Flow cytometric evaluation of circulating endothelial cells: A new protocol for identifying endothelial cells at several stages of differentiation

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Several factors may influence the analysis of endothelial cells (ECs) by flow cytometry: separation of mononuclear cell, washing and centrifugation steps, panel of monoclonal antibodies, and the lack of standardization of gating technique. Therefore, the reliable quantification of ECs remains a technical challenge. The purpose of this study is to define a new flow cytometric protocol to characterize and quantitate ECs. In previous investigations, increased numbers of circulating ECs have been found in sickle cell disease. The patients with sickle cell disease might provide useful material for the study. We performed flow cytometry on whole blood from 20 normal controls and 31 patients with sickle cell disease (20 patients with steady-state disease and 11 patients with vaso-occlusive crises) using a lyse/no-wash procedure, specific and non-specific antibody combinations (CD146, CD144, CD34, and CD117), and broad gating. This protocol produced much higher values for the number of circulating ECs (a mean of 2,396.55 ± 658.37 ECs/mL in controls vs 6,709.80 ± 1,772.32 ECs/mL in the steady-state group, or 18,213.50 ± 8,451 in the vaso-occlusive crises group, P < 0.001 for both), and also showed variable EC size and granularity, which may reflect activated, or early release ECs. This novel protocol performed comparably in terms of reproducibility, reliability, and dilution linearity with a previously described protocol. This approach has significant advantages for the characterization and quantitation ECs compatible with the pathophysiology. Using the specific antibodies, CD146 and CD144, together may give more informative EC data than the general approach used.

Introduction

Endothelial cells (ECs) are a population of cells in peripheral blood [1]. These cells exhibit nonspecific hematopoietic stem cell and/or progenitor cell markers (CD34, CD117, or AC133), as well as specific endothelial markers (CD144, or CD146) [1–3]. The characterization and quantification of EC in the peripheral blood can provide a diagnostic or therapeutic advantage for diseases associated with endothelial damage because ECs are angiogenic and replace dysfunctional endothelium [4–7]. Under steady-state conditions in humans, the number of ECs in the circulation are relatively low (104 to 106 of all mononuclear cells present) [8–10]. Significant site-to-site variation has been observed with the current flow cytometry methods used to determine the percentage and absolute numbers of ECs. Washing and centrifugation steps, panel of monoclonal antibodies, and the gating technique may influence the determination. At present, there is no standardized method for determining the number of circulating ECs, and there are no data on their in vivo lifespan or maturation process [10–13]. To investigate ECs, some authors have used adherence cultures of total mononuclear cells or mononuclear cells preselected by antibodies (inserted in magnetic microbeads) that bind to surface markers such as CD133, CD34, or CD31 [1,3,11,14–18]. Most authors have used the monoclonal antibodies, CD146 or CD144, for identification of ECs, but not their combination. The most widely used EC marker, CD146 (melanoma cell-adhesion molecule), also labels melanoma cells, smooth muscle cells, intermediate trophoblasts, and activated T-cells [19]. The surface marker CD144 (vascular endothelial cadherin) labels ECs and fetal liver hematopoietic tissue cells [20]. These measurements show a wide variation in EC number because of rapid changes in endothelial surface markers with activation or maturation [8, 11–13]. In addition, we hypothesized that some ECs might be lost during the separation of mononuclear cells from other peripheral blood cells. Moreover, analyzing the mononuclear cell region of flow cytometry may not identify all circulating ECs because they may have different size and granularity. The goal of this study was to develop a new and improved flow cytometry protocol to characterize and quantitate circulating ECs. First, we compared the performance characteristics of our protocol with a protocol that is generally used by investigators. Second, we studied whether our protocol could measure changes in a disease where endothelial activation is evident (sickle cell disease).

