Circulating endothelial cells and their progenitors in acute myeloid leukemia

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Abstract. Acute myeloid leukemia (AML) is an aggressive hematological malignancy characterized by the accumulation of immature myeloid progenitor cells in the bone marrow. Studies are required to investigate the prognostic and predictive value of surrogate biomarkers. Given the importance of angiogenesis in oncology in terms of pathogenesis as well as being a target for treatment, circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) are promising candidates to serve as such markers. The aim of the present study was to quantify CECs and EPCs in patients with AML at initial diagnosis and following induction chemotherapy, and to correlate these findings with the response to treatment in AML patients. The present study included 40 patients with *de novo* AML and 20 age- and gender-matched healthy controls. CECs and EPCs were evaluated by flow cytometry at initial diagnosis and after induction chemotherapy (3+7 protocol for AML other than M3 and all-trans-retinoic acid plus anthracycline for M3 disease). CECs and EPCs were significantly higher in AML patients at diagnosis and after induction chemotherapy than in controls. After induction chemotherapy, CECs and EPCs were significantly decreased compared with the levels at initial diagnosis. Patients who achieved complete response (n=28) had lower initial CEC and EPC levels compared with patients who did not respond to treatment. These results suggest that CEC levels are higher in AML patients and may correlate with disease status and treatment response. Further investigations are required to better determine the predictive value and implication of these cells in AML management.

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Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous disease with accumulation of acquired genetic alterations in hematopoietic progenitor cells that disturb the normal mechanisms of cell growth, proliferation and differentiation (1). In recent years, there has been an increased understanding of the role of angiogenesis in the progression of AML (2). Although circulating endothelial cells (CECs) and their progenitors, endothelial progenitor cells (EPCs), were first described >30 years ago through light microscopy, the development of specific monoclonal antibodies against these cells has only recently provided the opportunity to investigate the pathophysiology of these cells (3). Previously, the immune-beads technique and/or flow cytometry were used to investigate the significance of CECs in a variety of diseases, including infections as well as cardiovascular, inflammatory and autoimmune syndromes, and cancer (4). These rare CECs (which encompass <1 cell in 1,000 circulating blood cells) are probably derived from vessel wall turnover and are quite stable overtime (5). The majority of these cells exhibit characteristics of mature, terminally differentiated cells (5). EPCs are a sub-population of CECs that express antigens suggestive of a stem-like or progenitor-like phenotype (6). These putative EPCs may migrate to sites of vasculogenesis and angiogenesis, and may participate in new blood vessel formation by stimulating vasculogenesis (7). The lack of a universal definition, a unified phenotypic characterization and standardized methods of detection makes comparisons very difficult, and between-studies interpretations of EPCs should be analyzed cautiously (5).

Multiple studies have focused on CECs as a non-invasive angiogenesis marker and their role as predictors of the clinical response in cancer patients receiving both antiangiogenic and standard chemotherapy for breast, lung and hepatocellular cancer, among others (8). Furthermore, the levels of CECs have been correlated to tumor stage and prognosis (5-16).

Notably, CECs and EPCs may be a valuable tool for prediction of graft-versus-host disease in allo-transplantation, as confirmed by Almici *et al* (9). Furthermore, their kinetics may be helpful in monitoring the mobilization of hematological cells prior to transplantation (10); however, their kinetics differs

by the use of different chemotherapy agents (11). Additionally, CECs and EPCs could be used as a novel marker for minimal residual disease in chronic myeloid leukemia, as reported by Wu *et al* (12). Wierzbowska *et al* suggested that endothelial cells may enhance the survival and proliferation of leukemic blasts and mediate chemotherapy resistance in AML (13).

The aim of the present study was to quantify CECs and their progenitors EPCs in patients with AML by flow cytometry at initial diagnosis and after induction chemotherapy, and to correlate these findings with the patients' response to treatment.

Patients and methods

Patients. The present study is a retrospective case-control study including 40 patients with *de novo* AML, who presented to the Hematology/Oncology Clinic of South Egypt Cancer Institute (SECI), Assiut University (Assiut, Egypt) between May 2014 and October 2015, and 20 healthy controls. The study was approved by the Institutional Review Board of SECI. Written informed consent was obtained from all cases and controls.

