Quantification of circulating endothelial and progenitor cells as biomarkers for dengue virus infection

[1,4]Mohamed F. Elshal, [1]Taha A. Kumosani, [2]Essam I. Azhar, [3]Alia M. Aldahlawi

[1]Department of Biochemistry, Faculty of Sciences, Immunology Unit. KFMRC, King Abdulaziz University, Jeddah, Saudi Arabia
[2]Special Infectious Agent Unit (Biosafety Level 3) – King Fahd Medical Research Centre, King Abdulaziz University, Jeddah, Saudi Arabia
[3]Department of Biological Science, Faculty of Sciences, King Abdulaziz University, Jeddah, Saudi Arabia
[4] Department of Molecular Biology, Genetic Engineering and Biotechnology Institute, Sadat City University, Egypt.
Melshal2002@yahoo.com

Abstract: Dengue fever (DF) has emerged as a global health problem which is characterized by abrupt onset of plasma leakage. Endothelial dysfunction has been associated with vascular permeability leading to plasma leakage. The presence of circulating endothelial cells (CEC) has recently been recognized as a useful marker of endothelial dysfunction, whereas endothelial progenitor cells (EPCs) were found to be implicate in endothelial repair. The aim of this study is to assess the use of CECs and EPCs as biomarkers for vascular damage in patients infected with dengue virus confirmed by real time reverse transcriptase polymerase chain reaction (RT-PCR), IgM or IgG anti-dengue antibodies. Forty two blood samples were collected from patients with suspected dengue virus infection. Viral RNA was extracted from each serum sample then it was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) to detect dengue virus (DV). Anti-dengue immunoglobulin-M (IgM) and IgG antibodies was determined using capture ELISA Kits. Peripheral blood mononuclear cells (PBMCs) were stained with anti-human CD45, CD34, KDR (VEGFR-2) and CD146 antibodies to identify CEC by the phenotype $CD45^{-}CD146^{+}$, and EPCs as $CD45^{-}CD34^{+}VEGF\text{-}2^{+}$ cells. Mean CECs numbers was significantly higher in DV positive patients than healthy controls (ANOVA, $P<0.0001$). DV PCR-positive patients showed increased numbers of CECs compared to IgM and/or IgG positive cases and healthy subjects ($p<0.01$ and $p<0.0001$, respectively). Additionally, mean EPCs numbers was significantly higher in patients positive for DV by PCR than healthy controls and IgM and/or IgG positive cases and healthy subjects ($p<0.01$ and $p<0.001$, respectively). EPCs in IgM/IgG positive patients were significantly lower than that of Health Controls ($p<0.01$). In conclusion, the significant increases in both CECs and EPCs numbers in DV PCR-positive than DV IgM or IgG positive cases and healthy controls suggest that they might provide early, noninvasive methods to improve current diagnostic strategies of DV infection.
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1. Introduction

Dengue fever (DF) has emerged as a global health problem in the last two decades in countries of the tropical and subtropical regions (Kholedi et al., 2012). It is caused by dengue virus (DV) and is transmitted between people by the mosquitoes *Aedes Aegypti* and *Aedes albopictus*, which are found throughout the world. Today about 2.5 billion people, or 40% of the world's population, live in areas where there is a risk of dengue transmission is endemic in tropical and subtropical areas worldwide including west and south of Arabian Peninsula (Kholedi et al., 2012; Madani et al., 2013). The World Health Organization (WHO) estimates that 50 to 100 million infections occur yearly, including 500,000 DHF cases and 22,000 deaths, mostly among children (Murray et al., 2013).

Virus transmission requires a coincidence of large numbers of vector mosquitoes, large numbers of people with no immunity to one of the four virus types and the opportunity for contact between the two. DV are single-stranded, enveloped RNA flaviviruses of the Flaviviridae family that are classified into four serotypes (DEN-1, DEN-2, DEN-3, and DEN-4). Although these viruses are closely related, however infection with one of DV serotypes does not grant cross-protective immunity against other serotypes. Meanwhile, secondary infection with a heterogonous serotype is implicated in increase in the overall replication of the virus and a higher risk of severe dengue and an increased frequency of hospitalization as a result of antibody-dependent enhancement (Guabiraba and Ryffel, 2014).

Symptoms of infection usually begin 4 - 7 days after the mosquito bite and typically DF last 3-10 days. The clinical manifestations of patients infected with any of these four types are ranging from classical DF to life–threatening dengue hemorrhagic fever (DHF) that

is characterized by abrupt onset of vascular leakage and the dengue shock syndrome (DSS).

