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Bone Marrow Microenvironment; Assessment of Changes in Acute Leukemia at Diagnosis and in Remission

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ABSTRACT

This study included 40 patients with Acute Leukemia (15 Acute Lymphoblastic and 25 Acute Myeloid) at diagnosis comprising Group I and during remission comprising Group II. 15 bone marrow donors for transplantation were taken as control comprising Group III.

All cases were subjected to morphological, cytochemical, and immunophenotypic analysis for diagnosis and typing of acute leukemia.

All cases were examined for assessment of different bone marrow microenvironment (BMM) elements: Fibronectin by Radial Immunodiffusion, TNFα and L-selectin by ELISA technique in plasma samples of bone marrow aspirate. Bone marrow biopsies were done for 10/15 (66%) of ALL cases in Group I and II, and 9/25 (36%) of AML cases in Group I and 5/25 (20%) during remission in Group II as well as for controls.

Results: Fibronectin mean level in plasma of BM in Group I at diagnosis was found to be significantly decreased ($p<0.05$) as compared to control Group III i.e. Group III was 2.5 folds higher frequencies of Group I, while during remission, it was more than its level at diagnosis but still lower than control.

L-selectin mean level of BM plasma in acute leukemic patients at diagnosis was found to be markedly increased as compared to control i.e. Group I was 25 folds of Group III, while during remission, it was less than its level at diagnosis but still higher than control i.e. Group II was nearly 5 folds of Group III.

TNFα mean level in plasma of BM was significantly increased at diagnosis reaching 9 folds the mean levels of controls, while it was decreased markedly after chemotherapy, i.e group II was nearly 2 folds of group III. There was no difference in TNFα levels between ALL and AML.

As regard stromal cell pattern: There was an increase in reticular cells in variable degrees, in addition to fibroblasts and macrophages which showed elevation in 90% of cases. Adipocytes were reduced in only 25% of cases. In Group II during remission only few cases showed minimal increase in reticular cells and fibroblasts while all the remaining cases showed normal reticular cells, fibroblasts, macrophages and adipocytes.

Conclusion: The study of bone marrow microenvironment in acute leukemia showed how the malignant process can cause a significant disturbance in the equilibrium of this microenvironment. Also, the use of L-selectin in BM plasma is recommended as a useful prognostic marker in acute leukemia.

Key Words: *BM microenvironment - Fibronectin - L-selectin - TNFα.*

INTRODUCTION

Normal hematopoiesis takes place in the bone marrow and is the result of interaction between hematopoietic progenitor stem cells and the surrounding microenvironment **[1]**.

The bone marrow microenvironment (BMM) plays an important role in promoting hematopoietic progenitor cell proliferation and differentiation as well as the controller progress of these developing hematopoietic cells **[2]**.

The normal hematopoietic microenvironment (HM) in the bone marrow consists of a heterogeneous population of hematopoietic and nonhematopoietic stromal cells, their extracellular biosynthetic products, and hematopoietic cytokines **[3]**.

The cells include myofibroblasts, other fibroblastoid cells, endothelial cells, osteogenic precursors, adipocytes and macrophages. These cells produce a complex array of extracellular matrix (ECM) molecules consisting of proteoglycans and their constituent sulfated gly-

cosaminoglycans, chondroitin, heparin, fibronectin, thrombospondin and glycoprotein **[4]**.

In addition, hematopoietic progenitors express cell adhesion molecules (CAMs) classified into six superfamilies: Integrins, selectins, sialomucins, immunoglobulins, cell surface proteoglycans and cadherins **[5]**. Cells comprising the HM also provide a source of many hematopoietic cytokines, either secreted or membrane bound, including GM-CSF and stem cell factor **[6]**.

Fibronectin is a two-subunit, multidomain subendothelial extracellular matrix protein that is also found in platelet alpha-granules and plasma. Fibronectin extracellular matrix plays a critical role in the microenvironment of cells. Loss of this matrix frequently accompanies oncogenic transformation, allowing changes in cel1 growth, morphology, and tissue organization **[7]**.

L-selectin is a member of selectin family adhesion molecules; it is expressed on surface of all classes of leucocytes and plays an important role in homing and function of these cells. L-selectin dependant attachment to endothelium is observed in vivo as well as in vitro under rotating or flow conditions, suggesting that L-selectins is involved in the initial attachment of leucocytes to the endothelium **[8]**.

