

Circulating Endothelial Cells in Acute Myeloid Leukemia

¹Khaled Moustafa Ibrahim, ²Shadia Hasan Ragab, ¹Nahed Abdel Alwahab Mohammed, ²Nahed A. Emara, ²Nagwa Abdel Alghafar, ¹Eman Kandeal and ²Al shaymaa Ahmed Ibrahim

¹Department of Clinical Pathology National Cancer Institute Cairo University.

²Department of Clinical and Chemical Pathology National Research Center, Cairo, Egypt.

Abstract: INTRODUCTION: The circulating endothelial cells (CEC) are proposed to be a non-invasive marker of angiogenesis. The level of CEC in peripheral blood (PB) of acute myeloid leukemia (AML) patients has not been investigated prior to this study. The aim of this work was to study aCEC and EPC levels in patients with AML and compare their levels with healthy subjects to prove their role in tumorigenesis and correlate these findings with clinical and hematological data of patients and used as prognostic factors. METHODS: CEC were quantified by utilizing flow cytometry procedures in 50 AML patients at the time of diagnosis and 20 healthy controls. RESULTS: The numbers of aCEC and CEPC were significantly higher in the AML patients than in the controls ($P < 0.0001$ and $P < 0.001$, respectively). CONCLUSION: CEC levels are higher in AML and correlate with disease status. Further investigation should be undertaken to better determine their prognostic value.

Key words: Acute myeloid leukemia-CEC-aCEC-CD34

INTRODUCTION

AML is an aggressive disorder that results from a block in the differentiation of hematopoietic progenitor cells along with uncontrolled proliferation (Gregory *et al*, 2009).

In NCI, Cairo University; AML accounts for approximately 349 (41.5%) out of the 840 newly diagnosed cases with acute leukemia registered in the time period between January 2002 and December 2003. The male to female ratio was 1.37:1 with a median age of 22 years and a range between ≤ 10 and 80 years (NCI Cancer Registry, 2003).

The AML has the lowest survival rate of all leukemias, so assessment of the prognostic factors in AML is very important (Phekoo *et al*, 2006). Recently, there is mounting evidence that circulating endothelial cells play an important role in neovascularization and tumor growth.

In the past years, the concept of angiogenesis has evolved from a simple model of the formation of new blood vessels from the preexisting vasculature into a multifaceted process in which, beyond local activation and division of endothelial cells, bone marrow-derived endothelial progenitor cells (EPCs) contribute to neovascularization. It was postulated that EPCs are mobilized from the bone marrow into the circulation and subsequently home to sites of tumor neovascularization where they differentiate into endothelial cells and contribute to angiogenesis (Roodhart *et al*, 2010).

Many studies have demonstrated the presence of mature circulating endothelial cells (CECs) in the peripheral circulation (Beerepoot *et al*, 2004). However CECs are extremely rare events in normal peripheral blood, representing somewhere between 0.01% and 0.0001% of peripheral mononuclear cells (Khan *et al*, 2005). Circulating endothelial cells present in peripheral blood comprised of mature resting (rCEC) and activated (aCEC) circulating endothelial cell (Wierzbowska *et al*, 2005) and endothelial progenitor cell (CEPC) (Steurer *et al*, 2008).

EPCs have properties similar to those of embryonic angioblasts (Beerepoot *et al*, 2004) which can be defined as migratory endothelial cells with the capacity to circulate, proliferate and differentiate into mature endothelial cells (Khan *et al*, 2005) but which have not yet acquired characteristic mature endothelial markers and have not yet formed a lumen (Zampetaki *et al*, 2008). The majority of circulating EPCs reside in the bone marrow in close association with hematopoietic stem and progenitor cells (HSPC) and the bone marrow stroma that provides an optimal microenvironment (Ria *et al*, 2008).

Miller *et al* studies have suggested that EPCs may promote local angiogenesis by secreting angiogenic growth factors in a paracrine manner (Miller *et al*, 2007). Their mobilization, recruitment and homing mechanisms are regulated by various chemokines and cytokines and several physiological and pathological conditions can influence the number of circulating progenitor cells (Jung *et al*, 2008).

