

Clinical significance of 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester-labeled microspheres for detecting endothelial progenitor cells in human peripheral blood

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Abstract. The aims of the present study were to establish a single-platform flow cytometry method using 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled microspheres as the reference for determining endothelial progenitor cell (EPC) number and to evaluate the efficacy of this detection method. Single-platform flow cytometry was used to count cell numbers using CFSE-stained fluorescent microspheres as the internal reference and the EPC numbers in specimens using this novel method were compared with an *in vitro* clonogenic counting assay. The results of the two counting methods were consistent and compared with the *in vitro* clonogenic counting assay, the time and cost of the novel method was markedly reduced, as were the corresponding technical requirements. The present findings indicated that single-platform flow cytometry, with CFSE-labeled microspheres as the reference, provides faster and improved detection of EPCs in human peripheral blood specimens, with reduced time and cost, making it more suitable for routine clinical application.

Introduction

The occurrence, development and metastasis of tumors are closely related to angiogenesis due to the fact that blood vessels provide the necessary oxygen supply, nutrition, metabolite and avenue for metastasis to maintain the rapid

growth of tumors (1-3). Angiogenesis, which is the generation of novel blood vessels, occurs by two completely different processes (4-6). In the first, the required vascular endothelial cells arise from the sprouting of existing blood vessels. In the second, they are derived from recruited endothelial precursor cells, which are a type of blast cell with the potential to differentiate into clonal endothelial cells *in vitro* as well as participate in cardiovascular generation *in vivo* (7). On their surface, they characteristically express cluster of differentiation (CD)34, vascular endothelial growth factor receptor 2 (VEGFR2) or kinase domain receptor (8,9).

A large number of basic and clinical studies have indicated that the number of endothelial progenitor cells (EPCs) is closely related to tumor size, prognosis and therapy response (10-13). Evidence from animal models suggests that the EPC level in the peripheral circulation has some relevance to tumor volume (13). The number of EPCs in circulation has been identified to alter with anti-tumor and anti-angiogenesis therapies. Igraja *et al* (14) suggested that the EPC level in the peripheral blood of patients with lymphoma was related to the efficacy of the therapy. This hypothesis was supported by the fact that the EPC level in patients with complete remission decreased, while EPC levels continued to rise or did not change in those with partial remission or no response to therapy. In addition, it was revealed that tumor size and angiogenesis were associated with the number of EPCs in lymph nodes. Ho *et al* (15) indicated that, in patients with advanced non-surgically treated hepatocellular carcinoma (HCC), the EPC level in circulation was significantly higher compared with patients with resectable HCC, suggesting that the number of EPCs in the peripheral circulation may be used to determine the prognosis of HCC patients.

Currently, EPC detection methods include clone counting and characteristic index-based flow cytometry, of which the latter may be divided into dual-platform counting and single-platform counting (14,16,17). Dual-platform counting, which involves two parallel tubes and two devices, exhibits large variations in results. Conversely, single-platform counting uses commercialized fluorescent microspheres, which are expensive and easily adhere. Artificially synthesized fluorescent microspheres have a different sedimentation rate than cells, leading to unreliable results (18-20). In our previous

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study, CFSE-labeled cells were used to replace commercial fluorescent microspheres (21). We suggested that these fluorescence-labeled cells were stable and did not easily adhere; thus, the test results were reliable (21).

Due to the clinical value of EPCs, establishing an improved complete EPC counting method is crucial. The present study used single-platform flow cytometry technology with CFSE-labeled cell fluorescent microspheres as the internal control to determine the number of EPCs in peripheral blood and subsequently verify the reliability of this technology from a biological standpoint. Furthermore, this recently developed technology was used to detect the changes in EPC number following tumor anti-angiogenic therapy. Subsequently, the clinical value of using CFSE-labeled cell microspheres with single-platform flow cytometry for determining EPC number in peripheral blood was verified.

