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FICOLL DENSITY GRADIENT ISOLATION METHOD VS. DIRECT FLOW CYTOMETRIC QUANTIFICATION OF EPCs

Cristiana Bujor[a], A. Anghel[a], Corina Samoilă[a], L. Tămaş[a], Gabriela Otiman[b], E. Şeclăman[a]

[a]University of Medicine and Pharmacy "Victor Babeş" Timişoara, Biochemistry Department, Piaţa Eftimie Murgu, Nr. 2, Timişoara, 300041, Romania

[b]University of Medicine and Pharmacy "Victor Babeş" Timişoara, Cardiology Department, Piaţa Eftimie Murgu, Nr. 2, Timişoara, 300041, Romania

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SUMMARY

Endothelial progenitor cells (EPCs) are bone-marrow derived cells involved in vascular homeostasis. In the present study, we assessed and compared two methods for EPCs isolation and quantification. The first method consisted of peripheral blood mononuclear cells (PBMC) isolation by Ficoll density gradient technique, followed by immunomagnetic labeling with specific antibodies beads (CD34, CD133 and VEGFR2/KDR) in order to enrich the target EPC subpopulation from the isolated PBMC. The selected immunolabelled cells were analyzed by flow cytometry. In the second method, whole blood was directly stained with specific antibodies and analyzed by flow cytometry. The aim of the study was to assess the efficiency and advantages of each method for EPCs quantification and also to evaluate the effect of immunomagnetic labeling with specific antibodies beads on EPCs quantification. The results have shown that there is not a significant difference between the EPCs number obtained by the two methods.

Keywords: endothelial progenitor cells; Ficoll density gradient isolation, immunomagnetic labeling, EPC quantification

INTRODUCTION

Endothelial progenitor cells (EPCs) are a heterogeneous group of cells characterized by the expression of surface antigens specific to both hematopoietic stem cells and endothelial cells [1]. These bone-marrow derived cells are involved in vascular homeostasis, vascular endothelium reparation and neovasculogenesis by differentiation in endothelium, smooth muscle and cardiomyocytes [2].

Under various pathological conditions the number and the proliferative potential of EPCs change comparative with the normal physiological conditions. The surface markers profile changes during the mobilization from bone marrow, migration and endothelial repair process [3, 4].

One subpopulation of "early" progenitor cells $CD133^+$ differentiate into $CD34^+CD133^+$ cells with a more pronounced angiogenic potential. These double positive cells are the most primitive progenitor cells for endothelial cells implicated in the vascular repair process [5, 6], and are also called circulating progenitor cells (CPC). However, the CD34 and CD133 markers are found on other types of cells and for that reason many studies recognize a later, more differentiated EPCs subpopulation represented by $CD34^+CD133^+VEGFR2^+$ cells, which are considered to be the so called „true"-endothelial progenitor cells and produce the mature endothelial cells [7, 8, 9, 10].

This heterogeneity may reflect the different developmental stages of EPCs, starting with the mobilization from the bone marrow induced by the injured endothelium and finishing with new endothelial cells maturation [11, 12].

There are several types of methods and markers combinations used to quantify EPCs subpopulations, but a generally accepted combination of markers and a standardized protocol for EPC isolation and identification with regards to reagents or flow cytometry gating strategy is missing.

In the present study, we assessed and compared two methods for EPCs isolation and quantification. The first method consisted of peripheral blood mononuclear cells (PBMC) isolation by Ficoll density gradient technique, followed by immunomagnetic labeling with specific antibodies beads in order to enrich the target EPC subpopulation from the isolated PBMC. The selected immunolabeled cells were stained with specific antibodies and the cells were analyzed by flow cytometry.

In the second method, we used whole blood which was directly stained with specific antibodies and analyzed by flow cytometry. The aim of the study was to assess the efficiency and advantages of each method for EPCs quantification and also to evaluate the effect of immunomagnetic labeling with specific antibodies beads on EPCs quantification.

MATERIALS AND METHODS

SUBJECTS

The study comprised 10 healthy volunteers, selected as individuals who had no sign of acute illness or infection, no immunological disease, no history of recent surgery, no uncontrolled hypertension and no established cardiovascular disease and were recruited upon obtaining informed consent. All participants didn't have any previously known abnormalities in glucose homeostasis. The study was approved by the local Ethical Committee and all subjects have given their written consent.

ISOLATION AND QUANTIFICATION OF EPCs

FICOLL DENSITY GRADIENT METHOD

A 10 mL sample of venous blood was used for the isolation of mononuclear cells in Ficoll density gradient according with the manufacturer's standard protocols. Briefly, the blood was diluted 3 times with phosphate – buffered saline solution (PBS), carefully added to 15 ml of Ficoll-Paque ^{TM} Plus (GE Healthcare) and centrifuged 40 min at 400xg at 20°C. The mononuclear cells are isolated, washed 2 times with PBS and resuspended in 300 µl PBS. $CD34^+$, $CD34^+/CD133^+$, $CD34^+/CD133^-$ and $CD34^+/CD133^+/VEGFR2^+$ enriched populations were obtained from peripheral blood mononuclear cells by single or two steps immunomagentic techniques (Miltenyi Biotec GMBH).