Patients and Methods

Subjects and sample collection

Thirty-one patients with sickle cell disease (11 women and 20 men; aged 17–45 years) and 20 healthy volunteers (11 women and 9 men; aged 20–27 years) were enrolled in the study. The sickle cell patient group was divided into two subgroups: individuals with painful vaso-occlusive crises (3 women and 8 men) and those with steady-state disease (pain free and without an acute clinical event in the 30 days before sampling; 8 women and 12 men). A peripheral blood sample

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TABLE I. Quantification of Circulating Endothelial Cells

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Vaso-occlusive crises mean ± SD (cells/mL) (n = 11)</th>
<th>Steady state mean ± SD (cells/mL) (n = 20)</th>
<th>Controls mean ± SD (cells/mL) (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECs</td>
<td>18213.5 ± 8451.2b</td>
<td>6709.6 ± 177.3c</td>
<td>2396.5 ± 658.3c</td>
</tr>
<tr>
<td>Mature ECs</td>
<td>4699.2 ± 5515.2</td>
<td>1257.3 ± 917.4</td>
<td>596.5 ± 563.2</td>
</tr>
<tr>
<td>Late progenitor ECs</td>
<td>9875.6 ± 692.36a</td>
<td>3986.05 ± 1960.78b</td>
<td>1066.7 ± 533.12bc</td>
</tr>
<tr>
<td>Early progenitor ECs</td>
<td>3638.18 ± 1546.86a</td>
<td>1465.80 ± 635.79hb</td>
<td>733.35 ± 463.97c</td>
</tr>
</tbody>
</table>

ECs, endothelial cells.

*P < 0.05; **P < 0.01; ***P < 0.001 between Vaso-occlusive group and steady state group.

**P < 0.05; ***P < 0.001 between Steady state and Controls.

***P < 0.001 between Vaso-occlusive group and Controls.

was collected from each subject and placed into an EDTA tube via a 21-gauge needle. Anticoagulated blood samples were kept at 4°C and analyzed by flow cytometry within 4 hr of venesection. The exclusion criteria for this study were active infection; acute coronary syndrome; diabetes mellitus; smoking; menstruation during the study; the use of statins, hydroxyurea, or estrogen; and overt heart failure. Patients with intravascular instrumentation were also excluded. The institutional review board of the Baskent University, Ankara, Turkey, approved this research program. All subjects provided informed consent.

Immunophenotyping of ECs

Flow cytometry. Peripheral whole blood cells were prepared by a lyse/no-wash procedure [21] and were then evaluated by flow cytometry (Coulter Epics XL-MCL, Beckman Coulter, FL). The use of this procedure minimizes the cell loss, making it possible to perform the measurement of the all ECs in the blood samples. A multistep manual technique was used to detect and quantify ECs. Assessment of a minimum of 60,000 cells/peripheral blood sample was considered informative. A new panel of monoclonal antibodies (anti-CD146, CD144, CD34, and CD117) was used to detect ECs. The antibodies were conjugated with fluorescein isothiocyanate (FITC-CD146, US Biological, MA), phycoerythrin (PE-CD144, Beckman Coulter, Marseille, France), Texas red (ECD-CD34, Beckman Coulter), and cyanin-5 (PC-5-CD117, Beckman Coulter). The 7-aminoactinomycin-D viability dye (7-AAD, Beckman Coulter) was used to measure EC viability. Cells were identified as early progenitor ECs if they were CD146+CD144+CD34+CD117+ cells. Cells were identified as late progenitor ECs if they were CD146+CD144+CD34+CD117- cells. Finally, cells were identified as mature ECs if they were CD34−CD117−CD146−CD144+ cells. Flow cytometry data were analyzed with EXPO 32 ADC software (Beckman Coulter, Miami, FL).

Gating and quantification of ECs. Four-color flow cytometry was used to count ECs. We first broadly selected all the cells on a forward scatter (FS)/side scatter (SS) plot, and then looked for CD146 + 144+ cells on a second 2D plot. A region of doubly positive cells with known light scatter properties was labeled ECs. Further gating was made to display the two progenitor markers in another 2D plot. ECs were introduced first to CD34/SS (late and early progenitor ECs), then to CD117/SS (early progenitor ECs). ECs were also observed by forward scatter FS/SS to determine their size and granularity. The percentage of ECs was determined as a percentage of total events (after the exclusion of debris) (Fig. 1). The absolute EC count in the sample was then calculated by multiplying this percentage by the absolute white cell count provided by the hematology analyzer (Cell Dyne 3700, Abbott Laboratories, Chicago, IL) after correction to exclude nucleated red cells [21]. Nucleated cells, platelets, debris, and nonspecific binding were excluded from the analyses by isotopic control and consecutive gating. Each tube was also subjected to real-time viability testing with 7-AAD to identify nonviable cells.