Materials and methods

Preparation and identification of artificial cell microspheres. A total of 50 μg (1 vial) of CFSE (Molecular Probes; Thermo Fisher Scientific Inc., Waltham, MA, USA) was dissolved in 18 μl of dimethyl sulfoxide to prepare the original solution with a final concentration of 5 mmol, which was stored at -20°C . Subsequently, 1 g of paraformaldehyde (PFA, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in 90 ml of distilled water and 10 ml of 10X phosphate-buffered saline (PBS) was added to prepare a 1% PFA solution. The THP-1 human acute leukemia cell line (Cell Bank of Shanghai Institute, Shanghai, China) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Chalfont, UK). THP-1 cells were washed with PBS three times and resuspended to a concentration of 1×10^6 cells/ml. Subsequently, 1 μl CFSE was added for each ml of cell suspension (to a final concentration of 5 $\mu\text{mol/l}$) followed by incubation at 37°C for 10 min. The original medium was added until a volume that was five times the original volume was achieved to terminate the marking procedure. The mixture was placed in an ice bath between 0 and 8°C for 5 min, followed by washing three times with fresh medium. Cells were resuspended in PBS supplemented with 1% PFA, with a cell concentration of 1×10^6 cells/ml and stored at 4°C until subsequent use. Non-marked THP-1 cells were used as a control. The prepared cell mixture, with artificial cell microspheres, was subsequently evaluated using flow cytometry.

Single-platform flow cytometry for determining the number of EPCs in peripheral blood. A total of 10 ml of human peripheral blood (anticoagulated with 1.8 mg/ml EDTA-K2) obtained from healthy volunteers was harvested for the extraction of mononuclear cells and the sample was divided into four parts, with respective volumes of 5, 2.5, 1.25 and 0.625 ml. Samples underwent negative selection, in which CD45 antibody-coated magnetic beads (Dynabeads; Thermo Fisher Scientific, Inc.) were added and the mixture was subjected to a magnetic field to adsorb cells that were able to bind the CD45 antibody-coated magnetic beads, thus removing non-EPC components. CD34, CD133 and KDR, commonly used membrane markers to define EPCs, were detected in cells by flow cytometry as described

previously (4). Subsequently, the target cells were pre-treated with an Fc-receptor-blocking reagent (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) to prevent non-specific binding and were incubated with an APC-conjugated-CD34 antibody (cat. no. 340441; 1:167; BD Biosciences, San Jose, CA, USA), a phycoerythrin-conjugated anti-KDR antibody (cat. no. FAB357p; 1:100; R&D Systems, Inc., Minneapolis, MN, USA) and a phycoerythrin-conjugated anti-CD133 antibody (cat. no. 130-080-801; 1:100; Miltenyi Biotec GmbH) at 4°C for 40 min. A total of 10,000 CFSE-labeled microspheres were added to the test sample and washed with PBS three times. The sample was thoroughly mixed before counting. Red blood cells were lysed with ammonium chloride (BD Biosciences, San Jose, CA, USA) and a total of 10^6 events were recorded on a FACS Calibur cytometer (BD Biosciences). Data were analyzed with CellQuest software (version 5.2.1; BD Biosciences).

The absolute number of cells inside the test sample (ND) was calculated using the following formula: Absolute number of cells = target cell number / number of cell microspheres \times added number of cell microspheres.