All patients were subjected to: i) A thorough history evaluation and clinical examination, with careful assessment of clinical signs relevant to leukemia, including hepatomegaly, splenomegaly, lymphadenopathy, gums or skin infiltration; ii) a complete blood test, which was performed using fully automated blood counters and peripheral smear examinations; iii) a bone marrow examination and cytochemistry, including detection of myeloperoxidase, esterases, acid phosphatase and periodic acid-Schiff; and iv) immunophenotyping using mouse monoclonal antibodies for diagnosing AML at a 1:1,000 dilution, including anti-cluster of differentiation (CD)34 (cat. no. 340430; BD Biosciences, Franklin Lakes, NJ, USA), anti-CD13 (cat. no. 555394; BD Biosciences), anti-CD33 (cat. no. 340680; BD Biosciences), anti-CD117 (cat. no. 555714; BD Biosciences), anti-CD15 (cat. no. 332778; BD Biosciences), anti-intracellular myeloperoxidase (cat. no. 340580; BD Biosciences), anti-CD14 (cat. no. 550787; BD Biosciences), anti-human leukocyte antigen-antigen D related (cat. no. 560896; BD Biosciences), anti-CD41 (cat. no. 555466; BD Biosciences), anti-CD61 (cat. no. 555753; BD Biosciences) and anti-glycophorin A (cat. no. 340947; BD Biosciences). Blood samples from the patients and the control group were subjected to assessment of EPCs and CECs using flow cytometry.

Remission induction regimens. All AML patients other than those with M3 disease received the 3+7 protocol, which is a combination of intravenous (IV) chemotherapy that includes 7 days of cytarabine (100 mg/m^2/day by continuous infusion) and 3 days of adriamycin (45 mg/m^2, IV). AML M3 patients received the European acute promyelocytic leukemia regimen (14), which consisted of adriamycin (45 mg/m^2/day, IV) as 15-30 min infusion for 3 days (since other anthracyclines were not available) plus cytarabine (200 mg/m^2/day, IV) as 24-h continuous infusion on days 1-7, plus all trans-retinoic acid (ATRA; 45 mg/m^2/day, or 25 mg/m^2/day for patients <20 years old) in 2 divided doses, starting on day 1. The response to treatment was defined according to the revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia (15).

Flow cytometric detection of CECs and EPCs. Venous blood samples (2 ml) were collected at the time of clinical assessment in pyrogen-free ethylenediaminetetraacetic acid tubes. CECs and EPCs were evaluated using a panel of mouse anti-human monoclonal antibodies at a 1:1,000 dilution: Fluorescein isothiocyanate (FITC)-labeled anti-CD144 (cat. no. 560874; BD Biosciences), phycoerythrin (PE)-conjugated anti-CD133 (cat. no. 130080801; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), peridinin chlorophyll protein (PerCP)-conjugated anti-CD34 (cat. no. 555842; BD Biosciences) and allophycocyanin (APC)-conjugated anti-CD45 antibodies (cat. no. 555485; BD Biosciences). Briefly, blood samples (50 μl) were incubated with 5 μl anti-CD144, anti-CD133, anti-CD34 and anti-CD45 antibodies for 15 min at room temperature in the dark. Subsequently, red blood cells were lysed, and resuspended in phosphate-buffered saline. Flow cytometric analysis was performed using a FACSCalibur flow cytometer with CellQuest Pro software (BD Biosciences). In total, 50,000 events were analyzed, and FITC- , PE-, PerCP-, and APC-conjugated mouse anti-human immunoglobulin G (cat. nos. 556649, 554680, 349044, and 550854, respectively; 1:1,000 dilution; BD Biosciences) were used as isotype-matched negative controls for each sample. The gating strategy to detect CECs and EPCs was based on CD45 staining to exclude hematopoietic cells. CECs were identified as cells lacking CD45 expression, which were positive for CD144 and CD34, and negative for CD133 (CD45-/CD144+/CD34+/CD133-), while EPCs were identified as cells that were negative for CD45, and positive for CD144, CD34 and CD133 (CD45-/CD34+CD144+/CD133+) (Fig. 1). The number of CECs and EPCs were expressed as per 50,000 cells.

Statistical analysis. Data analysis was performed with SPSS version 16 software (SPSS, Inc., Chicago, IL, USA). The statistical differences between the groups were examined using the Mann-Whitney U test and the Wilcoxon signed-rank test, while the *t*-test and the χ^2 test were used for analysis of continuous and categorical parameters. Due to the relatively small sample size and the requirement to indicate the uncertainty around the estimate of the mean, the standard error (SE) was calculated as follows: $SE=SD/\sqrt{N}$, where N is the sample size and SD is the standard deviation. $P\leq0.05$ was considered to indicate a statistically significant difference. The Spearman's rank correlation coefficient was used to examine the correlations among the different studied parameters.