A key step for tumor growth and metastasis is the establishment of new blood vasculature. Angiogenesis is the sprouting of new blood vessels from already established vessels; this is in contrast to vasculogenesis, which refers to the de novo formation of blood vessels from endothelial progenitors.

The role of BM-derived EPC in tumor vasculogenesis remains a controversial topic. Nonetheless, it raises the question: What is the role of putative BM-derived EPCs that are recruited to tumors? Accumulating evidence suggests that BM derived cells do not contribute significantly to form tumor endothelium directly but rather contribute by stimulating tumor angiogenesis through paracrine mechanisms (Ahn and Brown, 2009).

Wierzbowska *et al* studies suggest that endothelial cells may enhance the survival and proliferation of leukemic blasts and mediate chemotherapy resistance in acute myeloid leukemia (AML). In peripheral blood of AML patients there's an increase in the amount of CECs which correlates with disease status and response to therapy supporting the idea that CEC could represent a marker of angiogenesis (Wierzbowska *et al*, 2005). The aim of this work was to study aCEC and EPC levels in patients with AML and compare their levels with healthy subjects to prove their role in tumorigenesis and correlate these findings with clinical and hematological data of patients and used as prognostic factors.

MATERIAL AND METHODS

The study was conducted on fifty patients with de novo acute myeloid leukemia that were selected from National Cancer Institute, Cairo University.

Their ages ranged from 18 to 83 years with mean 35.6 years and they were 27 females and 23 males.

The study also included twenty healthy volunteers, age and sex matched served as a control group. Their ages ranged from 19 to 61 years with mean 37.2 years. They were 11 females and 9 males.

The AML patients were diagnosed according to clinical presentation, morphological studies, immunophenotyping, and cytogenetics analysis. Diagnosis of AML was based on the presence of blast cells $\geq 20\%$ in BM film according to WHO proposal, together with MPO positive staining and immunophenotyping results consistent with AML (Jaffe *et al*, 2001).

All patients were subjected to the following:

- 1- Full history taking
- 2- Physical examination: with careful notation and assessment of clinical signs relevant to leukemia as hepatomegaly, splenomegaly, lymphadenopathy, gums or skin infiltration.
- 3- Complete blood pictures were performed by the fully automated blood counters Sysmex SE9000.
- 4- Bone marrow examination for:
 - FAB classification
 - Cytochemistry: such as MPO, SBB, esterases, acid phosphatase and PAS when indicated.
 - Immunophenotyping of blast cells using the panel of fluorescein isothiocyanate (FITC)/ phycoerythrin (PE) conjugated monoclonal antibodies (MoAbs) that were used for diagnosing AML includes:
 - Common progenitor marker: CD34, HLA-DR.
 - Myeloid markers: CD13, CD33, CD117, CD15 and intracellular myeloperoxidase (MPO).
 - Monocytic marker: CD14.
 - Other markers: CD61 and glycophorin A.

Cells were considered positive for a certain marker when $\geq 20\%$ of cells expressed it except for CD34 and intracellular MPO where its expression by 10% of cells was sufficient to confer positivity (Jaffe *et al*, 2001).

All the collected blood samples of the patients and the control group were subjected to assessment of both EPCs and aCECs using flow cytometry (lyse no wash technique)

Assay of Circulating Endothelial Cells:

Sample Collection:

Samples were collected by clean venipuncture into vacutainer tubes containing EDTA from cases and controls under strict sterile conditions.

Two ml of venous blood was obtained and all samples were collected at room temperature (24 - 26°C). Analysis was done on fresh samples.

Equipments:

- FCM (flow cytometry).
- 50 μ l automatic pipette.
- Vortex stirrer.

Principle of Flow Cytometry:

Monoclonal antibodies recognize specific molecules in the surface of some cells. These antibodies are artificially conjugated to fluorochromes. Cells are analyzed by flow cytometry after period of incubation with the antibodies. The cells expressing the marker for which the antibody is specific will manifest fluorescence. Cells which lack the marker will not manifest fluorescence.