The isolated cells subpopulations were labeled with antibodies: anti CD34-FITC human, anti CD133-APC human (Miltenyi Biotec) and anti VEGFR2/KDR-PE human (R&D Systems) according with the manufacturer recommendations. FACS analysis was performed as previously described and the results were presented as mean ±3%.

The EPCs selection using flow cytometry have been obtained by a gating strategy of $CD34^+$ cells, followed by a double selection of $CD34^+/VEGF\text{-}R2^+$ and then a selection of the $CD34^+/VEGF\text{-}R2^+/CD133^+$ cells. The results have been analyzed using WinMDI Version 2.9.

RESULTS

Patient characteristics

The baseline characteristics of the study subjects are summarized in Table 1. All included patients had no history of hypertension or other cardiovascular risk factors. The subjects included 57.7% males.

Table I. Baseline characteristics of subjects

Parameter	Subjects (n=10)
Age (years)	47 ± 8
Glucose	91.98 ± 12.27
Total cholesterol	159.64 ± 27.98
Triglycerides	93.56 ± 33.93
Blood pressure (Systolic)	121 ± 12
Blood pressure (Diastolic)	74± 10
Sex	M: 57.78%

Circulating endothelial progenitor cells

The blood samples were analyzed in parallel by both methods. Using Ficoll density gradient PBMC isolation method followed by immunomagnetic labeling, the $CD34^+$, $CD34^+/CD133^+$, $CD34^+/CD133^-$ and $CD34^+/CD133^+/VEGFR2^+$ enriched populations were quantified (Figure 1) considering 10 ml of whole blood.

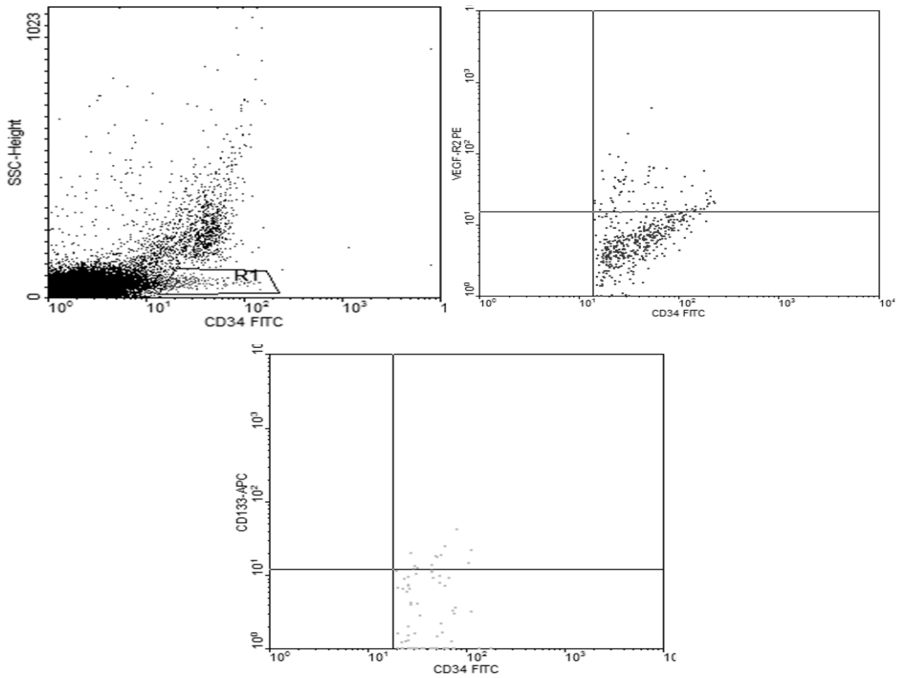

Figure 1. Gating strategy and EPCs subpopulations quantification after FICOLL density gradient isolation method

The cells number was calculated first as cell number/1 ml of whole blood, and then divided by 100 to calculate the cell number/100 µl. The cell number was expressed as10^6 events. The $CD34^+$ cells number accounted was 620 ±592, $CD34^+/CD133^+$ /$VEGFR^-$ cell number was 530 ± 584, $CD34^+$/ $VEGFR^{2+}$ cell number was 215 ± 194 and $CD34^+/CD133^+$/ $VEGFR^{2+}$ cell number was 75±82. Results were comparable with those obtained by direct flow cytometric quantification ($CD34^+$: 584±548, $CD34^+/CD133^+/VEGFR^-$: 547±584, $CD34^+$/ $VEGFR^{2+}$ / CD 133^-: 358±237, and $CD34^+/CD133^+$/ $VEGFR^{2+}$: 52±114).