The effect of TNFα on hematopoiesis can be either directly mediated or indirectly by inducing other cells to produce growth factor (GFS) such as GM-CSF from fibroblast and G-CSF and CSF-1 from monocytes **[9]**.

This study was carried out in order to investigate BMM of acute leukemia patients (at diagnosis and at remission) through assessing the BM levels of fibronectin and levels of L-selectin and TNFα, in addition to microscopic examination of BM biopsies to assess the stromal cells.

MATERIAL AND METHODS

Materials:

Study Samples were divided into three groups:

Group (I): 40 patients with de novo diagnosis of acute leukemia (24 males and 16 females) with a mean age 35 years (range 8-70). Patients of this group were subdivided according to FAB Classification into: (a) ALL (6 cases L_1 and 9 cases L_2) (b) AML (4 cases M_1 - 7 cases M_2 - 3 cases M_3 - 4 cases M_4 - 3 cases M_5 and - 4 cases M_7).

Group (II): The same 35 patients out of 40 during remission, after induction course of chemotherapy (21 males and 14 females), age range 8-60 years. 5 cases died during follow up (2 cases M_7 & 3 cases M_3).

Group (III) Control group: 15 healthy subjects (7 males and 8 females) with age range 18-36 years selected from bone marrow transplantation donors. All the cases were selected from National Cancer Institute and Nasser Institute from 2002 to 2003.

Sample Collection:

1- Peripheral blood samples were collected for routine lab investigations.

2- Bone marrow sample: This was done for all patients and controls by the standard technique **[10]**. Diagnosis was done based on morphology, cytochemistry and immunophenotyping. The remaining aspirate was transferred into a tube containing heparin and centrifuged at 1000 x g for 10min and plasma was divided into 6 sterile aliquots of 250-500ul and stored at -70ºC till use.

3- Bone marrow biopsy: BM biopsy was done only to fulfil the diagnosis whenever indicated (done only for 10 cases of ALL and 9 cases of AML) to study the stromal cells, using the standard technique according to Williams and Nicholson, 1963 **[11]**.

Methods:

Clinical examination was performed for all patients with emphasis on fever, pallor, purpura, ecchymosis, lymph node enlargement, hepatomegaly, splenomegaly and palpable masses. Full laboratory investigations: included CBC, ESR, bone marrow aspiration, routine laboratory and radiological investigations and bone marrow trephine biopsy for ALL cases and for some cases of AML. These investigations were done for leukemic patients at time of diagnosis and during remission.

Assay of Fibronectin:

Fibronectin in BM plasma samples was measured by Radial-Immunodiffusion technique by Mancini et al. **[12]**. The kit was manufactured

by BINDARID, NANORID kits. (The Binding site Ltd., R&D, Birmingham, UK).

Assay of SCD 62 L (L-Selectin) and TNFα:

L-Selectin and TNFα were measured by a two step sandwich ELISA technique by Diaclone Research, FRANCE **[13]** for in-vitro quantitative determination in bone marrow plasma. L-selectin is expressed in ng/ml and TNFα in pg/ml. Sensitivity of L-selectin <1ng/ml and TNFα <25pg/ml.

All assays were performed as per the manufacture's instructions. Each sample was assayed in duplicate. The plates were read at 450nm wavelength.

Statistical Analysis:

The collected data were tabulated and statistically analyzed (Minitab statistical software version, 1998):12-1. Chi-square as a non parametric test was used to assess the statistical significance of associations among categorical variables when assumptions for its application were fulfilled.

Pearson correlation coefficient (*r*) was used to assess the statistical significance of correlation among normally distributed quantitative variables. The value of *r* ranges from –1 to +1, if the value of r positive then the correlation is positive, whereas negative values of *r* indicate inverse or negative correlation.

Difference, associations and correlations were considered significant when the *p*-value of the corresponding test is less than or equal to 0.05.

RESULTS

Characteristic and Clinical Data of Patients and Controls: Pallor was the most common finding present in 80% of cases, 56% of cases were presented by fever, 48% were presented by purperic rashes and 32% were presented by ecchymosis. Splenomegaly was felt in 60% of cases hepatomegaly in 44% of cases and lymphadenopathy in 60% of cases.

Laboratory Investigations:

Hematological tests were performed for all cases. In patients at diagnosis; 82% of patients presented by anaemia and leucocytosis, while 18% of patients presented by leucopenia and 90% patients presented by thrombocytopenia.

