Research Article

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Flow cytometric detection of endothelial progenitor cells (EPC) in acute coronary syndrome

Akut Koroner Sendrom’ da Akım Sitometrik Olarak Endotelyal Progenitor Hücrelerin Saptanması

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Abstract

Aim: The aim of the present study is to establish a moAb combination and a study procedure for the accurate determination of circulating EPC using multicolor flow cytometry, as well as to find out whether there is a relation between disease severity and EPC count by comparing the circulating EPC count between the subgroups of patients presented with acute coronary syndrome.

Materials and methods: Absolute circulating EPC count was identified in 40 healthy subjects and acute coronary syndrome patients using multicolor flow cytometry with a single-tube panel consisting of CD45, CD31, CD34, CD309 and syto 16 monoclonal antibodies.

Results: Circulating EPC count was 11.33 (7.89–15.25) cells/μL in healthy control group and 4.80 (0.70–10.85) cells/μL in acute coronary syndrome patients.

Conclusion: In the present study, which investigated whether there is a difference between EPC counts of STEMI and USAP patients that have presented with ACS, EPC count was lower in both disease groups as compared to healthy controls. First the method must be standardized and then harmonization studies are required in order to use this method as a prognostic factor in cardiovascular diseases.

Keywords: Flow cytometry; Endothelial progenitor cell; Acute coronary syndrome.

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Introduction

It has been believed that vascular endothelial cells, damaged for a while or lost, could be regenerated only by adjacent endothelial cells [1]. However, this belief has
changed with the identification of endothelial progenitor cells (EPC) in the peripheral circulation, as well as by demonstrating their ability to differentiate into mature endothelial cells and exhibiting their role in vascular repair [2, 3].

Endothelial progenitor cells (EPCs) are bone marrow-derived cells that can differentiate into mature endothelial cells [4]. They mobilize during tissue ischemia and vascular injury and pass into circulation from the bone marrow (BM) as hemangioblasts and enable the reintegration of damaged endothelium [5]. Hypoxia, hematopoietic cytokines and granulocyte-macrophage colony-stimulating factor (GM-CSF) induce EPC release [6–8]. It is known that EPCs are at a place between stem cells and endothelial cells in terms of maturation [8]. EPCs are potential biomarkers of neovascularization, cardiac regeneration, vessel repair and aid in tumor growth, or in monitoring response to treatment [7]. There is correlation between decreased circulating EPC count and decreased endothelial repair capacity, which may lead to the development of cardiovascular disease [8, 9]. Cardiovascular risk factors such as age, diabetes mellitus (DM), hyperlipidemia and smoking unfavorably influence the number and functions of circulating EPCs [10–13]. The importance of this correlation in the development of atherosclerosis is also underlined. It has been demonstrated that EPC count is negatively correlated with endothelial dysfunction and cardiovascular events [14]. Today, it is being emphasized that EPCs can be considered as an independent risk factor for cardiovascular diseases [8, 11, 12].

Flow cytometry (FCM) is accepted as the gold standard for quantification of these cells, which are less in number in the circulation [13]. The advantages of FCM is the ability to perform rapid, automated and repeatable multiparameter analysis at the level of single cell [8, 13, 14]. The flow cytometry protocol is based on a monoclonal antibody combination and study procedure, which would accurately identify immunophenotype of EPCs, has not been standardized yet [16, 17]. The aim of the present study is to investigate moAb combination that will accurately determine the circulating EPC count using FCM, as well as to investigate whether there is a difference between EPC counts of the patients with ST-elevation myocardial infarction (STEMI) and the patients with unstable angina pectoris (USAP), who have been admitted to the coronary intensive care unit for acute coronary syndrome (ACS).

