Abstract

Endothelial progenitor cells (EPCs) are a type of white blood cells present in circulation at low numbers and are hypothesized to play a role in the development of cardiovascular disease. EPCs, defined as “triple positive cells” which express CD34, CD133 and CD309 (VEGFR2), were reported to decrease and/or be functionally impaired in smokers. To evaluate whether EPCs can be utilized as smokers' endogenous biomarker of effect in clinical studies, several technical challenges exist. Therefore, we assessed various experimental conditions that could impact EPC viability and other parameters that could influence enumeration of EPCs by flow cytometry. Among the parameters evaluated were flow cytometric conditions, the effect of time lag in processing blood, effect of co-agents used for blood collection, conditions used to detect EPCs and the effect of shipping. The method optimization involved collecting fresh blood from the healthy volunteers who were non-smoking controls. Lysing the red blood cells prior to labeling with specific antibodies against the cell differentiation markers (CD34, CD133 and CD309) improved resolution of white blood cells and enumeration of EPCs. While freshly collected blood is ideal for enumeration of EPCs, blood stored up to 24h at room temperature could be used. Among the co-agents used for blood collection, citrate and CPDA were better for enumeration of EPCs. Shipping the blood samples overnight at room temperature did not seem to have an impact on EPC numbers. We also showed that functional EPC colonies can be grown in culture. In summary, we have tested several conditions for quantifying EPCs and offer a simple and reproducible method that can be used in clinical studies.

Method Development for Enumeration of Endothelial Progenitor Cells (EPCs)

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Experimental Methods

Blood samples were collected from ten healthy human volunteers at a local clinical research unit (IPMC Research, Inc., Winston-Salem, NC) under the approval of a central institutional review board (Independent IRB, Plantation, FL). Fractionation and all manipulations of blood were performed under Wake Forest Institutional Health (WFIT) IRB approval.

EPC identification and flow analysis: Blood cells were labeled with 10µl of CD45- FITC, CD133 APC, and CD309 antibodies for the detection of triple-positive EPCs. For isotype controls, mouse IgG2a FITC, mouse IgG2b APC and mouse IgG1 PE were employed. The labeling was performed for 30min at 4°C. Labeled cells were washed with MACS running buffer (Miltenyi Biotec, Auburn, CA) and stained with 10µl of 7AAD for 10min at room temperature. Finally, cells were fixed with 500µl of 2% paraformaldehyde and flow cytometry was performed. Cells positively stained with 7AAD were considered to be dead cells, and hence only 7AAD negative cells were gated for other analysis. Data were analyzed using FlowJo Software (Tree Star, Ashland, OR).

Effect of blood storage on EPC enumeration: 20 ml of blood with CPDA (anticoagulant) was placed on a roller at room temperature. One ml aliquots were taken at 4h, 24h, 48h and 72h, and lysed with 3ml of RBC lysis buffer, and processed for EPC enumeration as described above.

Effect of anticoagulants on EPC enumeration: Blood was collected in CPDA, citrate, EDTA or heparin. Blood was processed at 4h and 24h, following collection and processing for detection of EPCs.

Effect of RBC lysis and blood volume on EPC enumeration: Varying volumes (100µl, 500µl, and 1000µl) of blood were used for labeling EPCs either before or after RBC lysis.

Effect of overnight shipping of blood on EPC enumeration: 15 ml aliquots of whole blood collected in CPDA were shipped overnight at a density of 0.2 million cells/µl. After 7 days of culture, the EPC colonies were stained with Dil-Ac-LDL followed by 2% paraformaldehyde for 15 min. Colonies were further labeled with 10 µg/ml lectin followed by DAPI (nuclear stain). Positive staining with Dil-Ac-LDL and the lectin indicated EPCs. Microscopy was performed using a Nikon Eclipse fluorescence microscope at 20x magnification.

Culture of EPCs: Fresh PBMCs were plated onto a 6-well human fibronectin-coated plate at a density of 5 million cells/ml using M199 medium containing 20% FCS and antibiotics. After 48h, non-adherent cells were removed and plated onto human fibronectin-coated chamber slides at a density of 2 million cells/chamber. After 7 days of culture, the EPC colonies were stained with Dil-Ac-LDL followed by 2% paraformaldehyde for 15 min. Colonies were further labeled with 10 µg/ml lectin followed by DAPI (nuclear stain). Positive staining with Dil-Ac-LDL and the lectin indicated EPCs. Microscopy was performed using a Nikon Eclipse fluorescence microscope at 20x magnification.

Culture of EPCs: Fresh PBMCs were labeled with 10µl of CD45-FITC, CD133-APC and CD309 antibodies for the detection of tri-positive EPCs. The triple positive (CD34+ CD133+ CD309+) cells were labeled with propidium iodide and 7AAD for 10min at room temperature. The percentage of the positive population and brightness highlighted numbers on the far right parameter represent the detection rate of triple-positive EPCs calculated for one million lymphocytes from an individual donor.

Summary and Conclusions

• Simple method to enumerate EPCs from whole blood samples was developed.
• While freshly collected blood is best suited for EPC enumeration, blood stored for 24h at room temperature yields measurable numbers of EPCs.
• The anticoagulants citrate and CPDA appear to yield higher numbers of EPCs compared to EDTA and heparin.
• Preliminary data suggest that labeling of EPCs with specific antibodies after removing RBCs may result in better flow cytometric resolution of blood cells and reproducible enumeration of EPCs.
• Overnight shipping of blood samples at room temperature does not appear to impact EPC viability or enumeration.
• Functional EPC colonies were cultured using freshly collected PBMCs.
• This pilot study demonstrates the feasibility of enumerating EPCs in clinical samples and further studies may be required for full optimization of the method.

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