Specific Investigations:

1- *Assay of bone marrow plasma level of Fibronectin (mg/L):*

Table (1) shows the bone marrow plasma levels of Fibronectin (mg/l) in all groups. The mean level of Fibronectin in acute leukemia patients at diagnosis (67±10.58) was found to be significantly decreased as compared to the mean level in control (173±67.5) i.e Group III was 2.5 folds higher frequencies of Group I.

During remission, the mean level of Fibronectin (117±27.13) was more than its level at diagnosis but still lower than control i.e. Group III was nearly 1.5 folds of Group II. There was no significant difference in the mean levels of Fibronectin in ALL & AML neither at diagnosis nor during remission (Table 2).

2- *Assay of Bone Marrow Plasma Level of L-selectin (ng/ml):*

Table (3) shows the BM plasma levels of L-selectin (ng/l) in all groups: The mean level of BM plasma L-selectin in acute leukemic patients at diagnosis (107.87±133.28) was found to be markedly increased as compared to the mean level in controls (4.29±1.82) i.e. Group I was 25 folds of group III.

During remission, the mean level of BM plasma L-selectin (29.91±13.08) was less than its level at diagnosis but still higher than in controls i.e. Group II was nearly 5 folds of Group III.

Table (4) shows comparison in BM plasma L-selectin levels between ALL and AML cases at diagnosis and during remission identifying no significant statistical difference neither at diagnosis nor during remission.

3- *Assay of Bone Marrow Plasma Level of TNF-α (pg/ml):*

Table (5) shows the BM plasma levels of TNFα (mg/L) in all groups. The mean level of BM plasma TNF-α in acute leukemic patients at diagnosis (388.63±189.83) was found to be significantly increased as compared to the mean levels in controls (42.13±10.56) i.e. Group I was 9 folds higher frequencies of Group III.

During remission, the mean level of TNF-α (94.84±33.91) was less than its level at diagnosis but still higher than its level in controls i.e. Group II was nearly 2 folds of Group III.

Comparison of BM plasma TNF-α level between ALL and AML at diagnosis and during remission identified no significant statistical difference (Table 6).

Evaluation of BM Stromal Cells: BM Trephine biopsies were performed for 10/15 (66%) of ALL cases at diagnosis and during remission, and 9/25 (36%) of AML cases at diagnosis and 5/25 (20%) during remission.

Microscopic findings of BM trephine biopsies in Group I at diagnosis:

- Bone marrow was hypercellular in 5/19 cases (26%), hypocellular in 6/19 cases (32%) and normocellular in 8/19 cases (42%).
- The stromal cell pattern of BM biopsies demonstrated an increase in reticular cells in variable degrees (slight, moderate, marked) in all examined cases. Also, fibroblasts and macrophages showed variable degrees of elevation in 90% of cases. Adipocytes were reduced in only 25% of cases.
- Fibrosis was present only in AML M_7 cases (4 cases).

Microscopic Findings of BM Trephine Biopsies in Group II During Remission:

- One case showed hypocellularity while all the remaining cases were normocellular.
- The stromal cell pattern of BM biopsies showed minimal increase in reticular cells and fibroblasts in few cases while all the remaining cases showed normal reticular cells, fibroblasts, macrophages and adipocytes.

Table (7) illustrates coefficient correlation between the studied levels of FN, L-Selectin, and TNFα and total leucocytic count, % of blasts in peripheral blood and in bone marrow.

- There was significant positive correlations between L-Selectin levels and total leucocytic count, % blasts in both peripheral blood and BM ($p<0.05$).
- There was no significant correlation between levels of FN as well as TNFα and any of the studied parameters ($p>0.05$).

Table (1): Bone marrow plasma levels of fibronectin in all groups (normal mean value = 213mg/l).

Fibronectin	Group I	Group II	Group III	*t* value	*p* value
Range	40-90	58-150	100-290	t_1=12.99	*
Mean	67.75	117.31	173	t_2=2.18	*
± SD	10.58	27.13	67.50	Paired$_t$=17.55	*

SD = Standard deviation.
t_1 = Control versus leukemic patients at diagnosis.
t_2 = Control versus leukemic patients during remission.
Paired t = Leukemic patients at diagnosis vs themselves during remission.
* = Statistically significant *p*-value ($p<0.05$).

Table (2): Comparison between ALL and AML patients versus control as regard fibronectin levels (expressed in mg/L).