Results

A total of 40 adult AML patients were included in the present study. The sociodemographic and laboratory characteristics of the AML patients and the controls are shown in Table I. There was no significant difference in the mean age or gender percentages. In total, 30% of the patients had M2, while 22% had M4 and 22% had M3 disease.

At diagnosis (baseline levels), CECs and EPCs were significantly higher in AML patients than in the controls. Regarding CECs and EPCs kinetics after induction chemotherapy, the

Table I. Comparative analysis between patients with acute myeloid leukemia and controls regarding sociodemographic characteristics and several laboratory parameters.

Parameter	Patients (n=40)	Controls (n=20)	P-value
Age, years (range)	54 (23-68)	48 (24-56)	0.681[a]
Gender (male/female)	24/16	13/7	0.091[b]
WBCs (10^9 cells/l)	45.32±3.47	6.62±0.41	<0.001[c]
Platelets (10^9 cells/l)	49.39±4.83	231.25±15.70	<0.001[c]
Hemoglobin (g/dl)	7.49±0.25	12.99±0.18	<0.001[c]

[a]*t*-test analysis. [b]χ^2 analysis. [c]Mann-Whitney U test data represented as means ± standard error of the mean. P≤0.05 was considered to indicate a statistically significant difference. WBCs, white blood cells.

Table II. CECs and EPCs in AML patients at presentation and after induction chemotherapy vs. controls.

Characteristics	AML patients at presentation (n=40)	AML patients after induction chemotherapy (n=40)	Controls (n=20)	[a]P-value	[b]P-value	[c]P-value
CECs	102.64±6.14	83.18±3.47	24.09±1.78	<0.001	<0.001	<0.001
EPCs	32.64±1.87	26.67±1.18	4.86±0.34	<0.001	<0.001	<0.001

Data represented as means ± standard error of the mean. P≤0.05 was considered to indicate a statistically significant difference. [a]P-value of AML patients at presentation vs. controls (Mann-Whitney U test). [b]P-value of AML patients after induction chemotherapy vs. controls (Mann-Whitney U test). [c]P-value of AML patients at presentation vs. AML patients after induction chemotherapy (Wilcoxon signed-rank test). CECs, circulating endothelial cells; EPCs, endothelial progenitor cells; AML, acute myeloid leukemia.

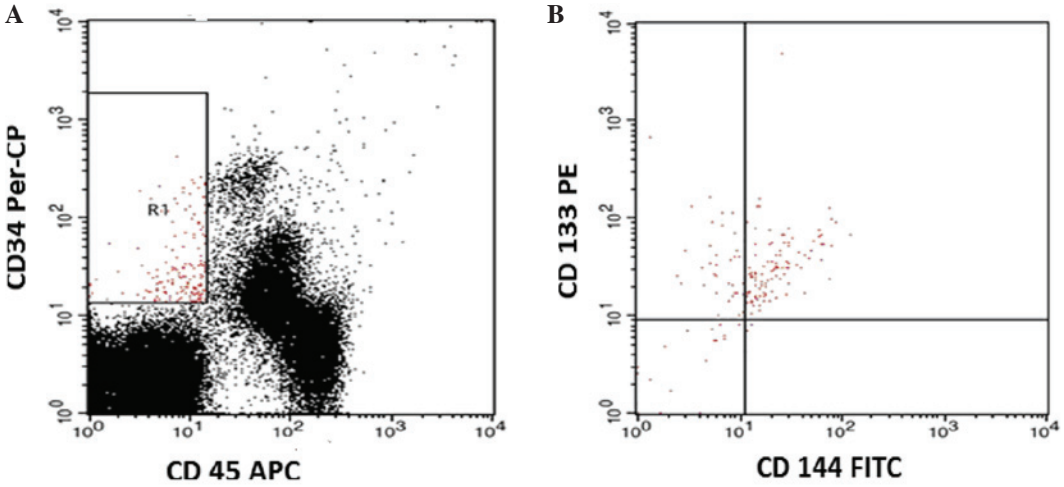

Figure 1. Flow cytometric detection of CECs and EPCs. (A) The analysis gate (R1) included CD34+ CD45- cells. (B) The expression of CD144 and CD133 in the R1 gate was detected compared with the negative isotype control (data not shown). CECs were defined as CD45-, CD34+, CD144+ and CD133- cells, while CEPs were identified as CD45-, CD34+, CD144+ and CD133+ cells. CECs, circulating endothelial cells; EPCs, endothelial progenitor cells; CD, cluster of differentiation; PerCP, peridinin chlorophyll protein; APC, allophycocyanin; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

levels of these cells were significantly decreased in AML patients compared with their levels at diagnosis, but the levels were still significantly higher than those in the controls (Table II). After induction chemotherapy, 28 patients (70%) achieved complete response (CR) to treatment, while 12 (30%) did not achieve CR. The mean baseline levels of CECs and EPCs in AML patients who achieved CR were significantly lower than in those who did not achieve CR in response to induction treatment. In addition, the levels of CECs and EPCs after induction chemotherapy in AML patients were significantly lower in those patients who achieved CR than in those who did not achieve CR (Table III). There were significant positive correlations between total leukocyte count and bone marrow blast count with both CECs and EPCs (Fig. 2). There was no significant correlation between CEC or EPC levels and hemoglobin levels; however, CECs were correlated with

