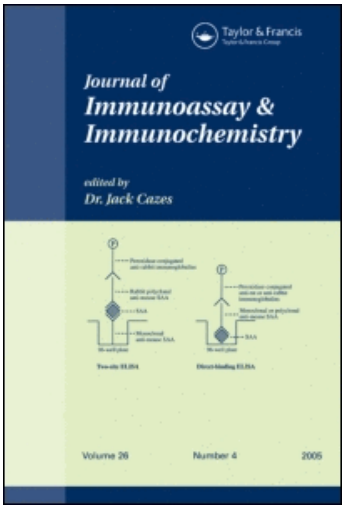

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Flow-Cytometric Identification, Enumeration, Purification, and Expansion of CD133⁺ and VEGF-R2⁺ Endothelial Progenitor Cells from Peripheral Blood

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Abstract: A flow cytometric method for identifying, purifying, and expanding endothelial progenitor cells (EPC) derived from peripheral blood is reported. During our experiments, we found that isolation of viable EPC is not possible by using the standard flow cytometric protocols, since erythrocyte lysing influences cell survival. Furthermore, erythrocyte lysing has a high impact on quantative analysis of EPC with 20% lower numbers compared to no-lyse data. The viability of EPCs was tested with a colony forming test after both lysis based FACS of EPCs and without lysing. CD133 and VEGF-R2 revealed as positive markers for EPC selection and 7-amino actinomycin D (7-AAD) to eliminate dead cells. The few purified CD133$^+$ and VEGF-R2$^+$ cells showed strong colony-forming capacity in a human stem cell methylcellulose based medium (colony assay) when isolated by the no-lyse protocol. The colonies showed the typical shape of early EPC-colonies with round immature cells in the centre and dendritic or spindelcell-shaped peripheral cells, which were also immunologically identified as EPC-derived. Compared to this, erythrocyte lysing reagents destroyed even all sorted EPCs.

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Summarizing the presented data suggest that the use of erythrocyte lysing reagents is neither suitable for cloning nor for counting of endothelial progenitor cells, and no-lyse protocols should be used.

Keywords: Endothelial progenitor cells, Flow cytometry, No-lyse, Expansion

INTRODUCTION

It has been shown that endothelial progenitor cells (EPC) are capable of contributing to re-endothelialization and neo-vascularisation in tissue injury and that injected EPC home to sites of ischemia and augment neovascularization.[1] However, it is supposed that a small number of EPC circulate permanently in the peripheral blood and that a variety of vascular diseases may have an influence on the mobilization of EPC from the bone marrow to the blood.

To understand the pathogenesis of vascular diseases and to elucidate how risk factors contribute to vascular changes, reliable methods to quantify EPC in peripheral blood are needed. Flow cytometry is described as a rapid and convenient way to assess the number of EPC. However, recent publications revealed some critical protocol steps in flow cytometric enumeration of hematopoietic stem cells like fixation,[2] lysing of erythrocytes, and centrifugation.[3,4] Some authors even recommend use of no-lyse protocols.[4,5] The same protocol steps may also disturb EPC enumeration and isolation.

EPC are bone marrow-derived cells which exhibit both characteristic stem cell and endothelial surface markers and properties.[6] They were defined as cells positive for the haematopoietic stem cell markers, such as CD34 or CD133,[7] and an endothelial marker protein such as VEGFR2.[3] Circulating EPCs express, with different intensity, a variety of markers, which are typical for the endothelial lineage. These markers include platelet endothelial cell adhesion molecule-1 (CD31), CD146, VE-cadherin, von Willebrand factor, endothelial NO synthase, and, on stimulation, E-selectin.[8–10] In general, early EPCs in the bone marrow, or immediately after their migration into the systemic circulation, are positive for CD133/CD34/VEGFR-2, whereas the more differentiated circulating EPCs are positive for CD34/VEGFR-2/CD31/VE-cadherin, obviously lose CD133 and begin to express von Willebrand factor.[11]

We have developed a new method of early EPC-counting in the peripheral blood by volumetric flow cytometric measurement, which delivered highly reliable values in different patients and volunteers. Furthermore, we have proven the stem cell properties of our counted cells by showing their colony forming potential in a stem cell methylcellulose assay. By the way, we have studied the influence of the erythrocyte-lysing procedure on the recovery of endothelial progenitor cells in flow cytometric analysis and on the culturing potential.