Materials and Methods

Patients and controls

The study comprised 40 healthy controls (20 females, 20 males, age range: 26–50 years) and 50 patients with CAD (15 females, 35 males, age range: 38–72 years). All healthy subjects were interviewed for their medical history, and donors presenting at least one of the following parameters out of the normality ranges were excluded: blood pressure, glycemia, cholesterol level [13]. Smokers, healthy women within 15 days from menstruation, pregnant, individuals fasted within 12 h, subjects with endometriosis, with active duodenal or gastric ulcer, positive for HIV, HCV, HBV, donors that had undergone drug treatments in the preceding 48 h or with present or previous neoplastic, infectious, inflammatory or cardiovascular disease, were also excluded. Blood was collected in tri-potassium ethylene diamine tetra-acetic acid (K3EDTA) tubes (Becton Dickinson-BD, CA, USA). In order to avoid endothelial cell contamination due to phlebotomy, the blood sample of 3 mL taken into the first tube was used for complete blood count, whereas the blood taken into the second tube was used for flow cytometry. Fifty patients at the age of 38–72 years, who have visited the emergency room for chest pain and have been admitted to the coronary intensive care unit being diagnosed with acute coronary syndrome, were assigned to the patient group. Patients were divided into two subgroups. The first group consisted of patients presented with typical chest pain and had ST elevation in two consecutive leads on the electrocardiogram (ECG) together with elevated cardiac enzymes, who have been defined as ST-elevation myocardial infarction (STEMI, n = 25). The second group consisted of patients presented with typical chest pain lasting for at least 15 min within the last 24 h, had ST segment and T wave alteration in at least two consecutive leads on ECG without elevation of the cardiac enzyme, who have been defined as unstable angina pectoris (USAP, n = 25). After the comparison of baseline characteristics and EPC count between the patient group and the control group, EPC count was compared between the STEMI and USAP groups. The study protocol was approved by Ankara Numune Training
and Research Hospital, Local Ethics Committee (Ethics committee number is 2010-023).

The patients with fasting glucose level ≥126 mg/dL or the patients receiving oral antidiabetic drugs or insulin were considered to have DM; the patients with systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg or the patients receiving antihypertensive drug were considered to have hypertension (HT). In addition to complete blood count and biochemical analyses routinely studied in the clinic, blood sample was taken also for EPC count both just after the patients have been admitted to the intensive care unit for acute coronary syndrome and prior to the treatment and put into the tubes containing K3EDTA.

Flow cytometry

A cocktail of fluorochrome-labeled mAb is used to identify EPCs, allowing highly specific definition of EPCs. Peripheral blood was evacuated into a 4-mL vacutainer (BD Vacutainer®, CA, USA) tube as an anticoagulant and processed for 2 h after collection. Nonspecific antibody binding was blocked using 20 μL Fc-receptor (FcR) blocking reagent and 200 μL of mouse serum (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at room temperature before staining with conjugated antibodies. Five-color staining was performed for quantification of EPCs could be done accurately. MoAbs used in the panel included CD45 PC7 (Beckman Coulter, CA, USA), CD31PE (BC, CA, USA), CD34 (BC, CA, USA), ECD, CD309 (VEGFR-2/KDR) APC (Miltenyi Biotec, Germany), and nuclear staining syto16 FITC (Dako, Germany). [Anticoagulated venous blood sample was dispensed in 200 μL aliquots into two polypropylene test-tubes (BD, USA), which were 12×75 mm in size.] After mixing gently, the tubes were incubated with suitable fluorochrome-conjugated MoAbs at a concentration recommended by the manufacturer for 15 min at room temperature in the dark. Stained whole blood samples were subjected to red blood cell lysis with 2 mL of lysing solution (Optilyse, BC, USA), and then shaken and incubated for 15 min at room temperature in the darkness. Prepared samples were stored at room temperature in the dark and then analyzed within 1 h. Nucleated cells derived from whole blood specimens were evaluated using a FC 500 (BC, USA) device with identical set up parameters between samples. A total of 1×10⁶ cells were counted for each sample. Results of the analyses were considered informative when adequate number of cells (approximately 1000 events) was collected in the EPCs enumeration gates. FCM data was analyzed using BC Cell Quest software program. Gating strategies and whole blood analysis were as following: non-viable cells, platelets, debris and non-specific binding in the CD45/ (side scatter) SSC dot plot were excluded from the analysis by syto16® nuclear staining and isotype control and consecutive gating. Syto16®, CD31+ nucleated cells were retrieved from the CD45 dimmer/negative region. EPCs with CD34+ KDR+ immunophenotype were determined among these cells (Figure 1). The absolute EPCs count was derived from absolute white blood cell count obtained in a hematology analyzer (LH 750, BC, USA), and the percentage of EPCs was determined by FCM using the following formula: percentage of EPCs×white blood cell (WBC) count/100 [18]. Before each run, the flow cytometer was cleaned according to the manufacturer’s instructions. Passage between the samples was prevented using FC500 device. A range of internal quality assurance procedures including daily compensation (Flow-set, BC, USA) of optical alignment (Flow-Check, BC, USA) of the flow cytometer and the use of fluidic stability was applied. We The accuracy and precision of cell count were tested using international quality controls purchased from United Kingdom National External Quality Assessment Scheme (UK NEQASLI, Sheffield, UK) (z score range –2.0 to 2.0).