Table III. CECs and EPCs in acute myeloid leukemia patients at presentation and after induction chemotherapy, and their correlation with treatment response.

Endothelial cells	Patients who achieved CR (n=28)	Patients who did not achieved CR (n=12)	P-value[a]
CECs at presentation	92.66±5.72	125.92±14.71	0.011
EPCs at presentation	29.85±1.72	39.15±4.32	0.021
CECs after induction chemotherapy	75.13±4.58	93.16±5.01	0.005
EPCs after induction chemotherapy	21.43±1.55	32.53±3.16	0.001

[a]Mann-Whitney test data represented as means ± standard error of the mean. P≤0.05 was considered to indicate a statistically significant difference. CECs, circulating endothelial cells; EPCs, endothelial progenitor cells; CR, complete response.

Table IV. Spearman's correlation between CECs and EPCs in acute myeloid leukemia patients at presentation and the investigated parameters.

Parameter	WBCs (x10^9/l)	BM blasts (%)	Hemoglobin level (g/dl)	Platelet count (x10^9/l)
CECs				
r (correlation coefficient)	0.66	0.62	0.05	0.36
P-value	0.001	0.001	0.76	0.022
EPCs				
r (correlation coefficient)	0.49	0.46	0.28	0.096
P-value	0.002	0.003	0.08	0.556

P≤0.05 was considered to indicate a statistically significant difference. CECs, circulating endothelial cells; EPCs, endothelial progenitor cells; WBCs, white blood cells; BM, bone marrow.

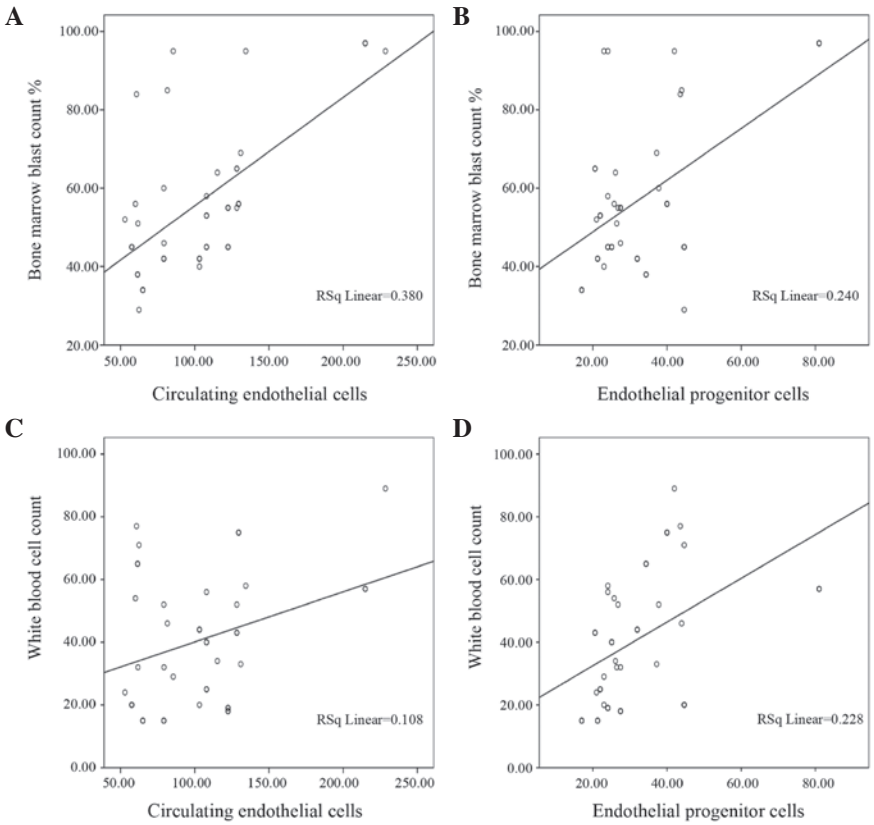

Figure 2. Spearman's correlations between (A and C) circulating endothelial cells and (B and D) endothelial progenitor cells in acute myeloid leukemia patients at presentation with (A and B) bone marrow blast count and (C and D) white blood cell count.

platelet counts (Table IV). No correlation of CECs or EPCs with patients' age, gender or French-American-British (FAB) classification were observed.