EXPERIMENTAL

Samples

EDTA-blood samples were obtained from volunteers as part of a routine diagnostic or screening procedure after informed consent had been obtained. The blood from 16 healthy volunteers (11 women and 5 men; mean age 41 years, SD 18.9) was included in the study. The cell culture experiments were performed with 4 EDTA-blood samples of cytokine-mobilized donors for allogeneic peripheral blood stem cell apheresis. Only fresh blood samples were used for all experiments, except the cytokine mobilized blood, which was stored at 4°C for not more than 6 hours. Informed consent was obtained from all volunteers; the study protocol was approved by the local Ethics Committee of the University of Münster.

Sample Preparation

100 μL EDTA-blood samples were pretreated with FcR blocking reagent (Miltenyi Biotec, Bergisch-Gladbach, Germany). Following, the samples were incubated for 30 minutes in the dark with the phycoerythrin (PE)-conjugated monoclonal antibody against human VEGF R2 (R&D Systems, Wiesbaden, Germany) and with the biotin-conjugated monoclonal human CD133 antibody (Miltenyi Biotec, Bergisch-Gladbach, Germany). After washing with PBS, the samples were incubated with the fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody against biotin (Miltenyi Biotec, Bergisch-Gladbach, Germany) and with the viability staining reagent 7-AAD (Biocience, San Diego, USA) in a second step. Isotype-identical antibodies served as controls (Serotec, Düsseldorf, Germany). After incubation, cells were washed with PBS, lysed with IO-Test 3 lysing solution (Beckmann Coulter, Marseille, France) and resuspended in PBS.

For the cell sorting and culturing experiments, 5 mL EDTA-whole blood samples were stained as described above. To investigate the effect of erythrocyte lysing procedures on the stem cells, one half of the samples was treated with the erythrocyte lysing procedure like the samples for the flow cytometric analysis only and, in the other half, mononuclear cells were isolated by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) before staining.

Volumetric Flow Cytometry

The double-labeled samples were diluted 1:18 by adding 900 μL of phosphate-buffered saline (PBS, PAA Laboratories, Pasching, Austria) to a tube. The tubes were analyzed cytometrically within 2 h of preparation on a

PAS III flow cytometer (Partec GmbH, Münster, Germany) equipped with a 20 mW 488-nm argon ion laser. The threshold was set at the lower end of the forward scatter (RN1). Gates were set around the region containing the leukocytes (R1) (Figures 1a and 1b). Cells inside these gates were further