Fibronectin levels	ALL		AML		Control
	Diagnosis	Remission	Diagnosis	Remission	
Range	55-90	90-150	40-88	58-135	100-290
Mean	68.53	124.13	67.28	119.7	173
± SD	11.34	21.76	10.31	24.03	67.50
t value at diagnosis			0.16		
During remission			2.11 (NS)		

SD = Standard deviation.
NS = Nonsignificant statistically.

Table (3): Bone marrow plasma levels of L-selectin in all groups (normal mean value = 3.0±0.9ng/ml).

L-Selectin	Group I	Group II	Group III	*t* value	*p* value
Range	28-889	8-65	2-8	t_1=7.70	*
Mean	107.87	29.71	4.15	t_2=5.06	*
± SD	133.28	13.08	1.53	Paired$_t$=7.99	*

SD = Standard deviation.
t_1 = Control versus leukemic patients at diagnosis.
t_2 = Control versus leukemic patients during remission.
Paired t = Leukemic patients at diagnosis vs themselves during remission.
* = Statistically significant *p*-value (p<0.05).

Table (4): Comparison between ALL and AML patients versus control as regard L-selectin levels in BM plasma (expressed in ng/ml).

L-Selectin	ALL		AML		Control
	Diagnosis	Remission	Diagnosis	Remission	
Range	28-180	11-53	36-889	8-65	2-8
Mean	109.41	30.27	118.94	29.30	4.15
± SD	49.09	12.62	164.68	13.73	1.53
t value at diagnosis			0.41		
During remission			0.58 NS		

SD = Standard deviation.
NS = Nonsignificant statistically.

Table (5): BM plasma levels of TNF-α (pg/ml) in all studied groups.

TNF-α	Group I	Group II	Group III	*t* value	*p* value
Range	175.00-760.0	145.00-175.80	30.00-65.00	t_1=7.52	*
Mean	388.63	94.840	42.133	t_2=3.58	*
± SD	189.83	33.911	10.855	Paired$_t$=8.37	*

SD = Standard deviation.
t_1 = Control versus leukemic patients at diagnosis.
t_2 = Control versus leukemic patients during remission.
Paired t = Leukemic patients at diagnosis vs themselves during remission.
* = Statistically significant *p*-value (p<0.05).

Table (6): Comparison between ALL and AML patients versus control as regard TNFα levels in BM plasma.

TNFα level	ALL		AML		Control
	Diagnosis	Remission	Diagnosis	Remission	
Range	235-760	29.2-175	175-750	46-135	30-65
Mean	368.27	104.06	400.84	87.93	42.13
± SD	190.66	40.88	192.19	26.63	10.86
t value at diagnosis			0.07		
During remission			0.15 NS		

SD = Standard deviation.
NS = Nonsignificant statistically.

Table (7): Correlation coefficient (*r*) of Group I (acute leukemic patients diagnosis).

	TLC	% PB Blasts	% BM Blasts
Fibronectin:			
r	-0.065	0.021	0.026
p	0.692	0.897	0.884
L-Selectin:			
r	0.610*	0.560*	0.653*
p	0.016	0.030	0.008
TNF-α:			
r	0.136	-0.002	0.090
p	0.403	0.989	0.607

DISCUSSION

Leukemias are a group of disorders of uncertain etiology characterized by an abnormal proliferation of the leucopoietic tissue of the body, they are almost invariably fatal although remission may occur **[3]**.

This study was conducted to evaluate some elements in plasma of BMM of acute leukemia patients (at diagnosis and during remission) through assessing the levels of fibronectin by radial immuno-diffusion technique and levels of L-selectin and TNFα by Elisa technique in addition to microscopic examination of BM trephine biopsies to assess the stromal cells.

The present study was conducted upon 40 patients; 15 cases acute lymphoblastic leukemia (ALL) and 25 cases acute myeloid leukemia (AML) in addition to 15 healthy subjects of matched age (donors of BM transplantation) as control group. In this study, we found that the levels of plasma BM Fibronectin (FN) in acute leukemic patients (both ALL & AML) were lowered than control group. During remission, the FN levels were increased but still lower than its level in normal control subjects.

In agreement with our findings, Gee et al. **[14]** demonstrated that blood plasma fibronectin was markedly reduced in newly diagnosed acute leukemic patients (ALL and AML) than in controls. And its level was corrected to near normal level with treatment. So it predicted the onset of relapse in these patients and follow up till complete remission.

