Cell blood count (CBC) and Di-dimer test by latex agglutination technique, were also performed.

2.4. Flow cytometry analysis of MPs

For absolute counts i.e. absolute numbers MPs per microliter of plasma, 30 μl of Flow CountTM fluorospheres (Beckman-Coulter, Marseille, France) is added to each tube. The total no. of MPs is calculated using the formula:

Microparticles/μl = Events in microparticle gate*[C/Flow count bead events]; C = Flow count Fluospheres assayed concentrations that are provided by the manufacturer as shown in previous study (Patil et al., 2013).

2.5. Statistical analysis

Elevated MP levels were defined as levels > 2 standard deviations (SD) from the mean of controls. MP levels were of the different groups were also compared versus controls using the 2 tailed Student's *t*-test. Statistical significance was assumed at $p < .05$, 95% confidence interval (CI). Data was analyzed using SPSS statistical software.

3. Results

3.1. Characteristics of patients and controls

The age of the patients ranged from 10 to 32 years; (mean = 24 years). The patients were classified according to WHO 2009 guidelines (World Health Organization, 2009) as

- 41 DWOS
- 18 DWS
- 1 DSS

The characteristics of the patients and controls are given in Table 1.

The platelet counts in our patients ranged from 4000 to 180,000 platelets/μl. Thrombocytopenia was seen in all DWS as well as few DWS patients. The Median platelet counts on Day 1–3, Day 4–7 and Day > 8 of patients with DWOS were 95500, 60000 and 46000; DWS were 92000, 44000 and 20000 and for 1 patient with DSS was 40000, 40000 and 29000 platelet/μl respectively. D Dimer test was positive in 35 patients on Day 6–7; 11 were strong positive (5 DWS, 1 DSS, and 5 DWOS). Both platelet count and D Dimer tests showed no statistical correlation to severity.

3.2. Platelet MPs

PMP levels in majority of our patients were found to be lower (536 ± 111 PMPs/μl) than that observed in healthy subjects (731.5 ± 377.3 PMPs/μl; cut off: > 1486 PMPs/μl) (Patil et al., 2013). Profound thrombocytopenia was seen in 13 patients out of whom 8 were without bleeding manifestations. Profound thrombocytopenia patients had a platelet count < 20000, range: 4000–20000 platelets/μl. Patients with profound thrombocytopenia with no bleeding manifestations had statistically elevated PMPs ($p < .001$, 95% CI: 922.53–2909.12) i.e. mean ± SD = 2270.6 ± 990.1 PMPs/μl when compared to those of the patients with profound thrombocytopenia having bleeding manifestations (mean ± SD = 354. 8 ± 88.3 PMPs/μl) (Fig. 1). Patients with no bleeding symptoms had either elevated PMPs or those with very low PMPs had a moderate platelet levels as seen in Fig. 1.

3.3. Red blood cell MPs

RBC MPs were statistically high in patients with DWS (Cut off: > 2200 MPs/μl) ($p < .0001$, 95% CI: 2946–4706) in the 2nd phase when compared to patients with DWOS (< 1100 MPs/μl) (normal range: 211–355 MPs/μl; cut off: > 427 MPs/μl) (Patil et al., 2013). This is seen earliest on Day 4 of infection. The MP profile of our controls was similar to that of DWOS. The scatter plot for the same is shown in Fig. 2.

In DWS patients, no matter how high the MPs rise, they gradually decrease as infection progresses and normalize as the patient's health improves. However, in the one case of DSS, the high levels of RBC MPs elevated even further from 5549 to 7414 RBC MPs/μl (Fig. 2). This patient expired 2 days after the last sample.

3.4. Activated endothelial cell derived MPs

No statistical significant difference was observed in different severities.

4. Discussion

This is the first report on the role of cell-derived MPs in DVI from India and there are only two such studies worldwide. One study showed that MPs harbored a viral envelope protein, NS1 protein on their surfaces and elevated RBC MPs directly correlated with disease severity, differentiating DHF from classical DVI (Punyadee et al., 2015). Another study showed increased expression of IL-1β in platelets and PMPs which enhances endothelial permeability in vitro (Hottz et al., 2013).

In our study, we had few patients with profound thrombocytopenia; generally if they have bleeding manifestations, platelet transfusion is recommended. However, for patients with thrombocytopenia without bleeding having a platelet count of < 20000 platelets/μl, the clinicians are in a dilemma whether prophylactic platelet transfusion should be given or not. We observed that patients with thrombocytopenia without bleeding had statistically elevated PMP levels. A low platelet count is known to cause bleeding manifestations. PMPs which are known to be procoagulant are derived from activated platelets, carrying all the surface antigens of platelets and thus functioning like platelets. It is possible that platelet MPs support stable hemostatic function in these patients. Thus such patients with a low platelet count but elevated PMP levels may be protected and not suffer from hemorrhagic manifestations