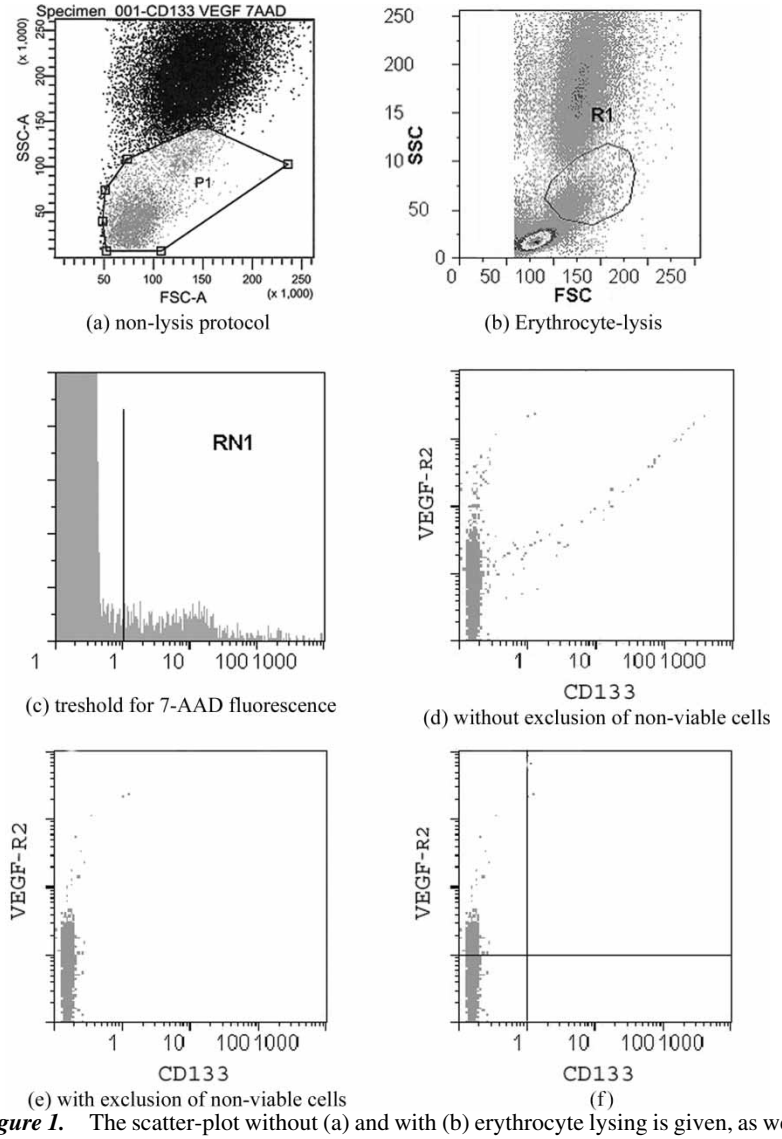

Figure 1. The scatter-plot without (a) and with (b) erythrocyte lysing is given, as well as the exclusion of non-viable cells by the use of 7-AAD (c). If non-viable cells were excluded, the background noise of unspecific stained cells disappears completely (d-e). The quadrant-gate to isolate VEGF and CD133 positive cells is also shown in the last histogram (f). The upper right quadrant shows the double positive EPCs.

analysed with regard to the green, orange, and red fluorescence. A threshold was set for the orange fluorescence and cells exhibiting fluorescence in this channel (7-AAD) were excluded from further analysis (Figure 1c).

Quadrants were set to analyse the red and green fluorescence as shown in Figure 1f. The right upper quadrant contained the double positively stained cells (Q4) (Figure 1f). If non-viable cells were not excluded, a fat smear of unspecifically stained cells along the diagonal will impede any evaluation (Fig. 1d).

As the volume of samples aspired by the PAS III flow cytometer is exactly known, no counting beads are required, and the leukocyte, respectively, the EPC concentration was provided directly by the instrument and was multiplied by the dilution factor.[1]

Fluorescence Activated Cell Sorting (FACS)

Directly after staining, cells were analysed and sorted on a FACSAria cell sorter (BD Biosciences, USA).

Cultivation and Characterization of Early EPC

The CD133$^+$ and VEGF-R2$^+$ cells from the cell sorter were cultured in a human methylcellulose base medium (R&D Systems, Minneapolis, USA) supplemented with β-EGF, IL-3 and SCF. All cell cultures were maintained at 37°C with 5% CO_2 in a humidified atmosphere. After 2 weeks, colonies were counted by two or three independent investigators.

For further characterisation, cytospins of colonies were made. Cells were stained with 4′6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and unconjugated monoclonal antibodies against von Willebrand Faktor (vWF; Dako, Glostrup, Denmark). Immunodetection was visualized by FITC-labelled goat-anti-mouse-antibody (Dako, Glostrup, Denmark).

RESULTS

Identification of CD133$^+$ and VEGF-R2$^+$ Cells by Flow Cytometry

Early EPCs are very rare in the peripheral blood. For the detection of these cells, it is necessary to decrease the background noise in the flow cytometric analysis to a minimum. The most effective criterion, in this way, is the exclusion of non-viable cells by staining with 7-AAD, since they are markable by unspecific binding of fluorescent antibodies. If they were not excluded from our experiments, a smear of unspecifically stained cells along the diagonal impedes any evaluation of rare EPCs (Figure 1d). After

exclusion of 7-AAD stained cells, the unspecific smear disappears completely (Figure 1e). Other strategies to reduce background activity, such as inclusion of CD19 and CD3, as well as definition of a dump channel, did not improve the measurement significantly.

Reproducibility of EPC Quantification

To evaluate the reproducibility of the new method for early EPC-measurments, EDTA-blood samples were taken from 16 healthy volunteers. After staining with CD133, VEGF-R2, and 7-AAD and, after erythrocyte lysis, cells were analysed by 5 channel flow cytometry (FCS, SSC, Phycoerythrin-fluorescence, FITC-fluorescence and 7-AAD-fluorescence). The $CD133^{+}$, $VEGF\text{-}R2^{+}$ and 7-AAD- cells were counted and the original concentration in the peripheral blood specimen was calculated by a volumetric method. The measurement was repeated after 7 days and after 14 days (see Table 1). The mean concentration of early EPC in the 16 healthy volunteers was 154/mL (SD+/−78). The values measured in samples of the same volunteer at different times showed relative constant enumeration of early EPC in the peripheral blood. Some changes in concentration should be physiologic, e.g., after intensive sport activity.[11] Anyway, the results of our measurements are reproducible (Table 1).

