Indoleamine 2,3-dioxygenase and regulatory T cells in acute myeloid leukemia

Iman Mansour, Rania A. Zayed, Fadwa Said, Lamyaa Abdel Latif

Clinical and Chemical Pathology Department, Faculty of Medicine, Cairo University, El Saraya Street, Infront of El Manial Palace, Cairo 11451, Egypt

Background and objectives: The microenvironment of acute myeloid leukemia (AML) is suppressive for immune cells. Regulatory T cells (Tregs) have been recognized to play a role in helping leukemic cells to evade immunesurveillance. The mesenchymal stem cells (MSCs) are essential contributors in immunomodulation of the microenvironment as they can promote differentiation of Tregs via the indoleamine 2,3-dioxygenase (IDO) pathway.

The aim of the present work was to evaluate the expression of IDO in bone marrow derived MSCs and to study its correlation to percentage of Tregs.

Methods: Thirty-seven adult bone marrow samples were cultured in appropriate culture medium to isolate MSCs. Successful harvest of MSCs was determined by plastic adherence, morphology, and positive expression of CD271 and CD105; negative expression of CD34 and CD45 using flowcytometry. MSCs were examined for IDO expression by immunocytochemistry using anti-IDO monoclonal antibody. CD4+ CD25+ cells (Tregs) were measured in bone marrow samples by flowcytometry.

Results: MSCs were successfully isolated from 20 of the 37 bone marrow samples cultured. MSCs showed higher expression of IDO and Tregs percentage was higher in AML patients compared to control subjects (P = 0.002 and P < 0.001, respectively). A positive correlation was found between IDO expression and Tregs percentage (P value = 0.012, r = 0.5).

Conclusion: In this study, we revealed an association between high IDO expression in MSCs and elevated levels of Tregs which could have an important role in the pathogenesis of AML, providing immunosuppressive microenvironment.

Keywords: Mesenchymal stem cells, T regulatory cells, Acute myeloid leukemia, Indoleamine 2, 3-dioxygenase

Introduction

Acute myeloid leukemia (AML) arises from a series of genetic alterations in a hematopoietic stem cell (HSC) leading to uncontrolled cell growth. It has been postulated that the hematopoietic microenvironment (HM) is implicated in the pathogenesis of AML with defects in HSCs themselves arising secondarily.1,2

Mesenchymal stem cells (MSCs) are the key component of the HM as they are essential element of both healthy and leukemic HM.3 MSCs are capable of promoting growth, survival and drug resistance of leukemic cells by providing the necessary cytokines and cell contact-mediated signals to leukemic stem cells (LSCs).4,5 These LSC are resistant to chemotherapy and are the cause of disease relapse.6,7

MSCs have immunosuppressive effects on cells of the innate and adaptive immune responses. They can inhibit B cell function and differentiation,8 inhibit dendritic cells generation from monocytes,9 inhibit production of the proinflammatory cytokines such as IL-2, IFN-γ, and TNF-α and promote production of IL-10.10–12 Also, MSCs affect T cells, MSCs inhibit T cell proliferation in response to various stimulants,13,14 inhibit the production of IL-2, TNF-α by T cells,15 induce the differentiation of CD4+CD25hiFOXP3+ T regulatory cells (Tregs) and maintain their inhibitory function.16,17 MSCs immunosuppression is mediated by different mechanisms including indoleamine 2,3-dioxygenase (IDO), nitric oxide, transforming growth factor β1, prostaglandin E2, and IL-10.18

Tregs are immune suppressive T-cells; they inhibit T cell proliferation and cytokine production.19 IDO plays important role in Tregs regulation by enhancing the suppressive phenotype and preventing Tregs reprogramming into non-suppressive helper-like cells thus promoting stabilization of Tregs.20,21 IDO may help tilting the tumor microenvironment from hostile to supportive for tumor cells and may be considered as one of the mechanisms of leukemia escape from immune control and so IDO inhibition can be
regarded as a potential target in anti-leukemia therapy.\textsuperscript{22–24}

We studied the expression of IDO in bone marrow derived MSCs and its correlation to percentage of regulatory T cells to evaluate its role in providing immunosuppressive microenvironment in AML.