Evidence is increasing that a small group DV patients may develop severe clinical manifestations, including bleeding, organ impairment, and endothelial dysfunction with increased capillary permeability causing hypovolaemic shock that can lead to cardiovascular collapse (Yacoub et al., 2014). The pathogenesis of plasma leakage is not well understood, however it was proposed that it could be the result of the interplay between pathogens, host immune response, and endothelial cells that were found to elicit immune-enhancing responses to dengue virus infection (Dalrymple and Mackow, 2012). In the mean time, DV exerts immune attack against endothelial cells leading to their activation and expression of cytokine, chemokine and adhesion molecules that lead to endothelial cell dysfunction, sloughed off endothelial cells, and increased capillary permeability (Avirutnan et al., 2006; Murphy and Whitehead, 2011).

Measurement of circulating endothelial cells (CECs) shed from damaged endothelium been documented in a variety of vascular disorders (Blann et al., 2005). A related circulating cell population is endothelial progenitor cells (EPC), which originate from the bone marrow rather than from vessel walls. Seen in small numbers in healthy individuals, their numbers tend to increase following vascular injury (Kong et al., 2004). So far, experiments have established the ability of EPC to form colonies *in vitro*, suggesting a role in both angiogenesis and in the maintenance of existing vessel walls (Hristov and Weber, 2008).

Therefore, the objective of this study is to assess vascular damage in patients infected with dengue virus confirmed by real time reverse transcriptase polymerase chain reaction (RT-PCR) by detection and quantification of blood biomarkers of vascular damage namely CEC and EPC using flow cytometry. As well as, to determine any potential correlation between CEC and EPC counts with the different diagnostic status based on PCR, IgM or IgG.

2. Patients and Methods:

2.1. Patients

Total of 42 blood samples were collected from patients presented for diagnosis of suspected dengue virus infection at Special Infectious Agent Unit (Biosafety Level 3) – King Fahd Medical Research Centre of KAU in Jeddah during the period between January 2013 and December 2013. Patients' clinical and laboratory data including age, sex, duration of fever, complete blood counts (CBC) were collected. Ten milliliter blood specimens were collected in lithium heparin Vacutainer tubes (Becton Dickinson, Rutherford, NJ) and used within 12 h of sampling for flow cytometry protocol. While plasma samples were separated from blood within 1 to 3 h following blood collection and were aliquoted and stored at -80°C until use. Written consents prior to participation were taken from patients or one of their relatives. Twenty age matched healthy volunteer were used as negative control.

2.2. RT-PCR Assay for Dengue

Viral RNA was extracted from each serum sample using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Then reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect dengue virus as described before (Lanciotti et al., 1992).

2.3. Assay for Dengue IgM and IgG

The presence of anti-dengue immunoglobulin-M (IgM) and IgG antibodies was determined using capture ELISA Kits (Dengue Duo IgM & IgG Rapid Strip, Panbio Diagnostic, Queensland, Australia) according to the manufacturer's instruction.

2.4. Assays for CECs and EPCs

Peripheral blood mononuclear cells (PBMCs) were stained with anti-human CD45 antibody and anti-human CD146 antibody (BD Biosciences) to identify CEC as $CD45^{-}CD146^{+}$ cell population as previously described previously by Elshal et al. (2009). While EPCs were identified by the phenotype $CD45^{-}CD34^{+}VEGF\text{-}2^{+}$ cells as described previously by Peichev et al. (2000). Mouse isotype controls corresponding to each antibody were used to determine nonspecific binding. Analysis was done using a Flow cytometry and data were acquired using a Navios flow cytometer with Navios analysis software (Beckman Coulter). Cells were plotted according to forward scatter (FSC) and side scatter profiles (SSC) and a regions were drawn around cell population containing the lymphocyte. Normal CEC count by flow cytometry was 7.4 ± 3.4% and EPC 0.4 ±0.2% (Elshal et al., 2009; Peichev et al., 2000).

2.5. Statistical analysis:

Data were analyzed using SPSS version 11.5. Descriptive statistics were done by number and percent as well as mean, median and range. Unpaired student's t-*test* was used for comparison between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical characteristics

A total of 42 patients, 28 male and 14 female, with median age of 27 and range of 3-83 years were included in the present study. The average interval between the beginning of symptoms and blood sampling was 4 days (range 3–5 days). The clinical diagnosis of DV infection was determined based on dengue genomic RNA, IgM antibodies and IgG antibodies. From the 42 samples tested, 58.2% (24/42) of the sera were found to be positive for DV infection based on the IgM antibody, IgG antibody and PCR

tests. Of these, 42% of the samples (18/42) were PCR positive, 47.1% (20/42) were IgM and IgG positive for DV (table 1).