Performance Characteristics

Reproducibility. Reproducibility was assessed for our EC assay protocol (protocol 1) and for a previously described protocol [1] (protocol 2). Protocol 2 uses monoclonal cell isolation, lysis, and wash procedures during preparation, and a gating region around mononuclear cells during analysis. Therefore, human umbilical venous ECs from the HUVEC cell line (ECV304, American Type Culture Collection, Rockville, MD) were cultured [22]. Purified ECs (10^7/mL) were added to a peripheral whole blood sample of a healthy individual. Ten replicates from this single sample were processed and acquired by the same technician for each method. For the two protocols, the same nucleated cell count was used for all the calculations.

Reliability. Seven replicates from peripheral whole blood samples from a healthy individual were processed and acquired at two different times (initially and 2 hr after initial assessment) by one staff (intra-reliability) trained in flow cytometry. The test was repeated for protocol 2 with three replicates from this single sample. The sample required evaluation within 2 hr to prevent cell death. The test could not be done more than three times from a single sample with protocol 2 because of cell loss during the lysis and wash procedures.

Linearity studies and recovery rate. To determine linearity, a whole peripheral blood sample from a healthy individual was diluted with the assay buffer (phosphate buffered solution), making serial dilutions such as 1/1, 1/2, 1/4, and 1/8, and then quantitated. The recovery rate was calculated based on:

Percent recovery rate = [(measured value − (added value))/(Added value)] × 100

Morphologic characteristics of ECs in circulation

Positive and negative CD34 selection was performed on a peripheral whole blood sample from each of the 20 patients with steady-state sickle cell disease to identify both CD34+ ECs and CD34− ECs. This testing was performed with a MidiMACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were subjected to centrifugation and stained with a complex of low-density lipoprotein cholesterol from human plasma and acetylated-Dil (DiAc-LDL, Molecular Probes, Eugene, OR), and lectin from Ulex europaeus (EUA I, FITC labeled ulex-lectin, Sigma, St. Louis, MO). The cells were then observed with an immunofluorescence microscope (Nikon Eclipse E 600, Tokyo, Japan), and microphotographs were taken with a camera (Nikon Coolpix 4500, Tokyo, Japan). Cells that stained positive were identified as ECs. The size and granularity of the ECs at the CD146/CD144 FS and SS gates were investigated via flow cytometry.

In vitro tube test

Peripheral blood samples from patients with steady-state sickle cell disease were incubated in a 5-mL Falcon tube at 37°C in 5% CO2 for 12 hr, and ECs were analyzed before and after incubation.

Statistical analysis

Assay performance characteristics were analyzed by calculating intra-method coefficients of variation (CV), a measure of the proportion of variance attributable to individuals. Reliability and correlation between the two protocols was estimated with a Pearson correlation test. A correlation of 1 indicates perfect agreement, whereas a value of 0 indicates agreement similar to chance, and negative values show agreement worse than chance. Means and standard deviations (SD), as well as the range, which includes a 95% confidence interval (CI), were also calculated. Linear regression analysis was applied for dilution tests.

Differences in ECs counts between the groups were analyzed by the Mann–Whitney U test. Calculations were made with SPSS software (Statistical Package for the Social Sciences, version 11.5, SPSS, Chicago, IL).

A P value < 0.05 was considered significant.

Results

Flow cytometry and EC morphology in study groups

Both groups of patients with sickle cell disease (with steady-state disease and with vaso-occlusive crises) had significantly more ECs, including early progenitor ECs and...
late progenitor ECs, than did control subjects ($P < 0.001$, for all; Table I). Patients with vaso-occlusive crises had significantly more ECs, including early progenitor ECs and late progenitor ECs, than those who did not ($P < 0.001$). Both groups of patients with sickle cell disease had significantly higher numbers of mature ECs ($P = 0.027$ for patients with steady-state disease and $P < 0.019$ for those with vaso-occlusive crises) than did controls. No significant difference was found in the numbers of mature ECs between the groups with sickle cell disease ($P > 0.05$).