Identifying EPCs and determining the number of EPCs in peripheral blood. A total of 10 ml of human peripheral blood was collected and divided into four parts, with volumes of 5, 2.5, 1.25 or 0.625 ml. A single karyoplast was obtained by the density centrifugation method and planted onto a human fibronectin-coated culture plate and cultured in M199 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS, 10 ng/ml VEGF, 100 ng/ml penicillin and 100 ng/ml streptomycin for 2 days. Following culturing for 2 days, the mature endothelial cells had adhered to the wall and the non-wall-adherent cells were collected and re-planted onto human fibronectin-coated culture plates for final counting. The medium was changed once every 3 days and the non-wall-adherent cells were washed off with PBS 7 days later. Subsequently, 2.4 mg/l of 1,1'-dioctadecyl-3,3,3'-tetramethyl-indocarbocyanin perchlorate-labeled-acetylated-low density lipoprotein (Thermo Fisher Scientific, Inc.) was added to the cultured cells, followed by incubation at 37°C in an atmosphere containing 5% CO_2 for 12 h. Cells were fixed with 2% PFA for 30 min, followed by washing with D-Hank's solution (GE Healthcare Life Sciences) twice. A total of 10 $\mu\text{g/ml}$ fluorescein isothiocyanate-Ulex Europaeus Agglutinin-I (Sigma-Aldrich; Merck KGaA) was added and the mixture was incubated at 37°C for 1 h. Cells were observed under a fluorescence microscope (magnification, $\times 40$; CKX53; Olympus Corporation, Tokyo, Japan) and cells with positive dual-staining were considered to be differentiated EPCs.

The number of clones was determined under a microscope and the number of EPCs was calculated using the following formula: EPC concentration = number of colonies / original collected blood volume.

Detecting the changes in EPC number in the peripheral blood of patients with cancer prior to and following the administration of anti-angiogenic agents. A total of 20 patients with solid tumors (10 cases of liver cancer, 6 cases of osteosarcoma and 4 cases of stomach cancer) were selected according to the standards for clinical treatment with anti-angiogenic agents.

Patients were enrolled between March 2014 and February 2015 and were aged 25 to 59 years old, with a male: female ratio of 3:2. Patients had no underlying conditions, history of surgery or allergies. The inclusion criteria were as follows: Clear diagnosis of solid tumors, no myocardial infarction and intracranial hemorrhage within a month, no organ infarction and deep venous thrombosis, no significant systemic infection, no chemotherapy radiotherapy history nearly a month and no other cancer treatment, including targeting medical treatment. The exclusion criteria were: Neutrophil count $<1.5 \times 10^9/l$ or platelet count $<100 \times 10^9/l$, women of childbearing age who serum pregnancy test was positive or long-term use of immunosuppressive agents after organ transplantation. The Ethics Committee of Gongli Hospital approved the study protocol, and written informed consent was obtained from all participating subjects. A total of 20 ml blood was harvested prior to and following treatment and the testing method was the same as described above.

The absolute number of cells in the test sample (ND) was calculated using the following formula: Absolute number of cells = target cell number/number of cell microspheres \times added number of cell microspheres. The method for the *in vitro* clonogenic counting assay was the same as described above.

Statistical analysis. Data were analyzed using Statistical Package for the Social Sciences (SPSS) software (version 13.0; SPSS Inc., Chicago, IL, USA). One-way analysis of variance with Dunnett's post test was used for statistical evaluation of significant differences among the groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Preparation and identification of artificial cell fluorescent microspheres. The cell fluorescent microspheres exhibited strong homogeneous fluorescence (Fig. 1; Q2) and the average fluorescence intensity was strong. Thus, CFSE-labeled cell beads were easily distinguished from non-labeled THP-1 cells (Fig. 1; Q1) and with maintained fluorescence, indicating that the obtained cell microspheres were feasible for the intended application.

Detection of EPCs in peripheral blood using single-platform flow cytometry. Regression curves for EPC number changed according to the reduction in the original blood sample volume (Fig. 2), indicating that this method was able to determine the number of EPCs in peripheral blood.

Detection of EPCs in peripheral blood using an *in vitro* clonogenic counting assay. Regression curves for EPC number altered according to the reduction in the original blood sample volume (Fig. 3), indicating that this method was able to determine the number of EPCs in peripheral blood. When comparing Figs. 2 and 3, there is a clear consistency between these two detection methods, indicating that single-platform flow cytometry may be used to feasibly and accurately determine the number of EPCs in peripheral blood.