Table 1: Clinical and laboratory characteristics of patients studied

Age, years, median (range)	27 (3-83)
Days of hospitalization, median (range)	3 (2-7)
General malaise, N (%)	42 (100)
Myalgias, N (%)	42 (100)
Bleeding, N (%)	9 (21)
IgM and IgG anti-DV positive, N (%)	20 (47)
Dengue RT-PCR positive, N (%)	18 (42)

3.2. Detection of CECs

For CEC detection, a flow cytometry analysis of peripheral blood stained with monoclonal antibodies against human CD146 and CD45 was used, and a region was drawn to include lymphocytes and monocytes populations (Figure 1A). We detected the presence of CECs according to the phenotype $CD45^-CD146^+$ (Figure 1B, C). The mean CECs numbers was significantly higher in dengue positive patients than healthy controls (ANOVA, $P<0.0001$). Patients with DV positive PCR showed increased numbers of CECs compared to IgM and/or IgG positive cases and healthy subjects ($p< 0.01$ and $p< 0.0001$, respectively) (Figure 2).

3.3. Detection of EPCs

Circulating EPCs were determined using Flow cytometry analysis after staining it with monoclonal antibodies against human CD34, KDR (VEGFR-2) and CD45. Two flow cytometric regions were used, first (R1) surrounded lumphocytes and monocytes (Figure 3A). The second region (R2) surrounds cells that are negative for CD45 cells. EPCs were characterized by the phenotype $CD45^-KDR^+CD34^+$ (Figure 3C, D). The mean EPCs numbers was significantly higher in patients positive for DV by PCR than healthy controls and IgM and/or IgG positive cases and healthy subjects ($P<0.01$ and $P<0.001$, respectively). EPCs in IgM/IgG positive patients were significantly lower than that of Health Controls ($P<0.01$) (Figure 4).

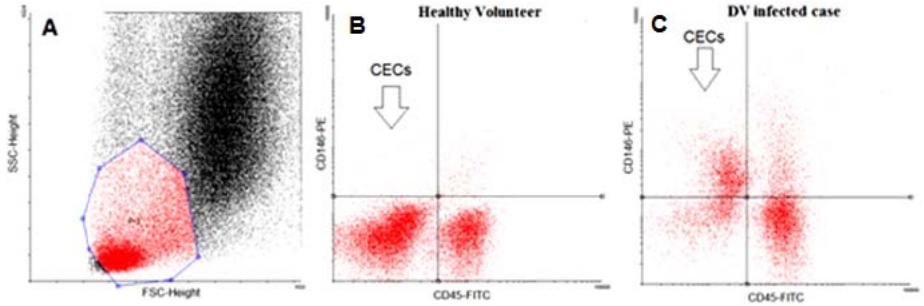

Figure 1: Representative flow cytometry plots of CECs. A: gate area surrounds lymphocytes and monocytes. B and C: CECs in healthy and DV infected case respectively.

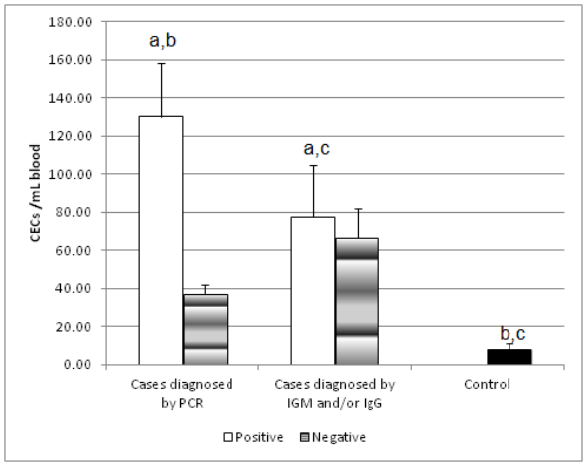

Figure 2: Mean numbers of CECs (a) Significant compared to healthy control, (b) Significant compared to positive IgM/IgG, (c) Significant compared to PCR.