\textbf{Subjects and methods}\n
The study was conducted in Kasr Alainy Hospitals, Cairo University on 37 subjects; 21 denovo AML patients with age range 18–60 years; 12 males and 9 females. Sixteen age- and sex-matched patients who came for diagnostic bone marrow aspirates to check the marrow cellularity prior to splenectomy operations were included in the study as a control group. The study was performed in accordance with the Helsinki Declaration, and the protocols were approved by the ethics committee of Cairo University. All participants provided informed consent before enrolment into the study.

\textbf{Sample collection}\n
From each participant bone marrow samples were collected; 0.5 ml on EDTA-anticoagulant for flowcytometric quantification of Tregs and 2 ml on heparin anticoagulant for isolation of MSCs and subsequent estimation of IDO expression by immunohistochemical staining.

\textbf{Flowcytometric analysis of Tregs}\n
For each sample, two tubes were prepared; the first tube was used as control tube containing 50 μl of the sample with no monoclonal antibodies added in order to allow the flowcytometer adjustment and to obtain the basic histogram showing the main cell population and adjust the auto-fluorescence region. In the second tube, 50 μl of the sample was mixed with 5 μl of FITC conjugated anti-CD4 monoclonal antibody and 5 μl of PE conjugated anti-CD25 monoclonal antibody and was incubated in the dark for 20 minutes. Erythrocytes were eliminated by adding 500 μl of Opti Lyse C Lysing solution. A minimum of 10 000 leukocytes was analyzed in each tube by means of FACScan flow cytometer (Becman Coulter Cytomics FC500). Forward and side scatter gates were established to exclude cell debris and clumps before analysis. The control tube was then introduced; the laser scatter was received on both forward and side scatter detectors showing the cell population on a basic histogram and adjustment of the auto-fluorescence on the corresponding PMT. The tube for CD4 and CD25 determination was then introduced into the flowcytometer. Lymphocytes were gated first and CD4+ cells were detected, then CD4+ cells were gated and we detected CD25+ population within this gate. Results were expressed as a percentage of cells expressing positive CD25 within the CD4 positive population.\textsuperscript{25}

\textbf{Isolation and identification of MSCs}\n
Bone marrow mononuclear cells were separated using ficoll-hypaque density gradient centrifugation, and the cell count was adjusted to 1.0 × 10^6 cells/ml. The cells were plated in 25 cm² tissue culture flask containing 5 ml complete culture medium (CCM: 1% l-glutamine, 2% antibiotic–antimycotic, 10% fetal calf serum in DMEM) with a density of 1.0 × 10^6 cells/cm² and the flasks were incubated at 37°C with 5% humidified CO\textsubscript{2}. Two days later the media and non-adherent cells were removed, 5 ml of fresh CCM was added to the flask and incubated. The cells were examined every other day by inverted microscopy and medium change was performed every 3 days until the cells reached 70% confluence. Cell harvest was performed at 70% confluence using Trypsin-EDTA and counted. 125 000 cells were suspended in 5 ml CCM and cultured in 25 cm² tissue culture flasks with CCM changed every 3 days until cells reached 70% confluence and cells were harvested and a second passage was performed. Cells harvested after second passage were identified as MSCs by plastic adherence property, fibroblast-like morphology and flowcytometric analysis; harvested cells were stained with monoclonal anti-CD271 and anti-CD105 as MSCs markers; anti-CD45 (panleucocytic marker) and anti-CD34 (HSCs marker) were used as exclusion markers.