- Data generated in flow cytometry is displayed using multiparameter acquisition and display software platforms.
- Histograms corresponding to each of the parameters of interest can be analyzed using statistical tools to calculate percentage of cells manifesting specific fluorescence and fluorescence intensity.
- This information can be used to look at fluorescence expression within subpopulations of cells in a sample (gating).

Reagents:

- Mouse antihuman CD133 PE monoclonal antibody: MACS,130-080-801.
- Mouse antihuman VEGFR2 FITC monoclonal antibody: R&D systems,FAB357F,LWS06.
- Mouse antihuman CD106 FITC monoclonal antibody: R&D systems.
- Mouse antihuman CD34 PE monoclonal antibody
- Mouse IgG / FITC - Dakocytomation, REF X0927, Lot 00038153.
- Mouse IgG / PE - Dakocytomation,REF X0925,Lot 00047465

CD133: is a glycoprotein that is expressed in hematopoietic stem cells, endothelial progenitor cells (EPC (Khan *et al*, 2005). VEGFR2: VEGF is a potent and specific endothelial cell mitogen that regulates blood and lymphatic vessel development and homeostasis. VEGFR-2 expression in adult endothelial cells appears to account for most of the mitogenic and chemotactic effects of VEGF (EBI, 2010). Khan *et al* studies have demonstrated that CD133 and VEGFR2 positive cells in the circulation have functional properties of EPCs (Khan *et al*, 2005).

So in our study, the cells that coexpressed CD133 and VEGFR2 were identified as EPCs.

CD34: is a monomeric cell surface antigen that is selectively expressed on human hematopoietic progenitor cells (Entrez gene, 2010). CD34 expressed on both progenitor and mature endothelial cells (Khan *et al*, 2005).

CD106: Vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin family of cell-cell adhesion receptors, is expressed principally on endothelial cells. Activated CECs were defined as those CECs that were positive for CD105 or CD106 (Khan *et al*, 2005).

So in our study, the cells that coexpressed CD34 and CD106 were identified as aCEC.

Reading and Interpretation of Results:

The number of cells that coexpressed the CD133 and VEGFR2 (EPC) emit fluorescence signals which were multiplied in PMT, then the computer analyzed the data and results were expressed as a percentage of cells. The number of cells that coexpressed the CD34 and CD106 (aCEC) was quantified in the same manner.

The percentage of positive cells was converted to absolute number of positive cells/ μ L using the following formula: percentage of positive cells X WBC count/100.

Statistical Analysis:

Data was analyzed using SPSSwin statistical package version 15 (SPSS Inc., Chicago, IL). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test (Fisher's exact test) was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test (non parametric t-test). A P-value < 0.05 was considered significant.

Spearman-rho method was used to test correlation between numerical variables and r value < 0.5 signifies very fair or no correlation, r = 0.5-0.75 signifies good correlation and r $\geq$ 0.75 signifies very good correlation.

Results:

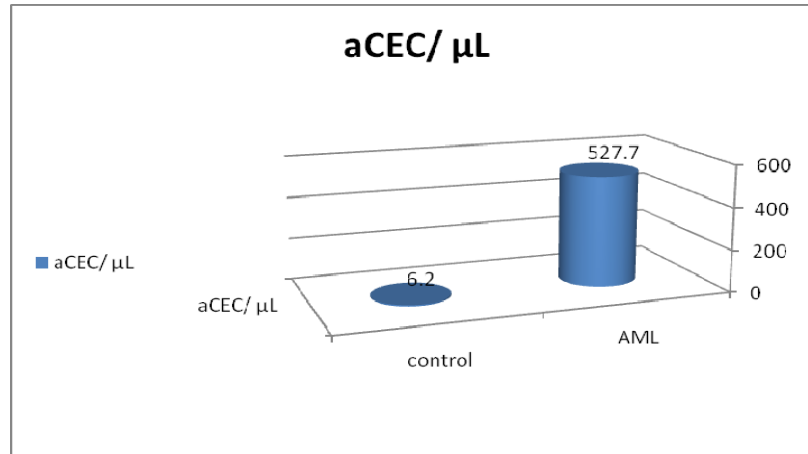
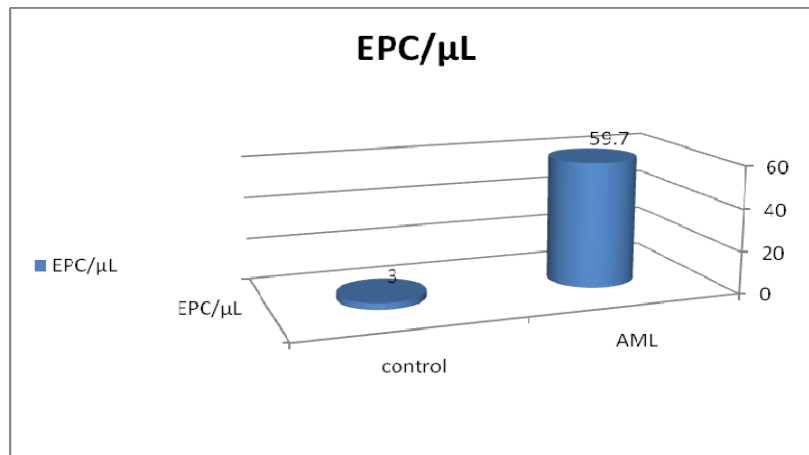
Fifty adult acute myeloid leukemia patients were included in this study between October 2009 and April 2010. Twenty healthy age and sex matched subjects were also included and served as control group. This control group involved 11females (55%) and 9 males (45%) and their ages ranged from 19-61 years with a mean 37.2 ± 13.8 years and median 33 years.

In Study Group:

In Table (1) and Fig (1 and 2).

Table 1: Comparison between AML group vs control group.

Variable	AML	Control	p-value
Age	35.6±15.6	37.2 ± 13.8	0.644
Sex	23 male (46%) 27 female (27%)	9 male (45%) 11 female (55%)	0.940
TLC ($10^9/L$)	32.1 ± 41.3	6.5±2.2	<0.001
aCEC%	1.16±1.2 %	0.09±0.12 %	<0.001
aCEC/ μL	527.7±1052.6	6.2±12.6	<0.001
EPC%	0.35 ± 0.74%	0.05 ± 0.06%	0.012
EPC/ μL	95.7± 129	3± 4.8	<0.001

**Fig. 1:** Comparison between mean level of aCEC/ μL in both patients and control.**Fig. 2:** Comparison between mean level of EPC/ μL in both patients and control.

aCEC mean level was 1.16 ± 1.2 % with range 0-4.4% and its absolute number mean was 527.7 ± 1052.6 cells/ μL with range 0-5749 cells/ μL .

EPC mean was 0.35 ± 0.74 % with range 0-4.6% and its absolute number was 95.7 ± 129 cells/ μL .

Quantification of aCEC% and EPC% and absolute number showed significantly elevated levels in AML patients compared to control group ($P < 0.001$).

In Table (2) and Fig (3 and 4).

Table 2: Correlation between hematological values and both CEC and EPC in AML patients.

Variable	aCEC	p-value	EPC	P-value
WBCs ($10^9/L$)				
< 50	336.6 ± 922.6	0.001	56.6 ± 92.7	0.003
≥ 50	1398 ± 1221		274 ± 373	
Hb (gm/dL)				
< 8	629.3 ± 1233.4	0.377	90.3 ± 125.7	0.382
≥ 8	433.8 ± 867.8		100.7 ± 240	

PLT ($10^9/L$) < 50 ≥ 50	955.8 ± 1721.9 361 ± 590	0.23	78 ± 95 102.5 ± 219.2	0.58
% of leukemic cells in PB < 50 ≥ 50	344.6 ± 562.4 1259.9 ± 1979	0.489	95.2 ± 210.3 97.7 ± 95.2	0.409
% of leukemic cells in BM < 75 ≥ 75	346.4 ± 554 648.5 ± 1278	0.874	101.8 ± 266.9 91.7 ± 1332.7	0.539