EPCs in the peripheral blood of patients with cancer were counted prior to and following anti-angiogenic agent administration, using single-platform flow cytometry and the *in vitro*

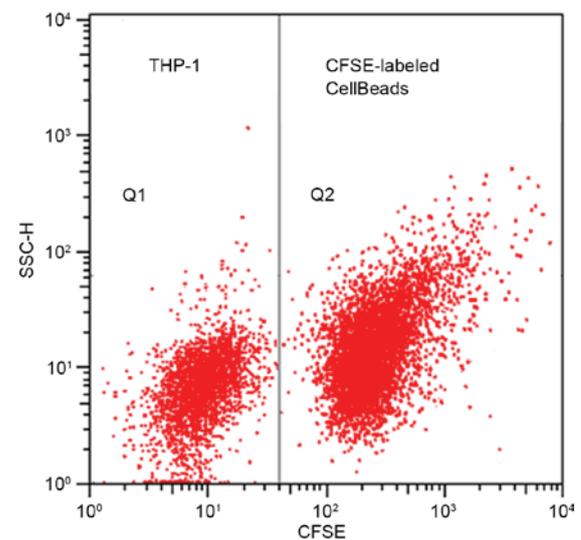


Figure 1. Number of EPCs detected using THP-1 human acute leukemia cells by single-platform flow cytometry. EPCs, endothelial progenitor cells; CFSE, 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester; Q1, content of target cells; Q2, content of CFSE-labeled fluorescent microspheres.

clonogenic counting assay. Changes in the EPC number in the peripheral blood of patients with cancer prior to and following the administration of anti-angiogenic agents were measured using single-platform flow cytometry (Fig. 4A). Following anti-angiogenic agent administration, the EPC number was reduced when compared with the number prior to anti-angiogenic agent administration and in 12 patients this was statistically significant ($P < 0.05$; Fig. 4A). In addition, the results using the *in vitro* clonogenic counting assay were consistent with the flow cytometry results and 10 patients exhibited a significantly decreased EPC number following anti-angiogenic agent administration ($P < 0.05$; Fig. 4B). Overall, the data indicated that the anti-angiogenic treatment was able to significantly reduce the number of EPCs in peripheral blood ($P < 0.01$; Fig. 4C).

Discussion

Current methods for determining EPC number may be divided into two categories. The first uses *in vitro* culture, in which cell differentiation is induced and cell clones that are formed are characterized as endothelial cells and counted. The second uses targeting to detect EPC-specific surface markers and this category may be divided into flow cytometry (16,17,22,23) and gene expression-based polymerase chain reaction (PCR) quantitative detection (24,25).

Although these two categories of detection methods have a large number of applications, they are essentially basic research methods and are difficult to apply to routine clinical testing, predominantly due to the following: Clone counting, although currently recognized as the most widely used EPC detection method, has the disadvantages of requiring time-consuming cell culturing, highly technical methods and is considered expensive; and flow cytometry, although it directly targets the indicators, is time consuming and requires expensive commercial fluorescent microspheres for quantitative analysis (26,27). In addition, the physical properties of