Ariel et al. **[15]** reported that in acute leukemia cases, low blood plasma level of FN was detected at diagnosis and relapse and returned to normal range after achieving chemotherapy. This level was not related directly to tumour load or neutropenia but correlated well with episodes of intercurrent infection. Also, they found that in severe infections, FN fell rapidly to very low levels and was sometimes not restored to normal up to two weeks later.

Brenner et al. **[7]** stated that decreased blood plasma fibronectin levels compared to the controls and to its levels after treatment, may be attributed to increased consumption of FN in expanded mononuclear phagocytic system present in the liver and spleen, and also to reduced hepatic synthesis.

It was concluded that measurement of plasma fibronectin may help in early detection of infection in acute leukemia and may be considered of prognostic value in follow up of treatment. However, as its level is influenced by sepsis, blood transfusion and chemotherapy, it can not serve as a treatment marker at least as a single isolated measurement **[14]**.

In the present study, there were no significant correlations between levels of fibronectin and any of the studied parameters of acute leukemia at diagnosis ($p>0.05$).

L-Selectin, a member of selectins family recognizes mucins. L-selectin found on all mature leucocytes, marrow progenitor cells and stroma express various combination of L-selectin and mucin. Leukemic blasts express a wide range of L- selectin which is important in homing of the blasts to the bone marrow **[16]**.

As regard L-selectin in the present study, the estimated BM levels of L-selectin in acute leukemic patients (both ALL & AML) at diagnosis were found to be significantly higher than its levels in controls ($p<0.05$). Also, we found that BM levels of L-selectin in leukemia patients who achieved complete remission (CR) were significantly lowered than their levels at diagnosis before starting chemotherapy ($p<0.05$), and did not significantly differ from that of controls ($p>0.05$).

There was no statistical significant difference in L-selectin levels between ALL and AML cases neither at diagnosis ($p>0.05$) nor during remission ($p>0.05$).

In agreement with our study, Olejnik et al. **[17]** demonstrated that serum L-selectin decreased significantly from diagnosis to the end of intensive chemotherapy and increased in relapse. These results suggested that monitoring of L-selectin may be useful for evaluating leukemic activity.

In the present study, there were significant positive correlations between L-selectin levels and total leucocytic count (r=0.610, p=0.016), percentage of blasts in peripheral blood (r=0.560, p=0.030) and percentage of blasts in bone marrow (r=0.653, p=0.008), while there was no significant correlation between levels of FN as well as TNF-α and any of the studied parameters (p>0.05).

In the present work; plasma BM levels of TNFα were significantly higher in acute leukemic patients at diagnosis than healthy controls. TNFα levels during remission were noticeably lowered but still slightly more than normal controls.

This is in agreement with Gessner et al. **[18]** who reported that 80% of plasma BM sample of acute leukemia patients had markedly higher TNF-α than control group during remission. It was concluded that the elevated plasma BM levels of TNFα are a useful marker to assess the disease activity, but not prognosis of acute leukemia.

Stromal cells are important elements of BMM that influence the development of hematopoietic cells through the production of cytokines and through the signals mediated by direct contact of progenitor cells with stromal cells **[19]**.

It is evident that leukemia cells interact with the BMM at many levels and mimic the action of normal early precursors to a variable extent **[4]**.

In the present study, microscopic examination of BM biopsies of cases included in the study, revealed disturbed BM cellularity in 58% of cases, (26% of cases were hypercellular and 32% were hypocellular) while the remaining 42% were normal in cellularity.

Also, we found disturbance is stromal cell pattern in BM biopsies: As regard reticular cells, we demonstrated increase in its number by variable degrees (slight, moderate, marked) in all examined cases in relation to normal control subjects. Also, fibroblasts and macrophages showed variable degrees of elevation in most cases (90%). Adipocytes were in normal distribution in 75% of examined biopsies and were reduced in the remaining 25%.

Our findings were in agreement with a previous study done by Giles et al. **[3]** comparing bone marrow biopsies, obtained from different categories of leukemia cases, with those from normal donors. They revealed normal number of macrophages but increased number of fibroblast and reticular cells.

In conclusion, we found that the different components of BMM are greatly affected by malignant transformation of the hematopoietic cells in acute leukaemia. L-selectin can be used as a prognostic marker in acute leukemia, while Fibronectin and TNFα can be used as markers for the disturbance of the hematopoietic microenvironment. Also, bone marrow trephine biopsies may be used as a tool to asses the BMM in acute leukaemia as stromal elements are essential factors in the development of haemopoetic cells.

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