Statistical analysis

Data were analyzed using SPSS for Windows 15 package program. Proximity of continuous variables to normality was investigated by Shapiro Wilk test. Descriptive statistics were presented as mean±standard deviation or median (minimum–maximum) for continuous variables and as case number and (%) for categorical variables. Significance of the difference between the groups was analyzed by Student’s t-test for mean values and by Mann-Whitney U-test for median values. Whether there is statistically significant correlation between continuous variables was investigated using Spearman’s correlation test. The results were considered statistically significant for p<0.001.

Results

Demographic characteristics of 40 healthy controls and 50 acute coronary syndrome cases, which have been investigated in the present study, are demonstrated in Table 1. The mean age was 40.3±4.4 years in the healthy control group and 56.9±10.3 years in the ACS group. While circulating EPC count was 11.33 (7.89–15.25) cells/μL in the
Table 1: Demographic characteristics of the cases according to the control and patient group.

| Variables          | Control group (n=40) | Patient group (n=50) | p-Value |
|--------------------|----------------------|----------------------|---------|
| Age, year          | 40.3 ± 4.4           | 56.9 ± 10.3          | <0.001  |
| Gender             |                      |                      | 0.125   |
| Female             | 20 (50.0%)           | 15 (30%)             |         |
| Male               | 20 (50.0%)           | 35 (70%)             |         |
| Hs-CRP             | 0.02 (0.02–0.33)     | 0.5 (0.02–7.5)       | 0.004   |
| HDL                | 40.8 (36–85)         | 27.5 (22–60.1)       | <0.001  |
| LDL                | 102.9 (61.0–181.6)   | 120.3 (65–216)       | 0.131   |
| Triglyceride       | 79 (33–545)          | 147 (50–860)         | <0.001  |
| Fibrinogen         | 345.5 (180–550)      | 355.8 (184.9–621.9)  | 0.131   |
| EPC                | 11.33 ± 11.84        | 3.82 ± 2.75          | <0.001  |

Hs-CRP, High sensitive CRP; HT, hypertension; EPC, endothelial progenitor cell.

Figure 1: EPC detection by flow cytometry, gating strategy. (A) Gate1: FSC vs. SSC to exclude debris. (B) Gate2: SSC vs. CD45 to exclude hematopoietic cells. (C) Gate3: Syto16 (DNA marker) vs. CD31 to exclude platelets (red). (D) CD34+KDR+ EPCs (blue).