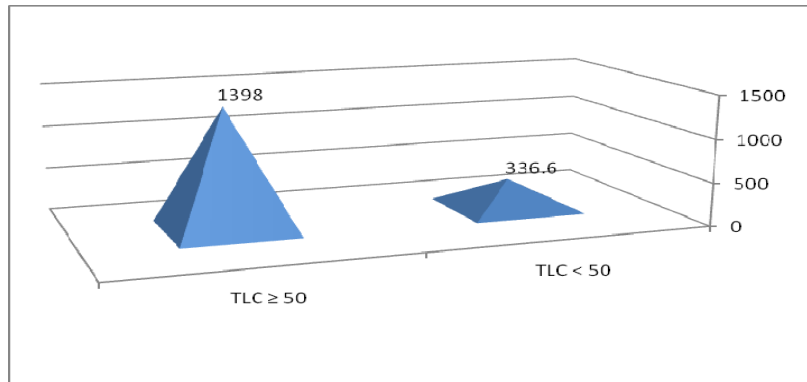


Fig 3: Comparison between mean level of aCEC /μL in AML patients with TLC < 50 ($10^9/L$) and ≥ 50($10^9/L$).

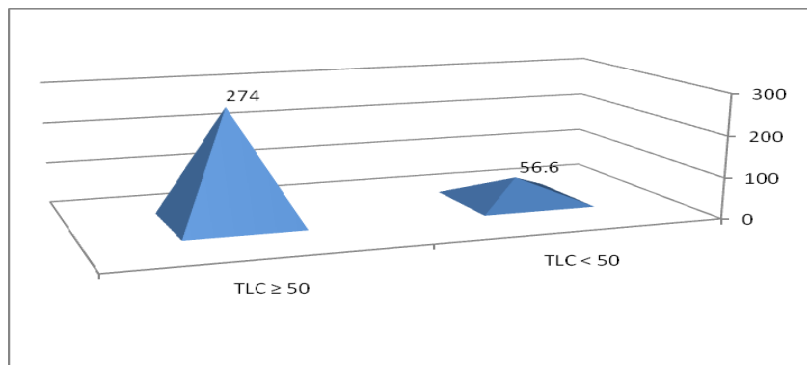


Fig 4: Comparison between mean level of EPC/μL in AML patients with TLC < 50 ($10^9/L$) and ≥ 50 ($10^9/L$).

There was highly significant positive correlation between TLC and both aCEC and EPC ($r=0.76$ and 0.65 respectively) with significant increase of aCEC and EPC in patients with WBC ≥ $50(10^9/L)$ more than those with < $50(10^9/L)$ ($p=0.001$ and 0.003 respectively).

As regard the hematological findings, no statistical significant differences in both aCEC and EPC levels were found between Hb < 8 gm/dl and Hb ≥ 8 gm/dl (p value = 0.37 and 0.38 respectively), platelets < $50(10^9/L)$ and those ≥ $50(10^9/L)$ (p value = 0.23 and 0.58 respectively), PB blast < 50% and those ≥ 50% (P -value = 0.48 and 0.40 respectively), BM blasts < 75% and ≥ 75% (p value = 0.87 and 0.53 respectively). In table (3)

Table 3: Correlation between aCEC and EPC and immunophenotyping in AML patient.

Immunophenotype	aCEC	P- value	EPC	P- value
Myeloid	519 ± 1131	0.149	61.8 ± 109	0.011
Myeloid with monocytic element	559 ± 696		231.2 ± 351.7	
CD34		0.055		0.015
+ve	978 ± 1553		167 ± 275.9	
-ve	274 ± 493.3		55.6 ± 109.23	

There was significant increase in level of EPC in Patients with monocytic elements more than patients with myeloid markers only ($p=0.011$), although no significant difference in aCEC level between the two groups ($p=0.149$). Both aCEC and EPC were significantly higher in patients with CD34 expression on their blast cells than in patients without CD34 ($p=0.055$ and 0.015).

In Control Group:

aCEC mean was 0.09 ± 0.12 % with range 0-0.5% and its absolute number mean was 6.2 ± 12.6 cells/ μ L with range 0-57.5 cells/ μ L.