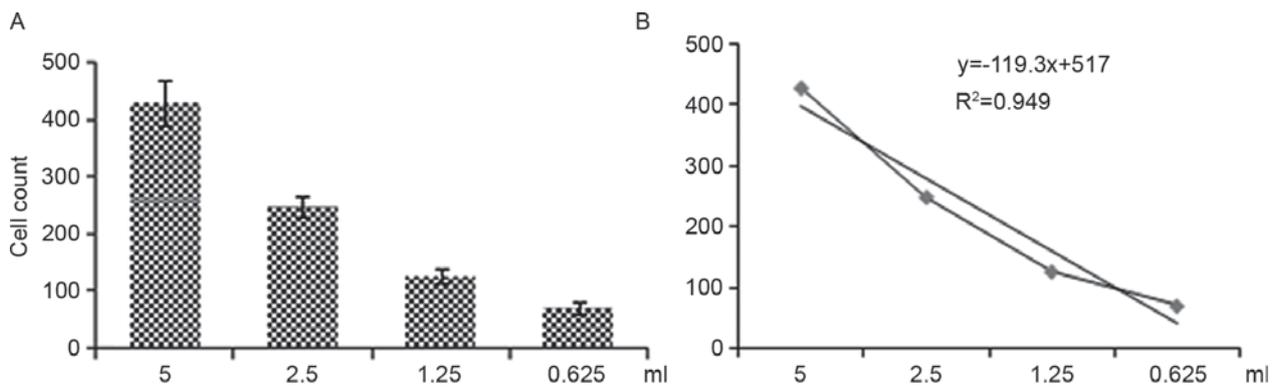


Figure 2. Detection of EPCs in 5, 2.5, 1.25 or 0.625 ml of peripheral blood from patients with cancer by single-platform flow cytometry. (A) Contents of EPCs in different original-volume blood samples. (B) Regression equation of EPCs content in different original-volume blood samples. EPCs, endothelial progenitor cells.

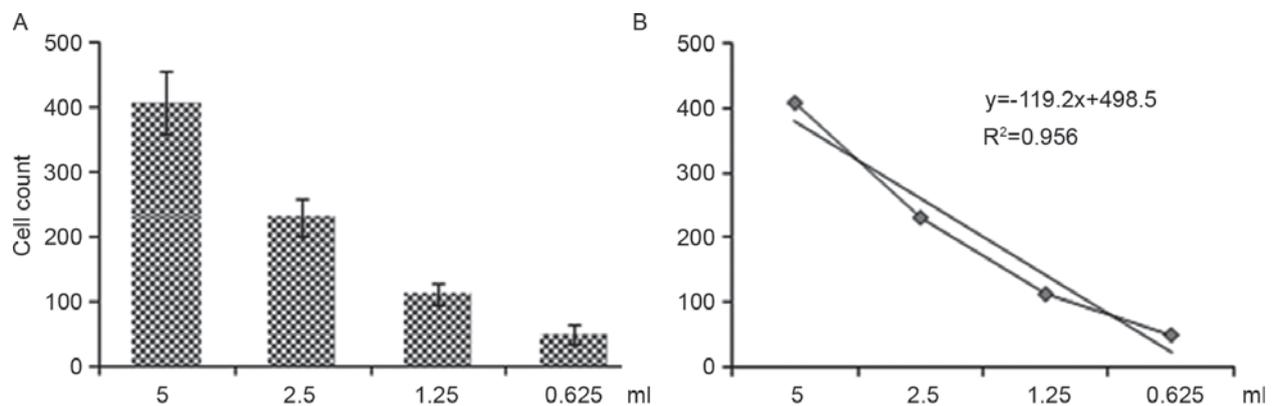


Figure 3. Detection of EPCs in 5, 2.5, 1.25 or 0.625 ml of peripheral blood from patients with cancer by *in vitro* clonogenic counting assay. (A) Detection of EPCs in peripheral blood by *in vitro* clonogenic counting assay. (B) Regression equation of EPCs contents in different original-volume blood samples. EPCs, endothelial progenitor cells.