Regarding EPC count in the STEMI and USAP groups of ACS patients, it was found higher in the USAP group as compared to the STEMI group (6.89 ± 6.25 vs. 3.82 ± 2.75, p = 0.011). The factors that might influence EPC count in each disease group are demonstrated in Table 2. Among these variables, hsCRP was significantly lower in the USAP group (p = 0.011), while HDL was significantly lower in the STEMI group (p = 0.035). The groups were similar in terms of other factors. No statistically significant difference was determined between the groups in terms of age, presence of DM or HT, smoking, serum low-density lipoprotein (LDL), and statin use. Fibrinogen and high-sensitive C-reactive protein (hsCRP) values as well were...
Table 2: STEMI and USAP groups characteristics.

| Variables  | STEMI (n=25) | USAP (n=25) | p-Value |
|-----------|-------------|-------------|---------|
| Age, year | 56.7±10.9   | 57.8±10     | 0.68    |
| Gender    |             |             | 0.277   |
| Female    | 10 (40%)    | 5 (20%)     |         |
| Male      | 15 (60%)    | 20 (80%)    |         |
| Smoking   |             |             | 0.353   |
| Yes       | 12 (48%)    | 10 (40%)    |         |
| No        | 13 (52%)    | 15 (60%)    |         |
| HT        |             |             | 0.151   |
| Yes       | 11 (44%)    | 6 (24%)     |         |
| No        | 14 (66%)    | 19 (76%)    |         |
| DM        |             |             | 0.014   |
| Yes       | 3 (12%)     | 5 (20%)     |         |
| No        | 22 (88%)    | 20 (80%)    |         |
| Statin    |             |             | 0.745   |
| Yes       | 10 (40%)    | 3 (12%)     |         |
| No        | 15 (60%)    | 22 (88%)    |         |
| EPC       | 3.82±2.75   | 6.89±6.25   | 0.001   |
| LDL       | 126.8±39.1  | 117.3±37.9  | 0.514   |
| HDL       | 27.9±7.3    | 29.9±8.1    | 0.035   |
| Homocystein | 17.0±8.0  | 12.4±3.3    | 0.043   |
| Fibrinogen | 312.45±135.20 | 321.54±119.20 | 0.571 |
| HsCRP    | 2.5±10.5    | 0.67±3.6    | 0.011   |

STEMI, ST-elevation myocardial infarction; USAP, unstable angina pectoris; HT, hypertension; DM, diabetes mellitus; EPC, endothelial progenitor cell; Hs-CRP, high sensitive CRP.

compared between the groups. The comparison revealed no difference between the groups in terms of fibrinogen (p=0.571), whereas hsCRP was found higher in the STEMI group as compared to the USAP group (p=0.011).

Discussion

In the present study, we used a method that identifies the EPCs by excluding HSC, CEC, thrombocyte and EMP, another nucleus-free cell-, which may be confused with EPCs, in one tube by means of gating from CD45-negative and dim+ cells by 5-color FCM and found EPC count significantly lower in the STEMI and USAP patients as compared to the healthy subjects (p<0.0001). Comparison between two patient groups revealed lower EPC count in the STEMI group versus USAP group (p=0.011). A standardized method of FCM for identification and quantification of circulating EPCs has not been developed yet. Therefore, different studies report different results. Accurate quantification of EPC is of great importance for its usage in clinical practice. Pre-analytic factors, different moAbs, and different sample preparation, marking and analysis techniques also influence the outcomes. There is no standardized protocol or gating strategy, which is operator-dependent [14, 19–22].