EPC mean was 0.05 ± 0.06 % with range 0-0.2% and its absolute number was 3 ± 4.8 cells/ μ L with range 0-18.8 cells/ μ L. (Table 1 and fig 1 and 2)

Discussion:

The circulating endothelial cells (CEC) are proposed to be a noninvasive marker of angiogenesis. Recent data suggest that endothelial cells may enhance the survival and proliferation of leukemic blasts and mediate chemotherapy resistance in acute myeloid leukemia (AML). In peripheral blood of AML patients, there's an increase in the amount of CECs which correlate with disease status and response to therapy (Wierzbowska *et al*, 2005)

Most of patients presented with anemia, thrombocytopenia and leucocytosis. This is in accordance with Anchorag (2006) and Hoffbrand *et al*, (2006), who mentioned that 90% of patients have clinically evident hematological abnormalities at diagnosis including anemia, leukocytosis with neutropenia and thrombocytopenia.

In the current study, we found that EPC was statistically highly significant ($P < 0.001$) in AML patients (mean= 95.7 ± 192 and median value= 12.6 cell/ μ L) than in control group (mean= 3 ± 4.8 and median value= 0.9 cell/ μ L). Also we quantified aCEC in AML patients that showed a high significant elevation (mean= 527 ± 1052.6 cell/ μ L, median value= 117 cell/ μ L and $P < 0.001$) than in control group (mean= 6.2 ± 12.6 cell/ μ L, median value= 1.9 cell/ μ L). These findings were in accordance with Wierzbowska *et al* (2005) that found that aCEC and EPC were statistically higher in AML patients than control group by seventeen fold and eight fold respectively.

Mancuso *et al* (2001) reported that resting and activated CECs are increased in newly diagnosed cancer patients and decline after cure underlines the crucial role of angiogenesis in both solid tumors and hematopoietic malignancies. He also reported that increase of activated CECs in cancer patients may be due to different biological reasons: CECs may derive from newly formed tumor vessels or, alternatively, represent ingress of proliferating endothelial cells from neighboring normal tissue or even from distant uninvolved vessels activated by tumor's derived cytokines.

There was highly significant positive correlation between TLC and both aCEC and EPC ($r=0.76$ and 0.65). This is in accordance with Wierzbowska *et al* (2005) and Rigolin *et al* (2007).

There was significant increase in level of EPC in patients with monocytic markers on their blast cells more than patients with myeloid markers only ($P=0.011$), although no significant difference in aCEC level between the two groups and no difference between aCEC and EPC between FAB subtypes.

However this can be explained from the theory that supports an idea about origin of EPC from a common precursor that give origin to endothelial progenitor cell and monocytic cell. This theory is supported by Zampetaki *et al* (2007), Patenaude *et al* (2010) and Kuwana *et al* (2006).

Naik, Igrejia and Lin *et al* studies have reported that EPCs are detected more frequently in the peripheral blood and in the tumors of patients with more invasive stage of disease (Naik *et al*, 2008) or recurrence (Igrejia *et al*, 2007 and Lin *et al*, 2007) indicating a possible involvement in tumor progression. Consistent with this studies have shown that EPCs promote metastasis (Gao *et al*, 2008; Kaplan *et al*, 2005 and Tayler *et al*, 2009) and determine sensitivity of tumors to chemotherapeutic (Bertolini *et al*, 2006; Capillo *et al*, 2003 and Shaked *et al*, 2008).

In conclusion, our findings suggest that in AML patients, EPCs and aCEC are showing high levels and their levels correlate with response to therapy and disease progression. These CECs may contribute to tumor neovasculogenesis and possibly to the spreading and progression of the disease. The study of CECs may have important implications not only for the understanding of the AML specific biological aspects but also for the translation of new antiangiogenic therapies to the clinic.

Further studies are needed to address many questions on the fundamental characteristics of CECs and their real role in tumor progression and resistance to chemotherapy. New therapies against CECs should be applied to the clinic and quantification of CECs should be considered as potential biomarker to antiangiogenic therapies.

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