these commercial microspheres are different from those of the cells, thus leading to unreliable results and the operators are required to constantly adjust to novel detection processes. Furthermore, the PCR-based method, which detects specific indicators, has poor specificity among its shortcomings (28). The present study used single-platform flow cytometry with microspheres prepared according to the method recently reported in Cytometry A, regarding the use of the fluorescent dye, CFSE, to uniformly label leukemia cells and impart them with fluorescence (21). A known number of fluorescent cells were added to the test specimens as an internal reference for detection by single-platform flow cytometry, allowing the determination of the number of other, undetected cells in the specimens. In our previous study, CFSE-labeled cells were used to replace commercial fluorescent microspheres (21). In addition, they may be clearly distinguished from cells not labeled with fluorescence and have the same density and uniform sedimentation rate as the test cells; thus, they may be used as an internal reference for quantitative analysis and the results will be reliable (21). Therefore, once EPCs were immunolabeled, the newly constructed cell fluorescent microspheres were added, resulting in a quantitative detection, with improved accuracy, of EPCs in human peripheral blood, with reduced testing costs (1/5-1/6 of the current commercial microspheres). However, further studies are

required to determine whether there are alternative cells or indicators, such as blood cells and dyes, that are more suitable than leukemia cells and CFSE for cell fluorescent microspheres, respectively.

In the present study, specific CD34, VEGFR-2 and CD133 antibodies were used to label EPC cells; however, no specific cell surface marker has been identified that is able to completely distinguish EPCs from hematopoietic cells. Previous results have indicated that mesenchymal stem cell-associated CD34⁻/VE-cadherin⁻/AC133⁺/Flk-1⁺ multipotent adult progenitor cells (MAPCs) may be converted to CD34⁺/VE-cadherin⁻/AC133⁻/Flk-1⁺ angioblasts by the action of VEGF and that these cells may be further differentiated into mature endothelial cells (23,29,30) and become involved in tumor angiogenesis and wound healing. The cell surface markers of EPCs have not been fully elucidated and there continues to be large variances in the reported quantities of EPCs present in the circulation and uncertainty regarding the best enrichment and isolation methods (31,32). Thus, the specific phenotype that may distinguish EPCs from hematopoietic cells or mature endothelial cells still requires further exploration, which provides the motivation for continued progression in the method described in the present study.

The number of EPCs is closely related to tumors due to the fact that tumor growth requires angiogenesis (33). Once