Not a single EPC-specific antigen has been defined until today. The antigen profile overlaps with CEC and HSC [8]. In the study panel, we used CD45, CD34, CD31 and VEGFR-2 (KDR) to distinguish EPCs from CEC and HSC. HSC expresses CD34 and sometimes weak KDR, but these cells are CD45-positive. However, EPCs are CD45 dim-positive or CD45-negative; therefore they can be easily distinguished. Some researchers exclude all leukocytes stained positive with CD45 in the EPC analysis [16, 17]; nevertheless, most of CD34+ cells are CD45-dim and the number of CD45-CD34+ and CD45-CD133+ cells is very low in the peripheral circulation [8]. In the present study, we performed the analysis by gating from CD45-negative and dim+ cells. CEC has KDR expression and sometimes weak CD34 expression and, through this aspect, may be confused with EPC. However, CEC contamination is so low in the CD34+KDR+ EPC population that could be ignored [13]. Some researchers emphasized that CD133, which is another stem cell antigen, enhances the specificity of EPCs as CD133 is not found on the surface of CEC [7]. CD133 is in vitro and in vivo downregulated during development of endothelial cell line. In addition, CD133+ EPCs are early EPCs and are 20 times lesser in the peripheral circulation and have also lower proliferation capacity than CD34+KDR+ late EPCs. They are poorly reliable for the quantification of EPCs [13], this is why we did not use CD133 in the present study. Fadini et al. [23] and Schmidt et al. demonstrated that clinical correlation of CD34+KDR+ EPCs is better than that of CD133+KDR+ and CD34+CD133+KDR+ cells and recommended it as the best EPC phenotype to be analyzed [13, 24]. Lanutti et al. determined no CD34+CD133+KDR+ cell in the peripheral circulation. Different from the other studies, we used syto16® DNA stain as was recommended by Mancuso et al. and excluded endothelial micro particles (EMP) and platelets, which are nucleus-free cells found in the CD45-negative region and may show antigenic overlap with EPCs [22]. Subsequently, we performed the analysis by gating from Syto16+CD31+ cells (Figure 1). Again, in the present study, we exposed the flow cytometer to long clean before each acquisition to prevent potential contamination. We counted at least 1×10⁶ cells for each sample to enhance the sensitivity.

Although these two subgroups have not been compared in the studies conducted in ACS patients, individual evaluation of each group revealed increased EPC count in such patients as compared to healthy controls [25–27]. Contrary to these studies, we determined decreased EPC count in both patient groups as compared to the
healthy controls. Healthy controls’ being at younger age and selected very carefully might have played a role in this result. Furthermore, the time of phlebotomy in ACS patients might have had a role. Earlier studies stated that EPC peaks on the 7th day of myocardial infarction [28, 29]. In the present study, we performed phlebotomy at the time of hospital visit before starting any treatment or procedure (the first 24 h at the latest). We found EPC count higher in the USAP group, which is one of the two subgroups of acute coronary syndrome, as compared to the STEMI group. This might have resulted from low number of atherosclerotic plaques in the USAP patients [25–27]. Among inflammatory markers, fibrinogen and high-sensitive C-reactive protein (hs-CRP) values were also compared between the subgroups of ACS for atherosclerosis, which is an inflammatory process. While there was no difference between the groups in terms of fibrinogen values, hs-CRP value was significantly higher in the STEMI group as compared to the USAP group. Hs-CRP is one of the important predictors of coronary artery disease and this may be explained by higher atherosclerotic burden and expectation of more inflammatory process in acute phase in STEMI patients as compared to USAP patients indicating that atherosclerotic burden is lower in the USAP group versus the other groups. This may explain why EPC count is higher in the USAP group.

It was demonstrated that cardiovascular risk factors such as HT, DM, hyperlipidemia and smoking decrease EPC count independently from the development of coronary artery disease [10–12, 21].

Comparing other independent variables that influence EPC count, no statistically significant difference was determined between the two groups in terms of age, HT, DM, smoking, statin use and serum LDL levels [26, 30].

The limitations of the present study are as following: magnetic beads could be used instead of Kröbling formula in the dual platform while calculating absolute EPC count. The number of healthy controls could be higher.

In the present study, primarily the subjects who have been selected in accordance with the exclusion criteria depending on the patients’ detailed anamnesis were enrolled in the healthy control group. The significance of the present study is that the analysis can be performed simply and without consuming too much time by excluding the platelet, debris, EMPs and HSC using a single tube. In the present study, we identified the circulating EPC count in healthy subjects and the patients with ACS using multicolor FCM. We investigated whether there is a difference between STEMI and USAP subgroups of ACS patients in terms of EPC count and found the EPC count lower in both disease groups as compared to the healthy controls. First the method must be standardized and then harmonization studies are required to use this parameter as a prognostic factor in cardiovascular diseases.

**Conflict of interest statement:** The authors declared no conflict of interest.

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