\textbf{Estimation of MSCs expression of IDO}\n
A total of 50 000 cells harvested after the second passage were suspended in 600 μl CCM and cultured in Labtek wells (two wells per each participant, one used for staining using monoclonal anti-IDO and the other used as a negative control), incubated at 37°C with 5% humidified CO\textsubscript{2}. Cells were examined 2–3 days later and after reaching 70% confluence, all the wells were fixed in ice cold methanol for 5 minutes and washed three times with PBS followed by cell permeabilization with 0.1% PBS-Tween for 20 minutes. Ultra V Block was applied and wells were incubated for 5–10 minutes at room temperature to block non-specific background staining. Staining of MSCs was done using mouse monoclonal antibody to IDO 1 mg/ml (Abcam: catalog No. ab55305) according to the manufacturer’s instructions. IDO expression was evaluated by two independent assessors using light microscopy. Reading of tissue slides was blind, and both assessors were unaware of clinical outcome. The total IDO immunostaining score was calculated by multiplying the proportion score by an intensity score. The proportion score reflects the estimated fraction of positively stained cells (score 0, none; score 1,
The intensity score represents the estimated staining intensity (score 0, no staining; score 1, weak; score 2, moderate; score 3, strong) giving a total score ranging from 0 to 12. IDO overexpression was defined as a total score > 4.6 Intensity score performed was strictly controlled by using negative controls for individual cases and control samples. In addition, each slide was compared, regarding intensity scoring to its negative control.

Statistical analysis
Data were statistically described in terms of mean, standard deviation, median, minimum, and maximum for quantitative data and frequencies (number of cases) and relative frequencies (percentages) for qualitative data. Comparison of quantitative variables was done using Mann–Whitney test when comparing two groups and Kruskal–Wallis when comparing more than two groups. For comparing categorical data, Chi square ($\chi^2$) test was performed. Exact test was used instead when the expected frequency is less than 5. Correlations were done between variables using spearman correlation coefficient. A probability value ($P$ value) less than 0.05 was considered statistically significant. All statistical calculations were done using SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 21.

Results
The study included 37 participants; 21 denovo AML, 12 males (57.1%) and 9 females (42.9%) with age range from 18 to 60 years (mean ± SD, 45.5 ± 13.9) and 16 control patients, 10 males (62.5%) and 6 females (37.5%) with age range from 16 to 60 years (mean ± SD, 43.5 ± 12.8). Bone marrow samples of only 12 cases and 8 controls gave successful culture of MSCs. The other 17 samples were excluded from the study due to the following: 14 samples were discarded due to contamination, one sample failed to show any growth of spindle shaped cells and two samples showed few adherent spindle cells, however these cells failed to grow further and expand in culture.

Flowcytometric analysis of the Tregs was performed to all samples ($n=37$). In AML patients ($n=21$), percentage of CD25+/CD4+ cells ranged from 4.2 to 20% with a mean ± SD of 9 ± 4.2% and median 8%. While among the controls ($n=16$), percentage of CD25+/CD4+ cells ranged from 0.2 to 4.7% with a mean ± SD of 1.99 ± 1.5% and median 1.4% with statistically significant difference between AML patients and control group ($P<0.001$) (Table 1 and Fig. 1).

Identification of MSCs was based on the presence of plastic adherent cells with spindle shaped fibroblast-like morphology (Fig. 2) that showed positive expression of CD271, CD105 and negative expression of CD45, CD34.

MSCs showed higher expression of IDO among AML patients than controls with $P$-value of 0.002. MSCs of three patients (25%) had score 4, of four

Table 1 | Tregs (CD25+/CD4+ cells) percentage in patients and controls ($n=37$)

| CD25+/CD4+ cells% | AML patients ($n=21$) | Controls ($n=16$) | $P$ value |
|-------------------|-----------------------|------------------|-----------|
| Range             | 4.2–20                | 0.2–4.7          | <0.001    |
| Mean ± SD         | 9 ± 4.2               | 1.99 ± 1.5       |           |

$P$ value < 0.05 is considered statistically significant.

![Figure 1](image1.png)

Figure 1 Phenotypic characterization of Tregs.
patients (33.3%) had score 8 and of five patients (41.7%) had score 12. All MSCs of the control patients had score 4. Mean IDO expression was shown in Table 2 and Fig. 3.

IDO expression in MSCs had positive correlation with Tregs percentage and bone marrow blasts percentage, with $P = 0.012$, $r = 0.5$ and $P = 0.011$, $r = 0.703$, respectively (Fig. 4).

**Discussion**

Successful isolation of BM-MSCs was determined by flowcytometric characterization of cells morphologically identified as MSCs by positive expression of CD271, CD105 and negative for CD45, CD34 according to the proposed criteria of the International Society for Cellular Therapy.