Table 1. Repeated measurement of early endothelial progenitor cells (EPC) by volumetric flow cytometry in 16 volunteers within a period of 7 days resp. 14 days. (Mean age 41 years, 11 women and 5 men)

	Early EPC/mL - measurement day1	Early EPC/mL - measurement day7	Early EPC/mL - measurement day14
Volunteer 1	140	210	140
Volunteer 2	140	70	90
Volunteer 3	210	140	90
Volunteer 4	210	140	120
Volunteer 5	70	70	90
Volunteer 6	210	210	140
Volunteer 7	140	140	90
Volunteer 8	70	0	60
Volunteer 9	280	490	210
Volunteer 10	280	420	280
Volunteer 11	70	70	120
Volunteer 12	90	60	90
Volunteer 13	90	60	120
Volunteer 14	30	90	60
Volunteer 15	0	120	90
Volunteer 16	60	60	70

Influence of Erythrocyte Lysing Procedure on Flow Cytometric Recovery

To evaluate the influence of the erythrocyte lysing procedure on the recovery of EPC, WBCs were separated from the peripheral blood specimens of two healthy volunteers by Histopaque-gradient centrifugation. Cells were then stained with anti-CD133 and anti-VEGF-R2, as well as 7-AAD. Contemporaneously, whole blood specimens from the same volunteers were stained with the same antigens and 7-AAD, followed by erythrocyte lysis. Each probe was analysed by 5-channel flow cytometry. The comparison of early EPC detection after erythrocyte-lyses and after density gradient centrifugation revealed a mean decrease of about 20% in the relative amount of CD133$^+$ and VEGF-R2$^+$ double positive and 7-AAD negative cells (Table 2).

Fluorescence Activated Cell Sorting (FACS)

EPCs were isolated by FACS, using two different protocols. In a first experiment, erythrocytes were eliminated from analysis by erythrocyte lysing and, in a second one, by isolation of WBCs by gradient centrifugation of peripheral blood. In both cases, about 300 million of the purified mononuclear cells were analysed by FACS. About 2,000 cells were sorted on the basis of immunostain with monoclonal CD133 and VEGF-R2 specific antibodies. In addition, dead cells were identified and excluded by 7-amino actinomycin D (7-AAD) stain. Figure 1 shows the strategy for isolation of EPCs by FACS analysis. The sorted cells showed simultaneous expression of the CD133-antigene and the VEGF-R2-antigene and were negative for the 7-AAD staining, which means they were alive.

Expansion of CD133$^+$ and VEGF-R2$^+$ Cells

Even though all other conditions were identical, the experiments with erythrocyte lysing instead of gradient centrifugation did not lead to any colony growth at all. In contrast to this, out of the 2,000 purified CD133$^+$

Table 2. Relative amount of CD133$^+$, VEGF-R2$^+$ and 7-AAD- cells from the mononuclear white blood cell fraction in peripheral blood probes measured by 5 channel flow cytometry

	No-lysis protocol	Erythrocyte lysis
Volunteer 1	0.01 %	0.008 %
Volunteer 2	0.02 %	0.015 %

and VEGF-R2$^+$ cells from the FACS after gradient centrifugation, 57 colonies were grown in a human methylcellulose based stem cell medium after 14 days incubation. These colonies showed the typical shape of early EPC-colonies with round immature cells in the centre and dendritic or spindelcell-shaped peripheral cells (Figure 2). Single colonies were aspirated and cytospins were prepared. Immunohistochemical staining for von Willebrand factor (vWF) was chosen for the characterisation of endothelial-like cells. The round cells in the centre of the colonies showed almost no specific staining for vWF, like the original seeded cells from the cell sorter whereas, the dendritic or spindelcell-shaped more at the periphery of each colony located cells were specifically stained with vWF. This proves the endothelial differentiation of the colonies.

DISCUSSION

EPCs are very rarely observed in peripheral blood. To determine their concentrations, it is necessary to identify these cells exactly, within millions of other cells. Cell fragmentation and aggregation disturb the detection of EPC. A major problem is further caused by the unspecific binding of the antibodies by non-viable cells. The most effective way to eliminate these disturbing influences is careful treatment of the specimen and the elimination of non-viable cells by staining with 7-AAD.

Discussion still goes on about which marker proteins should be used to characterise the EPC fraction in peripheral blood. According to the initial discovery, EPCs were defined as cells positive for both haematopoietic stem cell markers, such as CD34 and an endothelial marker protein as VEGF-R2. Because CD34 is not exclusively expressed on haematopoietic stem cells but, at a lower level, also on mature endothelial cells, further studies used the more immature haematopoietic stem cell marker CD133[7] and demonstrated that purified CD133$^+$ cells can differentiate to endothelial cells in vitro.[12] CD133, also known as prominin or AC133, is a highly conserved antigen with unknown biological activity, which is expressed on haematopoietic stem cells, but is absent on mature endothelial cells and monocytic cells.[13] Thus, CD133$^+$/VEGFR2$^+$ cells more likely reflect immature progenitor cells, whereas CD34$^+$/VEGFR2$^+$ may also represent shredded cells of the vessel wall. Therefore we decided to count the CD133$^+$/VEGFR2$^+$ cells.