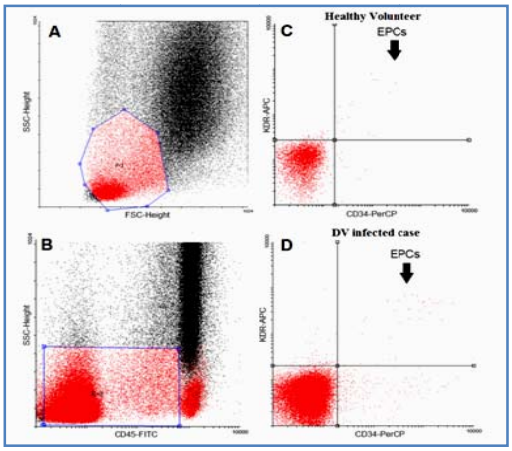

Figure 4: Representative flow cytometry plots of EPCs for healthy volunteer and DV infected case. A: R1 gates lymphocytes and monocytes. B, R2 gates cells negative for CD45. C and D: EPCs in healthy and DV infected case respectively.

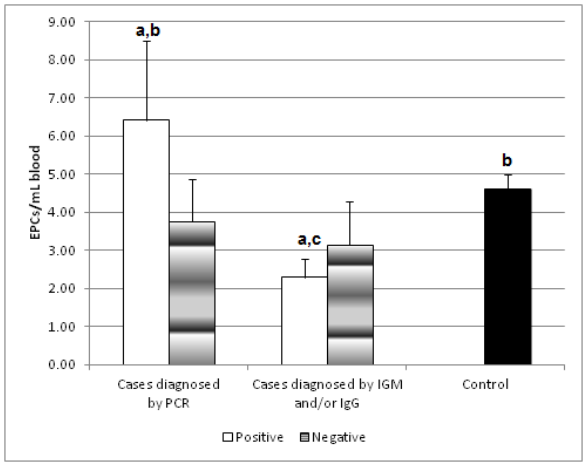

Figure 4: Mean numbers of EECs in the different studied groups. (a) Significant compared to healthy control, (b) Significant compared to positive IgM/IgG, (c) Significant compared to PCR.

4. Discussion:

DF is an acute, potentially life-threatening disease that is caused by DV. There are difficulties in the diagnosis of DF that may cause a small proportion of patients to develop DHF that is characterized by capillary extravasations, thrombocytopenia and acute mucosal bleeding. These complications of capillary leakage can quickly lead to hypotension, vascular dysfunction and cardiovascular collapse that finally may lead to their death (Yacoub et al., 2014).

Capillary leakage develops rapidly, usually over a period of hours, and resolves within 1 to 2 days, and usually becomes evident as early as the 3rd day of illness (Srikiatkhachorn et al., 2007), even before the time it takes to develop antiviral immune response in the form of IgM that is initially detectable between 5 to 6 days post onset of fever and/or early convalescent phase (more than 6 days of symptoms) (Sang et al., 1998).

Therefore the availability of a rapid, sensitive and specific method to detect dengue virus infection is one of the most important factors necessary for early detection and prevention of fatal capillary leakage complicated with bleeding.

Although numerous clinical studies found that early diagnosis of acute dengue fever patients could be obtained by detection of virus RNA using RT-PCR or by IgM capture enzyme-linked immunosorbent assay (Chow, 1997; Schilling et al., 2004), however, the use RT-PCR is advantageous in that it can detect infection within 5 days of illness onset, whereas IgM antibodies assay is preferred for those presenting later (Singh et al., 2006). Nevertheless, there is no assay that allows the screening and/or diagnosis of capillary leakage associated with DV infection. Therefore, we aimed to assess the use of CECs and EPCs as markers of vascular damage associated capillary leakage. CECs have been established as a specific and sensitive marker of vascular dysfunction and damage in a variety of diseases affecting the vasculature (Blann et al., 2005; Elshal et al., 2009), however there is scarce information about their status in DF.

In the present study, the mean CECs numbers was significantly higher in Dengue positive patients than healthy controls (ANOVA, $p<0.0001$). Patients with PCR-positive for DV showed increased numbers of CECs compared to IgM and/or IgG positive cases and healthy subjects ($p<0.01$ and $p<0.0001$, respectively). Additionally, PCR-positive for DV showed significantly higher ($p<0.0001$) CECs numbers than DV PCR-negative cases. Whereas no significant differences were found between positive and negative cases diagnosed by of IgM or IgG assays. This would suggest that endothelial damage is an early process that occurs even before the emergence of IgM, which could take from 5 to 10 days do develop as previously reported by Yap et al. (2011). These data also support previous findings that dengue virus targets the endothelium leading to severe endothelial dysfunctions that often cause disturbed permeability that consequently leads to capillary leakage, edema, hemorrhages and organ failure (Cardier et al., 2005; Lin et al., 2003). This increase in numbers of CECs indicates a disturbance in the endothelial monolayer of blood vessels which its integrity is a crucial process for proper endothelial function. Therefore it is sought that these detached endothelial cells may be replaced by proliferation and migration of adjacent endothelial cells. However, previous reports suggest another mechanism of repair of injured endothelium that is the mobilization and differentiation of bone marrow-derived progenitor cells to endothelial cells (Costiniuk et al., 2013; Tsai et al., 2012).