Some ECs showed high intensity, indicating that they contained more granules or were larger than other ECs (Fig. 1F,G). CD34+ EC cells also showed marked variations in granularity and size. Immunofluorescence microscopy revealed that some stained ECs resembled lymphocytes with scant cytoplasm, and others exhibited granules, a
large nucleus, and abundant cytoplasm (Fig. 2). We noted low numbers of CD34-cells that co-expressed the endothelial markers, CD146 and CD144. This result was supported by the finding that immunofluorescence microscopy showed circulating CD34− ECs co-labeled with Dil-AcLDL and ulex-lectin (Fig. 3). Specific performance data

Reproducibility: The mean number of ECs per mL was 6,214.0 ± 406.7; (95% CI 5,923.0–6,504.9) and 1,718.1 ± 632.8; (95% CI 1,265.4–2,170.7) by protocol 1 and protocol 2, respectively (Fig. 4). The CV was 15% for protocol 1 and 25% for protocol 2. A poor correlation was found
between protocols 1 and 2 ($r = -0.06, P = 0.8$). Reliability: We demonstrated that both protocol 1 and protocol 2 are reliable ($r = 0.86$ and $r = 0.87$, respectively). Linearity studies: Protocol 1 showed better linearity than protocol 2 ($R^2 = 0.97$ vs $R^2 = 0.69$, respectively). Recovery rate: The recovery rates for diluted samples were more than 10% for both protocols (range 24–34% for protocol 1 and 15–77% for protocol 2).

**In vitro tube test results.** EC granularity in peripheral blood increased after 12 hr of incubation (data not shown).

**Discussion**

Changes in the numbers of circulating ECs are becoming prognostic criteria for various clinical events, such as those associated with cardiovascular disease [24–26]. The quantification of circulating ECs could indicate the presence of endothelial injury, and is a simple method of evaluating endothelial-related physiologic and pathophysiologic states. Flow cytometry is a method of choice for quantification of ECs. Numerous attempts have been made to standardize flow cytometric protocols, but results from these studies show a high degree of variability among centers [8,27]. The reliable quantification of the ECs remains a technical challenge. We therefore tested a novel protocol of quantifying and functionally characterizing circulating ECs. Our protocol could reflect the pathophysiology of sickle cell disease, both during steady state and vaso-occlusive crisis. Performance studies demonstrated that our protocol showed superior reproducibility, reliability, dilution linearity, and recovery rate.

Here we list problems with the common protocol, and how our protocol overcomes these limitations: (1) **Mononuclear cell isolation:** ECs are a subpopulation of the mononuclear cell lineage, so analyses are made after mononuclear cell isolation [13,18]. However, cell isolation, centrifugation, and cell-washing procedures may cause the loss of cells and the exclusion of large, granulated ECs. To minimize EC loss, we avoided such procedures. Indeed we isolated fewer ECs using protocol 2 after cell-washing and separation. (2) **EC culture:** Mononuclear cells are analyzed after isolation by density-gradient centrifugation then cultured on fibronectin-coated plates [1]. However, it is unclear whether the in vivo and in vitro maturation processes match. Therefore, we analyzed ECs in peripheral blood rather than generating cultures. (3) **CD133+ or CD34+ cell selection:** Cell loss can occur during cell selection by magnetic microbeads [13,28]. ECs also develop from mesenchymal CD34-progenitor cells [29], and CD133- or CD34- ECs will be excluded by this procedure. Our protocol can identify CD34-ECs (4) **Incorrect gating strategy:** Analyzing only CD45- cells leads to the exclusion of possible CD45+ ECs. ECs are evaluated as progenitors, which resemble lymphocytes, and so cells in the lymphocyte region in FS and SS are gated during flow cytometry [26,30]. This approach might cause the exclusion of ECs with different morphologic features, as we saw with our data showing variable EC size and morphology. Using broad selection criteria on an FS/SS plot identified circulating ECs that were larger and more granulated than lymphocytes, and that carried early markers (CD34, CD117) associated with EC-specific markers (CD146, CD144). These findings are further evidence that ECs exhibit heterogenous phenotype. To prevent the exclusion of cells, flow cytometry analysis should use multiple monoclonal antibodies specific to ECs. (5) **Use of endothelial markers:** There are limited studies on EC identification with multiple cell surface markers, and few specific markers exist for ECs. Furthermore, the maturation process of ECs is not clearly understood, so the use of various marker combinations is necessary. The endothelial marker CD146, which is often used in EC analyses, is also expressed on active T lymphocytes [8,31]. The surface marker, CD144 (VE-cadherin), is expressed on both ECs and fetal hematopoietic stem cells [20,32,33]. For that reason, CD146/CD144 surface markers were used simultaneously to identify ECs during the flow cytometric analyses.