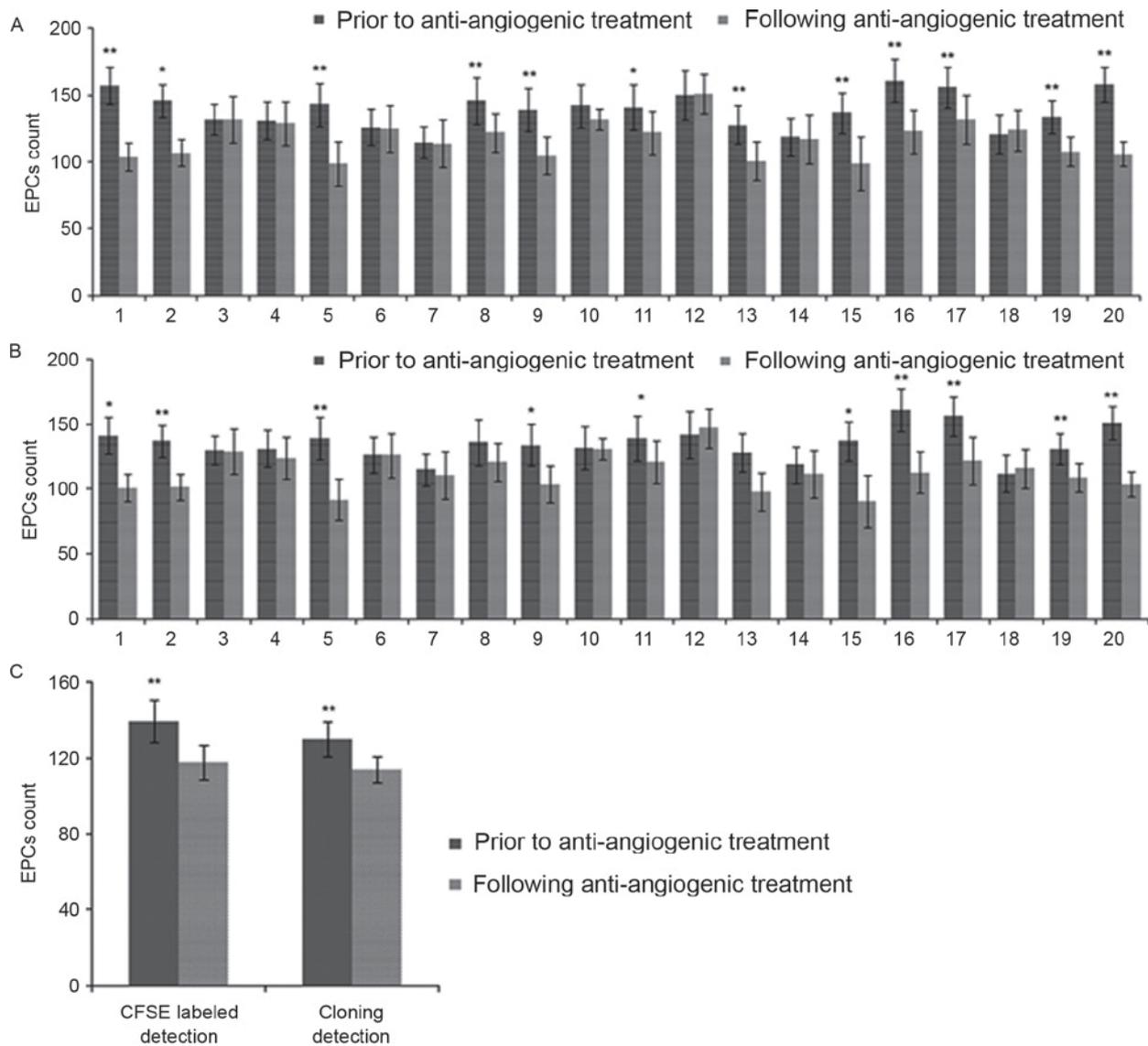


Figure 4. Changes of EPCs in peripheral blood of 20 patients with cancer prior to and following the administration of anti-angiogenic agents by (A) single-platform flow cytometry or (B) *in vitro* clonogenic counting assay. (C) Impact of anti-angiogenic agents on the number of EPCs. *P<0.05 and **P<0.01 vs. following anti-angiogenic treatment. EPCs, endothelial progenitor cells; CFSE, 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester.

a tumor reaches a size of 3 mm, the tumor cannot survive unless novel blood vessels are produced (5,6). Tumors are able to secrete specific factors that stimulate the bone marrow to increase EPC generation and to mobilize the generated EPCs into the peripheral blood, thus enriching local tumors with EPCs that participate in the formation of novel blood vessels (4,25,34). Therefore, EPCs are an important indicator of tumor growth and prognosis and determining the number of EPCs has important clinical significance for patients with cancer. The present study determined the content of EPCs in the peripheral blood of patients with cancer. Compared with the results of an *in vitro* clonogenic counting assay, the accuracy of our method was reasonable.

Small arterial lesions may cause long-term high blood pressure, leading to tissue ischemia of important target organs such as the heart, brain and other organs (3). Endothelial dysfunction is caused by the destruction of the dynamic balance between endothelial injury and repair, and hypertension and endothelial dysfunction enhance one another. A previous study found that

EPC was able to differentiate into mature endothelial cells to repair damaged endothelial cells (35). Therefore, monitoring the number of EPCs may have important clinical significance for cardiovascular and cerebrovascular diseases. Therefore, monitoring the number of EPCs may have important clinical significance for cardiovascular and cerebrovascular diseases. The present study demonstrated that single-platform flow cytometry based on CFSE-labeled cell microspheres has unique advantages in determining the number of EPCs, overcomes the shortcomings of other methods and was objective and accurate. This method may be widely used in clinical practice for fast and accurate analysis of EPCs in peripheral blood.

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