Bone marrow-derived endothelial progenitor cells known as EPCs constitute a linage of immature cells originate from the bone marrow, rather than from injured vessel walls and play a role in postnatal angiogenesis (Khurana and Simons, 2003). They share common characteristics with various precursor cells including haematopoietic stem cells (HSC), the haemangioblast; and non-HSC, and it have also been shown to differentiate into mature endothelial cells (EC) (Planat-Benard et al., 2004). EPCs are found in small numbers in healthy individuals, however, their numbers tend to increase in response to angiogenic stress and vascular injury that were found to induce mobilization of EPCs to in the peripheral circulation (Gill et al., 2001; Kong et al., 2004). The process of EPCs mobilization from bone marrow occurred as a result of stimulated secretion of cytokines/chemokines by various endothelial stress factors such as drugs and

behavioral practices (Van Craenenbroeck and Conraads, 2010), and insulting factors including viral infections (Krautkramer et al., 2014). After mobilization to peripheral circulation, EPCs home to sites of endothelial injury and ischemia, where they proliferate, differentiate and integrate into the endothelial layer or exert a paracrine function by producing vascular growth factors (Hristov and Weber, 2008). Many infectious diseases are associated with endothelial damage leading to organ failure. For example, sepsis induced multiple organ failure is a consequence of altered endothelial function induced by infection and subsequent host response (Rafat et al., 2007). Later on, studies identified the key role of endothelial progenitor cells in the outcome of severe sepsis (Fan et al., 2014).

In our data we found a significant increase in the mean number of EPCs ($P<0.0001$) in PCR-positive cases compared with PCR-negative cases and compared with healthy control ($P<0.01$). These findings may indicate that there is increased mobilization of EPCs in response to the viral infection. Conversely, both IgM and/or IgG testing characterizing late-stage disease, EPCs numbers were significantly lower than that of cases positive by PCR. The increase in EPCs number in PCR-positive cases would suggest rapid but transit mobilization of bone marrow EPCs as previously demonstrated by Gill et al. (2001).

On the other hand, the lower number of EPCs in IgM or IgG positive cases compared with PCR-positive cases may indicate that a negative impact of DV antibodies on EPCs (Lin et al., 2003), suggesting a narrow gap of time for vascular damage to be repair early in the acute phase, and accompany virus spreading phase. Previous studies reported that the effect of DV isolates on primary methylcellulose progenitor cells cultures had no inhibitory of colony formation. However, after an initial 8-day liquid culture, inhibition of colony formation was observed suggesting that dengue virus impaired progenitor cell growth (Murgue et al., 1997). Another report suggests that the gradual decrease in EPCs following vascular trauma is attributed to their homing to sites of injuries (Lin et al., 2003).

These data support our findings of the negative effects of DV on EPCs is a late process that may coincide with the emergence of IgM humoral immune response. However, this not precludes the possibility of EPCs homing to sites of virally affected vasculature. Therefore, it would be of great interest to analyze the number of apoptotic EPCs to verify if the cause of loss in numbers of EPCs is due to cell death or other factors.

Conclusions:

Endothelial dysfunction in dengue infection considered the main cause of plasma leakage, edema and life threatening hemorrhage. The detached endothelial cells from the intimal monolayer in response to endothelial injury consists the major source of CECs, which we found significantly higher in DV positive patients than healthy controls. The increase in CECs was found both in PCR positive and in IgM and/or IgG positively diagnosed DV patients, however, CECs was higher in PCR-diagnosed than IgM or IgG diagnosed cases, suggesting it as an early biomarker for DV even before IgM titer increases. In the mean time, EPCs, which are produced from the bone marrow in response to vascular insults, was found increased early in the course of the disease even before detection of IgM or IgG DV antibodies. These data may suggest that determination of CECs and EPCs might provide early, noninvasive methods to improve current diagnostic strategies of DV infection.

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Corresponding author

Mohamed F. Elshal,
Department of Biochemistry, Faculty of Sciences, King Abdulaziz University, Jeddah, Saudi Arabia
Email: melshal2002@yahoo.com

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