If a correlation between the quantity of circulating ECs and pathologic conditions could be established, circulating ECs could be useful in the diagnosis of vascular disease, in the explanation of pathophysiologic factors, in the prognostic evaluation of disease progression, and/or in the evaluation of treatment efficacy. In this study, we used sickle cell disease as a clinical model to develop a standardized EC analysis method. Sickle cell disease is a vascular disease that causes tissue ischemia and exaggerated endothelial turnover, both of which are characterized by evident endothelial injury and regeneration [34]. In different patient groups, the number of ECs/mL of peripheral blood may vary between zero and 1,500; most studies found 1 to 100 circulating ECs/mL in healthy people [34–40]. We found much higher numbers of circulating ECs in both sickle cell patients and controls, probably due to methodological differences in sample preparation and cell quantification.

A critical point in flow cytometry is the nonspecific binding of monoclonal antibodies to debris, thrombocytes, erythroblasts, and sickled erythrocytes. In our 4-color immunologic study, such nonspecific binding was excluded by the use of CD146/CD144/CD34/CD117 monoclonal antibodies with a consecutive gating strategy. We used CD34-negative cells as a positive control to identify false-positive binding. Debris, thrombocytes, and erythroblasts were excluded at the bottom of the FS-SS gate. Binding of isotype controls to cells may be the result of dead or dying cells. To minimize this type of nonspecific binding we also used 7-AAD to exclude dead cells.

ECs are of various sizes, shapes, and granularity in sickle-cell patients. The phenotypic heterogeneity of circulating ECs depends on the pathologic situation. The reason for this phenotypic heterogeneity is not known, but might originate in different vascular beds, or it may be a response of the endothelium to various pathologic stimuli [10]. How-
ever, our findings suggest that heterogeneity can also occur in the same pathophysiologic situation, a finding that should be carefully considered during EC analysis.

Studies on the maturation process of circulating ECs usually rely on culture experiments, which may not represent the physiological state [13,41,42]. To evaluate the maturation process of ECs, flow cytometry was performed after the initial tube test and 12 hr later. The cells had developed into brighter and/or more mature cells at 12 hr. Thus, EC maturation may occur more rapidly than was previously thought, and the timescale of alterations in maturation may be expressed in hours. Studies in peripheral blood samples from patients with sickle cell disease, which contain a large number of ECs and cytokines, can provide insight into these processes in vitro [28]. Future studies that evaluate the maturation of cells from healthy subjects and in different pathophysiologic conditions may be useful.

The cell surface markers and maturation results presented in this report challenge previously published results [10–13]. Our data provide clinical support for the concept that cell surface markers are not always indicators of cell maturation. These markers are probably functional, rather than simply an expression of cell maturity. Sickle cell disease may be a good choice for further investigation.

In conclusion, we demonstrate a new flow cytometric protocol for identifying ECs that is reliable and reproducible. The major advantages of this protocol are the lack of washing/centrifugation steps, and the direct analysis of the whole blood, not requiring the use of mononuclear cells. Analyses should be performed with a consecutive gating strategy using broad gates, and by using anti-CD146/CD144/CD117 monoclonal antibodies. The use of 7AAD is useful to exclude dead cells that can nonspecifically bind to the CD144 and CD146 antibodies. This process may also be applicable to patients with other clinical manifestations of endothelial injury.

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