In our study, the low affinity nerve growth factor receptor (LNGFR; CD271) was used in characterization of MSCs as several studies described CD271 as...
the most selective marker for the characterization and purification of human BM-MSCs. MSCs selected by CD271 expression have a 10- to 1000-fold higher proliferative capacity in comparison to MSCs isolated by plastic adherence. These observations led to the recent commercialization of CD271 as a preferred marker for the purification of a homogeneous population of cells that contains all the BM-MSCs activity. In a study by Jarocha et al., comparison of different strategies of MSCs isolation revealed advantage to expand MSC directly from purified CD105 + and CD271 + cells.

Tregs were characterized in bone marrow samples by measuring positive CD25 expression on gated CD4+ cells. Although the expression of Foxp3, a member of the forkhead/winged-helix family of transcriptional factors, is considered an optimal marker for Tregs. Nevertheless, some studies used only CD4 and CD25 or other parameters such as absolute counts and percentages in total lymphocytes. Also, FOXP3 protein expression may not be stable, depending on FOXP3 gene methylation, and can disappear in 10–15% of FOXP3 + Tregs (so-called exFOXP3 cells) in mice. Moon et al. showed that CD4 + CD25high/CD4 and CD4 + CD25highFoxp3+/CD4 cell populations were significantly correlated (P < 0.0001). Our results showed a significant increase in the percentage of Tregs in AML patients in comparison with the control group (P < 0.001). These finding are in agreement with Moon et al. who found that the AML and high-grade MDS groups had significantly increased Tregs populations in both peripheral blood and bone marrow compared to the control groups. This can be explained by the previous studies which have shown that AML cells secrete factors, which inhibit T-cell activation and proliferation and limit proinflammatory T helper-1 cytokine production. This suppressive effect is reversed, however, when Tregs and other T lymphocytes were removed from the microenvironment in vitro, leading to augmented immune responses to AML. This can also be explained by the effect of MSCs in the marrow microenvironment on generating CD4 + CD25 + FOXP3+ cells. Ivanova-Todorova et al. found a stable tendency towards an increase in CD4 + CD25 + FOXP3 + cells number in the presence of MSCs compared with the respective control cultures without MSCs. It was also stated that CD271 + MSCs secrete higher levels of cytokines and have greater immunosuppressive properties.

Our results showed a significant increase in MSCs IDO expression in AML patients when compared to the control group (P = 0.002). The increase in IDO expression in AML patients in comparison to the controls may be explained by the ability of leukemic cells to induce changes in MSCs. Civini et al. showed that there is a dynamic relationship between BM-MSCs and leukemia cells. They confirmed that BM-MSCs affect leukemia cells and found that leukemia cells change the profile of cytokines produced by BM-MSCs to a proinflammatory signature through soluble factors and does not require direct contact between BM-MSCs and leukemia cells.

A positive correlation was found between IDO expression and Tregs (r = 0.5, P = 0.012), which may be explained by the role of IDO in inducing the conversion of CD25-FOXP3-T cells into CD25 + foxp3+. The percentage of CD4 + CD25 + cells was significantly increased in IDO + AML patients compared to IDO-AML patients or controls, which may also explain the correlation that was found in our study between blast cell percentage and the percentage of Tregs further supporting the proinflammatory effect of leukemia cells on BM-MSCs.

Data from several studies proved the poor prognostic effect of elevated Tregs on outcome of AML; Tregs at diagnosis were lower in patients who had achieved CR compared with those with persistent leukemia or death. Studies in murine models have shown that the frequencies of Tregs are increased in AML in vivo, that these Tregs have suppressive functions on Teffs in vitro, and that removing/depleting Tregs improves the function of Teffs in vitro and improves treatment outcome.

In conclusion, our study revealed an association between high IDO expression in MSC and elevated levels of Tregs which has an important role in the pathogenesis of AML and supposed to affect treatment outcome. Current treatments for AML have not changed for several decades and have not resulted in satisfactory outcomes. Modulating the immune system may improve survival in patients with AML because the immune system is highly active against leukemic cells. It would be interesting to determine whether inhibiting IDO in conjunction with chemotherapy would result in better outcomes since it persistently breaks the tolerance to tumors, thus eliciting an effective immune response.

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