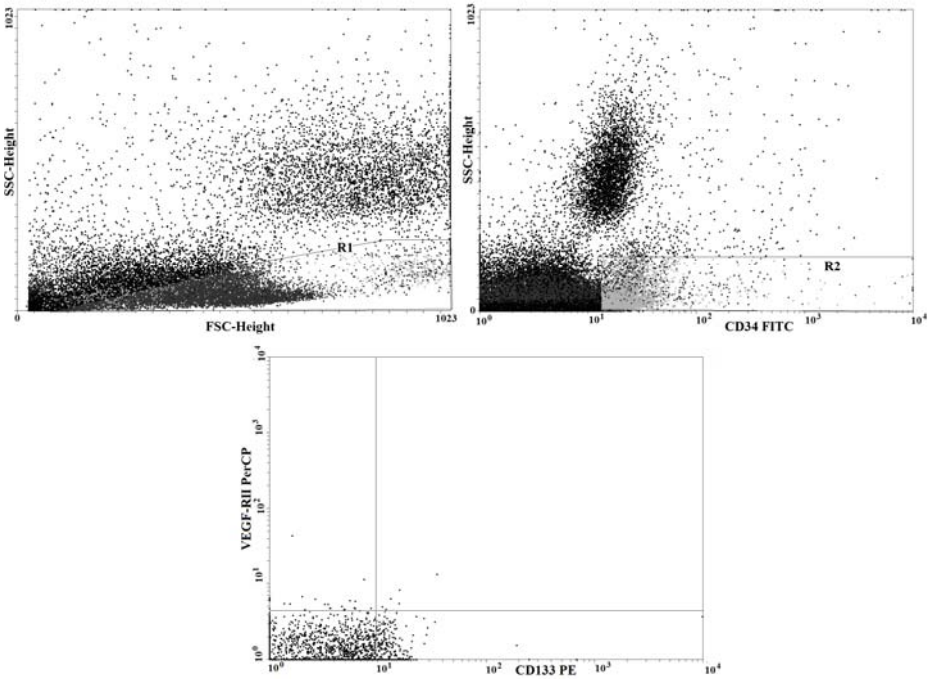

Figure 2. Gating strategy and EPCs subpopulations quantification by direct flow cytometry method

DISCUSSION

A combination of CD34, CD133 and VEGFR2 was used in different studies [13, 14, 15] to characterize and quantify the "true" EPCs. In the present study the same combination of membrane markers was used. The isolation of PBMC from whole blood by Ficoll density gradient method was reported by different studies to be suitable for further cell culture analysis [14] by the preplating of the isolated PBMC in fibronectin-coated dishes for 48 hours followed by the replanting of nonadherent cells [14, 16]. It will give rise to colonies of endothelial progenitor cells with endothelial repair potential.

Due to the difficulties to analyze rare events such as circulating progenitor cells by flow cytometry in the blood [16], the study has applied Ficoll method in order to improve the quantification process. The results have shown that isolation of PBMC fraction from whole blood and immunolabelling of the interest cells with magnetic beads have increased the sensibility of detection. It has eliminated the problems of non lysing samples which were accounted in the direct flow cytometry procedure. However, the procedure may give false positive results due to the beads, dead cells or cells clumps. The quantification strategy by Ficoll technique was laborious, requiring at least 10 hours to the end of the cells quantification process. If the procedure is interrupted after PBMC isolation step and the cells are stored ON at 5°C this could increase the number of non viable cells with direct consequences on results.

Isolation of PBMC fraction from whole blood with Ficoll and immunolabelling of the interest cells with magnetic beads has reduced the number of cells analyzed by flow cytometry and because the start volume of the blood sample was 10 ml was difficult to calculate the EPC cells number/ml to be compared with data from the literature reports [17]. To compare the two methods we used the cells number/10^6 events and it was difficult to calculate in the case of Ficoll method considering that only 2-3000 events were acquired by flow cytometry.

The results of the study have shown that both methods could be used successfully to account EPCs and the further applications of these cells should orientate the strategy towards one or another: the Ficoll isolation of PBMC followed by immunomagnetic labeling and flow cytometry is more suitable for isolation and culture of EPCs to study functionality and migratory capacity of EPCs while the direct flow cytometry method is more rapidly and uses small blood sample volume to determine the number of EPCs cells /10^6 events but it is technically demanding due to a high noise level and staining procedures as long as non-specific binding of fluorochrome-matched isotype controls may be 0.5% of the analyzed cells (the endothelial progenitor cells percentages exceeding the reported percentage from PBMC (0.0001% - 0.01%) [18, 19].

CONCLUSION

The results of the study have shown that both methods could be used successfully to account EPCs. The Ficoll isolation of PBMC followed by immunomagnetic labeling and flow cytometry is more suitable for isolation and culture of EPCs while the direct flow cytometry method is more rapidly and uses small blood sample volume to determine the number of EPCs cells /10^6 events.

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