During our experiments, we made an interesting observation: the erythrocyte lysis prevented the CD133$^+$ and VEGF-R2$^+$ cells from growing in the stem cell medium. The erythrocyte lysing process seems to be aware of the integrity of most of the EPC, but seems to prevent the cells from cell dividing circle. This could be explained by the destruction of cell membrane proteins, which are necessary for long time survival and cell division. Cells, in this way attenuated, seem to be incapable of growing in a medium, especially after the

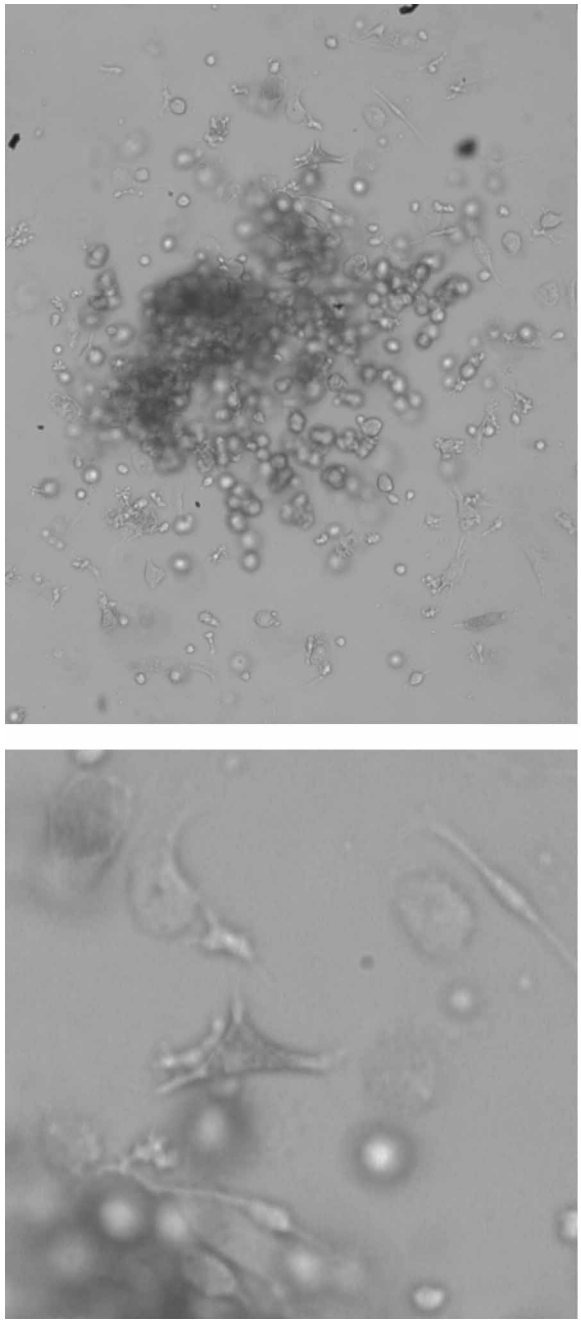

Figure 2. The colonies grown from the purified CD133$^+$ and VEGF-R2$^+$ cells showed the typical shape of early EPC-colonies with round immature cells in the centre and dendritic or spindle cell shaped peripheral cells (see magnification).

stressing FACS-process. Similar observations have been described for haematological stem cells to appear as a result of the erythrocyte-lysing procedure.[3,14]

Furthermore, the erythrocyte-lyses seem to have an influence on the relative amount of EPC in the leukocyte fraction. In the probes treated with lysing solution, the relative EPC count was about 20% lower than in the comparable probes in which the erythrocytes have been separated by gradient centrifugation. It is known that erythrocyte-lysing procedures affect the determination of progenitor cells in whole blood.[15,16] The relative high grade loss of EPC demonstrates the important role of erythrocyte lysing procedures and leads to underestimation of total cell concentration. Further studies have to be performed to determine the exact amount of underestimation.

Nevertheless, the fact, that the detected and isolated $CD133^+$ and VEGF-$R2^+$ cells have shown colony-forming capacity proves their stem cell properties and the shape of the colonies; the expression of vWF prove their epithelial character.

CONCLUSION

Our presented protocol for the flow cytometric measurement of EPCs in the peripheral blood can be used as a diagnostic or prognostic tool; we propose this protocol as the standard for the EPC quantification. As shown in a former study, flow cytometry is the superior method to enumerate EPC in peripheral blood, because the cell culture is more susceptible to external influences and is more time consuming.[17]

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