Discussion

AML is a hematological malignancy of the bone marrow characterized by a mutation in hematopoietic stem or progenitor cells, which develops into a highly proliferative accumulation of dysfunctional and immature myeloid cells (2). When interpreting studies on CECs and EPCs, attention must be paid to the different definitions and phenotypic characterization of CECs in each study, due to the lacking of a universal definition of these cell population (16), and to the different methods of CECs and EPCs detection, such as flow cytometry and the CELLSEARCH® system (16). The cell population definition used in the present study was based on the markers most widely accepted in flow cytometric analysis. It is widely accepted that CD45 expression can be used to exclude haematopoietic cells from the analysis, while endothelial cells are identified by the expression of CD146, which is an endothelial-specific marker, and CD31 (17). Furthermore, at the present time, the sole antigen that appears to be expressed in EPCs and subsequently downregulated in mature CECs is CD133 (3).

In the current study, both CECs and EPCs were higher in AML patients than in the control group, both at diagnosis and after induction chemotherapy, which may indicate that angiogenesis may have a role in the maintenance of AML. This may indicate that widespread vascular damage and disruption occur in the endothelium of AML patients. The EPCs may increase to allow reconstitution of the endothelial layer and to maintain re-endothelialization and vascular repair. There was no correlation of CECs or EPCs with patients' age, gender or FAB classification, which may indicate that angiogenesis is a common feature in all subtypes of AML. The positive correlations between CECs and EPCs with both total leukocytes count and bone marrow blast count may indicate that the levels of CECs and EPCs are correlated with the tumor mass. These findings are in accordance with those by Wierzbowska *et al*, who observed that the levels of CECs and EPCs were significantly higher in AML patients than in the control group by a 17- and 18-fold, respectively (13). This is in concordance with previous studies in multiple myeloma (MM), metastatic carcinoma, myelodysplastic syndrome, gastrointestinal stromal disease (GIST), hepatocellular carcinoma, breast cancer, non-Hodgkin's lymphoma (NHL), myelofibrosis and chronic lymphocytic leukemia (5-17).

In the present study, the CEC and EPC levels decreased after induction chemotherapy compared with pre-chemotherapy levels in AML patients, but the levels were still higher than those in the controls. This reduction in CEC and EPC levels may support the clinical relevance of these cells in reflecting the tumor mass. The higher levels of CECs and EPCs in AML patients after induction chemotherapy compared with those in the controls may be due to the fact that these cells were measured precisely at the time of very active bone marrow recovery, as they were counted at the day of bone marrow aspirate performed for the evaluation of response, usually 3-4 weeks after chemotherapy. The EPCs may mobilize from the bone marrow with hematopoietic cells, and these elevated mobilized EPCs possibly matured to CECs. The lower CEC and EPC levels in patients who achieved CR compared with patients who did not achieve CR either at presentation or after treatment indicate that CEC and EPC levels may be used to detect treatment response, and they could be used to reflect the level of minimal residual disease in AML. There is evidence that EPCs are mobilized from the bone marrow simultaneously with hematopoietic progenitor cells (18). Endothelial cells may enhance the survival and proliferation of leukemic blasts and may mediate chemotherapy resistance in hematological disease, at least at the preclinical level (19,20).

The present results are in concordance with other studies. In a study of imatinib-resistant GIST patients, the authors detected changes in CECs, which differed between patients with clinical benefit and those with progressive disease (21). Another study of low-dose cyclophosphamide administered continuously in combination with celecoxib in adult patients with relapsed or refractory aggressive NHL demonstrated that CECs and EPCs declined and remained low in responders (22). Similarly, CEC and EPCs were observed to be correlated with disease activity (serum M protein and β2 microglobulin) and with response to thalidomide therapy in MM (23). Conversely, CECs/EPCs did not prove to be useful pharmacodynamic biomarkers (24). In a phase I study of enzastaurin (a protein kinase Cβ inhibitor) administered in combination with gemcitabine and cisplatin to patients with advanced tumors, the single agent enzastaurin had no effect on any of the angiogenesis biomarkers analyzed (CECs and messenger RNA expression of CD133 and CD146 in peripheral blood) (25).

In conclusion, the present study revealed that CEC levels are higher in AML and correlate with disease status and response to treatment. Further investigation should be undertaken to better determine their predictive value and implication in AML management.

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